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Preparation of anti-Pefloxacin Antibody and Development of an Indirect Competitive Enzyme-Linked Immunosorbent Assay for Detection of Pefloxacin Residue in Chicken Liver

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Pefloxacin has been increasingly used in veterinary medicine to treat microbial infections. To avoid using a labor-intensive instrumental method to detect the residue of pefloxacin in food, a simple and convenient indirect competitive enzyme-linked immunosorbent assay method has been developed in this study. The antibody generated from immunogen cationized bovine serum albumin−pefloxacin showed high sensitivity toward pefloxacin with an IC_{50} value of 6.7 ppb in buffer and was suitable for a screening assay to detect the residue of pefloxacin in food products. The antibody has been assessed using rapid enzyme immunoassays to exploit its specificity. The antibody prepared shows cross-reactivity with a few other (fluoro)quinolones including fleroxacin (116%), enrofloxacin (88%), and ofloxacin (10%). The assay measured drug residue in chicken liver spiked with pefloxacin with an interassay coefficient of variation of 13.6% or less and an intra-assay coefficient of variation of 10.9% or less. The average recovery rates at 0.5, 5, 10, 50, and 100 ppb were in the range of 86−106% for interassay and in the range of 87−103% for intra-assay, respectively.

KEYWORDS: Pefloxacin; ELISA; drug residue; antibody; immunoassay; cross-reactivity

INTRODUCTION

(Fluoro)quinolones are the most important group of synthetic antimicrobials that are widely used in the veterinary industry to treat and prevent various infectious diseases. Because of concerns related to drug residues entering the food chain and contributing to bacterial resistance (*1*−*4*), more and more countries are setting MRLs (maximum residue levels) and withdrawal periods for (fluoro)quinolones. According to different food resources and different drugs, the MRLs of (fluoro)-quinolones are set in the range of 30−1500 μg/kg (*5*, *6*). In China, the species of animal, usage, dosage, and withdrawal period for (fluoro)quinolones have been determined by the Ministry of Agriculture of the People's Republic of China (no. 278, 2003.5.22). In the case of pefloxacin (**Figure 1**), a withdrawal period of at least 28 days is required before livestock can be slaughtered for food purposes. In order to monitor pefloxacin residue levels in livestock products, simple and rapid analytical methods are required.

Traditionally, residue analysis has relied upon classical analytical methods such as chromatography, either gas or liquid, coupled to various detectors including UV absorbency, mass spectrometry, or fluorescence detection (*7*−*14*). These methods require extensive sample preparation as well as highly trained individuals to operate sophisticated instruments and interpret complicated chromatograms or spectral results. Consequently, these traditional methods, although highly accurate, are time-consuming, costly, and generally not suitable for use in the field. For regulatory purposes, a simple and rapid analytical method can serve as a screen to detect the presence of analytes such as pefloxacin, which could be confirmed by instrumental methods. The ELISA (enzyme-linked immunosorbent assay) is the most suitable method for screening of drug residues in the veterinary field due to its rapidity, mobility, and high sensitivity with detection limits in the ppb range. Commercial immunoassays available to detect residues of (fluoro)quinolones are limited to ciprofloxacin and enrofloxacin. So far, the (fluoro)quinolones that have been studied to develop immunoassays include enrofloxacin (*15*, *19*, *20*), sarafloxacin (*16*, *22*), ciprofloxacin (*18*), norfloxacin (*20*, *22*), nalidixic acid (*20*), and flumequin (*20*, *21*). The aim of the present work is to develop an antibody that is able to recognize pefloxacin residue in food and food products. To our knowledge, no research on an immunoassay to detect pefloxacin residue has been reported in the literature so far.

