JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLES

Development of an Indirect Competitive ELISA for the Detection of Furazolidone Marker Residue in Animal Edible Tissues

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Due to its carcinogenicity and mutagenicity, furazolidone has been prohibited completely from being used in food animal production in the world since 1995. To monitor the illegal abuse of furazolidone, a polyclonal antibody-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) was developed for the determination of tissue-bound furazolidone metabolite 3-amino-2-oxazolidone (AOZ). The highly specific antibody was targeted for PAOZ, the benzaldehyde derivative of AOZ. The 50% inhibition values (IC_{50}) of 0.91 μg/L for AOZ was achieved with the most sensitive antibody Ab-B1 by altering ELISA conditions. In the ELISA, sample extraction and cleanup were performed by an is MAX cartridge following combined hydrolysis of the tissue-bound AOZ and derivatization of the homogenized tissues with benzaldehyde. The limitsof detection (LOD) calculated from the analysis of 20 known negative tissue samples (swine liver, swine muscle, chicken liver, chicken muscle,and fish muscle) were 0.3−0.4 μg/kg (mean + 3 SD). Recoveries of AOZ fortified at the levels of 0.4, 1, and 5 μg/kg ranged from 55.8 to 96.6% in the tissues. The coefficients of variation were less than 20% over the range of AOZ concentrations studied. The linear detection range was between 0.1 and 25.6 μg/L. Validation of the ELISA method with swine muscle and liver from furazolidone-treated pigs was carried out using HPLC, resulting in a similar correlation in swine muscle ($r = 0.99$) and in swine liver ($r = 0.98$). The results suggest that this ELISA is a specific, accurate, and sensitive method of detecting AOZ residues in animal edible tissues.

KEYWORDS: Furazolidone; AOZ; antibody; ELISA; residue; edible tissues

INTRODUCTION

Furazolidone, *N*-[5-nitro-2-furfurylidene-3-amino]-2-oxazolidinone (**Figure 1**), is a synthetic broad-spectrum antibiotic used widely as a feed additive for the prevention and treatment of gastrointestinal infections caused by *Escherichia coli* and *Salmonella* in swine, poultry, and cattle. The use of furazolidone has been prohibited completely in food animal production worldwide since 1995, due to its carcinogenicity and mutagenicity (*1*–*3*); however, it is still illegally and widely used now. Residue screening techniques are the most efficient ways for monitoring illegal abuse of furazolidone. Before the ban of furazolidone, most established methods aimed at the detection of residues of furazolidone itself in food-producing animals (*4*). However, furazolidone is characterized by its rapid metabolism in vivo in less than a few hours. Studies conducted with ^{14}C-

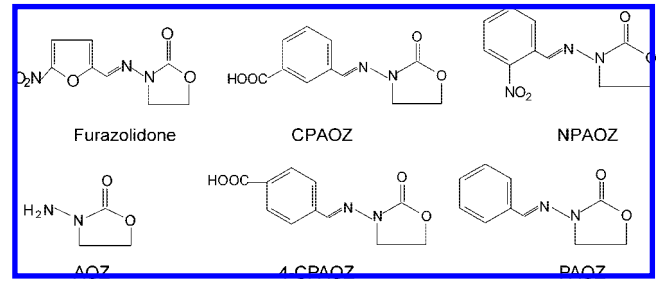

Figure 1. Chemical structures of the banned nitrofuran furazolidone, its marker residue AOZ, the former target derivative NPAOZ, the former immunogen hapten CPAOZ, the immunogen hapten 4-CPAOZ, and the target analyte PAOZ.

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10.1021/jf0726684 CCC: $40.75
Published on Web 02/09/2008

labeled furazolidone had shown that protein-bound residues were formed, some of which contained the intact side chain 3-amino-2-oxazolidone (AOZ; **Figure 1**) released from bound furazolidone residue by mild acid hydrolysis. Furthermore, the AOZ residue is stable in tissues and persists for at least 6 weeks in pig tissues after drug withdrawal (*5*). As a result, effective analytical detection of furazolidone could be monitored by the determination of bound furazolidone metabolite AOZ. AOZ is a very small molecule (M_r 102), which is not UV absorbing, and it is eluted too quickly out of a column. It could be further derivatized with 2-nitrobenzaldehyde (2-NBA), forming stable nitrophenyl derivatives, 3-[[2-nitrophenyl]methylene]amino-2-oxazolidinone (NPAOZ; **Figure 1**), prior to the analysis of AOZ (*6–9*).

Several methods have been released for the determination of AOZ by HPLC-UV (*6*), LC-MS (*7*), and LC-MS/MS (*8, 9*). However, these instrumental analytical procedures were usually not fit for high-capacity screening analysis of tissue-bound furazolidone residues. There is now an urgent need for a rapid, high-capacity, and sensitive screening method for AOZ residues. The indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) has become the most popular method for the detection of drugs in animal tissues due to its high sensitivity, low cost, and ability to screen large numbers of small-volume samples.

