JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Development and Validation of an Indirect Competitive Enzyme-Linked Immunosorbent Assay for the Screening of Tylosin and Tilmicosin in Muscle, Liver, Milk, Honey and Eggs

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ABSTRACT: Incorrect use of tylosin and tilmicosin could result in allergy and select resistance. To monitor the illegal use of these antibiotics in animals, a monoclonal-based indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) has been established. Several haptens were synthesized and conjugated to carrier protein. Female Balb/c mice were inoculated with the four different conjugates to produce monoclonal antibodies according to the schemes of immunization. Aftercell fusion and culture several times, nine hybridoma cell lines were isolated. Only one, 3C4 that has isotype IgG2a, was selected for detailed study. The cross-reactivity of the monoclonal antibody 3C4 to tylosin and tilmicosin was 100% and 51% respectively. The standard curves based on the tylosin and tilmicosin matrix calibration ranged from 2.5 to 40 μg L^{-1}, with an IC_{50} value of 6.1 μg L^{-1} and 12.1 μg L^{-1}, respectively. The limits of detection of the ic-ELISA ranged from 5.1 μg kg^{-1} to 13.8 μg kg^{-1} in edible animal tissues. The recoveries were 74.1% to 120.7% with less than 18.6% of the coefficient of variation when tylosin and tilmicosin were spiked in various biological matrices with the concentrations of 25.0−200.0 μg kg^{-1}. Good correlations between the results of the ic-ELISA and high performance liquid chromatography were observed in the incurred tissues. These results suggest that the ic-ELISA is a sensitive, accurate and low-cost method that would be a useful tool for the screening of the residues of tylosin and tilmicosin in muscle, liver, milk, honey and eggs.

KEYWORDS: *tylosin, tilmicosin, monoclonal antibody, indirect competitive enzyme-linked immunosorbent assay, muscle, liver, honey, egg, milk*

INTRODUCTION

Tylosin (Figure 1) is a macrolide antibiotic used exclusively in veterinary medicine, since it is highly active against most Gram-positive and -negative bacteria as well as mycoplasma and Chlamydia.[1] Tilmicosin (Figure 1) is a semisynthetic derivative of tylosin, which has an antibacterial spectrum similar to that of the parent drug and, furthermore, enhances activity against *Pasteurella multocida* and *Actinobacillus pleuropneumoniae*.[2,3] They are widely used in animal production for the treatment and prevention of respiratory and enteric infections of cattle, sheep, swine and poultry.[1] Incorrect use of these antibiotics could result in their residues in edible tissue of animals that would cause allergy and select resistance. Therefore, tylosin and tilmicosin have been banned as feed additives in the European Union since 1999.[1] Within China, maximum residue limits (MRLs) have been established.

To ensure that macrolides are used only in approved situations and to control their use in food-producing animals, it is very important that targeted samples are taken at the slaughterhouse and screened for the presence of residues. Many methods have been developed to monitor tylosin and tilmicosin residues in living animals and animal products, such as microbiological assay,[4] high performance liquid chromatography (HPLC),[5−8] and high performance liquid chromatography coupled with mass spectrometry (HPLC−MS/MS).[9−14] However, the microbiological assay is lengthy and not sufficiently specific for analytical purposes. Most of the chromatographic methods are expensive, time-consuming, and unsuitable for analysis of many samples. Therefore, a simple, quick and low cost screening method is required for routine determinations of tylosin and tilmicosin in edible tissues of animals. Indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) is the most popular method for detection of drugs in animal tissues due to its high sensitivity, low cost, and ability to screen large numbers of samples.

During the past decade years, some attempts have been made to prepare antibodies against tylosin and tilmicosin. However, some of these antibodies were used to detect metabolites in fermentation and to search for new therapeutic agents;[15] some were used to localize tilmicosin in porcine lung and to determine tilmicosin in avian respiratory tissue;[16,17] some were used to investigate potential tissue binding sites of tilmicosin;[18] others were used for detection of tylosin in feedstuff.[19] In recent years, many test kits have existed on the market to measure and determine tylosin in animal tissues. Nevertheless, there is not much information available about ic-ELISA techniques that could be useful at the same time for determination of tylosin and tilmicosin in different animal species and in a wide range of tissues. Clearly, the need for

Received: September 15, 2011
Revised: December 2, 2011
Accepted: December 2, 2011
Published: December 3, 2011

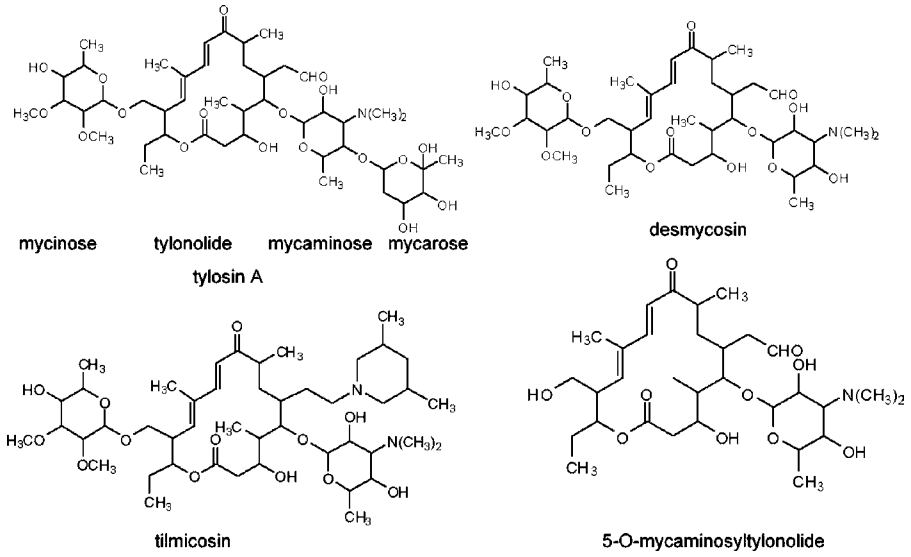

Figure 1. Chemical structure of tylosin A, desmycosin (DES), tilmicosin, and 5-*O*-mycaminosylonolide (OMT).

