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Determination of Multiresidue of Avermectins in Bovine Liver by an Indirect Competitive ELISA

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An ELISA was developed to detect multiresidues of avermectins (AVMs) including abamectin (ABM), ivermectin (IVM), and eprinomectin (EPR) in bovine liver. The modified ABM, 4′-*O*-succinoyl-ABM was conjugated to bovine serum albumin as the immunogen for the preparation of polyclonal antibodies to AVMs and conjugated to ovalbumin as the coating antigen for the ELISA. Serum with the highest antibody titers to AVMs, which had a cross-reactivity of 100% with ABM, 145.4% with EPR, and 25% for IVM, was selected for the development of an indirect competitive ELISA. The ELISA could detect ABM, IVM, and EPR residues in bovine liver tissues, with a limit of quantitation of 1.06 ng/mL for all three AVMs. Optimal pH, ion strength, organic solvent, and duration of incubations were investigated to increase the sensitivity of the ELISA. Recoveries of these drugs ranged from 53.8% to 80.6% with inter-assay coefficients of variation (CV) of 3.4−17.9% and intra-assay CV of 5.5−14.7%. Analysis results of field samples by the ELISA were consistent with those by a previously developed HPLC method. The ELISA can be used as a rapid method for screening of AVMs residues in bovine liver.

KEYWORDS: Avermectins; multiresidue; ELISA; bovine liver

INTRODUCTION

Avermectins (AVMs) are insecticidal/miticidal compounds derived from the soil bacterium, *Streptomyces avermitilis*. Four AVMs, namely, abamectin (ABM), ivermectin (IVM), doramectin (DOR), and eprinomectin (EPR), are widely used in food-producing animals to control parasitic diseases. The AVMs consist of two components, >80% B1a and <20% B1b, and molecular structures of the main component (**B1a**) are shown in **Figure 1**. When the drugs are administered to cattle, they are distributed mainly in the liver, which is the target tissue for residue analysis. The maximum residue limits for AVMs in bovine liver recommended by WHO/FAO are 100 ng/g for ABM, IVM, and DOR and 2 μg/g for EPR (*2*).

Due to their widespread use and potential toxicity, analysis for residues of these drugs in edible tissues is of major importance. Several analytical methods for monitoring of AVM residues in bovine liver (*1*, *3*−*5*), in milk (*6*), and in salmon muscle (*7*) have been reported. Liquid chromatography/mass spectrometry (LC/MS) was used for the confirmation of the AVMs (*8*−*10*). However, these methods involved complex sample preparation procedures and need expensive equipment. Immunoassays have recently become popular to use for detecting AVM residues in animal tissues (*11*−*17*), and they are rapid and have good sensitivity and specificity. However, none of the immunoassays could analyze multiple AVMs, and no ELISA method to detect multiresidues of AVMs has been reported. The aim of this study was to develop an ELISA based on a polyclonal antibody to detect multiresidues of AVMs in bovine liver.

MATERIALS AND METHODS

Reagents and Solutions. ABM (purity 98%) and DOR (purity 94.3%) were obtained from Pfizer Co. (New York, NY), IVM (purity 95%) was sourced from CAU Newtech Development Co. (Beijing, China), and EPR (purity 96%) was a gift from Prof. Ming Wang (China Agricultural University, Beijing, China). Freund's complete adjuvant, bovine serum albumin (BSA), and ovalbumin (OVA) were purchased from Gibco Chemical Co. (Carlsbad, CA).

Buffers used in the ELISA were as follows: the coating buffer was 0.05 M carbonate (pH 9.6), made up with 1.59 g of sodium carbonate and 2.93 g of sodium bicarbonate in 1 L of purified water; the assay buffer was phosphate-buffer solution (PBS), containing 0.01 M phosphate (pH 7.2) and 0.145 M NaCl; the washing buffer was PBS containing 0.05% Tween 20; the blocking buffer consisted of the coating buffer and 0.5% casein. Standard ABM stock solutions (10 μg/mL) were prepared in pure methanol and stored at −20 °C. Standard working solutions (0.5, 1, 1.5, 2.5, 4.5, 9.0, 13.5, and 40.5 ng/mL) were made from the stock solution in the assay buffer and stored at 4 °C before use.