The first polyclonal rabbit antibody raised against NPAOZ, nitrophenyl-derivatized AOZ hapten, was produced in FoodBRAND, an EU-funded Research and Development project (QLK1-CT1999-001142) and used in ELISA for the determination of AOZ in prawn tissue (*10, 11*). An ELISA test kit had been manufactured by R-Biopharm AG (Darmstadt, Germany) based on the first polyclonal rabbit antibody. In 2005, an ELISA method based on the use of a specific monoclonal antibody for the quantification of AOZ in tissues was developed (*12*). Although these polyclonal and monoclonal antibodies incorporated into an immunoassay did meet current criteria of monitoring AOZ residue, the preparation of matrix-matched standards from blank samples prior to analysis might be less favorable (*11, 12*). Additionally, these polyclonal and monoclonal antibodies were produced with the same immunizing hapten, 3-[[3-carboxyphenyl]methylene]amino-2-oxazolidinone (CPAOZ; **Figure 1**) and allowed the detection of AOZ (in the form of NPAOZ) following derivatization with 2-NBA. Furthermore, a high coefficient of variation (38.5%) was obtained in the previous study (*12*). This led us to the current study aiming to develop a reliable ELISA method that provided detection of AOZ residues by adopting PBS calibration standard rather than matrix calibration standard.

This paper describes an alternative ELISA method for detection of AOZ residues following derivatization with benzaldehyde. In this study, the polyclonal antibodies produced with immunizing hapten, 3-[[4-carboxyphenyl]-methylene]amino-2-oxazolidinone (4-CPAOZ; **Figure 1**), were highly sensitive to PAOZ (**Figure 1**), phenyl-derivatized AOZ hapten. Benzaldehyde, a new derivatizing agent, was substituted for the traditional 2-nitrobenzaldehyde. This ELISA method has a tendency to incorporate into the ELISA test kit, which would provide a reliable ELISA method for screening analysis of AOZ residues using PBS calibration standard and a new derivatizing agent.

MATERIALS AND METHODS

Materials. 3-Amino-2-oxazolidinone (AOZ) and 3-{[(2-nitrophenyl)methylene]amino}-2-oxazolidinone (NPAOZ) were purchased from WITEGA Laboratorien (Berlin, Germany). *N*-[Phenzlidene]-3-amino-2-oxazolidinone (PAOZ) was synthesized at the National Reference Laboratory of Veterinary Drug Residues (Wuhan, China). 4-Carboxybenzaldehyde was purchased from Daqing New Century Fine Chemicals Co., Ltd. (Daqing, China). Furazolidone, nitrofurantoin, nitrofurazone, and furaltadone were purchased from Shangdong Jintai Co., Ltd. (Ji'nan, China). Bovine serum albumin (BSA), ovalbumin (OVA), goat anti-rabbit IgG−horseradish peroxidase, 3,3′,5,5′-tetramethylbenzidine (TMB), *N*-hydroxysuccinimide (NHS), *N*,*N*-dicyclohexylcarbdiimide (DCC), complete Freund's adjuvant (CFA), and incomplete Freund's adjuvant (IFA) were supplied by Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO), polyoxyethylenesorbitan monolaurate (Tween-20) and *N*,*N*-dimethylformamide (DMF) were purchased from Linfei Biotec Cp., Ltd. (Wuhan, China). The solid-phase extraction cartridge, Oasis MAX (3 cm^3, 60 mg), was obtained from Waters (Milford, MA). All other chemicals were of analytical grade. Landrace-Large white crossbred pigs were supplied by the Huazhong Agricultural University Breeding Pig Stack Farm (Wuhan, China). New Zealand white rabbits were supplied by Hubei Centers for Disease Prevention and Control (Wuhan, China). ELISA was measured by enzyme immunoassay microplate reader MagenllanCE 2.5 (Sunrise, Austria). HPLC analyses were performed with an Agilent HP1100 series (Agilent, Palo Alto, CA). UV–vis spectra of conjugates were obtained by an Agilent UV–vis spectrophotometer 8453. Mass analyses were performed by Thermo-TSQ ACCESS (ThermoFisher).

Synthesis of AOZ Derivative (4-CPAOZ). 3-[[4-Carboxyphenyl]-methylene]amino-2-oxazolidinone (4-CPAOZ) was synthesized through the reaction of AOZ hapten and 4- carboxybenzaldehyde. A mixture of 1.02 g (10 mmol) of AOZ in 10 mL of water and 3.0 g (20 mmol) of 4-carboxybenzaldehyde in 3 mL of DMF was stirred at room temperature (25 °C) for 2 h and then filtered. The pale yellow solid layer was washed with water three times, dried under a flow of nitrogen at 45 °C, and then was characterized by infrared absorbance spectrum and HPLC-MS/MS analysis.

Preparation of 4-CPAOZ Conjugates. 4-CPAOZ was covalently attached to BSA to be used as immunogen or OVA to be used as coating antigen by the active ester method (*10, 13*). 4-CPAOZ was reacted with NHS to obtain active esters, and isolated esters were then reacted with proteins. In this procedure, 10.6 mg (100 mmol) of NHS and 20.6 mg (100 mmol) of DCC were added sequentially to a solution of 23.4 mg (100 mmol) of 4-CPAOZ dissolved in 2 mL of DMF at 4 °C in the dark. After the mixture had been stirred for 16 h, the activated hapten was centrifuged for 10 min (5000 rpm); 1.5 mL of the supernatant was added dropwise under stirring to 170 mg (2.5 mmol) of BSA dissolved in the solution of 10 mL of PBS (0.1 M, pH 8.0) and 1 mL of DMF. The conjugation mixture was then stirred at 4 °C for 5 h and centrifuged for 10 min (5000 rpm). The supernatant mixture was purified by exhaustive dialysis against PBS (0.1 M, pH 7.4) for 6 days and stored at −20 °C until use. The coating antigen conjugate was prepared by conjugation of the carboxylic derivative 4-CPAOZ with OVA as described above. The UV absorbance method was employed to determine whether the linking had been a success.