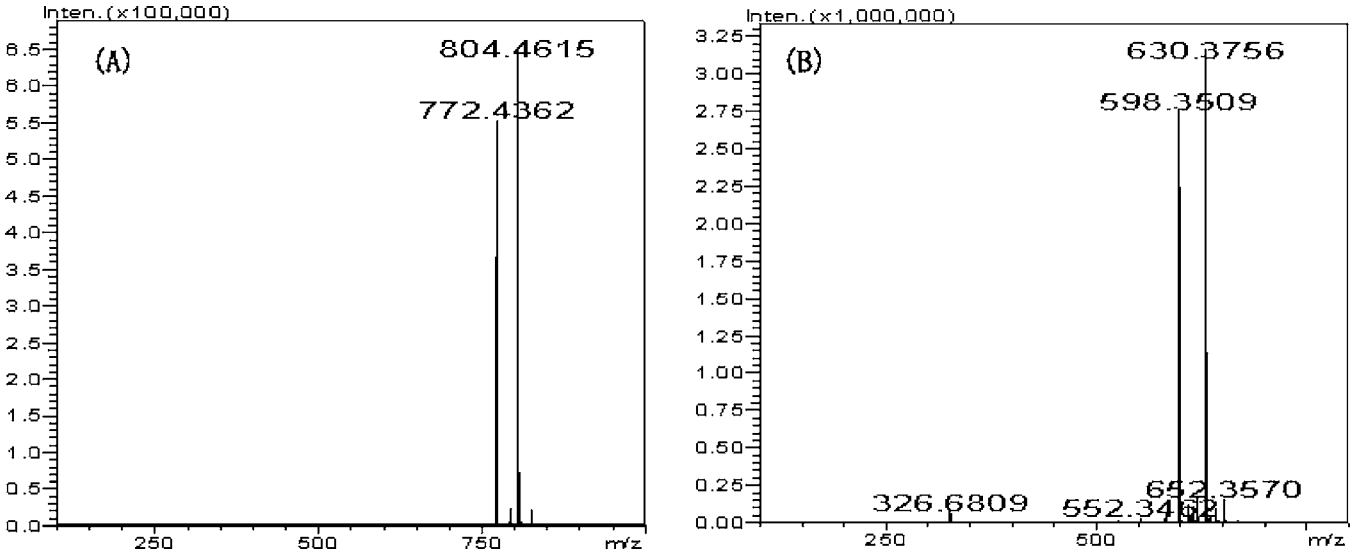

Figure 2. Full scan mass spectra of haptens: (A) DES, [M + H] + *m*/*z* 772.4362; (B) OMT, [M + H] + *m*/*z* 598.3509.

simple and widely applicable ELISA methods for the detection of macrolides residues persists.

In the present study, haptens with differences in the type and length of the spacer arm were synthesized in order to obtain high-affinity monoclonal antibodies (mAbs) to tilmicosin and tylosin. The most sensitive hapten and mAbs were applied to develop a high sensitivity and low cost ELISA for simultaneous determination of tylosin and tilmicosin in various biological matrices (muscle, liver, milk, honey and eggs) without complicated sample preparation and cleanup. The correlation between the ELISA and HPLC analysis on detecting tilmicosin and tylosin in edible tissues of animals was also investigated in this study.

MATERIALS AND METHODS

Materials. Tylosin tartrate was purchased from Xi'an Hengtong Pharmaceutical Co., Ltd. (Xi'an, China). Adipic dihydrazide (ADH), *O*-carboxymethoxylamine hemihydrochloride (CMO), morpholinoethyl sulfonic acid, urea hydrogen peroxide, hypoxanthine-thymidine, RPMI-1640, hypoxanthine-thymidine-aminopterin, dimethylsulfoxide, *N*-hydroxysuccinimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, dicyclohexylcarbodiimide, bovine serum albumin (BSA), ovalbumin (OVA), Freund's adjuvant (complete and incomplete), goat anti-mouse IgG horseradish peroxidase conjugate (HRP-IgG) and polyethylene glycol 1500 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were all of analytical grade.

Synthesis of Haptens and Antigens. The haptens desmycosin (DES) (Figure 1) and 5-*O*-mycaminosyltylonolide (OMT) (Figure 1) with different spacer structures were designed and synthesized. DES and OMT were conjugated to BSA to prepare the immunizing conjugates via linkers ADH and CMO. They were also conjugated to OVA via the same linkers to prepare the coating conjugates.