Antigens and Antibodies. The antigens were prepared according to the method described by Li and Qian (*18*). Briefly the haptens, 4′-

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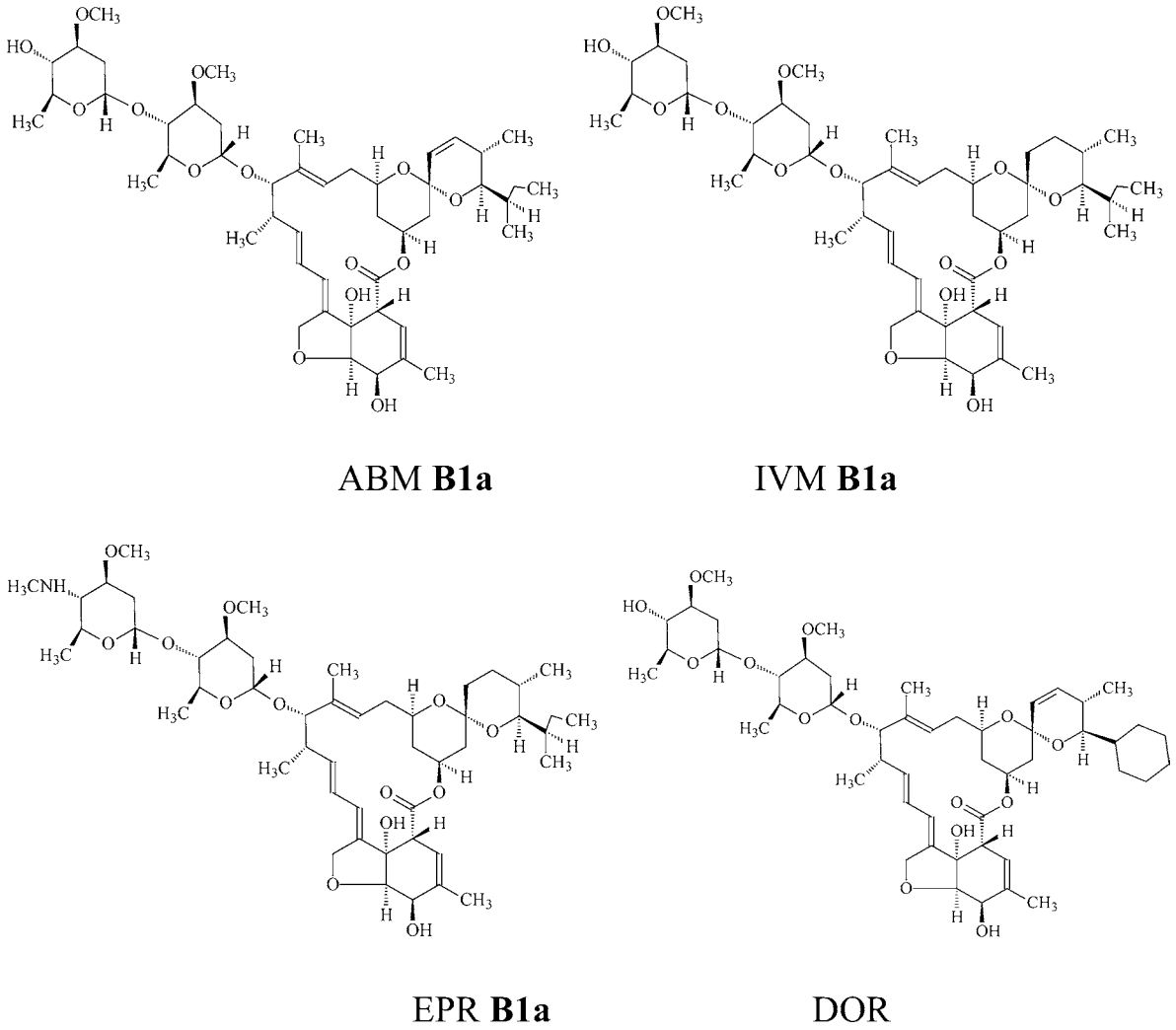

Figure 1. Molecular structures of the main component (B1a) of abamectin (ABM), ivermectin (IVM), doramectin (DOR), and eprinomectin (EPR).