Production of Polyclonal Antibodies. Twelve female New Zealand white rabbits weighing 2−2.5 kg, Freund's adjuvant, and multiple-site injection method were used for obtaining polyclonal antibodies. Four schemes (three rabbits per scheme) used for immunizing are shown in **Table 1**. Scheme A employed 1 mg of immunogen per injection with booster injections every 2 weeks. Scheme B employed 1 mg of immunogen with booster injections every 4 weeks. Scheme C employed 0.5 mg of immunogen with booster injections every 2 weeks. Scheme D employed 0.5 mg of immunogen with booster injections every 4 weeks. The antisera were purified by ammonium sulfate precipitation and tested by checkerboard (*14, 15*).

Indirect Competitive ELISA Procedure. The protocol for ic-ELISA was similar to that described previously (*14*). In general, 96-well immunoplates were coated with 100 μL of 4-CPAOZ-OVA conjugate (200 μg/L) in carbonate buffer (0.1 M, pH 9.6) overnight at 4 °C. The plates were washed three times with PBST and treated with 200 μL of 2% OVA in PBS for 1 h at room temperature. After 1 h of incubation, the plates were washed three times with PBST, and 50 μL of the antibody against PAOZ (1:250000 dilution in PBS) and 50 μL of various concentrations of standard PAOZ or the samples were added to each well for 2 h at 37 °C. After 2 h of incubation, the plates were

Table 1. Comparison of Immunization Schemes and Titer and IC_{50} Results of Polyclonal Antisera Produced with 4-CPAOZ-BSA Immunogen

antibody	scheme	immunogen dose (mg)	interval (weeks)	titer	IC_{50}(μg/L) for AOZ as PAOZ
Ab-A1	A	1	2	320000	5.81[a]
Ab-A2	A	1	2	320000	10.45
Ab-A2	A	1	2	160000	23.72
Ab-B1	B	1	4	640000	0.91[a]
Ab-B2	B	1	4	320000	4.63
Ab-B3	B	1	4	320000	9.37
Ab-C1	C	0.5	2	160000	11.56[a]
Ab-C2	C	0.5	2	no response	no response
Ab-C3	C	0.5	2	16000	15.83
Ab-D1	D	0.5	4	320000	3.14[a]
Ab-D2	D	0.5	4	320000	7.45
Ab-D3	D	0.5	4	no response	no response

[a] Standard curves for the most sensitive antibody in each scheme are illustrated in **Figure 5**.

washed three times with PBST and then reacted with 100 μL of HRP-labeled antirabbit IgG (1:10000 dilution in PBS) for 1 h at 37 °C. The plates were washed four times with PBST, and then 100 μL of TMB substrate solution was added to each well and incubated for 30 min at room temperature in the dark, followed by the addition of stopping solution (2 M, H_2SO_4). The absorbance at 450 nm was measured by a microplate reader. A linear dose–response standard curve was prepared by plotting log[PAOZ] versus percent binding.

Determination of Cross-Reactivities. Several nitrofuran analogues, NPAOZ, derivatizing agent benzaldehyde, and other antimicrobials were tested for cross-reactivities using the ic-ELISA procedure described above. The cross-reactivity values were calculated as follows: (IC_{50} of PAOZ/IC_{50} of competitor) × 100. All sensitivity and specificity data in this paper were calculated on the basis of underivatized AOZ (M_r 102), a more conventional expression than the derivatized form PAOZ (M_r 189).

Sample Preparation Procedure. One gram of the chopped samples (swine liver, swine muscle, chicken liver, chicken muscle, and fish) was weighed into glass centrifuge tubes. Fortification samples were prepared at this point by adding various concentrations of AOZ standard solution to the control tissues. Five milliliters of water, 0.5 mL of hydrochloric (1 M), and 0.1 mL of benzaldehyde in methanol solution (0.01 M) were added to each tube. Samples were shaken for 5 min and incubated for 16 h at 37 ± 2 °C. After their cooling, 5 mL of K_2HPO_4 (0.1 M) and 0.2 mL of $HClO_4$ were added to each tube, and samples were shaken violently for 5 min. The samples were centrifuged for 10 min at 5000 rpm, and the supernatant was transferred to a clean tube. The samples were adjusted to pH 7.0 with 5 M NaOH. Oasis MAX cartridges were placed on a vacuum manifold, and the cartridges were conditioned (3 mL of methanol followed by 3 mL of water). The supernatants were passed through the MAX cartridges, and the cartridges were washed with 3 mL of 2% ammonia (in water) followed by elution with methanol (3 mL). The eluates were collected, vortexed for 10 s, and evaporated to dryness on a heating block at 40 °C under nitrogen. They were redissolved in 1 mL of PBS and used for ELISA detection.

