Development of an Enzyme-Linked Immunosorbent Assay for the Determination of Maduramicin in Broiler Chicken Tissues

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Maduramicin is one of the most widely used coccidiostats in the world. A rapid and accurate analytical method for this drug should provide producers and users with an effective management tool. The current chromatographic methods are sensitive but labor-intensive. This paper reports the development of an enzyme-linked immunosorbent assay (ELISA) based on an immunoaffinity chromatography cleanup procedure for the analysis of maduramicin in broiler chicken tissues (including muscle, liver, and fat). Recoveries from fortified tissue homogenates at levels of 30.0−120.0 μg kg^{-1} ranged from 76.4 to 107.5% with coefficients of variation of 3.8−16.4%. The limits of detection were 1.0 ng g^{-1} in muscle, 2.8 ng g^{-1} in liver, and 1.5 ng g^{-1} in fat. The ELISA results from the analysis of incurred residue in tissue samples showed the cleanup procedure is viable.

Keywords: *Maduramicin; residue; broiler chicken; immunoaffinity chromatography (IAC); enzyme-linked immunosorbent assay (ELISA)*

INTRODUCTION

Maduramicin, discovered in the early 1980s, is a polyether ionophore, a major class of antimicrobials that have very potent broad spectrum anticoccidial activity (*1*) and improve feed utilization by ruminants (*2*). The chemical structure is shown in Figure 1. Maduramicin is registered for use as a feed additive at concentrations of 5−6 mg kg^{-1} to control coccidiosis in broiler chickens worldwide and has been marketed in all of the poultry-producing countries (*3*). Poisoning can often occur in broilers when they are fed maduramicin above these recommended levels, and the residues in broiler tissues can also do harm to humans and animals. Because of its great toxicity and narrow margin of safety, rapid and convenient methods for monitoring maduramicin in broiler chicken tissues are needed. Maduramicin is commonly found in the form of a nonvolatile ammonium or sodium salt, making it difficult to analyze by gas chromatography. Also, because maduramicin does not possess a chromophore, it cannot be readily detected spectrophotometrically without prior derivatization. To circumvent this limitation, a high-performance liquid chromatography (HPLC) method was first developed for the analysis of maduramicin in feed (*4*). Martinez and Shimoda (*5*) and Asukabe et al. (*6*) made fluorescent derivatives of the various polyether ionophores (including maduramicin) to detect them in liver tissue and animal feeds, respectively. Both methods required extensive sample purification prior to derivatization, and additional purification of the derivatized mixture was necessary before separation by HPLC and fluorescence detection (*7*−*9*). Moreover, the method requires the conversion of maduramicin to a fluorescent derivative, which is very difficult because maduramicin has little ultraviolet absorbency. The dosage of maduramicin for broiler chickens is low, and the residue in tissues is lower, so separation and analysis are difficult (*10*).

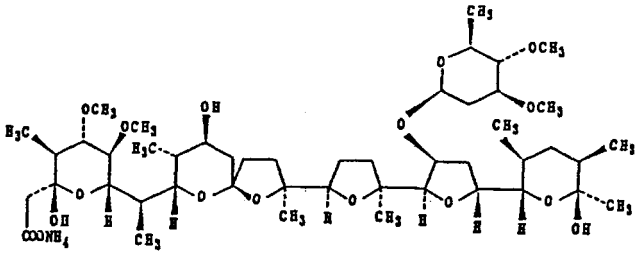

Figure 1. Molecular structure of maduramicin.

