ARTICLES

Development of an Indirect Competitive ELISA for Ciprofloxacin Residues in Food Animal Edible Tissues

Jiuhua Duan and Zonghui Yuan*

Institute of Veterinary Pharmaceuticals, Huazhong (Central China) Agricultural University, Wuhan, Hubei 430070, People's Republic of China

An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed to detect ciprofloxacin (CPFX) in food animal edible tissues. CPFX was converted by an active ester method into conjugates CPFX−bovine serum albumin (CPFX−BSA) and CPFX−human serum albumin (CPFX−HSA), which both allowed production of CPFX−specific rabbit antisera. In the ELISA, CPFX−HSA was coated onto the microtiter plate, followed by incubation with standard CPFX and anti-CPFX antibody. The indirect competitive ELISA revealed that the antisera have no cross-reactivity with penicillin, gentamicin, neomycin, sulfadiazine, and chlortetracycline. The antisera cross-reacted with enrofloxacin and norfloxacin about 69.8 and 44.6% as much as they did with CPFX. This ELISA was highly sensitive (0.32 ng/mL) to CPFX determination. Recovery of CPFX at 40 μg/kg was 75.58% in pork, 81.29% in chicken, and 84.30% in milk. The coefficients of variation varied from 3.7 to 9.2% over the range of CPFX concentrations studied. The linear detection range was between 1.6 and 1000 ng/mL. The results suggest that this ELISA is a specific, accurate, and convenient method for the detection of CPFX residues in food animal edible tissues.

Keywords: *Ciprofloxacin; ELISA; food animal; residues*

INTRODUCTION

Ciprofloxacin (CPFX) is a synthetic antibacterial agent that belongs to the fluoroquinolone group. It has important applications in veterinary medicine for the treatment of bacterial infection (*1*). With increasing use, the CPFX residues in animal edible tissues could cause serious public health problems. An EC Regulation has set a maximal residue limit (MRL) of 30 μg/kg for the sum of CPFX and enrofloxacin in animal edible tissues (*2*). So far, several methods have been described for the detection of CPFX in animal edible tissues, including HPLCs and microbiological methods (*3*−*5*). Those systems are time-consuming and require extensive sample cleanup. Even the HPLCs had detection limits of >10 ng/mL (*5*−*7*). Recently, enzyme-linked immunosorbent assays (ELISAs) have been developed due to the increasing interest in the identification or quantification of various drug residues in biological samples (*8*). The most common metabolite of enrofloxacin in food animals is CPFX, so our interests were concentrated on the CPFX residue determination. The procedure presented here employed an indirect competitive ELISA system to determine CPFX residues in food animal edible tissues.

MATERIALS AND METHODS

Instruments. ELISAs were carried out in 96-well polystyrene NUNC microtiter plates (Maxisorp) and measured with enzyme immunoassay reader model DG3022A (Nanjing Electric Apparatus Co.). Centrifugation was performed with centrifuge model LD5-2A (Beijing Medical Centrifuge Co.). The UV−vis spectra of conjugates were obtained by UV−vis scanning spectrophotometer UV-265FW (Shimadzu Co.). The reaction mixture was dialyzed with Spectrapore membrane tubing (Spectrum Medical Industries Inc.). The reaction mixture was shaken by a shaking incubator model THZ-C (Jiangshu Laboratory Apparatus Co.).

Chemicals. 1-Ethyl-3[(3-dimethylamino)propyl]carbodiimide methiodide (EDC), *N*-hydroxysuccinimide (NHS), human serum albumin (HSA), 3,3′,5,5′-tetramethylbenzidine (TMB), goat anti-rabbit IgG−horseradish peroxidase, incomplete Freund's adjuvant (IFA), and complete Freund's adjuvant (CFA) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was provided by Military Medical Institute (Beijing, China). Ovalbumin (OVA) was provided by Sino-America Biotec Co. CPFX, norfloxacin, enrofloxacin, penicillin, neomycin, gentamicin, chlortetracycline, and sulfadiazine were provided by National Control Institute of Veterinary Bioproducts and Pharmaceuticals (Beijing, China). Alu-Gel-S was provided by Veterinary College (Huazhong Agricultural University, China), Dimethylformamide (DMF) was provided by Beijing Chemical Co. All other chemicals and organic solvents used were of chemical grade.

