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An Enzyme-Linked Immunosorbent Assay for Determination of Dicyclanil in Animal Tissue

Xiaolin Hou [a], Shoujun Cao [b], Yan Lu [a], Yinliang Wu [c], Ross C. Beier [d] & Yingjian Sun [a]

[a] College of Animal Science and Technology, Beijing University of Agriculture, Beijing, China

[b] Department of Animal Science, Beijing Vocational College of Agriculture, Beijing, China

[c] Ningbo Academy of Agricultural Sciences, Ningbo, China

[d] USDA, Agricultural Research Service, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit, College Station, Texas, USA

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Immunoassay

AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETERMINATION OF DICYCLANIL IN ANIMAL TISSUE

Xiaolin Hou,[1] Shoujun Cao,[2] Yan Lu,[1] Yinliang Wu,[3] Ross C. Beier,[4] and Yingjian Sun[1]

[1]*College of Animal Science and Technology, Beijing University of Agriculture, Beijing, China*

[2]*Department of Animal Science, Beijing Vocational College of Agriculture, Beijing, China*

[3]*Ningbo Academy of Agricultural Sciences, Ningbo, China*

[4]*USDA, Agricultural Research Service, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit, College Station, Texas, USA*

Dicyclanil is a toxic, pyrimidine-derived insect growth regulator used in veterinary medicine for the prevention of myiasis. A competitive indirect enzyme-linked immunosorbent assay was developed firstly for the determination of dicyclanil in animal tissue. The antigen was prepared using the glutaraldehyde method and polyclonal antibodies were obtained by immunizing rabbits with a dicyclanil-bovine serum albumin conjugate. The antibody obtained was specific for dicyclanil with an IC_{50} of 9 ng/mL. Recoveries from sheep tissue and liquid milk were in the range of 52.5–72.4%, with coefficients of variation between 4.7% and 11.9%. The results were further confirmed by high performance liquid chromatography–tandem mass spectrometry.

Keywords: Animal tissues; Dicyclanil; Enzyme-linked immunosorbent assay; Polyclonal antibodies

INTRODUCTION

Dicyclanil (4,6-diamino-2-(cyclopropylamino)-5-pyrimidinecarbonitrile) is a pyrimidine-derived insect growth regulator, which is used in veterinary medicine for the prevention of myiasis or fly-strike in Europe, the Middle East, and Africa. The target animal species is sheep with a recommended therapeutic dose at 30 to

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Xiaolin Hou and Shoujun Cao contributed equally to this work.

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Address correspondence to Dr. Yingjian Sun, College of Animal Science, Beijing University of Agriculture, 102206, Beijing, P. R. China. E-mail: yjsun51@sina.com

Table 1. JECFA and EMEA approved MRL values for dicyclanil in sheep tissues

Tissue	MRL (CVMP 2000) (μg/kg)	MRL (JECFA 2003) (μg/kg)
Liver	400	125
Kidney	400	125
Muscle	200	150
Fat	50[a]	200

[a]The MRL for fat has been recommended to be increased to 150 μg/kg (CVMP 2000).

100 mg/kg body weight (BW) for one application per season (CVMP 2000). Due to its toxicity, the maximum residue limit for dicyclanil has been defined by JECFA as parent dicyclanil (JECFA 2000; Codex Alimentarius Commission 2011) and the current MRLs for dicyclanil are listed in the Codex Alimentarius as updated in 2011 (Table 1) (Codex Alimentarius Commission 2011). To protect consumers from potential exposure risks and control its application in food animals, it is necessary to monitor dicyclanil residues in food animal tissues.

Several analytical techniques have been developed to detect dicyclanil, such as high-performance liquid chromatography (HPLC) (Fan et al. 2009) and HPLC–MS/MS (China Standardizing Committee 2008; Hou et al. 2013). These instrumental methods are used to confirm the presence of dicyclanil, but they are not practical for screening large numbers of samples, because they require time-consuming extraction, use of complicated equipment, and they are costly. ELISA methods are cost-effective, portable, sensitive, and suitable for screening large numbers of samples. We have developed a competitive indirect enzyme-linked immunosorbent assay (ELISA) to screen for dicyclanil residues in food animal tissues, and then used UPLC–MS/MS to confirm the positive results (Hou et al. 2013).