DES was synthesized based on a modified method according to Hamill et al.[20] Briefly, 5 g of tylosin tartrate was dissolved in 200 mL of 0.005 mol L^{-1} H_2SO_4 (pH 2.0) and incubated at 80 °C for 4 h under stirring. After extraction with 200 mL of dichloromethane the aqueous phase was collected and adjusted to pH 8.8 using 1.0 mol L^{-1} NaOH. The alkaline mixture was extracted with 60 mL of dichloromethane. Then the dichloromethane extract was washed twice with 100 mL of

dx.doi.org/10.1021/jf2037449 | J. Agric.Food Chem. 2012, 60, 44–51

distilled water and evaporated to dryness in vacuo. The residue was purified by column chromatography (dichloromethane:methanol =10:1 (v/v)) and confirmed by mass spectra (Figure 2), for which the mass analyses were performed by LC/MS-IT-TOF (Shimadzu Corp., Kyoto, Japan). The mass spectrometer was equipped with an electrospray ionization source and operated in positive ionization mode. Mass spectroscopic analyses were carried out under full-scan mass spectrometer with a mass range of 100−800 Da. Nitrogen was used as the nebulizer gas at a flow rate of 1.5 L min^{-1}. The capillary and skimmer voltages were set at 4.5 and 1.6 kV, respectively. The curved desolvation line and heat block temperature were both set at 200 °C. OMT was synthesized and characterized essentially as described for DES, except that the pH of H_2SO_4 was 1.3 and the incubation conditions were 60 °C for 60 h.

DES and OMT were conjugated to BSA to prepare the immunizing conjugates and to OVA for preparation of the coating conjugates via linkers CMO according to the modifyed procedure of Kolosova et al.[21] The procedure for DES and OMT was the same. Briefly, 154 mg of DES or OMT, 21.9 mg of CMO and 8.4 mg of $NaHCO_3$ were dissolved in 8 mL of 50% (v/v) methanol solution and incubated 2.5 h at room temperature. The mixture was evaporated, and the residue was dissolved in 4 mL of dichloromethane and 2 mL of ethanol. Five grams of sodium sulfate anhydrous was added for dehydration. The liquid layer was separated and evaporated to dryness in vacuo to obtain the product DES−CMO or OMT−CMO. 44.2 mg of DES−CMO or OMT−CMO, 6.9 mg of *N*-hydroxysuccinimide and 12.4 mg of dicyclohexylcarbodiimide were dissolved in 3 mL of *N*,*N*-dimethylformamide, and the reaction mixture was incubated overnight at room temperature. 110 mg of BSA or 90 mg of OVA was dissolved in 17 mL of 0.1 mol L^{-1} phosphate buffered saline (PBS) (pH 8.0). The solution of the active ester was slowly added under stirring to the protein solution. The reaction mixture was kept at 4 °C overnight, and then centrifuged at 3,000 rpm for 10 min. The supernatant was purified by exhaustive dialysis against PBS and then stored at −20 °C.

DES and OMT were also conjugated to BSA or OVA via linkers ADH according to the modified procedure of Silverlight et al.[17] Briefly, 40 mg of BSA or OVA was dissolved in 2 mL of 0.1 mol L^{-1} morpholinoethyl sulfonic acid buffers (pH 4.7). 172 mg of ADH was dissolved in 6 mL of morpholinoethyl sulfonic acid buffer and added to the OVA together with 40 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The mixture was stirred for 1 h at room temperature and then was purified by exhaustive dialysis against PBS. Then, 8 mg of DES or OMT was added to 5 mL of ADH-derivatized OVA, and the mixture was incubated at 4 °C overnight and separated as described for the CMO linkers.

Verification of conjugate synthesis and estimation of the hapten/protein ratio were performed on an 8453 UV−visible spectrophotometer (Agilent, USA). The number of DES (OMT) residues conjugated to the carrier molecules was estimated according to the ultraviolet absorbance spectra of haptens, carrier proteins, and conjugates as follows: $[\varepsilon_{conjugation} - \varepsilon_{protein}]/\varepsilon_{hapten}$, where ε is the absorbance coefficient of analytes. The estimated incorporation rates are shown in Table 1. It can be primarily confirmed that the conjugates were successfully prepared.

Table 1. Ratio of Haptens and Carrier Proteins

hapten	conjugate	binding ratio to carrier protein
DES	DES−CMO−BSA	5.3
	DES−CMO−OVA	3.2
	DES−ADH−BSA	11.4
	DES−ADH−OVA	7.1
OMT	OMT−CMO−BSA	7.9
	OMT−CMO−OVA	4.6
	OMT−ADH−BSA	7.5
	OMT−ADH−OVA	5.9

Preparation of Monoclonal Antibodies. Female Balb/c mice (6−8 weeks old), which had been purchased from Hubei Center for Disease Control and Prevention (Wuhan, China), were inoculated with the immunizing conjugates (DES−ADH−BSA, DES−CMO−BSA, OMT−ADH−BSA and OMT−CMO−BSA). The immunization schemes are shown in Table 2. First, the immunogens were prepared for injection by emulsification of the conjugates in 500 μL of sterile isotonic saline and 500 μL of Freund's adjuvant. This cocktail was mixed vigorously until a homogeneous suspension was obtained. Complete adjuvant was used for the first injection, and incomplete adjuvant was used for the subsequent injections. The immunogen emulsion was injected subcutaneously into multiple sites on the back of each mouse. Blood was collected, and titers of antisera were determined by indirect ELISA. The mice giving the best dose−response curve were selected for fusion.