Validation. Immunoassay validation was carried out using limit of detection (LOD), the recovery (percent) of the fortified AOZ, and coefficients of variation (CV). The standard PAOZ solution was diluted in PBS to obtain five-point standard curves (0.1, 0.4, 1.6, 6.4, and 25.6 μg/L for AOZ). The determination of LOD was based on 20 blank samples accepting no false-positive rates (average + 3 SD) (*11, 12*). Twenty different swine, chicken, and fish samples were purchased in retail outlets in Wuhan, which have previously been proved to be free of AOZ using HPLC determinations of NPAOZ derivatives at the National Reference Laboratory of Veterinary Drug Residues (Wuhan, China). The recovery (percent) was determined by the analysis of the above samples fortified with AOZ at the levels of 0.4, 1, and 5 μg/kg and calculated as follows: (concentration measured/concentration fortified) × 100. The precision of the ELISA was analyzed by repeated determination of the samples' intra-assay and interassay CVs at the levels of 0.4, 1, and 5 μg/kg for 5 days.

Comparison of ELISA and Reference Method. To compare the ELISA and HPLC methods for AOZ analysis, an animal-feeding experiment was carried out. Twelve pigs were treated with feed containing 100 mg/kg furazolidone for a period of 7 days, followed by withdrawal periods of 0, 1, 2, or 4 weeks (three pigs per group). The same samples (liver and muscle) were subjected to this ELISA procedure and HPLC analysis according to the procedure of Conneely et al. (*16*), respectively. The HPLC system contained a model SCL-10ADvp controller unit, a model SIL-10ADvp autoinjector, and a model SPD-10Avp UV–visible detector. All separations were performed using a Zorbax XDB-C_{18} column (250 mm × 4.6 mm, 5 μm). The column temperature was maintained at 25 °C, and UV detection was at 275 nm. The HPLC mobile phase used for NPAOZ separation was acetonitrile/KH_2PO_4, 0.01 M, pH 7.4 (25:75), pumped at a flow rate of 0.8 mL/min.

RESULTS

Characterization of 4-CPAOZ and Conjugates. The identification of hapten AOZ derivative (4-CPAOZ) was performed by infrared spectrophotometer (Digilab FTS 3000) and LC-MS/MS (Thermo-TSQ ACCESS). The IR spectrum is shown in **Figure 2**. The broadband centered in the range of 2500−3000 cm^{-1} was caused by the presence of the OH. The band near 1703 and 1735 cm^{-1} is due to two CO double bonds, and a band near 1477−1602 cm^{-1} came from the carbon−carbon bonds in the aromatic ring. As shown in the full-scan mass spectra of 4-CPAOZ (**Figure 3**) [M + H] m/z 234.39 indicated the molecular weight of 4-CPAOZ. The infrared absorbance spectrum and mass analysis demonstrated 4-CPAOZ was synthesized successfully. The identification of 4-CPAOZ−protein (BSA or OVA) conjugates was carried out by UV spectrophotometer (Aglient 8453). The UV absorbance spectrum of 4-CPAOZ-BSA (λ_{max}, 292 nm) was different from the UV absorbance spectra of BSA (λ_{max}, 278 nm) and 4-CPAOZ (λ_{max}, 296 nm). **Figure 4** suggests that derivative 4-CPAOZ would be likely to conjugate to the protein carrier successfully.

Characterization of Polyclonal Antibodies. Comparison of immunization schemes and titer and IC_{50} values in the presence of PAOZ (IC_{50} calculated as AOZ) for the polyclonal antibodies produced with 4-CPAOZ-BSA is shown in **Table 1**. It is found in **Table 1** that increasing doses of 4-CPAOZ−BSA immunogen could promote the titer of antibodies and that lengthening the interval between booster immunizations could increase the sensitivity of the antibodies to PAOZ. PAOZ standard curves for the most sensitive antibody in four immunization schemes are illustrated in **Figure 5**. Our efforts were focused on ELISA based on the most sensitive antibody Ab-B1 in immunization scheme B. The specificity of antibody Ab-B1 was evaluated with PAOZ, NPAOZ, AOZ, benzaldehyde, furazolidone, nitrofurazone, furaltadone, nitrofurantoin, and various other veterinary drugs (**Table 2**). Antibody Ab-B1 showed 5.73% cross-reactivity (CR) with NPAOZ and 6.15% CR with furazolidone, the parent nitrofuran, which exhibited negligible cross-reactivity with derivatizing agents benzaldehyde (<0.01%), AOZ (<0.01%), and various other veterinary drugs (<0.01%). The results presented in **Table 2** and **Figure 5** demonstrate that antibody Ab-B1 was highly specific and sufficiently sensitive to PAOZ for practical detection of AOZ.