Our group has reported the development of an enzyme-linked immunosorbent assay (ELISA) without immunoaffinity chromatography (IAC) treatment for maduramicin in broiler chicken tissues (*11*). Four maduramicin-conjugated antigens were synthesized according to the mixed anhydride method (MA) and the active ester method (AE), and an antibody that recognized maduramicin was produced. The antibody exhibited little cross-reactivity with structurally similar ionophoric coccidiostats (salinomycin, monensin, lasalocid, etc). The method could detect <8.0 ng of maduramicin in a 1 g test sample. The sensitivity of our method was similar to that of Kennedy et al.'s method (*12*). Buffer extracts of chicken tissues (muscle, liver, and fat), which were fortified with maduramicin at concentrations from 0.01 to 1000 ng mL^{-1}, were analyzed using the method. Maduramicin recovery was quantitative in this range. The purpose of this study was to further modify the sample pretreatment procedure to improve the limit of detection of the method and enhance the accuracy of the method. This paper reports our successful development of an ELISA method based on pretreatment of tissue samples cleaned up on the IAC column.

MATERIALS AND METHODS

Chemicals and Supplies. The following chemicals and supplies were purchased: ammonium or sodium salt of maduramicn (Beijing Microchemistry Institute, Beijing, PRC), *N*,*N*-dimethylformamide (DMF) (Fluka, Buchs, Switzerland),

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methanol (Beijing Chemical, Beijing, PRC), *N*-hydroxysuccinimide (NHS) (Merck-Schwchoridr Co.), cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia, Uppsala, Sweden), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (Pierce, Rockford, IL), ethyl chloroformate (Beijing Chemical), and horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (Beijing Huamei Bio, Beijing, PRC). 1,6-Hexanediamine, bovine serum albumin, polyacrylamide gel, sodium dodecyl sulfate, and ovalbumin were from Sigma (St. Louis, MO). All other reagents were of analytical grade or better. All tissues used were obtained from broiler chickens fed with drug-free rations. The instruments used were a homogenizer, model AM-6 (Nihonseiki Kaisha, Japan), a Vortex mixer, model WH861 (Taicang Biochemical Instrument, Jiangsu, PRC), a magnetic stirrer, model 79HW-1 (Recheng Electrical Equipment, Zhenjiang, PRC), a rotary evaporator, model XZ-6 (Kelong Instrument, Beijing, PRC), a shaking apparatus, model SHZ-82 (Taicang Biochemical Instrument), a centrifuge, model LD4-2A (Beijing Medical Equipment, Beijing, PRC), a UV−vis spectrophotometer, model 751GW (Shanghai Analytical Instrument, Shanghai, PRC), a 96-well flat-bottom microtiter plate (Tianjin Biochemical, Tianjin, PRC), a Vmax Reader, model 5II (Shanghai Immune Instrument, Shanghai, PRC), and a glass column for packing immunosorbent, 10 × 0.17 cm i.d., with a fritted disk (porosity = 40−60 fm) sealed into the bottom and a 50-mL reservoir connected to the top with ground-glass joints (Beijing Chemical).

Buffers. Phosphate-buffered solution (PBS, 0.01 mol L^{-1}, pH 7.4−0.15 mol L^{-1} NaCl) was prepared by dissolving 0.27 g of KH_2PO_4, 2.86 g of Na_2HPO_4, 0.2 g of KCl, and 8.8 g of NaCl in 900 mL of distilled water. This solution was adjusted to pH 7.4 with 2.0 M NaOH and diluted to 1 L with water. The coating solution was obtained by dissolving 1.59 g of Na_2CO_3 and 2.92 g of $NaHCO_3$, adjusted to pH 9.6, and diluted to 1 L with water. Blocking buffer contained PBS and 0.1% (w/v) gelatin. Washing and assay solution contained PBS and 0.01% (v/v) Tween 20 (PBST).

Maduramicin Conjugates Preparation. Maduramicin conjugated to bovine serum albumin (M-BSA) or ovalbumin (M-OVA) had been prepared in our previous study (*11*). Estimation of the extent of conjugation was accomplished by determining the protein concentration by thin-layer SDS-PAGE assay (*13*) and Bio-Rad protein assay kit (*14*).