Spleen cells of the immunized mice were fused with myeloma cells Sp2/0 at a ratio of 10:1 according to the standard procedure.[22] Hybridoma from wells having a positive response in the ELISA described below were cloned twice by limiting dilution and expanded to guarantee its monoclonal origin. After cell culture, the cultured hybridoma was intraperitoneally injected into mice to produce ascites. The class and subclass of the isotypes of the secreted antibody were determined by using a mouse monoclonal antibody isotyping kit (Proteintech Group, Inc., Chicago, IL, USA).

The MAbs raised against each of the immunizing conjugates were screened against each of the corresponding coating antigens in a checkerboard for the best dilution of the coating conjugate and antiserum. The sensitivity of each mAb was primarily assessed by the B/B_0 values of 500 μg L^{-1} analyte (tylosin, tilmicosin, DES and OMT) which was measured by the ic-ELISA method. The extent of cross-reactivity (CR) was assessed by determining the IC_{50} values in the ic-ELISA. Several macrolide compounds, such as roxithromycin, tylosin, tilmicosin, erythromycin, kitasamycin, spiramycin, azithromycin, josamycin, oleandomycin, avermectin and ivermectin, which were purchased from China Institute of Veterinary Drug Control (Beijing, China), were selected to test for CR. The concentrations of the standard solutions of the compounds covered the range from 0.01 to 100,000 μg L^{-1}. The CR values were calculated as follows: CR = (IC_{50} of tylosin/IC_{50} of competitor) × 100%. The detailed procedure of ELISA is described in the section Indirect ELISA and ic-ELISA. The one with the lowest IC_{50} was selected for the rest of this study.

Indirect ELISA and ic-ELISA. The protocol used for indirect ELISA was similar to that described previously by Kim et al.[23] Briefly, 96-well Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with coating conjugates in 100 μL of coating buffer (0.05 mol L^{-1} carbonate buffers, pH 9.6) overnight at 4 °C. The plates were washed three times with PBS containing 0.1% Tween-20 (PBST) and were incubated with 200 μL of 1% OVA in PBS at 37 °C for 0.5 h. The plates were then washed with PBST, followed by the addition of 100 μL of antiserum in each well. After 0.5 h of incubation at 37 °C, the plates were washed with PBST and then incubated with 100 μL of HRP-IgG (1:5,000) at 37 °C for 0.5 h. After washing the plate with PBST, 100 μL of substrate solution was added to each well. The samples were incubated for 15 min at room temperature in the dark, followed by the addition of the stop solution (2 M H_2SO_4). The absorbance at 450 nm was measured on a Tecan Sunrise 2.5 microplate reader (SUNRISE, Austria).

The protocol used for the ic-ELISA was similar to that described previously.[23] Briefly, 96-well Maxisorp microtiter plates were coated with 100 μL of coating conjugates. After washing and blocking, 50 μL of the antibody and 50 μL of varying concentrations of standard analyte or the samples were added to each well. The plates were then incubated, washed, and measured with a microplate reader as described above. A linear standard dose response curve was obtained by plotting log [analyte] versus percentage of binding.

Sample Preparation. Samples, such as porcine muscle, chicken, bovine muscle, ovine muscle, fish, porcine liver, ovine liver, chicken liver and honey, were minced and homogenized. Two grams of each homogenized sample was weighed into a 50 mL polypropylene centrifuge tube. Ten milliliters of 0.3% metaphosphoric acid solution containing 40% (v/v) methanol was added. The samples were mixed with a vortex mixer for 5 min and then centrifuged at 4,000 rpm for 5

Table 2. Comparison of Immunization Schemes, Titer of Antisera Produced with Different Immunogen

scheme	immunogen	dose (μg)	titer			
			1	2	3	4
I	DES−ADH−BSA	50	12800	25600	*a*	*a*
II		100	*a*	12800	12800	*a*
III	DES−CMO−BSA	50	32000	64000	32000	64000
IV		100	16000	16000	*b*	*a*
V	OMT−ADH−BSA	50	6400	6400	6400	12800
VI		100	*a*	6400	*a*	*b*
VII	OMT−CMO−BSA	50	*a*	*a*	>25600	*a*
VIII		100	*a*	*a*	*a*	>25600

[a]Used to prepare immunized spleen cells. [b]The mouse was dead.

Table 3. Titer and Sensitivity to DES, OMT, Tylosin and Tilmicosin of Each Monoclonal Antibody

	B/B_0 values								
	OMT−CMO−BSA			DES−ADH−BSA			DES−CMO−BSA		
hybridoma cells	5A8	1B6	2E12	5F11	2G11	3B10	4E12	3C4	4G11
titer (1:$X \times 10^3$)	4	16	32	16	16	8	1	128	16
analyte (500 μg L^{-1})									
tylosin	0.21	0.88	0.94	0.40	0.67	*a*	0.09	0.06	0.53
tilmicosin	0.12	0.81	0.71	0.11	0.12	*a*	0.66	0.09	0.78
DES	0.12	0.67	0.64	0.15	0.11	*a*	0.11	0.06	0.39
OMT	0.13	0.67	0.66	0.34	0.15	*a*	0.62	0.45	*a*

[a]Cannot be inhibited.

min. One milliliter of the supernatant extract was taken out and adjusted to pH 9.0 with 1 mL of Tris-NaCl buffer. After extraction with 5 mL of chloroform, the organic phase was separated and evaporated under nitrogen flow at 45 °C. The residue was reconstituted in 2 mL of pH 6.0 PBS, and fat was removed using hexane. The water solutions were analyzed by ELISA. Milk samples were prepared basically as described above, except that the volume of chloroform was 4.0 mL and the residue was reconstituted in 1.0 mL of PBS pH 6.0. Egg samples were prepared essentially as described for muscle samples, except that the volume of 0.3% metaphosphoric acid solution containing 40% (v/v) methanol was 18.0 mL.