MATERIALS AND METHODS

Chemicals and Materials. Bovine serum albumin (BSA), ovalbumin (OVA), 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC), and Freund's complete and incomplete adjuvants (cFA and iFA) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Hydroxysuccinimide (NHS) was provided by Cxbio Biotechnology Ltd. (Shanghai,

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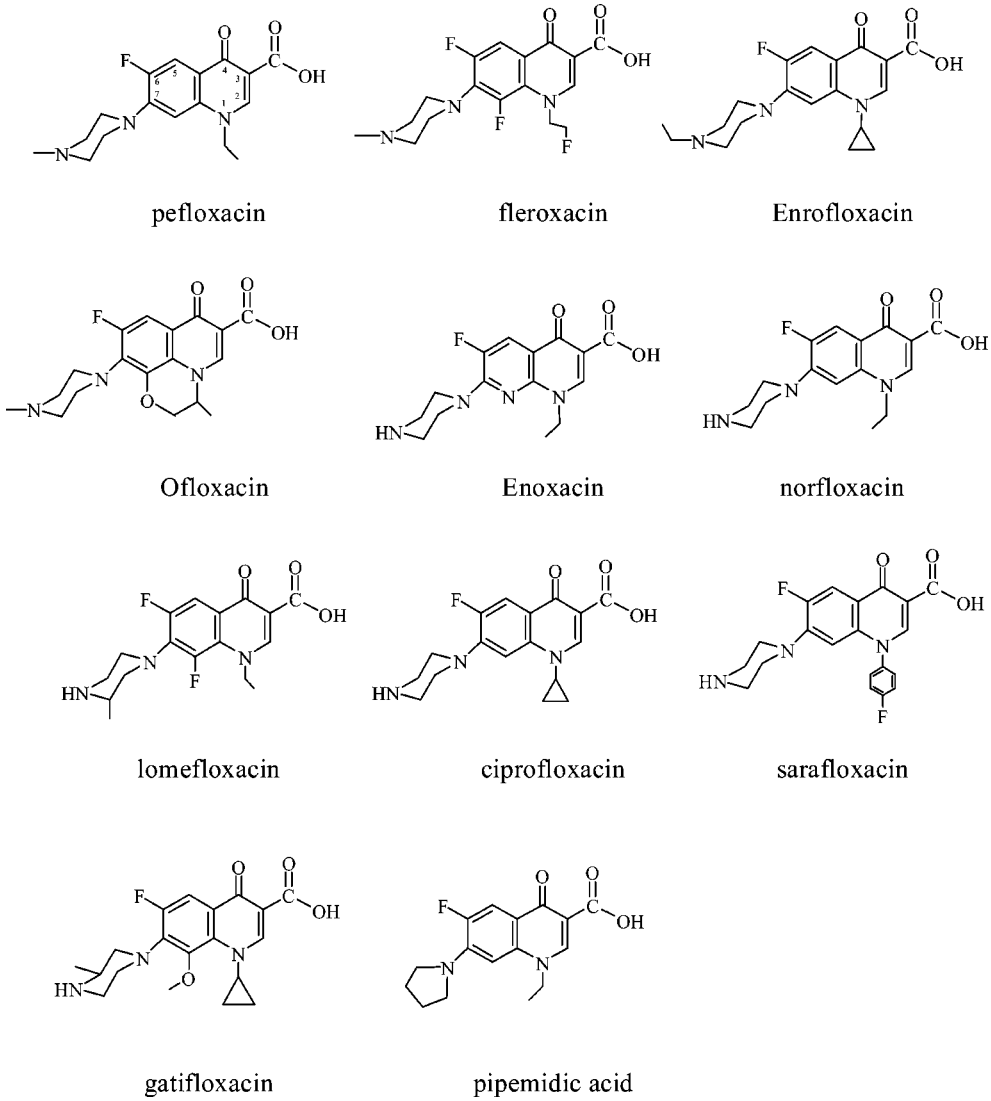

Figure 1. Structures of pefloxacin and related (fluoro)quinolones evaluated in this study.

China). 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Amresco (Solon, OH). Pefloxacin, fleroxacin, enrofloxacin, ofloxacin, enoxacin, norfloxacin, lomefloxacin, ciprofloxacin, sarafloxacin, gatifloxacin, and pipemidic acid were purchased from the Institute of Veterinary Medicine of China (Beijing, China). Goat anti-rabbit IgG-horseradish peroxidase conjugate was provided by the Military Medical Institute (Beijing, China). *o*-Phenylenediamine (OPD) was purchased from Xinjingke Biotechnology (Beijing, China). Dimethylformamide (DMF), ethylenediamine dihydrochloride (EDA), hydrogen peroxide (30%), and other reagents used were chemical grade from Guangmang Chemical Co. (Jinan, China).