Synthesis of M—C_6NH_2—Ovalbumin Conjugate (M-C_6-OVA). The synthesis was based on the method of Wong (*15*) and the previous paper (*11*). One gram of maduramicin ammonium was dissolved in 10 mL of toluene containing 100−160 μL of triethylamine. One hundred microliters of ethyl chloroformate was diluted in 15 mL of toluene and added to the cold maduramicin solution and allowed to stir at 4 °C for 30 min. 1,6-Hexanediamine (1.23 g) was dissolved in 10 mL of toluene and added. After 1.5 h of reaction at 4 °C, ice-cold water was added to remove salts and clarify the toluene solution. The product was removed by anhydrous sodium sulfate and evaporated to dryness at 55 °C. Maduramicin with 1,6-hexanediamine (M-C_6NH_2) was purified by silica gel column chromatography using a consistent solvent (methanol/ethyl ester/triethylamine, 80:18:2, v/v/v) as the mobile phase. The product was stored in toluene at 4 °C. M-C_6NH_2 was dissolved in 1 mL of DMF and added to 30 mL of BSA or OVA solution at a 40:1 molar ratio of ligand to protein. NHS and EDC were separately dissolved in 5 mL of PBS (pH 7.6) and added to the protein ligand mixture in sequence. The reaction solution was stirred at room temperature for 18 h. Excess reagent was removed by dialysis. This conjugate was compared to M-OVA as the coating antigen in constructing standard curves of PBST−10% methanol.

ELISA Development. The indirect competitive ELISA procedure used here was an adaptation and modification of the previously reported method (*11, 15*). The coating antigens (M-C_6-OVA or M-OVA) were diluted in PBS and 0.05% (v/v) Tween20 (PBST). Polystyrene 96-well microtiter plates were coated with M-C_6-OVA or M-OVA (100 μL) for 18−20 h at 4 °C. The coated wells were washed four times with PBST. Blocking buffer (200 μL) was then added to the wells for 1 h at 37 °C and the wells were washed again. When sample analysis or standard curves were performed, antibody and maduramicin working solution or sample solution (50 μL each) were mixed for 1 h and added to the coated microwells, reacting for 1 h. After washing with PBST, horseradish peroxidase-conjugated goat anti-rabbit IgG solution was added and incubated for 1 h. Freshly prepared substrate OPD (1:1000 dilution with PBS, 150 μL) was added after washing with PBST to remove the unbound enzyme. The absorbance was determined at 490 nm after 2.0 mol L^{-1} of sulfuric acid (50 μL) was added. The optimal concentrations of coating antigens and antiserum titer were obtained by the determination of indirect noncompetitive ELISA. Incubation and quantitation were the same as described above.

Antibody Preparation. The antibody was obtained by immunizing New Zealand rabbits with M-BSA in Freund's complete adjuvant and biweekly boosts with Freund's incomplete adjuvant 4 weeks after the initial immunization. The immunoglobulin G (IgG) in the antiserum was purified by ammonium sulfate precipitation and diethylaminoethylcellulose anion-exchange chromatography (*16*) and stored at −20 °C.

Immunosorbent Preparation and Column Capacity Determination. Immunosorbent was prepared by coupling CNBr-activated Sepharose 4B to IgG (*17*). The coupling efficiency was determined with a UV−vis spectrophotometer. The immunosorbent solution was transferred to a column (glass column, 100 mm × 8 mm, G_3 or G_4 filter) for 1.0-mL bed volume. After washing with 10 mL of PBST, 5 mL of H_2O, and 5 mL of methanol in sequence, 20 mL of PBS−10% methanol solution containing 200 ng mL^{-1} maduramicin was added to the IAC column continuously at a flow rate of 0.8−1.0 mL min^{-1} by natural gravity. The flowing solution was collected in batch with several tubes and was determined by ELISA. The maduramicin-saturated column was washed with 10.0 mL of PBS with 10% (v/v) methanol and 5 mL of H_2O with 10% (v/v) methanol. Maduramicin was eluted with 4 mL of eluant (methanol/H_2O, 9:1, v/v) and detected by ELISA. The column was regenerated by washing with 5 mL of water and 10 mL of PBS and stored in PBS−0.02% sodium azide at 4 °C. The column capacity was determined and calculated according to the method developed by Davis et al. (*18*).