Validation of the ic-ELISA. Validation of the ic-ELISA was performed according to the European Decision 2002/657/EC.[24] The standard solution (tylosin or tilmicosin) was diluted in PBS to obtain a five-point standard curve (2.5, 5.0, 10.0, 20.0, and 40.0 μg L^{-1}). Twenty different frozen tissue samples, which had previously been proven by HPLC to be free of tylosin and tilmicosin, were purchased from local retail outlets. Each tissue was assayed using ELISA to determine the limit of detection (LOD). The determination of LOD was based on 20 blank samples accepting no false positive rates, with an average +3 standard deviation (SD).

The accuracy and precision of the method were represented by recovery and coefficient of variation (CV), respectively. The recovery (percent) of the spiked tylosin or tilmicosin was established using five spiked duplicate blanks at levels of 0.5 × MRL, 1 × MRL, 2 × MRL for three different analyses and was calculated using the following equation: (concentration measured/concentration spiked) × 100. CVs were determined by analyzing the above samples spiked with tylosin or tilmicosin at levels of 0.5 × MRL, 1 × MRL, 2 × MRL for five different analyses. Each concentration level was tested three times in a time span of 2 months.

Comparison of the ic-ELISA with HPLC. To test the detection capability and the accuracy of the developed ic-ELISA for incurred tissues, six pigs were administered 10 mg kg^{-1} tylosin intramuscularly, on alternate sides of the neck into the brachiocephalic muscles, every 24 h for 5 consecutive days, followed by a withdrawal period of 0 day, 2 days, or 7 days (2 pigs in each group). The negative control group contained three pigs that were administered tylosin-free saline solution intramuscularly. At each sample collection time, two pigs from the treated group and one from the control group were sacrificed, followed by the sampling of muscle from the injection site, loin and liver. These incurred samples were minced and homogenized, transferred into plastic bags and frozen at −20 °C until analysis. All samples were subjected to ic-ELISA and HPLC analyses according to the methods described by Prats et al.[8]

Statistical Analysis. Descriptive statistical parameters such as mean value, SD, and CV were calculated. Statistical analysis of the data was performed using Microsoft Excel 2003.

RESULTS

Characterization of the Monoclonal Antibodies. After cell fusion and culture several times, the hybridoma cells 5A8, 1B6, 2E12, 3B10, 5F11, 2G11, 4E2, 3C4 and 4G11 were rescreened and subcloned three times. The titers of MAbs were determined by indirect ELISA (Table 3). Of the nine MAbs, 3C4 showed the best sensitivity toward tylosin and the highest titer. Therefore, the antibody 3C4 was selected for the rest of this study. The subclass of the mAb 3C4 was determined by the mouse monoclonal antibody isotyping kit and was identified as isotype IgG2a.

As shown in Table 4, the mAb 3C4 showed CR toward tylosin (CR = 100%), tilmicosin (CR = 51%), DES (CR =187%), OMT (CR = 3.6%) and did not exhibit measurable

Table 4. Specificity of the Antibody 3C4

competitor	cross-reactivity (%)	competitor	cross-reactivity (%)
tylosin	100	oleandomycin	<0.1
tilmicosin	51	josamycin	<0.1
desmycosin	187	erythromycin	<0.1
5-*O*-mycam-inosyltylonolide	3.6	azithromycin	<0.1
		roxithromycin	<0.1
spiramycin	<0.1	avermectin	<0.1
kitasamycin	<0.1	ivermectin	<0.1

dx.doi.org/10.1021/jf2037449 | J. Agric.Food Chem. 2012, 60, 44−51

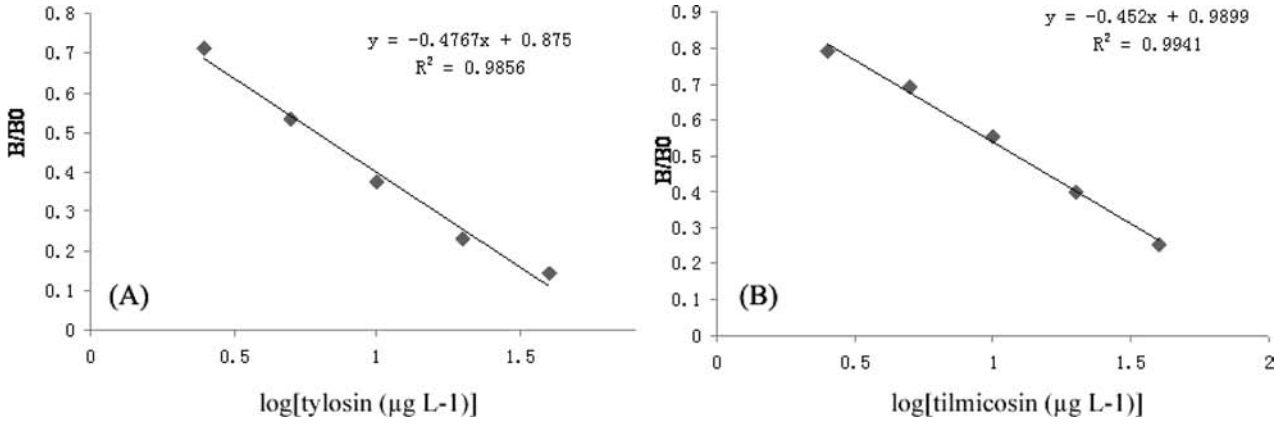

Figure 3. Standard curves of the method: (A) tylosin; (B) tilmicosin.