O-succinoyl-ABM, IVM, and DOR were obtained through three steps: (1) protection of the 5-hydroxy group as the *tert*-butyldimethylsily ether; (2) succinoylation of the 4′-hydroxy group using succinic anhydride; and (3) deprotection of the 5-hydroxy group. The 4′-*O*-succinoyl AVMs were conjugated to BSA as immunogens and to OVA as coating antigens by *N*-hydroxy succinimide.

Three groups of female New Zealand white rabbits (5/group) were inoculated subcutaneously with the prepared immunogen and Freund's complete adjuvant. After five inoculations at 3-week intervals, the animals were bled 1 week after the last injection. Antibody titers and cross-reactivity of the anti-sera were determined, and sera were stored at −20 °C until use.

ELISA Development. Optimal dilutions of the coating antigen and rabbit anti-ABM sera were determined using the checkerboard method with serial dilutions of the coating antigen (2.5, 5, and 10 μg/mL) and rabbit sera (1:10^2, 1:10^3, 1:5 × 10^3, 1:10^4, 1:5 × 10^4, 1:10^5, and 1:10^6). The competitive ELISA was a modified method by Shen et al. (*19*). Briefly, 100 μL of coating antigen (ABM-OVA) diluted in the coating buffer was added to each well of a microtiter plate and incubated at 4 °C overnight. Nonspecific binding sites were blocked with the blocking buffer at 37 °C for 2 h after the plates were washed with the washing buffer (three times). Then 100 μL of rabbit sera and 100 μL of standard AVMs in the assay buffer were added to the wells and the plates were incubated at 37 °C for 1 h. After washing, 100 μL of goat-anti-rabbit IgG-horseradish peroxidase was added before the plate was incubated at 37 °C for another hour. The plates were washed, and tetramethylbenzidine (TMB) was added. The plate was incubated at 37 °C for 15 min before the reaction was stopped by 100 μL of 2 M H_2SO_4. The optical density (OD) was measured at 450 nm. The OD values of the control and test wells were designated as B_0 and B. The inhibition was calculated as follows: % inhibition = $B/B_0 \times 100$.

AVMs are lipophilic drugs**,** so organic solvents were added to the buffer to increase the solubility of AVMs. Methanol, acetonitrile, acetone, isopropyl alcohol, and ethanol were tested and the optimal concentrations of these solvents were investigated with 5%, 10%, and 20% (v/v) solvent and 10 ng/mL AVMs.

The assay buffer with various pH (5.4, 6.4, 7.4, 8.4, and 9.4) and ion strengths (0, 0.15, and 0.5 M) were tested to maximize the sensitivity of the assay. Duration of incubations (blocking and antigen/antibody reaction) was also investigated. The optimum condition was evaluated by IC_{50}.

Sample Preparation. Bovine liver homogenate (2 g) in methanol (10 mL) was placed in a 50 mL polypropylene centrifuge tube and vortexed for 0.5 min and then gently mixed for 1 h. After centrifugation at 3000*g* for 10 min, the supernatant was collected and diluted by 20-fold with the assay buffer before being analyzed by the ELISA.

For the determination of recoveries, homogenates of blank bovine liver tissues free from AVMs were spiked with the mixed stock standard solution at 20, 50, and 100 ng/g. The spiked samples (n = 5) were mixed on a vortex mixer for 0.5 min and then allowed to stand at room temperature for 15 min. AVMs were extracted and analyzed as described above.