Preparation of Ciprofloxacin Conjugates. The antigens CPFX−BSA and CPFX−HSA were prepared according to an active ester method (*2*). In this procedure, 38.6 mg of CPFX, 206.4 mg of EDC, and 57.6 mg of NHS were added to 2.3 mL of DMF and incubated for 24 h at room temperature in the dark (solution 1). BSA (264 mg) was mixed with 23 mL of pH 7.0, 0.01 M PBS (solution 2). Solution 1 was added into solution 2 slowly and shaken (120 rpm) for 3 h at room temperature. The reaction mixture was dialyzed with Spectrapore membrane tubing against PBS for 6 days with 18 changes of the PBS solution during this period to remove free CPFX. The

* Author to whom correspondence should be addressed (telephone 0086 27 8728 0918; fax 0086 27 8728 5852; e-mail yuan5802@public.wh.hb.cn).

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solution was lyophilized and dialyzed, and the white conjugates were stored in a desiccator at −20 °C. A CPFX−HSA conjugate was also prepared similarly to CPFX−BSA. An $FeC1_3$ colorimetry and UV absorbance method were employed to determine whether the linking had been a success.

Antisera Production. As adjuvants Alu-Gel-S and CFA were used; 8 New Zealand male white rabbits (2 kg size) were immunized with CPFX antigen, using a multiple site injection method. Eight rabbits were grouped according to immunogen (CPFX−BSA and CPFX−HSA), initial dose (25 and 500 μg), and immunization interval (1 week and 2 weeks). Two milliliters of emulsion was made by mixing various doses of immunogen in 0.5 mL of normal saline, 0.5 mL of Alu-Gel-S, and 1 mL of CFA. About 0.05 mL of emulsion was injected intradermally at each site over the shaved area, and 0.6 mL of emulsion was injected subcutaneously on each shoulder. After the initial injection, the animals were boosted at 1 or 2 week intervals with immunogen in IFA emulsion, until a satisfactory titer was obtained. Blood was collected 7−10 days after every immunization, the antibody titer determined, and a portion of the antisera stored at −20 °C. The remainder was purified by ammonium sulfate precipitation, and the precipitate was dissolved in normal saline to the original serum volume. The precipitate was lyophilized and stored at −4 °C in 1 mL aliquots.

Indirect Competitive ELISA Procedure. The ELISA protocol applied to test the ability of CPFX−HSA (coating antigen) to recognize antibodies produced against CPFX−BSA (immunogen) was as follows: The concentration of coating antigen was optimized to microtiter plates in carbonate buffer (0.1 M, pH 9.6) and incubated overnight at 4 °C. The optimal dilution of coating antigen was determined by the checkerboard test. Plates were washed four times with PBST, and 0.25 mL of 2% OVA in PBS was added to each well to eliminate nonspecific binding by blocking the plastic surface where protein was not bound. After 2 h of incubation, plates were washed four times, and 1:1000 antisera and varying concentrations of standard CPFX (each 0.05 mL) were added. Shaking was performed at a speed of 200 rpm for 2 h at 37 °C, and 0.1 mL/well of goat anti-rabbit IgG−HRP peroxidase (1:1000) in PBST was added and incubated for 3 h at 37 °C. Plates were washed five times, and TMB substrate solution was added, followed by the addition of stopping solution (2 M H_2SO_4) after 30 min of incubation in the dark at 25 °C; absorbance at 450 nm was determined by an enzyme immunoassay reader. Percent binding was calculated from the absorbance obtained in the absence (b_0) and presence (b) of CPFX in standards. A linear dose−response standard curve was prepared by plotting log[CPFX] versus percent binding. Absorbance difference between presera and antisera was compared to determine titer (*9*). The antibody titer was defined as the reciprocal of the antisera dilution that at 450 nm gives an absorbance 0.1 greater than that of presera (*10*). In addition, the difference in absorbance between CPFX−HSA and HSA (negative control) coated plates (*11*) was compared, and PBST was used as a buffer negative control. All of these negative controls were considered to decrease the interference of the ELISA procedure.

Sensitivity of the Assay. The detection limit was defined as the lowest concentration of CPFX giving an absorbant value 3 times more than the blank sample control.

Precision of the Assay. The precision of the ELISA was analyzed by repeated determination of samples. Intra-assay and interassay coefficients of variation (CV) for samples of the absorbance at three different CPFX concentrations were obtained.

Specificity. Two different quinolone analogues and other antimicrobials were assessed for cross-reactivity with anti-CPFX antibody. Cross-reactivity was defined as (nanomoles of CPFX for 50% binding/nanomoles of other competitors for 50% binding) × 100 (*12*, *13*).

Recovery of CPFX. Given amounts of CPFX were added in samples and assayed in quadruplicate. In the recovery experiment, milk, chicken, and pork were homogenized with PBS at 5000 rpm and bathed in water at 84 °C for 5 min. After cooling to room temperature, solutions were centrifuged at 8000 rpm to remove the tissues. The acidic extracts were adjusted to pH 7.0 by NaOH, and the supernatant liquid was immediately used in ELISA.