Optimum ELISA Conditions. The selection of the optimum ELISA conditions was performed by ic-ELISA as described above. In this section, the antibody Ab-B1 and coating antigen (4-CPAOZ-OVA) optimum working concentration were studied (*15, 17*). The data presented in **Figures 6** and **7** demonstrate that the coating antigen concentration of 200 g/L

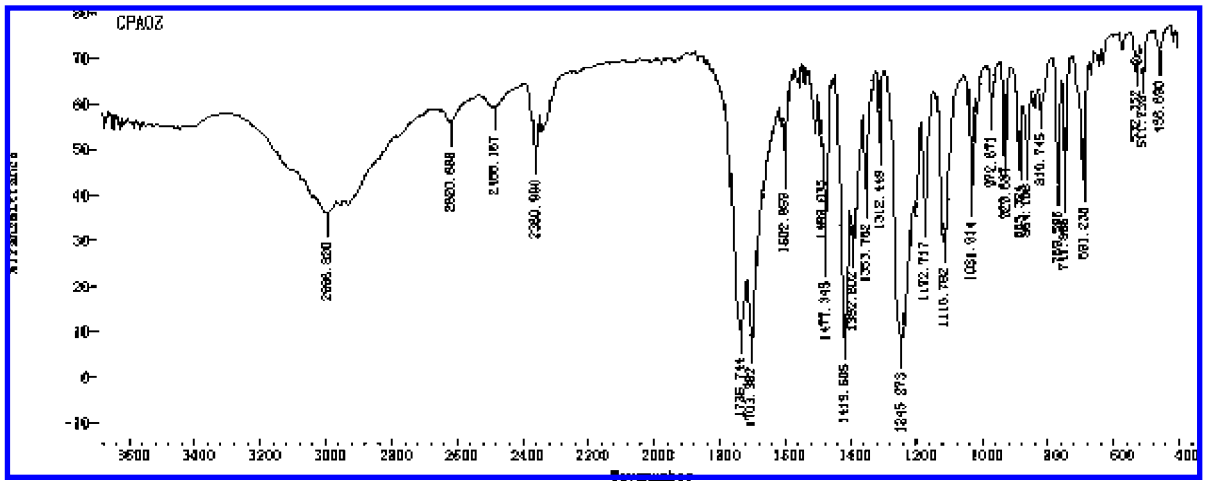

Figure 2. Infrared absorbance spectrum of 4-CPAOZ in KBr pellet.

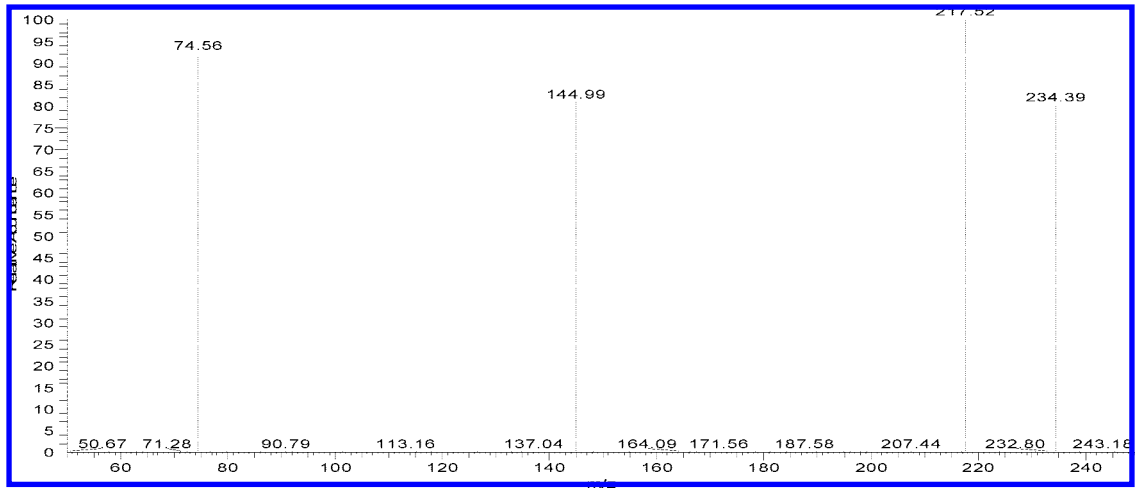

Figure 3. Full-scan mass spectrum of 4-CPAOZ. 4-CPAOZ was dissolved in methanol and injected into the MS system. Following the result, the four key fragment peaks (*m*/*z*) were 234.39, 217.52, 144.99, and 74.56. [M + H] *m*/*z* 234.39 was the molecular ion peak.

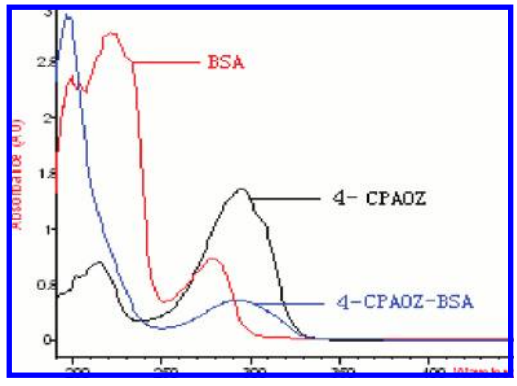

Figure 4. Ultraviolet absorbance spectra of BSA, 4-CPAOZ, and 4-CPAOZ-BSA. BSA, 4-CPAOZ, and 4-CPAOZ-BSA were dissolved in water. Following the result, the wavelengths of maximum absorbance of BSA, 4-CPAOZ, and 4-CPAOZ-BSA were 278, 296, and 292 nm, respectively.