Sample Extraction/IAC Cleanup. Tissue sample (muscle, liver, or fat, 6.0 g each) was transferred to a tube, and 10 mL of methanol was added. The mixture was homogenized for 30 s and transferred to a 100-mL graduated polypropylene centrifuge tube. After methanol was added to a total volume of 60 mL and 30 min of shaking, the mixture was frozen at −20 °C overnight and centrifuged to remove fat drops. The supernatant was evaporated with a rotary evaporator in a vacuum at 55 °C. To the remaining residue was added 20 mL of PBS−10% methanol, and this was subjected to the IAC cleanup procedure. The steps for adsorption, washing, and elution were the same as described under Immunosorbent Preparation and Column Capacity Determination. To the collected solution was added PBST to a volume of 20 mL, which was used directly in ELISA.

Calibration Curves and Fortification. The standard calibration curves of sample matrices without and with IAC treatment and of PBST−10% methanol for maduramicin, using M-C_6-OVA (1.25 μg mL^{-1}) as the coating antigen, covered a concentration range of $0-10^3$ ng mL^{-1}. Antibody was diluted 1:1600 with PBS (optimal dilution). Absorbance at 490 nm was inversely proportional to the concentration of maduramicin in the standards and samples. Absorbance values were normalized by dividing by the absorbance value of a negative control (0 ng mL^{-1} maduramicin). The normalized absorbance values (B/B_0) of the maduramicin standards were plotted against the values of the maduramicin concentration. Blank tissue samples were fortified with maduramicin at 30.0, 60.0, and 120.0 μg kg^{-1} and mixed thoroughly. After 10−15 min, the samples were extracted, cleaned up, and determined as described earlier.

Maduramicin Feeding Study. One-day-old AA broiler chickens (mean weight ± SD = 40.5 ± 2.1 g) were randomly

assigned to three treatment groups of 48 birds each. Throughout the experiment, the birds were supplied with water and an unmedicated diet that contained levels of critical nutrients recommended by the National Research Council (1984) (control diet) ad libitum. One of the treatment groups was given only the control diet (no-dose group). The other treatment group was fed the same control diet with the addition of the recommended dose of ammonium maduramicin (5.0 mg of ammonium maduramicin/kg of feed, 5.0 mg kg^{-1} group). The birds in each group were treated for 42 days. On day 43, five chickens from each of both treatment groups were killed by cervical dislocation, and their muscles, livers, and fat were immediately removed and frozen at −70 °C until processed. The remaining birds in each of the treatment groups were immediately given control feed and sacrificed 72 and 120 h later (5 birds/group/time period). The birds were handled as described previously.

Determination of Maduramicin in Broiler Chicken Tissues from the Incurred Residue Study by ELISA. Each set of samples consisted of a kind of tissue (muscle, liver, or fat) obtained from one bird from each treatment group per withdrawal time and a control tissue sample spiked to 1.0 μg kg^{-1}. One complete set of samples was analyzed at a time, and five complete sets were evaluated. Tissues were thawed at room temperature, homogenized, and stored at 4 °C prior to analysis. Tissue extracts were subjected to IAC cleanup procedure for ELISA as described above. The maduramicin concentrations were calculated by using the standard curve made in PBST−10% methanol and M-C_6-OVA.