MATERIALS AND METHODS

Reagents

Dicyclanil, melamine, cyromazine, and sulfamethazine were obtained from the China Institute of Veterinary Drug Control (Beijing, China). Bovine serum albumin (BSA), ovalbumin (OVA), and Freund's adjuvant were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and formic acid of HPLC grade were obtained from Fischer Chemical Limited (Beijing, China). Ultra-pure water was produced from a Millipore-Q Plus water-purification system obtained from the Millipore Corp. (Bedford, MA, USA). Other chemical reagents were all of analytical grade or better and were obtained from Beijing Chemical Company (Beijing, China). The standard stock solutions (1 mg/mL) were prepared by dissolving the standard substance in methanol. The final calibration standards were prepared by dilution of the standard stock solutions in phosphate-buffered saline (PBS). A similar procedure was used to create a dilution series to determine cross-reactivity.

Phosphate-buffered saline (PBS, pH 7.4) consisted of 137 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4 and 2.7 mM KCl. The wash buffer (PBST) was PBS buffer plus 0.05% Tween 20. The coating solution was 0.05 M carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6). The blocking buffer was PBS

containing 5% nonfat milk powder and 0.05% (*v*/*v*) Tween 20. The substrate solution contained 0.02% 3,3′,5,5′-tetramethylbenzidine (TMB) and 0.01% H_2O_2 in 0.1 M sodium citrate buffer (pH 5.0). The stopping solution was 2 M H_2SO_4.

Synthesis of Dicyclanil Conjugates

The synthesis scheme to produce the dicyclanil conjugate is shown in Figure 1. Dicyclanil (20 mg) was dissolved in 2 mL of pyridine, and 50 mg of OVA was dissolved in 5 mL of double distilled water. The dicyclanil solution was slowly added to the OVA solution, and 1 mL of a 1% glutaraldehyde solution was slowly added into the dicyclanil-OVA solution. The dicyclanil-glutaraldehyde-OVA reaction mixture was stirred for 24 h at room temperature. The mixture was dialyzed against a 0.9% NaCl solution for 3 days, with the dialyzing medium changed twice a day to remove unreacted small molecules. Finally, the mixture was centrifuged at 3000 g for 10 min. The supernatant containing the dicyclanil-OVA conjugate was collected and used as the coating antigen. Conjugation was confirmed using a SpectroQuest UV-2800 spectrophotometer (UNICO, Dayton, NJ, USA). The coating antigen was stored at −20°C until use. The immunogen, dicyclanil-BSA, was synthesized in an analogous way as the coating antigen.

Production of Polyclonal Antibodies

Three rabbits were immunized with the dicyclanil–BSA (0.1 mg per animal, calculated as protein) conjugate in Freund's complete adjuvant subcutaneously at multiple sites on the dorsal region. The rabbits were then boosted with dicyclanil–BSA (0.1 mg per animal) in Freund's incomplete adjuvant at 15-day intervals. The rabbits were then exsanguinated seven days following the fifth booster by heart puncture under general anesthetic and then euthanized by lethal injection before recovery. The anti-serum was prepared by allowing the blood to clot overnight at 4°C, followed by centrifugation at 5000 g for 10 min to remove particulate material. Antibody titers and cross-reactivity of the anti-serum were determined, and the obtained crude serum was purified using the saturated ammonium sulfate precipitation method and sodium azide was added as a preservative at a final concentration of 0.02% (*w*/*w*). The purified serum was divided into aliquots and stored at −70°C for later use.