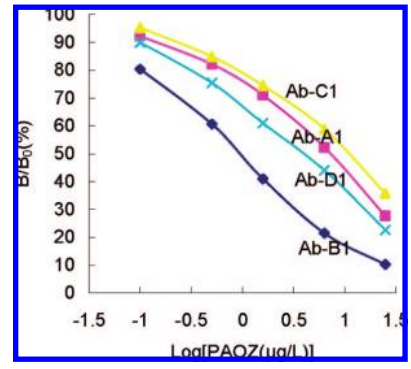

Figure 5. PAOZ standard curves based on PBS solution for the most sensitive antibody in four immunization schemes. B/B_0 is the normalized response relative to the zero standard. Log[PAOZ] is the logarithm concentration of PAOZ. The regression curve equations of the antibodies Ab-A1, Ab-B1, Ab-C1, and Ab-D1 were obtained as follows: $y = -26.853x + 70.92$ ($r = 0.98$), $y = -30.367x + 49.401$ ($r = 0.99$), $y = -24.496x + 75.213$ ($r = 0.98$), and $y = -28.059x + 64.775$ ($r = 0.99$), respectively.

and an antibody Ab-B1 dilution of 1:250000 were needed to get to the linear portion of the response curve. These conditions were fixed for the rest of the experiment.

Validation. On the basis of the determination of 20 different blank samples, the LODs in swine liver, swine muscle, chicken liver, chicken muscle, and fish were 0.19 ± (3 × 0.06), 0.17 ± (3 × 0.07), 0.24 ± (3 × 0.05), 0.15 ± (3 × 0.07), and 0.18 ± (3 × 0.05) μg/kg [mean ± (3 × SD)], respectively. They were all <0.4 μg/kg. The results of recoveries and interassay variability coefficients of the above samples fortified with AOZ at the levels of 0.4, 1, and 5 μg/kg are presented in **Table 3**. The test was repeated five times with three replicates per concentration. **Table 3** shows that the recoveries of AOZ were in the range of 55.8−96.6% at three levels in the above samples,

Table 2. Cross-Reactivities of the Most Sensitive Antibody Ab-B1 with Various Drugs

competitor	IC_{50} (μg/L)	cross-reactivity[a] (%)
PAOZ	1.05	100
NPAOZ	18.3	5.73
furazolidone	17.1	6.15
AOZ	>100000	<0.01
benzaldehyde	>100000	<0.01
nitrofurantoin, nitrofurazone, furaltadone	>30000	<0.01
tetracycline, oxytetracycline, ampicillin, enrofloxacin, sulphamethazine, metronidazole, streptomycin	>100000	<0.01

[a] Cross-reactivity (%) = (IC_{50} of PAOZ/IC_{50} of competitor) × 100.

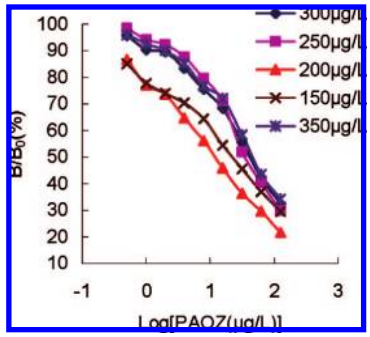

Figure 6. Influence of various concentrations of coating antigen (150, 200, 250, 300, and 350 μg/L) on competition reaction. B/B_0 is the normalized response relative to the zero standard. Log[PAOZ] is the logarithm concentration of PAOZ.

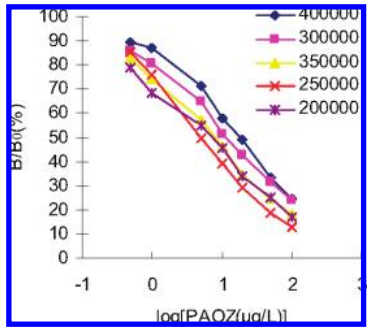

Figure 7. Influence of various dilutions of antibody Ab-B1 (200000-, 250000-, 300000-, 350000-, and 400000-fold in PBS) on competition reaction. B/B_0 is the normalized response relative to the zero standard. Log[PAOZ] is the logarithm concentration of PAOZ.

whereas those of the interassay variability were <20%.

Comparison of the ELISA and HPLC Analyst. Figures 8 and **9** demonstrate the performance of the ELISA in comparison with the confirmatory HPLC method for the determination of AOZ in swine liver and muscle, respectively. All of the samples (liver and muscle) were obtained from 12 pigs treated with feed containing 100 mg/kg furazolidone for a period of 7 days, followed by withdrawal periods of 0, 1, 2, or 4 weeks. **Figure 8** illustrates that the correlation coefficient for both methods was 0.98 (r) in the determination of swine liver, with a regression coefficient of 1.05. **Figure 9** illustrates that the correlation coefficient for both methods was 0.99 (r) in the determination of swine muscle, with a regression coefficient of 1.03. In addition, three control pigs were subjected to this ELISA procedure, with the results of 0.11, 0.22, and 0.21 μg/kg in swine muscles and 0.1, 0.21, and 0.15 μg/kg in swine livers, respectively, which were <0.4 μg/kg of the LOD. The results imply good agreement between AOZ concentrations determined by two methods and prove the reliability of the ELISA for the determination of real samples.

DISCUSSION

The synthesis of haptens was the key step in a procedure of research on rapid immunoassay. In a previous study, attempts to prepare functional antibodies against furazolidone metabolite AOZ were not successful (*12*). In our work, we designed an immunizing hapten to prepare functional antibodies against the free AOZ. However, it was not successful (data not shown). The reason for the lack of an assay sensitive to free AOZ could be the light molecular weight and low chemical stability of the free AOZ during the conjugation procedure. Thus, the derivatization of tissue homogenates was a necessary step for the determination of AOZ in food animal tissues.