RESULTS AND DISCUSSION

Maduramicin Conjugates Preparation. Maduramicin ammonium salt is an organic molecule with a molecular weight of 934.14. Because of its small size, it was conjugated to the carrier protein BSA to make it an immunogen. This M-BSA was one of the maduramicin conjugates that had been prepared in a previous study (*11*) and was used to immunize New Zealand rabbits. The M-OVA was compared to the coating antigen of M-C_6-OVA in constructing a maduramicin standard curve of the PBST−10% methanol matrix. The molecular weight of the protein can be determined by thin-layer SDS-PAGE assay, and the numbers of maduramicin linking to BSA or OVA were calculated by comparison of the molecular weights of the protein before and after conjugation. A Bio-Rad protein assay kit was used to determine the amount of free amino in BSA or OVA, so the binding ratio of maduramicin to protein was obtained by comparing the total free amino to the rest after conjugation. The results of both methods were similar. M-BSA and M-OVA conjugates were determined and calculated to contain 8.6 and 10.8 mol mol^{-1} of protein, respectively, and M-C_6-OVA contained 10.3 mol.

Importance of Coating Antigen. The two coating antigens (M-C_6-OVA and M-OVA) were used to construct standard curves, and the results are shown in Figure 2. Compared to the M-C_6-OVA curve, the M-OVA curve is shallow, and the displacement of antibody binding to M-OVA cannot be accomplished completely by maduramicin. However, the standard curve using M-C_6-OVA as coating antigen has a linear response to maduramicin concentrations (1−100 ng mL^{-1}). It was found that using a six-carbon bridge between maduramicin and the coating protein was sufficient to generate a linear dose response to maduramicin and to improve the specificity and sensitivity of the ELISA.

IAC Columns. The coupling efficiency of antibody IgG to CNBr-activated Sepharose 4B, calculated by the following equation, was nearly 99.5%, which resulted in the immunosorbent with IgG loading of 5 mg mL^{-1} gel.

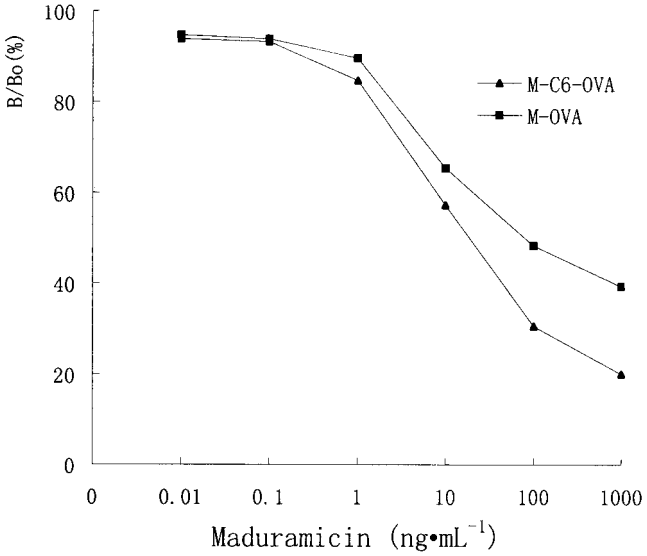

Figure 2. Comparison of maduramicin standard curves, made in PBST−10% methanol and the coating antigens M-C_6-OVA (1.25 μg mL^{-1}) and M-OVA (1.25 μg mL^{-1}), respectively, after B/B_0 transformation of OD readings (490 nm). In the range of 1−100 ng mL^{-1}, the standard curve of M-C_6-OVA has a linear response to maduramicin concentration.

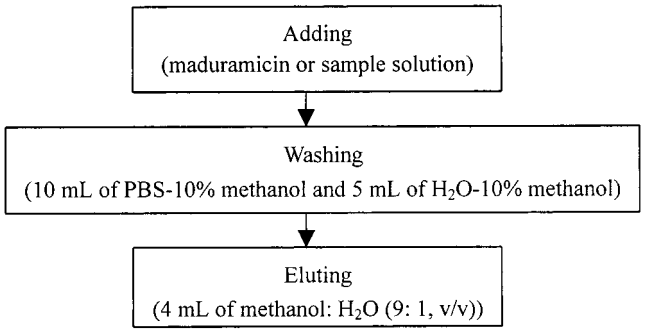

Figure 3. IAC cleanup procedure.

$$Z = \frac{A - B}{A} \times 100\%$$

Z is the coupling efficiency, A is the total of IgG, and B is the amount of uncoupled IgG.