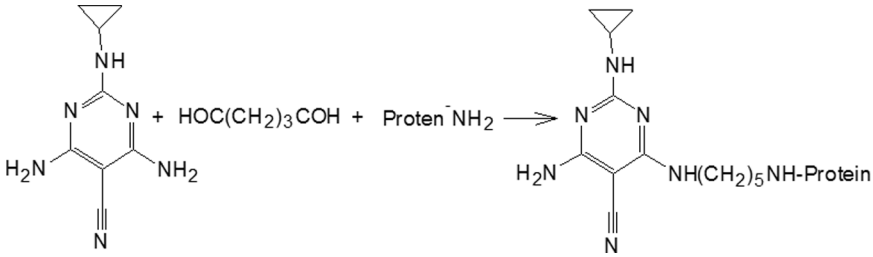


Figure 1. Synthesis scheme and structure of the dicyclanil protein conjugate.

Development of the Indirect ELISA

The checkerboard procedure was used to determine the optimal dilution of coating antigen and antibody used in the indirect ELISA. The microplates were coated with dicyclanil-OVA coating antigen at 1.0, 2.0, and 5.0 ng/mL in the coating solution (100 μL/well) with incubation at 4°C overnight. Plates were washed with PBST three times and blocked with 200 μL/well of blocking buffer for 2 h. The blocked plates were then washed three times with PBST. An antibody dilution series of 1×10^3, 1×10^4, 5×10^4, 1×10^5, 5×10^5, and 1.0×10^6 was added, and the plates were incubated at 37°C for 2 h. The plates were washed three times, and then goat anti-rabbit IgG-HRP (1:4000, 50 μL/well) was added, followed by incubation at 37°C for 2 h. The plates were washed three times with PBST, TMB substrate solution was added (100 μL/well), and the plates were incubated at 37°C for 30 min. The color development was stopped by adding 100 μL/well of 2 M H_2SO_4, and the absorbance was measured at 450 nm.

Development of the Indirect Competition ELISA

The procedure was identical with the aforementioned indirect ELISA except for the addition of a competition step after adding the coating antigen and blocking with blocking buffer. A competitive curve was obtained by plotting the normalized signal B/B_0 against the logarithm of analyte concentration, where B_0 is the signal without analyte and B is the dicyclanil signal of each sample or standard.

The limit of detection (LOD) was expressed as the lowest amount of dicyclanil in a sample that was detected, which was based on the mean value of six blank samples plus three times the mean standard deviation. The limit of quantification (LOQ) was the lowest amount of dicyclanil in a sample that was quantified with an acceptable precision and recovery, which was based on the mean value of six blank samples plus ten times the mean standard deviation. The accuracy and precision of the method were represented by the recovery and coefficient of variation (CV), respectively. The precision of the ELISA method was obtained by analyzing five replicates at each spiked level.

Antibody Specificity and Cross-Reactivity

The specificity of the antibody was obtained from cross-reactivity studies with structurally related compounds to dicyclanil, including melamine, cyromazine, and sulfamethazine. The IC_{50} value for each evaluated compound was based on its inhibition curve.

The cross-reactivity (CR) values were calculated according to the following equation:

$$CR = IC_{50}(\text{dicyclanil})/IC_{50}(\text{Analogs}) \times 100\%$$

where CR values used IC_{50} values with units of ng/mL and CR_{molar} values used IC_{50} values with units of nmol/mL (Xu et al. 2009).

Sample Preparation

Fresh milk (1 mL) was extracted with 9 mL of PBS-methanol (8:2, *v*/*v*) and 5 mL of hexane in a KQ-500E ultrasonic bath (Kunshan Ultrasonic Instrument Co., Ltd, Jiangsu, China) at 40 kHz and 500 watt for 10 min. The mixture was centrifuged at 8000 g for 5 min at 4°C, and the lower layer was used for direct analysis. For sheep tissues (liver, kidney, or muscle), a 1 g homogenized tissue sample was extracted with 5 mL of PBS-methanol (2:8, *v*/*v*) and 4 mL of hexane. The mixture was sonicated for 10 min, and then centrifuged at 4000 g for 5 min. The lower layer (1 mL) was diluted to 4 mL with PBS and used for analysis. Dicyclanil was fortified at concentrations of 0.05, 0.1, and 0.4 mg/kg, respectively, with each matrix analyzed to validate the analysis performance. The samples were then treated as previously described to estimate the accuracy, repeatability, and sensitivity.