Instrumentation and Supplies. ELISA was performed in polystyrene 96 well microtiter plates (Bio Basic Inc.) and spectrophotometrically read with an automatic microplate reader KHB ST-360 from Shanghai Zhihua Medical Instrument Ltd. UV data were collected on a U-4100 spectrophotometer from Hitachi Co. Centrifugation was carried out with a refrigerated centrifuge (Biofuge Stratos, Heraeus). Protein dialyses were performed using dialysis tubes from Aibo Economic & Trade Co., Ltd. (Jinan, China).

Buffers. For the preparation of all buffers and reagents for the immunoassays, ultrapure deionized water was used. Phosphate-buffered saline (PBS, pH 7.4) consisted of 138 mM NaCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, and 2.7 mM KCl. The wash buffer (PBST) was a PBS buffer containing 0.05% Tween 20. As a coating buffer, 0.05 M carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) was used. The blocking buffer was PBS + 1% of OVA + 0.05% (v/v) Tween 20. The substrate buffer was 0.1 M sodium acetate/citrate buffer (pH 5.0). To prepare the substrate solution, 10 mg of OPD was dissolved in 25 mL of sodium citrate buffer and this solution plus 5 μL of H_2O_2 [30% (w/w)]. The stopping solution was 2 N HCl.

Preparation of Cationized BSA (cBSA) and Cationized OVA (cOVA). In this procedure, carboxylic acid groups of the carrier proteins of BSA and OVA were converted into primary amine groups with an excess of EDA. A solution of 1 g of BSA (15 μmol) and 56 mg of EDC (300 μmol) in 20 mL of PBS (0.1 M, pH 7.4) was added slowly into a solution of 18 mg of EDA (300 μmol) in 20 mL of PBS (0.1 M, pH 7.4) under stirring. The mixture solution was incubated continuously for 2 h at room temperature and then dialyzed [molecular weight cutoff (mwco), 12000−14000 Da] under stirring against PBS (0.1 M, pH 7.4) to remove free EDA. Cationized BSA and OVA were defined as cBSA and cOVA, respectively. The solution was lyophilized, and the white solid (cBSA) obtained was stored at −20 °C before use in the next reaction. The cOVA was prepared in a similar method.

Preparation of Immunogen. The immunogen cBSA−pefloxacin and the coating antigen cOVA−pefloxacin were prepared by carbodiimide-modified active ester method as described in the literature (*23*). In this procedure (**Figure 2**), a solution of 10.0 mg of pefloxacin (23 μmol), 44.7 mg of EDC (230 μmol), and 13.4 mg of NHS (117 μmol) in 3.0 mL of DMF was incubated for 24 h at room temperature in the dark. To this solution, 52.8 mg of cBSA (0.8 μmol) dissolved in 20 mL of PBS (0.1 M, pH 7.4) was added slowly under stirring, followed by incubation at room temperature for 3 h. The reaction mixture was dialyzed (mwco, 12000−14000 Da) under stirring against PBS (0.1 M, pH 7.4) for 3 days with frequent change of the PBS solution to remove the uncoupled free hapten. The solution was lyophilized, and cBSA−pefloxacin conjugate obtained was stored at −20 °C. A cOVA−