The first AOZ hapten structure enlarged with 3-carboxybenzaldehyde has been reported (*11*). Some polyclonal and monoclonal antibodies specific for NPAOZ, the nitrophenyl derivative of AOZ, were produced. ELISA for the detection of AOZ residue following derivatization with 2-nitrobenzaldehyde has been developed. However, the existing ELISA method showed a high CV (38.5%) and was needed to prepare matrix-matched standards from blank samples prior to analysis (*12*). The reason for these could be incomplete derivatization reaction between 2-nitrobenzaldehyde and AOZ. Thus, in this work an effort was made to seek a new derivatizing agent. A different immunizing hapten was designed in this work. An AOZ hapten structure enlarged with 4-carboxybenzaldehyde was reacted with carrier proteins and was applied to experimental animals. It was found that the antibodies produced with 4-CPAOZ−BSA conjugates exhibited high affinity toward PAOZ, the phenyl derivative of AOZ (**Table 1**), and were more sensitive and specific for PAOZ (IC_{50} = 1.05 μg/L) than NPAOZ (IC_{50} = 18.3 μg/L). Furthermore, negligible cross-reactivity with derivatizing agent benzaldehyde (<0.01%) was obtained (**Table 2**), and the results of recoveries of fortification (**Table 3**) demonstrated that the free AOZ bound covalently to benzaldehyde in vitro easily. In addition, benzaldehyde is inexpensive, less toxic, and obtained easily in comparison with 2-nitrobenzaldehyde. Therefore, the development of an ELISA method for the detection of tissue-bound AOZ residues following derivatization with benzaldehyde became possible. A low CV (<20%) was obtained in **Table 3**. The existing ELISA showed a high CV (38.5%) (*12*). This indicated that the new technique is more reliable than the existing one.

Four schemes for immunizing were performed to see the effects of immunogen dose (0.5 and 1 mg) and interval (2 and 4 weeks) on immunization. The titer of antibodies produced by the high level of immunogen dose (1 mg) was higher than that by 0.5 mg at the same immunization interval. However, the sensitivity of antibodies obtained from the rabbits with a 4 week immunization interval was higher than that with a 2 week immunization interval at the same level of immunogen dose (**Table 1**). Thus, increasing the dose of immunogen could promote the titer of antibodies, and lengthening the interval between booster immunizations could stimulate production of higher affinity antibodies.

The validation of the ELISA method could be characterized by the LOD, the recovery (percent) of the fortified tissues, and CVs. The LOD was defined as the means observed concentration plus three standard derivations (mean + 3 SD) based on 20 blank samples, accepting no false-positive rates (*11, 12*). As Diblikova et al. reported, the assay LOD for AOZ was 0.185 ± 0.045 μg/kg in shrimp based on 20 blank samples, accepting

Table 3. Recoveries and Coefficients of the Samples Fortified with AOZ in Tissues ($n = 15$)[a]

	mean recoveries ± SD[b] (%)			interassay variability coefficient (%)		
sample	fortified, 0.4 μg/kg	fortified, 1 μg/kg	fortified, 5 μg/kg	fortified, 0.4 μg/kg	fortified, 1 μg/kg	fortified, 5 μg/kg
swine liver	96.6 ± 18.4	79.9 ± 14.3	64.1 ± 11.9	19.1	17.9	18.6
swine muscle	93.8 ± 12.4	79.6 ± 14.8	57.4 ± 6.6	13.2	18.6	11.5
chicken liver	92.1 ± 10.9	76.0 ± 13.1	56.5 ± 8.2	11.9	17.2	14.5
chicken muscle	91.1 ± 10.9	70.6 ± 12.3	55.8 ± 9.1	12.1	17.4	16.2
fish muscle	93.1 ± 10.8	72.5 ± 11.7	59.0 ± 9.6	11.7	16.1	16.2

[a] The test was repeated five times with three replicates per concentration. [b] Standard deviation.

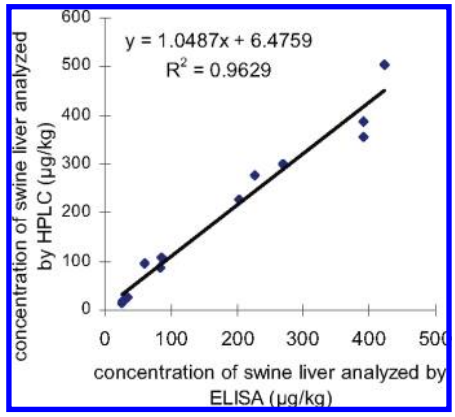

Figure 8. Correlation of AOZ assay between the confirmatory HPLC and the ELISA method in swine liver samples obtained from pigs treated with feed containing 100 mg/kg furazolidone. The regression curve equation was obtained as follows: $y = 1.0487x + 6.4759$, with a correlation coefficient of 0.98.