Analysis of Field Samples. Liver tissues from 10 cattle were purchased from local supermarkets. Each liver sample was divided into two portions: one analyzed by the ELISA and the other by a HPLC method described by Ali et al. (*1*). HPLC method could be used for detection and differentiation of EPR, ABM, IVM, and DOR. The HPLC system consisted of a Waters Model 2695 pump, a 474 scanning

Table 1. Evaluation of Organic Solvents

			inhibition (%)			
		OD value	ABM	IVM	DOR	EPR
acetonitrile	5%	1.582	50	81	87	41
	10%	1.820	54	83	78	38
	20%	2.032	53	99	95	50
methanol	5%	1.569	45	74	73	31
	10%	1.939	36	86	76	33
	20%	1.878	40	97	78	48
acetone	5%	1.586	40	73	78	32
	10%	1.445	41	76	82	37
	20%	1.657	32	82	86	47
isopropyl alcohol	5%	1.853	57	79	86	38
	10%	2.067	55	75	84	45
	20%	2.356	58	79	85	52
Ethanol	5%	1.613	55	66	63	30
	10%	1.873	54	65	71	45
	20%	2.128	57	77	89	51

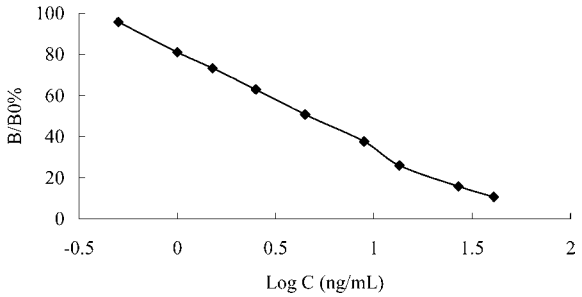

Figure 2. Standard curve of ABM by ELISA.

fluorescence detector (Waters Co., Milford, MA), and an Inertsil ODS reversed column (5 μm, 4.6 mm × 250 mm i.d., GL Sciences Inc., Tokyo, Japan). The HPLC conditions were as follows: flow rate, 1.0 mL/min; injection volume, 40 μL; column temperature, 30 °C; mobile phase, methanol:water (98:2, v/v); detector excited wavelength, 365 nm; emission wavelength, 475 nm.

RESULTS AND DISCUSSION

Polyclonal Antibody (PAb) Preparation and Characterization. AVMs have three hydroxy groups, which could be modified to conjugate with a carrier protein, but only at the 4′-OH, antibodies against AVMs could be obtained (*20*). In this study, the 4′-OH group of ABM, IVM, and DOR was modified for the production of anti-AVM PAbs. The sera from three groups of rabbits immunized with protein conjugated ABM, IVM, and DOR showed high titers by the homologous ELISA. One of the sera against ABM, which have the highest specificity and sensitivity, was chosen to determine cross-reactivity with the AVMs and develop the ELISA method. The cross-reactivity of the anti-sera was 100% for ABM, 145.4% for EPR, 25.0% for IVM, and 12.3% for DOR.

Assay Condition Optimization. Since AVMs are highly lipophilic, a water-miscible organic solvent in the assay buffer is necessary to dissolve the AVMs. Different concentrations (5%, 10%, and 20%, v/v) of methanol, acetonitrile, acetone, isopropyl alcohol, and ethanol were tested (**Table 1**). High inhibition was obtained with methanol, acetonitrile, and isopropyl alcohol. Because organic solvents may interfere with antigen−antibody binding, the lowest concentration of organic solvents with adequate AVM solubility was selected. Based on the IC_{50} values, methanol at 10% was selected for the assay.

In the determination of optimal pH of the assay buffer, IC_{50} values of 62.3, 12.3, 10.9, 9.5, and 8.6 ng/mL were obtained with pH values 5.4, 6.4, 7.4, 8.4, and 9.4, respectively. The results indicated that the assay was more sensitive under alkaline conditions than under slightly acidic conditions. Since AVMs are not stable in highly alkaline conditions, the optimal pH of the assay buffer was set at 8.4.

IC_{50} values of 11.4, 10.8, and 6.9 ng/mL were obtained with NaCl concentrations of 0, 0.15, and 0.5 M in the assay buffer, respectively. Thus, 0.5 M NaCl was added to the assay buffer.

Incubation with the blocking buffer for 30, 60, and 120 min resulted in IC_{50} values of 11.2, 12.4, and 8.6 ng/mL, respectively. Similar inhibition (7.5−8.7 ng/mL) obtained antigen/antibody reaction times of 30, 60, and 90 min. Based on the test results and time consideration, 2 h blocking and 30 min antigen/antibody reaction were selected for the ELISA.