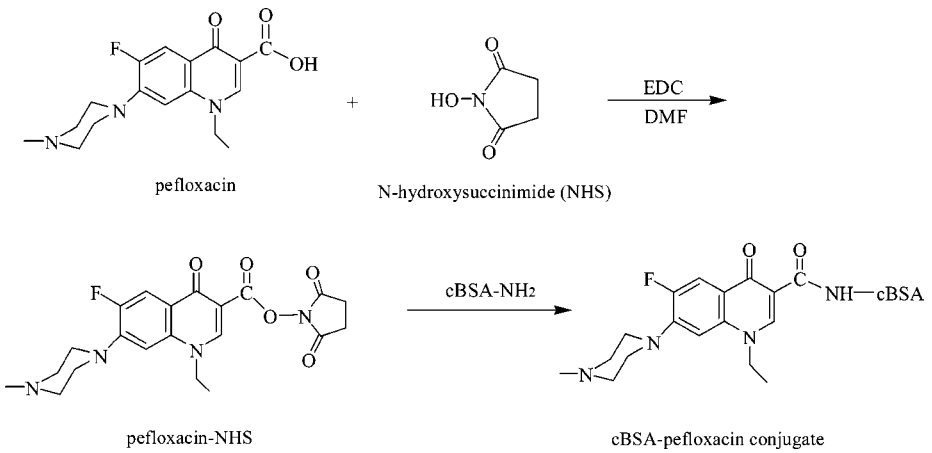

Figure 2. Synthesis of cBSA–pefloxacin conjugate.

pefloxacin conjugate was prepared in a similar method. An UV absorbance method was employed to determine whether the linking had been a success.

Immunization of Rabbits. Two Male New Zealand white rabbits were subcutaneously immunized at multiple sites in the back with cBSA−peflxoacin conjugate. The initial immunization was subcutaneously injected with 1 mg of conjugate in 0.5 mL of NaCl (0.9%) and 0.5 mL of Freund's complete adjuvant. Subsequent booster injections [0.5 mg of conjugate in 0.5 mL of NaCl (0.9%) plus 0.5 mL of Freund's incomplete adjuvant] were performed 15 days later and then at 12 day intervals. One week after each booster, serum titers were determined by ELISA. The antiserum obtained after each booster was prepared by allowing the blood to clot overnight at 4 °C, followed by centrifugation to remove particulate material. Ten days after the last boost, all rabbits were then exsanguinated by heart puncture under general anesthetic and euthanized by lethal injection before recovery. The serum was separated from blood cells by storage of the blood overnight at 4 °C and centrifugation with 13000 rpm/min for 20 min. The crude serum obtained was purified by saturated ammonium sulfate (SAS) precipitation method (purified three times using 50, 33, and 33% (v/v) of SAS, respectively), and sodium azide was added as a preservative at a final concentration of 0.02% (w/w). The purified serum was then aliquotted and stored at −70 °C.

Antibody Titer Determination by Indirect ELISA. The titer of the antibody was tested by indirect ELISA, using a procedure described below. The microplates were coated with coating antigen cOVA−pefloxacin at 5 μg/mL (50 μL/well) by overnight incubation at 4 °C. Plates were washed with wash buffer three times and blocked with 250 μL/well of blocking buffer, followed by incubation for 1 h at room temperature. Plates were washed three times again, the appropriate dilution of the antisera was added, and the plates were incubated for 2 h at room temperature. Plates were washed three times, and goat anti-rabbit IgG-HRP (1:3000, 50 μL/well) was added, followed by incubation for 2 h at room temperature. Plates were washed three times and OPD substrate solution was added (50 μL/well), and the plates were incubated for another 30 min at room temperature. The color development was halted by adding stopping solution (50 μL/well), and absorbances were measured at 492 nm. Absorbances were corrected by blank reading. Preimmune withdrew serum (the serum before immunization) was used as a negative control, and the antibody titer was defined as the reciprocal of the dilution that resulted in an absorbance value that was twice that of the background.

Development of Indirect Competitive ELISA. The checker board procedure was used to optimize the coating antigen and the primary antibody concentrations. To each well of a 96 well plate, 100 μL of 10 μg/mL of cOVA−pefloxacin solution in bicarbonate buffer (0.05 M, pH 9.6) was added and incubated overnight at 4 °C. The plate was washed with wash buffer three times and blocked with 250 μL/well of blocking buffer, followed by incubation for 1 h at room temperature. After the blocking solution was removed and the plate was washed three times, 100 ng of primary antibody was added to each well followed by the addition of buffer or competitor in buffer, and the plate was incubated for 2 h. The plate was washed three times and goat anti-rabbit IgG-HRP (1:3000, 50 μL/well) was added, followed by incubation for 2 h at room temperature. The plate was washed three times, OPD substrate solution was added (50 μL/well), and the plate was incubated for another 30 min at room temperature. The color development was halted by adding stopping solution (50 μL/well), and absorbances were measured at 492 nm. Absorbances were corrected by blank reading. Preimmune withdrawn serum was used as a negative control.