The dynamic column capacity was 3750 ng of maduramicin mL^{-1} of gel, and the specific column capacity was 469 ng mg^{-1} of immobilized IgG. Preparation of IAC columns with high capacity could be achieved by selecting purified antibody with high titer or improving the displacement level of antibody. The latter, however, would decrease the specific column capacity (*19*). The results confirmed high-titer antibody not only improved dynamic column capacity but also increased specific column capacity. Thus, high capacity could reduce eluant volume and improve the concentration of drug in the eluate in the IAC cleanup procedure shown in Figure 3.

Extraction and Cleanup. Methanol was used for extraction, not only because it can extract maduramicin from tissues quantitatively and precipitate protein well but also because this extract can easily be subjected to the IAC cleanup procedure. Maduramicin has extremely low water solubility. Therefore, PBST (or water)−methanol was used throughout the cleanup procedure

Table 1. Parameters of Maduramicin ELISA Standard Curves of Different Matrices

parameter	PBST−10% methanol	tissue samples without IAC treatment			tissue samples with IAC treatment		
		muscle	liver	fat	muscle	liver	fat
I_{50}[a]	32.3	44.2	60.0	47.3	33.3	35.3	37.1
slope	2.20	1.95	1.80	1.75	2.20	1.95	1.95

[a] I_{50} is the concentration of 50% inhibition (ng mL^{-1}) to antigen−antibody conjugation.

Table 2. Recoveries of Maduramicin in Fortified Tissue Samples

sample	added (μg kg^{-1})	recovery[a] (%)	CV (%)
muscle	30.0	79.0 ± 10.0	17.2
	60.0	89.2 ± 5.7	6.4
	120.0	107.5 ± 8.3	7.8
liver	30.0	76.4 ± 12.0	16.4
	60.0	89.2 ± 4.8	5.4
	120.0	88.9 ± 3.9	3.8
fat	30.0	82.0 ± 10.0	12.2
	60.0	90.7 ± 4.8	5.3
	120.0	88.3 ± 5.6	6.3

[a] Values are mean ± standard derivation, $n = 4$.

to increase the solubility of maduramicin in aqueous media and reduce the adsorption of maduramicin on glassware. Prewetting glassware (especially the reservoirs of IAC columns) with a few milliliters of methanol can improve the recoveries of maduramicin. Methanol is usually used in eluting haptens from immunosorbent during the IAC cleanup procedure, and maduramicin can be completely eluted from a maduramicin-saturated column of 1-mL bed volume with only 4 mL of methanol. Thus, only one solvent was used in the preparation of the sample, which simplified the extracting and eluting procedures. Through theoretical calculation and experimental confirmation, 3 mL of eluant was enough for the cleanup of maduramicin, but due to many conditions in practical operation, 4 mL of eluant was used for washing IAC columns.

Determination and Fortification. The standard calibration curves of sample matrices without and with IAC treatment and PBST−10% methanol for maduramicin were linear in the concentration range of 1−100 ng mL^{-1}. The parameters of these standard curves shown in Table 1. The earlier-reported ELISA (*11*) without the IAC treatment did not work well in the analysis of maduramicin in broiler chicken tissues because of matrix interference. At the same maduramicin standard concentration in the range of 1−100 ng mL^{-1}, the B/B_0 value gained by ELISA (*11*) without IAC treatment was ∼5% higher than that gained by ELISA with IAC treatment. On the other hand, the conjugation of maduramicin with antibody was obviously influenced by matrix interference, and the sensitivity and accuracy were reduced. The IAC columns were prepared in the present paper. The curves based on matrices of purified tissue extracts showed no interference existed after the IAC cleanup procedure. Therefore, the recoveries were calculated according to the standard curve of PBST−10% methanol using M-C_6-OVA while determining residue in tissues.