Validation of the ELISA Method by UPLC–MS/MS

The dicyclanil-fortified samples were confirmed by UPLC–MS/MS to validate the immunoassay (Hou et al. 2013). The fortified sample was extracted with 1% trichloracetic acid in water-acetontrile, cleaned up using hexane and MCX cartridges, and analyzed by UPLC-MS/MS with electrospray ionization in the positive-ion mode (ESI^+) (Hou et al. 2013).

RESULTS AND DISCUSSION

Complete Antigen Synthesis

The appropriate immunogen was required for development of an ELISA method for the detection of dicyclanil. No method was available for the synthesis of the complete antigen for dicyclanil. In this study, dicyclanil was used as the hapten due to its unique structural features of having two amino groups on a pyrimidine ring, and it was coupled to BSA or OVA using glutaraldehyde as the linker. During the synthesis, the reaction was carried out with an equal number of moles of dicyclanil and glutaraldehyde. The glutaraldehyde solution was slowly added to the dicyclanil-OVA (BSA) mixture, which allowed the dicyclanil molecule to primarily couple with only one glutaraldehyde molecule. The UV spectrum of the product demonstrated that the conjugation was successful (Figure 2). The dicyclanil-OVA conjugate was used as the plate coating antigen. Similar results were obtained for dicyclanil-BSA, and this conjugate was used to immunize rabbits to generate a polyclonal antibody to dicyclanil. The hapten-protein conjugation ratio was 24:1 and 22:1, respectively, for BSA and OVA, by calculating UV absorbance at 240 and 280 nm.

ELISA Analysis

Three New Zealand white rabbits were immunized five times and produced specific antibodies to dicyclanil. Furthermore, the polyclonal antibodies were isolated to improve sensitivity of the antibodies. All three rabbits produced specific antibodies to dicyclanil and the most sensitive antibody was selected for further experiments.

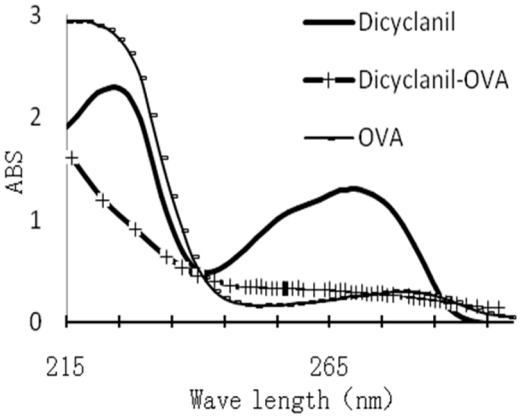


Figure 2. The UV scanning spectrum diagrams of dicyclanil, OVA, and dicyclanil-OVA.

The developed ELISA is a semi-quantitative method, and is the first developed ELISA method for the detection of dicyclanil in food animal tissues. After the crude serum was purified by the saturated ammonium sulfate precipitation method, the antibody concentration was 22 mg/mL as determined by UV-spectrometry. The optimal dilutions for the coating antigen and the selected antibody were 1.0 ng/mL and 1:20,000, respectively. The antibody showed high sensitivity to dicyclanil with a 50% of inhibition concentration (IC_{50}) at 9 ng/mL; the calibration inhibitory curve was $y = 96.32 - 49.23 \log x$ ($R^2 = 0.9712$) (Figure 3). The corresponding quantitative range was 2–35 ng/mL based on a lower and higher inhibition cut-off of 20% and 80%, respectively.