CR (CR < 0.1%) with other antibiotics such as spiramycin, kitasamycin, oleandomycin, erythromycin, josamycin, azithromycin, roxithromycin, avermectin and ivermectin.

The Standard Curve for the ic-ELISA. The optimum ic-ELISA conditions were determined as 0.5 μg mL^{-1} of coating antigen (DES−CMO−OVA) concentration, a 1:1.28 × 10^5 ratio of antibody (3C4) dilution. These were the best conditions to obtain the linear portion of the response curve. These conditions were fixed for the rest of the experiment. The standard curves (shown in Figure 3) based on the tylosin (A) and tilmicosin (B) matrix calibration range from 2.5 to 40 μg L^{-1}, respectively. The IC_{50} value of the method for tylosin was 6.1 μg L^{-1} and the one for tilmicosin 12.1 μg L^{-1}.

Validation of the ic-ELISA Method. Based on the results from twenty different blank samples, the LODs of the method for tylosin matrix calibration ranged from 5.1 μg kg^{-1} to 8.5 μg kg^{-1} and the one for tilmicosin 6.9 μg kg^{-1} to 13.8 μg kg^{-1} (shown in Table 5) in various biological matrices. The recoveries and CVs of the above samples spiked with tylosin and tilmicosin at the levels of 0.5 × MRL, 1 × MRL, 2 × MRL are listed in Table 6. The recoveries of tylosin and tilmicosin were in the range of 74.1% to 120.7%. The CVs were less than 18.6%.

Table 5. LOD of This Method Based on Different Matrix Calibration

	LOD (μg kg^{-1})	
biological matrix	tylosin matrix calibration	tilmicosin matrix calibration
porcine muscle	6.2	9.1
porcine liver	6.3	10.4
chicken	7.1	8.9
chicken liver	8.5	13.8
egg	5.7	8.1
ovine muscle	5.1	8.0
ovine liver	5.2	8.6
bovine muscle	5.4	6.9
milk	5.7	11.1
fish	5.3	7.3
honey	6.4	9.5

Comparison of the ELISA and HPLC Analyses. All samples from the nine treated pigs were subjected to the ic-ELISA and HPLC analyses. Figure 4 shows an excellent correlation between ELISA and HPLC results. These results suggested that ic-ELISA is reliable for the detection of tylosin and tilmicosin residue in edible tissues of animals.

Table 6. Recoveries and Coefficients of Variation (CVs) of the Samples Spiked with Tylosin and Tilmicosin in Tissues (n = 15[a])

		tylosin spiked		tilmicosin spiked	
tissue	spiked level (μg kg^{-1})	mean recovery ± SD[b] (%)	CV (%)	mean recovery ± SD[b] (%)	CV (%)
porcine muscle	50	91.6 ± 6.2	6.8	95.2 ± 11.4	12.0
	100	75.1 ± 8.4	11.2	88.6 ± 14.8	16.7
	200	88.8 ± 2.9	3.3	85.9 ± 13.6	15.8
porcine liver	50	89.5 ± 10.8	12.1	87.3 ± 12.8	14.7
	100	81.5 ± 10.2	12.5	101.8 ± 18.9	18.6
	200	94.3 ± 2.3	2.4	100.7 ± 7.6	7.5
chicken	50	102.2 ± 3.3	3.2	99.9 ± 5.5	5.5
	100	85.2 ± 13.4	15.7	81.7 ± 12.5	15.3
	200	74.1 ± 3.2	4.3	74.9 ± 2.1	2.8
chicken liver	50	105.4 ± 9.9	9.4	95.6 ± 13.7	14.3
	100	90.9 ± 4.1	4.5	102.4 ± 13.7	13.4
	200	81.6 ± 10.6	13.0	82.0 ± 9.3	11.3
egg	50	90.1 ± 12.9	14.3	92.7 ± 12.4	13.4
	100	87.2 ± 4.5	5.2	88.2 ± 4.1	4.6
	200	86.6 ± 10.5	12.1	87.3 ± 10.0	11.5
ovine muscle	50	120.7 ± 5.5	4.6	120.0 ± 9.6	8.0
	100	98.3 ± 9.8	10.0	99.2 ± 6.4	6.5
	200	78.1 ± 5.8	7.5	76.7 ± 5.9	7.7
ovine liver	50	109.5 ± 10.8	9.9	109.3 ± 9.7	8.9
	100	87.7 ± 7.1	8.1	91.3 ± 5.9	6.5
	200	75.3 ± 5.9	7.8	80.5 ± 7.4	9.2
bovine muscle	50	97.5 ± 15.4	15.8	98.4 ± 13.0	13.2
	100	85.4 ± 9.6	11.2	80.3 ± 7.2	9.0
	200	81.5 ± 3.4	4.2	76.0 ± 5.0	6.6
milk	25	108.9 ± 18.6	17.1	111.2 ± 12.6	11.3
	50	110.6 ± 11.7	10.6	112.9 ± 10.3	9.1
	100	101.3 ± 12.2	12.0	101.5 ± 10.6	10.4
fish	25	118.4 ± 4.1	3.5	116.4 ± 6.2	5.3
	50	113.6 ± 9.4	8.3	109.4 ± 7.5	6.9
	100	96.8 ± 5.1	5.3	98.0 ± 5.0	5.1
honey	50	80.5 ± 7.6	9.4	85.3 ± 13.5	15.8
	100	89.4 ± 10.4	11.6	79.1 ± 5.6	7.1
	200	77.7 ± 4.7	6.0	83.7 ± 9.3	11.1