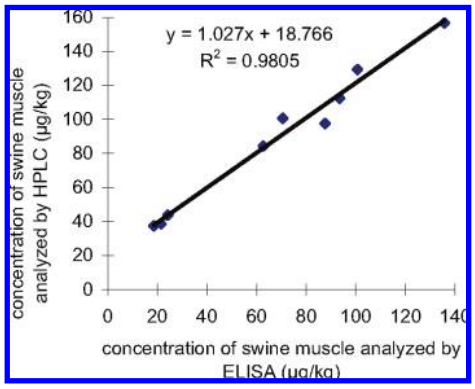

Figure 9. Correlation of AOZ assay between the confirmatory HPLC and the ELISA method in swine muscle samples obtained from pigs treated with feed containing 100 mg/kg furazolidone. The regression curve equation was obtained as follows: $y = 1.027x + 18.766$, with a correlation coefficient of 0.99.

no false-positive rates; the lowest mean recovery (51.1%) was found in pork sample, and the highest CV (38.5%) was found in beef samples (*12*). In our work, the LODs in swine liver, swine muscle, chicken liver, chicken muscle, and fish were 0.19 ± (3 × 0.06), 0.17 ± (3 × 0.07), 0.24 ± (3 × 0.05), 0.15 ± (3 × 0.07), and 0.18 ± (3 × 0.05) μg/kg [mean ± (3 SD)], respectively, and the recoveries of AOZ ranged from 55.8 to 96.6% at the levels of 0.4, 1, and 5 μg/kg in the above samples with low interassay variability (<20%). These results indicate that the detection capability of our ELISA method was similar to that of Diblikova et al.'s (*12*), and the precision of our ELISA method was superior to that described previously (*11*).

In a previous study, a matrix-matched standard curve was usually used to reduce potential matrix effects in the analytical procedure. The standard curve of the ELISA method developed by Diblikova relied on matrix-matched standards for the analysis of AOZ residues. Although good detection capability (0.4 μg/kg in tissues) was obtained, the preparation of matrix-matched standards from blank samples prior to analysis might be less favorable (*12*). The incorporation of the ELISA kits was not suitable because a blank matrix solution must be provided for the matrix-matched standard curve in the ELISA kit when various tissues are determined, such as liver, muscle, fish, and shrimp. For convenient incorporation into the ELISA kits, the standard curve based on PBS solution was performed in this work. Upon use of the PBS calibration standard curve, the good precision and accuracy of our ELISA method were observed (**Table 3**). In this work, we established a standard curve relying on a PBS solution rather than a matrix-matched solution. Thus, a commercially available kit is properly incorporated. However, the Oasis MAX cartridges were used to minimize matrix effects in our study. It was not fit for rapid screening and high throughput. Thus, the sample preparation procedure of our ELISA method would be simple and precise. There was now an urgent need for a new technology to minimize matrix effects. However, use of a matrix-matched standard curve to reduce potential matrix effects in the analytical procedure was less feasible.

The experiment of pigs treated with furazolidone at 100 mg/kg for a period of 7 days was carried out to compare the ELISA with the HPLC method and to assess the capability of determination of the real samples of the ELISA method. **Figures 8** and **9** indicate that an excellent correlation between the ELISA and the reference method was found in the determination of swine liver ($r = 0.98$) and swine muscle ($r = 0.99$). The results showed that this ELISA method was reliable for the determination of AOZ residue in real edible tissues.

This paper describes an alternative ELISA analytical technology for the determination of tissue-bound furazolidone metabolite, 3-amino-2-oxazolidone (AOZ), and presents an effective and less toxic derivatizing agent, benzaldehyde, which is a substitute for the traditional 2-nitrobenzaldehyde. The LOD of this ELISA method is <0.4 μg/kg in various tissues based on 20 blank samples, accepting no false-positive rates, which comfortably satisfies a minimum required performace limit (MRPL) for tissue-bound residues of AOZ at 1 μg/kg in the European Communities (*18*). The recoveries and coefficient of variation of AOZ from spiked tissues were also within acceptable scale. The presented ELISA method is similar to that of Diblikova et al., and the precision of our ELISA method was superior to that described previously (*12*). In addition, comparison between the ELISA and HPLC methods results in a good correlation. In conclusion, a new ELISA method for the detection of tissue-bound AOZ residues following derivatization with benzaldehyde is established, which has a potential for the development of a rapid test kit. This technology would provide an alternative method for the detection of AOZ residues.

ABBREVIATIONS USED

AOZ, 3-amino-2-oxazolidinone; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CPAOZ, 3-{[(3-carboxyphenyl)methylene]amino-2-oxazolidinone; 4-CPAOZ, 3-{[(4-carboxyphenyl)methylene]amino-2-oxazolidinone; DCC, *N*-dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; ic-ELISA, indirect competitive enzyme-linked immunosorbent assay; IFA, incomplete Freund's adjuvant; OVA, ovalbumin; NHS, *N*-hydroxysuccinimide; NPAOZ, 3-{[(2-nitrophenyl)methylene]-amino}-2-oxazolidinone; PAOZ, *N*-(phenylidene)-3-amino-2-oxazolidinone; TMB, 3,3′,5,5′-tetramethylbenzidine.

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Received for review September 9, 2007. Revised manuscript received January 9, 2008. Accepted January 14, 2008. We acknowledge the financial support of the Ministry of Science and Technology of the People's Republic of China (2001BA804A18-01), of which this work is a part.

JF0726684