Dicyclanil has been previously extracted using acetonitrile (China Standardizing Committee 2008) and 1% trichloracetic acid in water-acetontrile (Hou et al. 2013). However, organic solutions and highly acid solutions are inappropriate for ELISA analysis. In this study, PBS-methanol was used to extract dicyclanil. The IC_{50} for a series of PBS-methanol solutions was evaluated to determine the effect of methanol, and when the methanol concentration decreased to 20% in PBS, the interference to the

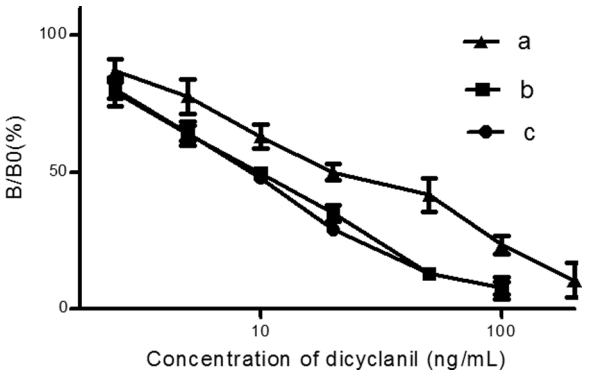


Figure 3. Comparison of matrix effects: (a) extraction of liver without hexane, (b) extraction of liver with hexane, and (c) dicyclanil in buffer.

ELISA analysis was small. To simplify sample pre-treatment, the extract was diluted with PBS to further decrease the methanol concentration before analysis.

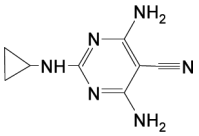
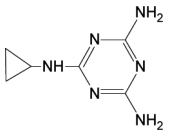
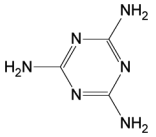
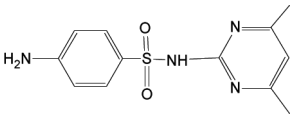
The limit of detection was 8 μg/kg and 7 μg/kg for sheep tissue and milk, respectively. The limit of quantitation (LOQ) was 25 μg/kg for sheep tissue and 20 μg/kg for milk. The antibody CR result toward the tested compounds showed high specificity for dicyclanil (Table 2).

The recoveries from spiked blank samples were determined by the ELISA method and are listed in Table 3 and Table 4. At the three spiked levels of 50, 100, and 400 μg/kg, the recoveries ranged from 52.5–72.4%, with CVs between 4.7% and 11.9%.

Validation of the ELISA Method with UPLC–MS/MS

To evaluate the quality of the developed ELISA, dicyclanil-fortified samples were analyzed by UPLC–MS/MS (Hou et al. 2013). The intra-assay recoveries of UPLC–MS/MS analysis were from 61.4% to 90.0%, with CVs of 4.0% to 12.5% (Table 3). The intra-assay results from the UPLC–MS/MS and ELISA methods correlated well, indicating the reliability of the developed ELISA method. The ELISA method correlated well with UPLC–MS/MS, and the ELISA method is simpler and faster, and can be used as a screening method for a large amount of samples.

Table 2. Cross-reactivity (CR) of the antibody to related compounds

Compound	Structure	IC_{50} (ng/mL)	Mol. wt.	CR (%)	CR_{molar} (%)[a]
dicyclanil		9	190.21	100	100
cyromazine		330	166.19	8	7
melamine		>1000	126.12	<1	<1
sulfamethazine		>1000	278.33	<1	<1
BSA		>1000	–	<1	<1

[a]Molar cross-reactivity (CR_{molar}) was calculated using units of nmol/mL for the IC_{50} concentrations of the chemicals (Xu et al. 2009).

Table 3. Intra-Assay performance for the ELISA and LC–MS/MS ($n = 5$) (%)

	Added (μg/kg)	Muscle		Liver		Kidney		Liquid milk	
		MR	CV	MR	CV	MR	CV	MR	CV
ELISA	50	52.5	6.7	61.1	5.4	60.2	11.9	72.4	9.2
	100	60.8	5.1	65.5	5.9	61.4	5.8	64.1	6.8
	400	62.4	4.7	67.8	8.7	67.2	6.6	70.5	8.7
LC–MS/MS	50	82.0	6.8	81.0	4.4	74.0	10.8	63.4	4.0
	100	82.4	7.1	90.0	10.9	82.5	4.7	65.9	12.5
	400	85.8	5.7	79.1	6.4	81.8	10.0	61.4	11.8

MR, Mean recovery; CV, coefficients of variation.