Chicken Liver Extract Preparation. Minced samples (5 g) of pefloxacin-free (determined by high-performance liquid chromatography) chicken liver were homogenized with 5 mL of extraction solvent consisting of a 1:5 (v/v) mixture of methanol and PBS adjusted to pH 7.4 with 6 N HCl. The homogenates were mixed on a vortex mixer for 30 s, vigorously shaken for 30 min, and then centrifuged at 10000*g* for 1 h. The supernatants were diluted by a factor 10 in the assay buffer before they were applied to the microtiter plate.

RESULTS AND DISCUSSION

Hapten Conjugation. As a small molecule, pefloxacin (MW = 333 < 1000 Da) (**Figure 1**) has to be conjugated with a carrier protein in order to stimulate the immune response of an animal to produce the anti-pefloxacin antibody. Among carrier proteins, BSA and OVA are two of the most often used ones, and usually, they give satisfying results. To convert carboxylic acid groups on the carrier protein to primary amine groups, BSA and OVA were treated with an excess of EDA as described previously (*15*, *16*). The cBSA prepared has a higher linking capacity with pefloxacin due to more primary amine groups available on cBSA than BSA (*16*). Moreover, the use of cationized carrier proteins can minimize cross-linking and increase their pI values to generate more immune responses as compared to their native forms (*23*). The free carboxylic acid group on pefloxacin was linked to amino groups on cBSA and cOVA using carbodiimide as reported in the literature (*15*, *23*). This reaction resulted in an amide bond between the pefloxacin and the carrier protein. UV absorbances were measured for cBSA, pefloxacin, and cBSA−pefloxacin, respectively. The absorbance peaks at 323 and 334 nm, coming from the absorbances of quinolone moiety of pefloxacin, have shifted to 319 and 331 nm of cBSA−pefloxacin in UV spectrometry, indicating the successful conjugation between pefloxacin and cBSA. The coating antigen cOVA−pefloxacin gives a similar UV pattern as cBSA−pefloxacin.

Antibody Characterization. The titer of antibody was determined by indirect ELISA as >512000 for both rabbits used in the immunization process, with the titer being defined as the reciprocal of the dilution that results in an absorbance value

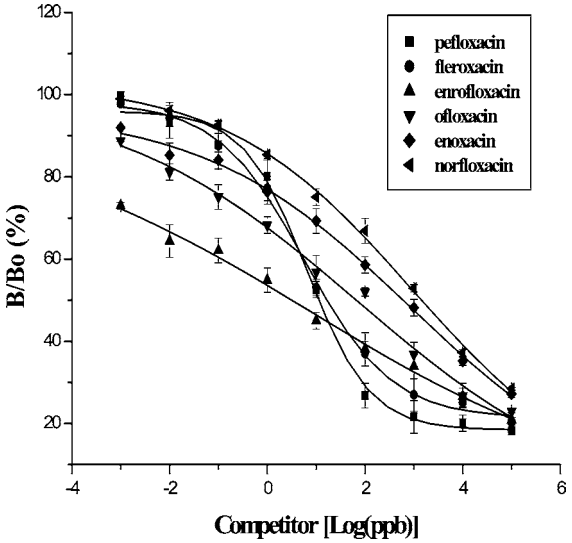

Figure 3. ELISA curves for polyclonal anti-pefloxacin antibody using pefloxacin (■), fleroxacin (●), enrofloxacin (▲), ofloxacin (▼), enoxacin (◆), and norfloxacin (left triangle) as competitors in PBS buffer solution. Each point represents the average of 10 replicates.