The results of fortification studies are shown in Table 2. Fortified at different levels, recoveries of maduramicin were 76.4−107.5%, with coefficients of variation (CVs) of 3.8−16.4%. The limits of detection, defined as the lowest concentration that can be determined to be statistically different from a blank, were 3.5 ng g^{-1} in muscle, 8.0 ng g^{-1} in liver, and 5.0 ng g^{-1} in fat (*11*). In this study the limits of detection were 1.0 ng g^{-1} in muscle, 2.8 ng g^{-1} in liver, and 1.5 ng g^{-1} in fat.

Table 3. ELISA Analysis of Maduramicin in Chicken Tissues from an Incurred Residue Study (μg g^{-1})

treatment	tissue	0 h	72 h	120 h
no dose	muscle	−[a]	−	−
	liver	−	−	−
	fat	−	−	−
5.0 mg kg^{-1}	muscle	0.27	0.042	−
	liver	1.08	0.095	−
	fat	1.12	0.19	0.05

[a] Below limits of detection: 1.0 ng g^{-1} in muscle, 2.8 ng g^{-1} in liver, and 1.5 ng g^{-1} in fat, $n = 5$.

Determination of Maduramicin in Chicken Tissues from the Incurred Residue Study. A positive control spiked tissue sample (1.0 μg kg^{-1}) was used and analyzed in parallel with each set of incurred residue samples to verify efficient maduramicin recovery and detection. The mean recovery of maduramicin from these samples was 90.5% (CV = 7.2%, $n = 5$), which indicated nearly quantitative recovery under these conditions.

Table 3 is a summary of the results from the incurred residue study. To include all of the data in the analysis, sample determinations that were below the method's limit of detection were designated zero. The incurred residue data obtained by using the ELISA method with IAC treatment provide information concerning the disappearance of maduramicin from tissues. Residues are present immediately following withdrawal from medicated feed and are below the limits of maduramicin residues (muscle, 0.24 mg kg^{-1}; liver, 0.72 mg kg^{-1}; and fat, 0.48 mg kg^{-1}) after 48 h approved by the China Ministry of Agriculture.

Conclusions. High recovery and sensitivity were due to the specificity of the IAC columns. The above analytical results of recovery were consistent with those determined by ELISA without a cleanup procedure. However, the latter method, in which the recovery of each tissue sample can be determined only by using the standard curve based on homologous tissue extract, was disturbed by matrix interferences and was inconvenient and unfit for practical determination. In this method, the recovery of maduramicin in various tissues can be determined on the basis of a PBST−10% standard curve due to the IAC cleanup procedure. The method is one of the simplest methods for determining maduramicin residue in broiler chicken tissues yet reported, with only one chromatographic separation step involved in the cleanup procedure. The ELISA results from the analysis of incurred residue in tissue samples showed the cleanup procedure is viable. This method is sensitive and reliable enough for determining maduramicin in various tissues.

This work demonstrates the high specificity of an antibody-mediated cleanup (AMC) procedure. It is certain that AMCs, such as the IAC cleanup, can simplify the preparation of samples and improve analytical quality. However, it is also shown that AMCs cannot always or completely eliminate matrix interference of samples. In the analysis of pesticide/drug residues in biological samples, even small nonspecific adsorption would have significant or deleterious effects on analytical performances, such as the limit of detection and

selectivity. Suitable coupling and washing steps are the key to a well-designed IAC cleanup procedure.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; IAC, immunoaffinity chromatography; HPLC, high-performance liquid chromatography; DMF, dimethylformamide; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-(3-dimethylaminopropyl)carbodiimide; OVA, ovalbumin; BSA, bovine serum albumin; M-OVA, maduramicin-conjugated ovalbumin; M-BSA, maduramicin-conjugated bovine serum albumin; M-C_6-OVA, maduramicin with 1,6-hexanediamine-conjugated ovalbumin; AMC, antibody-mediated cleanup.

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