Table 4. Inter-assay performance for the ELISA ($n = 3$) (%)

Added (μg/kg)	Muscle		Liver		Kidney		Liquid milk	
	MR	CV	MR	CV	MR	CV	MR	CV
50	58.7	9.2	55.6	8.4	62.9	8.9	64.7	10.1
100	58.4	7.2	63.7	5.5	66.4	6.6	61.6	9.2
400	62.7	2.7	66.7	7.1	66.8	9.5	71.3	6.4

Analysis of Field Samples

Ten sheep liver samples purchased from a local market were analyzed for dicyclanil by the ELISA method. Dicyclanil was found in only one of the samples. The result was confirmed by the UPLC–MS/MS method (Hou et al. 2013). The dicyclanil concentration measured by ELISA was 87 μg/kg and by UPLC–MS/MS was 95 μg/kg. The positive sample was safe for consumers because its dicyclanil residue level was below the MRL.

CONCLUSIONS

An anti-dicyclanil polyclonal antibody was prepared with high specificity. The corresponding developed ELISA was fast, sensitive, and simple; furthermore, the ELISA method was confirmed by UPLC–MS/MS. The ELISA method can be used for field screening of dicyclanil residues in milk and animal tissues.

REFERENCES

China Standardizing Committee. 2008. Determination of residue of dicyclanil in foodstuffs of animal original for import and export LC–MS/MS method. The people's Republic of China Entry-Exit Inspection and Quarantine Standard. SN/T 2153–2008 pp.

Codex Alimentarius Commission. 2011. *Maximum residue limits for veterinary drugs in foods*, updated at the 34th Session of the Codex Alimentarius Commission (July, 2011). http://www. (accessed April 19, 2012). Codex alimentarius. net/vetdrugs/data/MRL2_e_2011.pdf

CVMP (Committee for Veterinary Medicinal Products). 2000. Dicyclanil, EMEA/MRL/739/00-FINAL. The European Agency for the Evaluation of Medicinal Products, Veterinary

Medicines and Information Technology Unit. (accessed April 11, 2012). http://www.ema.europa.eu/pdfs/vet/mrls/073900en.pdf

Fan, Y.-M., X.-J. Yu, X.-J. Gu, J.-Y. Yin, Y.-J. Qiu, S.-B. Chen, J. Chen, et al. 2009. Determination of cyromazine and dicyclanil residues on greasy wool by high performance liquid chromatography. *Chin. J. Anal. Chem.* 1: 113–116.

Hou, X. D., Zhou, W. Huai, R. C. Beier, Y. Sun, Y. Lu, G. Wu, Z. Sun, and Y. Wu. 2013. Simultaneous determination of cyromazine and dicyclanil in animal edible tissues using UPLC–MS/MS. *Food Addit. Contam. Part A*. (In press) (http://dx.doi.org/10.1080/19440049.2012.760207)

JECFA (Joint FAO/ WHO Expert Committee on Food Additives). 2000. *Toxicological Evaluation of Certain Veterinary Drug Residues in Food,* 54th meeting of the JECFA, International Program on Chemical Safety, WHO Food Additive Series 45, Geneva: World Health Organization, pp. 75–89.

JECFA (Joint FAO/ WHO Expert Committee on Food Additives). 2003. Residues of some veterinary drugs in foods and animals. (accessed August 11, 2012). http://www.fao.org/ag/agn/jecfa-vetdrugs/details.html?substance=361

Xu, Z.-L., Y.-D. Shen, R. C. Beier, J.-Y. Yang, H.-T. Lei, H. Wang, and Y.-M. Sun. 2009. Application of computer-assisted molecular modeling for immunoassay of low molecular weight food contaminants: A review. *Anal. Chim. Acta* 647: 125–136.