Table 1. IC_{50} and Percentage of Cross-Reactivity of Selected (Fluoro)quinolones

compound	IC_{50}[a]	cross-reactivity[b] (%)
pefloxacin	6.7	100
fleroxacin	5.8	116
enrofloxacin	7.6	88
ofloxacin	67	10
enoxacin	820	~1
norfloxacin	920	~1
lomefloxacin	>1000	<1
ciprofloxacin	>1000	<1
sarafloxacin	>1000	<1
gatifloxacin	>1000	<1
pipemidic acid	>1000	<1

[a] IC_{50} was the competitor concentration where the absorbance value was decreased in half as compared to the absorbance value of no competitor. Data represent three separate experiments run on three different days. [b] The percentage of cross-reactivity is defined as the ratio of the test compound's IC_{50} to that of pefloxacin.

that is twice that of the background. The antibody was evaluated for its ability to bind to pefloxacin and other (fluoro)quinolones. The representative ELISA curves for pefloxacin, fleroxacin, enrofloxacin, ofloxacin, enoxacin, and norfloxacin are shown in **Figure 3**, and the IC_{50} values obtained from these experiments are summarized in **Table 1**. Three (fluoro)quinolones including pefloxacin, fleroxacin, and enrofloxacin show nearly equal relative affinity with IC_{50} values of 6.7, 5.8, and 7.6 ppb, respectively. Ofloxacin has a IC_{50} value of 67 ppb, whereas all of the other (fluoro)quinolones listed in **Table 1** have IC_{50} values of more than 800 ppb.

Specificity of the Antibody. The specificity of the antibody was evaluated by determination of the cross-reactivity toward various (fluoro)quinolones. The cross-reactivity studies were carried out by an indirect competitive ELISA by adding various free competitors at different concentrations (from 0.001 to 100000 ppb) to compete with binding of the antibody to the coating antigen. Cross-reactivity was measured by comparison of the IC_{50} of the competitor with that of pefloxacin. The anti-pefloxacin antibody shows fairly good specificity with significant cross-reactivity only toward a few drugs including flerofloxacin, ofloxacin, and enrofloxacin among commonly used (fluoro)quinolones (**Table 1**).

Bucknall et al. (*20*) designed an immunogen for norfloxacin using the secondary amino group located in the piperazinyl moiety of norfloxacin as a point to link with the carboxylic acid group on a carrier protein. This design exposes 4-quinolone carboxylic acid moiety, the common part for (fluoro)quinolone drugs (**Figure 1**), as the immunodominant area. Consequently, the antibody obtained shows fairly high cross-reactivity with other (fluoro)quinolones. They also prepared, however, the other three antibodies by immunogens synthesized through linking carboxylic acid groups of the haptens with amino groups of carrier proteins. These three antibodies demonstrate excellent specificities and show significant affinity only toward corresponding haptens (*20*). Bucknall's research supports a well-accepted rule in immunology that antibodies elicited to haptenic conjugates show a preferential recognition to the part of molecule that is furthest from the attachment site of the hapten to the carrier protein (*24*, *25*).

In our research, the immunogen was synthesized by the linkage of carboxylic acid group of pefloxacin with the amino group of the carrier protein (**Figure 2**). In this linkage, the furthest group of pefloxain from the linking point is the piperazinyl moiety. Therefore, it is not a surprise that fleroxacin (116%), enrofloxacin (88%), and ofloxacin (10%) show high cross-reactivities with the antibody because all of these drugs have methyl (pefloxacin, fleroxacin, and ofloxacin) or ethyl (enrofloxacin) groups connected to their piperazine moieties through a nitrogen atom (**Figure 1**). The other (fluoro)-quinolones such as enoxacin, norfloxacin, lomefloxacin, ciprofloxacin, sarafloxacin, gatifloxacin, and pipemidic acid demonstrated only minor or no detectable cross-reactivities (**Table 1**).

Holtzapple et al. (*16*) reported research concerning a monoclonal antibody against sarafloxacin. In their research, the immunogen used to prepare the antibody was synthesized by connecting a carboxylic acid group on sarafloxacin with an amino group on BSA. Their cross-reaction study shows that the antibody has a high cross-reactivity with the haptens, which have similar substituents located in position 1 [see pefloxacin in **Figure 1** for numbering of the (fluoro)quinolone ring] to sarafloxacin. In their study, the similarity in the piperazine moiety of haptens has only a minor influence on the specificities of the antibodies.