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

dx.doi.org/10.1021/jf2037449 | J. Agric.Food Chem. 2012, 60, 44−51

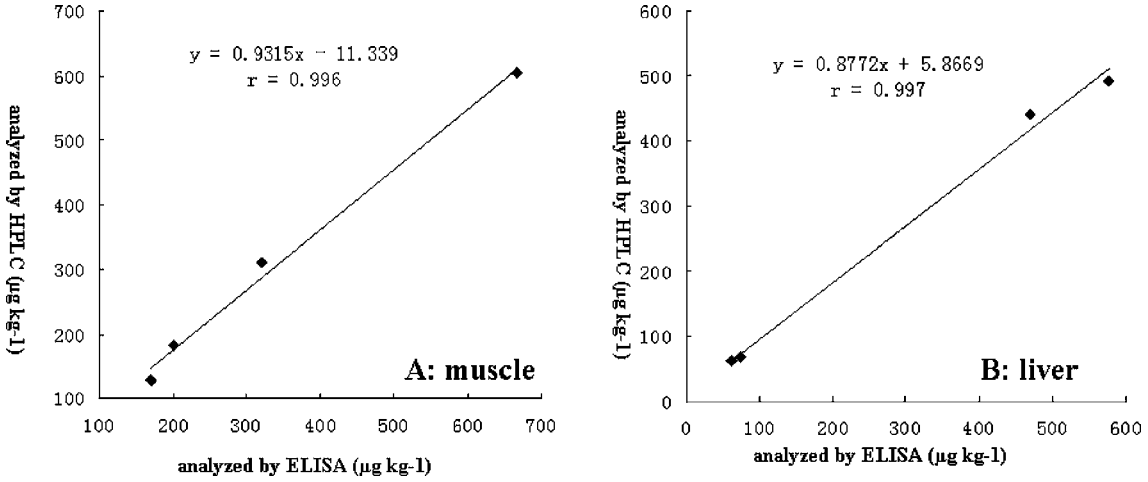

Figure 4. Correlation of tylosin assay between the HPLC and ELISA methods in swine muscle (A) and liver (B) samples.

DISCUSSION

The synthesis of haptens was the key step in a procedure of research on rapid immunoassay. In previous studies, Yao and Mahoney[15] selected 23-amino-OMT as hapten, which, attached to the protein carrier at C-23, presents the lactone ring and the amino sugar mycaminose to the antibody-forming B cells. A polyclonal antibody against this molecule demonstrated reactivity with 12-, 14-, and 16-membered macrolides that contained amino sugar moieties, regardless of the presence of neutral sugar residues. Jackman et al.[16] and Silverlight et al.[17] used desmycosin and lactenocin as haptens, whose polyclonal antibodies showed nearly the same CR with tylosin as with tilmicosin. Using spiramycin and tylosin as haptens, Situ and Elliott[19] produced polyclonal antibodies which showed nearly the same CR with tylosin as they did with tilmicosin. In an attempt to produce antibodies specific for tilmicosin, the hapten 23-demycinosyl-23-deoxy-23-(3-aminoprop-1-yl)-aminotilmicosin was synthesized by Berier et al.[18] In a word, a suitable hapten for immunization should preserve the structure of the target compound as much as possible. In this study, the haptens DES and OMT were designed and were anticipated to raise antibodies against tylosin and tilmicosin. The obtained mAb 3C4 exhibited strong CR to DES, tylosin and tilmicosin, but low CR to OMT. No CR was observed with other macrolides including avermectins, although they have a similar macrolactone ring. It was suggested that the antigenic determinant of this mAb is mycinose (C23), which is similar to the results of Silverlight et al.[17]

In some cases, spacer arms which contain 4−6 carbons have been shown to be optimal in obtaining high quality antibodies.[25] However, the short spacer arm CMO containing 2 carbons showed the higher titer and sensibility than the long one ADH containing 6 carbons in the present study (see Table 2 and Table 3). Compared with the above haptens, it seemed that the CMO was preferable to maintain the hapten DES to fully expose on the surface of carrier protein. This was helpful to expose a specific region of the hapten to the animals' immune systems. Furthermore, it was also probably related to the concentration of immunogens. The clonal selection theory suggests that a small quantity of antigen will preferentially bind with host lymphocytes and will trigger them to produce high-affinity antibodies. It was found in Table 2 that reducing the dose of immunogen from 0.1 mg to 0.05 mg could promote the titer of antibodies in almost all immunized mice. Similarly, the low incorporation rate of immunogen (DES−CMO−BSA) (shown in Table 1), which resulted in decreasing the concentration of haptens in fact, showed the higher titer of antibodies than the one of high incorporation rate (DES−ADH−BSA). These findings are in keeping with the study of Berek and Milstein.[26]

As shown in Table 3, the mAb 3C4 (immunogen DES−CMO−BSA) exhibited strong CR to DES, tylosin and tilmicosin and low CR to OMT. Although the mAb 2G11 (immunogen DES−ADH−BSA) and 5A8 (immunogen OMT−CMO−BSA) also exhibited strong CR to tilmicosin, DES and OMT, which could be better for broad specificity, they are low CR with tylosin. The main purpose of this study was to develop an ELISA for simultaneous determination of tylosin and tilmicosin in various biological matrices. Therefore, the mAb 3C4 was selected for further evaluation.