The ELISA was performed under the above-selected conditions to detect AVMs residues in bovine liver. The standard curve for ABM at 1.06−21.9 ng/mL was linear (**Figure 2**), with $R^2 = 0.9968$. The IC_{50} value was 4.8 ng/mL, and the limit of quantitation was 1.06 ng/mL.

Sample Extraction. In general, residues in samples should be diluted or purified to eliminate the matrix effect for determination by ELISA. Li and Qian froze samples to eliminate fat before the extract was diluted for analysis by ELISA (*13*), Mitsui et al. diluted the serum samples before analysis of IVM residues by ELISA (*12*), and Crooks and co-workers used liquid−liquid extraction to eliminate the matrix effect (*15*). In this study, we investigated the effect of extract dilution on the sensitivity of the ELISA. An IC_{50} of 4.8 ng/mL was obtained in the standard curve when the sample extract was diluted by 20-fold before ELISA analysis.

Recoveries and Variations. Recoveries and coefficients of variation (CV) are shown in **Table 2**. When AVMs were fortified at levels of 20, 50, and 100 ng/g, inter-assay recoveries of ABM ranged from 53.8 to 70.1% with CVs of 4.1−12.7%, recoveries of IVM ranged from 57.6 to 65.4% with CVs of 3.4−17.9%, and recoveries of EPR ranged from 68.7% to 80.4%

Table 2. Recoveries of Abamectin (ABM), Ivermectin (IVM) and Eprinomectin (EPR) from Spiked Samples Using ELISA ($n = 5$)

	inter-assay			intra-assay		
	spiked level (ng/g)	recovery (%)	CV (%)	spiked level (ng/g)	recovery (%)	CV (%)
ABM	100	64.5	9.8	100	57.3	11.7
	50	70.1	12.7	50	80.6	6.9
	20	53.8	4.1	20	65.4	6.6
IVM	100	61.91	3.4	100	70.9	14.7
	50	65.4	13.1	50	56.3	5.5
	20	57.6	17.9	20	78.5	8.8
EPR	100	79.4	3.7	100	69.1	7.5
	50	68.7	6.3	50	79.5	11.4
	20	80.4	6.9	20	63.2	7.1

Table 3. Recoveries of ABM, IVM, and EPR from Spiked Samples Using HPLC ($n = 5$)

	spiked level (ng/g)	recovery (%)	CV (%)
ABM	100	103.8	12.1
	50	87.4	14.7
	20	86.3	8.9
IVM	100	89.7	10.8
	50	83.2	8.4
	20	101.5	6.3
EPR	100	87.2	9.3
	50	86.9	6.9
	20	95.2	7.4

with CVs of 3.7−6.9%. Intra-assay CVs for ABM, IVM, and EPR were 6.6−11.7%, 5.5−14.7%, and 7.1−11.4%, respectively. Because of the low cross-reactivity of the PAb with IVM (25.0%), the measured concentration for IVM by the ELISA should be multiplied by a factor of 4. Since the cross-reactivity of the PAb to DOR was low (12.3%), the ELISA method here was not recommended to detect the DOR residue. The fortified samples were also detected by the HPLC method, and the results are shown in **Table 3**.

Analysis of Field Samples. Ten samples purchased from the local market were analyzed. ABM and IVM were not detected in these samples, but EPR was found in three samples. Because the ELISA method by itself could not differentiate the avermectin analogues, the findings should be confirmed by HPLC method. EPR concentrations measured by the ELISA were higher than those by the HPLC method by 20−36% (342.8, 441.6, and 992.0 ng/g by ELISA cf. 252.2, 367.7, and 751.6 ng/g by HPLC, respectively). The higher levels detected by ELISA were probably due to the high cross-reactivity of the PAb with EPR (145.4%). The EPR concentrations were below the MRL (2 μg/g) recommended by WHO.

Conclusion. The anti-AVM PAb had good cross-reactivities with ABM, EPR, and IVM. The PAb-based ELISA was sensitive and accurate, with a limit of quantitation of 1.06 ng/mL for the three AVMs. Analysis results of the ELISA method were consistent with those by a previously described HPLC method.

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