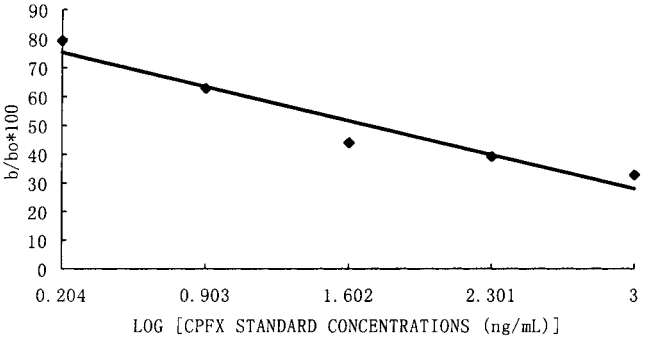

Figure 1. Semilog graph of indirect competitive ELISA standard curve for CPFX using standards.

Table 1. Intra-assay and Interassay Variability of ELISA Standard for CPFX

CPFX added (ng/mL)	intra-assay		interassay	
	% binding[a]	CV %	% binding[a]	CV %
1.6	79.6 ± 5.0	6.3	84.5 ± 7.8	9.2
40.0	44.2 ± 2.6	5.9	42.6 ± 3.3	7.8
1000	32.7 ± 1.2	3.7	33.4 ± 2.3	6.9

[a] Mean ± SD.

RESULTS

Five months after the initial immunization, both the rabbits immunized with CPFX−BSA and those immunized with CPFX−HSA immunogen produced antibodies and their titer reached 1:32000. For convenience, our efforts were concentrated on the ELISA based on the CPFX−BSA antibody. In the indirect competitive ELISA protocol, our research showed that in order to be in the linear portion of the response curve, which requires an absorbance reading of ∼1.0, a coating antigen concentration of 100 ng/mL and an antiserum dilution of 1:1000 were needed. These conditions were fixed for the rest of the experiment.

The assay had a detection limit of 0.32 ng/mL and a linear range of 1.6−1000 ng/mL at a 50% inhibition of ∼50 ng/mL. The standard curve is seen in Figure 1. The test was repeated three times with four replicates per concentration, and coefficients of variation for the intra-assay were in the range of 3.7−6.3%, whereas those of the interassay were 6.9−9.2% (Table 1).

Antibody specificity was determined by the indirect competitive ELISA in which CPFX competitors were presented in the assay to compete the binding of CPFX (*14*). In the assay, two quinolone derivatives, enrofloxacin and norfloxacin, had 69.8 and 44.6% cross-reactivity, respectively. The CPFX−BSA competitor gave 538.2% cross-reactivity, which was acceptable because the antibodies used in this system had been immunized by CPFX−BSA. Other antimicrobials showed no more than 0.01% cross-reactivity (Table 2). The results indicated that the antibody produced had high affinity for CPFX.

The recovery of middle concentration (40 μg/kg) was 75.58% in pork, 81.29% in chicken, and 84.30% in milk. The extraction used here showed adequate and reproducible recovery of CPFX.

DISCUSSION

The ability to obtain high-affinity antisera is critical for an ELISA procedure. In producing antibody to

Table 2. Cross-Reactivity of Anti-CPFX Antibody toward Some Competitors

competitor	cross-reactivity %	competitor	cross-reactivity %
CPFX	100	penicillin	<0.01
norfloxacin	44.6	neomycin	<0.01
enrofloxacin	69.8	gentamicin	<0.01
CPFX−BSA	538.2	chlortetracycline	<0.01
		sulfadiazine	<0.01

CPFX, an active ester method was employed to couple CPFX with carrier protein BSA or HSA. Because CPFX is a small molecule that cannot be used as an immunogen directly, EDC was used as a linking agent to couple the carboxyl group in the CPFX molecule structure with free amino groups on the protein BSA or HSA. The conjugates were assayed by an $FeCl_3$ colorimetry and a UV−vis scanning method. The color reaction showed that at the final dialysis, the CPFX concentration in the Spectrapore membrane tubing was significantly higher than that out of it. UV absorbance assay indicated an absorbance at 271 nm for CPFX−BSA, whereas the BSA had an absorbance at 275 nm. In addition, we used SDS-PAGE to distinguish between BSA and CPFX−BSA, but that resulted in failure. We suppose that the migration pattern of the conjugate band does not differ significantly from that of carrier BSA alone by SDS-PAGE, which separates on the basis of size (*15*). Our experiment indicated that in the immunization schedule, the proteins BSA and HSA were excellent carriers. Mixing Alu-Gel-S with CPFX−BSA or CPFX−HSA conjugate and emulsifying with Freund's adjuvant at the same volume not only improved the nonspecific immunization effect but also slowed the decomposing process of the immunogen in the animal body. We divided the rabbits into several groups to see how the changes of immunogen dose during the overall protocol and the interval between every two injections affected the immunization effect. No significant difference was observed.