Biological matrices, especially edible animal tissues, are complex and often contain elements that can interfere with the compounds of interest. It is usually necessary to purify and concentrate the analytes prior to analysis. In this study, the homogenized samples were submitted to an extraction process with a metaphosphoric acid−methanol mixture, and chloroform at alkaline pH was used to back-extract the supernatant extract. After shaking and centrifugation, the organic layer was collected and evaporated until dryness and finally resuspended in a pH 6.0 PBS used for ELISA analysis without further cleanup. However, solid-phase extraction (SPE) was employed in almost the extraction procedures in previous studies. SPE is usually time-consuming and causes an increase in analytical cost. For screening methods time and cost issues are more important than the removal of matrix interferences so that a simple extraction system might be more suitable than a more complex extraction with higher recoveries. Taking this into account, a modified sample preparation method that requires less time and effort than other methods was presented in this study. There was no need to use SPE; only simple extracts were enough to obtain good sensitivity, recovery and specificity.

On the other hand, several procedures have been described in the literature for the determination of macrolides, include tylosin and tilmicosin, in various foods and animal tissues. Prats et al.[8] developed a HPLC method for determination of tylosin residues in different animal tissues (muscle, liver and kidney tissues from calves, pigs and poultry, poultry and pig skin + fat, calf fat). Cherlet et al.[9] developed a HPLC−MS/MS method

dx.doi.org/10.1021/jf2037449 | J. Agric.Food Chem. 2012, 60, 44−51

for quantitation of tylosin in swine tissues (muscle, skin + fat, liver and kidney). Horie et al.[10] developed a HPLC−MS/MS method for determination of macrolide antibiotics in meat and fish. Thompson et al.[11] and Benetti et al.[12] developed a HPLC−MS/MS method to quantify and confirm tylosin residues in honey, respectively. Bogialli et al.[13] developed a HPLC−MS/MS method for determining macrolide antibiotic residues in eggs. Juan et al.[14] developed a HPLC−MS/MS method for determination of macrolide in meat and milk. However, none of them used the same extraction procedure to detect the macrolide residues in various biological matrices (muscle, liver, milk, honey and eggs). In this study, using almost the same extraction procedure, the developed ic-ELISA analytical technology could detect tylosin and tilmicosin in various biological matrices (muscle, liver, milk, honey and eggs), for which the average recoveries and CVs in all biological matrices were according to the European Decision 2002/657/EC.[24] However, these results (Table 6) also showed that the recovery in the highest spiked level was lower than that of the other low spiked levels in most of samples. It was possible that there were some complicated components in biological matrices, which made the sample extraction more difficult and the extraction recovery lower. Anyway, this method is suitable for extraction and cleanup, while the manipulation is rather simple.

To test the reliability of the developed ic-ELISA for incurred tissues, an animal-feeding experiment was carried out. According to the previous studies on tylosin residues,[27] after five intramuscular injections of tylosin in pigs at a dose of 10 mg kg^{-1}, the highest concentrations of tylosin residues were found at the injection site three (110−2540 $\mu g\ kg^{-1}$) and seven days (100−4100 $\mu g\ kg^{-1}$) after the withdrawal, respectively. Residues at the injection site depleted below the limit of quantitation (50 $\mu g\ kg^{-1}$) of the HPLC assay at ten and fourteen days after the last dose. In other tissues, tylosin residues declined faster than from the injection site. In this study, the concentration of tylosin detected by ic-ELISA and HPLC on the second day postadministration in loin muscle in 2 pigs was below or equal to 50 $\mu g\ kg^{-1}$, which is similar to the results of Prats et al.[27] Nevertheless, 7 days after treatment the tylosin concentration at the injection site in the present study was much lower than the one of the study of Prats et al.[27] This difference could have resulted from the medicine formulation for administration of tylosin. The present result suggested that the ic-ELISA is a reliable tool for the detection of tylosin residues in edible tissues of animals.

This paper would provide an alternative method for the determination of tylosin and tilmicosin in various biological matrices in routine monitoring. When the ELISA screening shows positive result, a chromatographic method such as LC/MS is indispensable for the identification. From a practical point of view, the method does not require working with SPE and can be carried out in any routine laboratory without requirements for special facilities. Furthermore, the comparison of this method to the gold standard HPLC demonstrated its reliablility. Overall, the developed ic-ELISA provides a rapid, accurate, and inexpensive way to detect the residues of tylosin and tilmicosin in various biological matrices and is suitable for routine diagnostics.

Abbreviations Used

MRL, maximum residue limits; HPLC, high performance liquid chromatography; HPLC−MS/MS, high performance liquid chromatography coupled with mass spectrometry; ic-ELISA, Indirect competitive enzyme-linked immunosorbent assay; mAb, monoclonal antibody; ADH, adipic dihydrazide; CMO, *O*-carboxymethoxylamine hemihydrochloride; OVA, ovalbumin; BSA, bovine serum albumin; HRP-IgG, goat anti-mouse IgG horseradish peroxidase conjugate; DES, desmycosin; OMT, 5-*O*-mycaminosyltylonolide; PBS, phosphate buffered saline; PBST, PBS containing 0.1% Tween-20; CR, cross-reactivity; SPE, solid-phase extraction; LOD, limit of detection; SD, standard deviation; CV, coefficient of variation

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