In the ELISA procedure, we used CPFX−HSA rather than CPFX−BSA as coating antigen to avoid the binding of carrier BSA to antibody against CPFX. A saturate binding and a competitive binding were also used to compare, but no significant difference was found.

This is the first report of an ELISA for the detection of CPFX in biological samples. In conclusion, we have developed a sensitive, specific, and convenient ELISA procedure for the routine screening of CPFX residues in food animal edible tissues.

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LITERATURE CITED

(1) Vancutsem, P. M.; Babish, J. G.; Schwark, W. S. The fluoroquinolone antimicrobials: structure, antimicrobial activity, pharmacokinetics clinical use in domestic animal and toxicity. *Cornell Vet.* **1990**, *80*, 173−186.

(2) Hammer, P.; Heeschen, W. Antibody-captured immunoassay for the detection of enrofloxacin in raw milk. *Milchwissenschaft* **1995**, *50*, 513−514.

(3) Walker, R. D.; Stein, G. E.; Hauptman, J. G.; MacDonald, K. H.; Budsberg, S. C.; Rosser, E. J. Serum and tissue cage fluid concentrations of ciprofloxacin after oral administration of the drug to healthy dogs. *Am. J. Vet. Res.* **1990**, *51*, 896−900.

(4) Aramayona, J. J.; Mora, J.; Fraile, L. J.; Garcia, M. A.; Abadia, A. R.; Bregante, M. A. Penetration of enrofloxacin and ciprofloxacin into brease milk, and pharmacokinetics of the drugs in lactating rabbits and neonatal offspring. *Am. J. Vet. Res.* **1996**, *57*, 547−553.

(5) Mengozzi, G.; Intorre, L.; Bertini, S.; Soldani, G. Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and intramuscular administrations in sheep. *Am. J. Vet. Res.* **1996**, *57*, 1040−1043.

(6) Kung, K.; Riond, J. L.; Wanner, M. Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and oral administrations of the drug. *J. Vet. Pharmacol. Ther.* **1993**, *16*, 462−468.

(7) Garcia Ovando, H.; Gorla, N.; Luders, C.; Poloni, G.; Errecalde, C.; Prieto, G.; Puelles, I. Comparative pharmacokinetics of enrofloxacin and ciprofloxacin in chickens. *J. Vet. Pharmacol. Ther.* **1999**, *22*, 209−212.

(8) McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. Mono- and polyclonal antibodies to the organophosphate fenitrothion 1. Approaches to hapten-protein conjugation. *J. Agric. Food Chem.* **1992**, *40*, 1466−1470.

(9) Singh, P.; Ram, B. P.; Sharkov, N. Enzyme immunoassay for screening of sulfamethazine in swine. *J. Agric. Food Chem.* **1989**, *37*, 109−114.

(10) Chu, F. S.; Fan, T. S. L. Fan. Indirect enzyme-linked immunosorbent assay for saxitoxin in shellfish. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 13−16.

(11) Mount, M. E.; Failla, D. L. Production of antibodies and development of enzyme immunoassay for determination of monesin in biological samples. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 201−205.

(12) Fleeker, J. R.; Lovett, L. J. Enzyme immunoassay for screening sulfamethazine residues in swine blood. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 172−174.

(13) Schneider, P.; Hammock, B. D. Influence of the ELISA format and the hapten-enzyme conjugate on the sensitivity of an immunoassay for s-triazine herbicides using monoclonal antibodies. *J. Agric. Food Chem.* **1992**, *40*, 525−530.

(14) Chen, T.; Dwyre-Gygax, C.; Hadfield, S. T.; Willetts, C.; Breuil, C. Development of an enzyme-linked immunosorbent assay for a broad spectrum triazole fungicide: hexaconazole. *J. Agric. Food Chem.* **1996**, *44*, 1352−1356.

(15) Kamps-Holtzapple, C.; Stanker, L. H.; Deloach, J. R. Development of a monoclonal antibody-based ELISA for the anthelmintic hygromycin B. *J. Agric. Food Chem.* **1994**, *42*, 822−827.

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