Huet et al. (*22*) reported research on two antibodies prepared by immunogens synthesized from sarafloxacin and norfloxacin with carrier proteins. Their study shows that the substituent, such as a piperazinyl group, connected with (fluoro)quinolone at position 7 (see **Figure 1**), is the most important structural factor to determine the cross-reactivity for a (fluoro)quinolone toward sarafloxacin antibody or norfloxacin antibody. Because they all lack the piperazinyl moiety contained in sarafloxacin and other fluoroquinolones, three (fluoro)quinolones including flumequine, oxolinic acid, and cinoxacin exhibit lower affinity toward these two antibodies.

In the (fluoro)quinolone family, current research could not reach a clear conclusion on where a substituent located (position 1 or position 7; see **Figure 1**) is a more important structural factor to determine the affinity of a drug toward an antibody. Although further careful designed research is necessary to clarify the issue, we believe that both electronic and three-dimensional structures should be considered before the problem can be elucidated clearly in the future.

ELISA Performance in Chicken Liver Extract. The applicability of the developed assay in the determination of

Table 2. Inter- and Intra-assay Variations of Chicken Liver Spiked with Pefloxacin

	interassay[a]				intra-assay[b]			
level (ppb)	*n*	measured (ppb)	recovery (%)	CV (%)	*n*	measured (ppb)	recovery (%)	CV (%)
0.5	12	0.44 ± 0.06	88	13.6	6	0.46 ± 0.05	92	10.9
5	12	5.3 ± 0.4	106	7.5	6	5.2 ± 0.3	103	5.8
10	12	8.6 ± 0.6	86	6.9	6	8.7 ± 0.4	87	4.6
50	12	45.7 ± 3.3	91	7.2	6	46.5 ± 3.0	95	6.5
100	12	89.7 ± 6.0	90	6.7	6	90.4 ± 5.6	90	6.2

[a] Interassay variation was determined by 12 replicates on 15 different days. [b] Intra-assay variation was determined by six replicates on a single day.

pefloxacin residue in chicken liver was tested, and the results obtained are shown in **Table 2**. In this research, samples for the recovery study were prepared by the dilution of the extract in PBS without any further treatment. To gain information about the matrix effect, a series dilution of chicken liver extract were measured by ELISA and compared with the results obtained in PBS buffer. It was found that the extract dilution 1:10 gives the inhibition curve almost the same as that of PBS buffer. The linear range of the percentage B/B_o vs log pefloxacin concentration competitive curve generated in PBS was 0.1−100 ppb (**Figure 3**). The assay has a detection limit of 0.16 ppb in buffer solution. The detection limit was determined by a concentration of pefloxacin where the absorbance value was decreased to 90% of the absorbance value of no competitor. Known amounts of pefloxacin were spiked into chicken liver and were analyzed using a calibration curve made in buffer. The liver sample spiked at 0.5, 5, 10, 50, and 100 ppb gave an average recovery rate of 88, 106, 86, 91, and 90% for interassay and 92, 103, 87, 95, and 90% for intra-assay, respectively. The CVs (coefficient of variation) ranged from 13.6−6.9% for interassay and 10.9−4.6% for intra-assay. Because 0.5 ppb is close to the detection limit of 0.16 ppb, larger variability and decreased accuracy are to be expected.

In summary, we have prepared a high-quality polyclonal antibody for pefloxacin for the first time. The ELISA based on this antibody shows high sensitivities toward pefloxacin, fleroxacin, and enrofloxacin with IC_{50} values less than 10 ppb. The feasibility to apply this antibody in a competitive ELISA to detect the residue of pefloxacin has been explored using chicken liver extract. The ELISA developed in this study could be used for the routing screening of pefloxacin residues in food animal edible tissues.

ABBREVIATIONS USED

MRL, maximum residue level; BSA, bovine serum albumin; OVA, ovalbumin; cBSA, cationized BSA; cOVA, cationized OVA; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; IC_{50}, concentration at 50% inhibition; Da, unit of molecular mass Dalton; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Tween 20; OPD, *o*-phenylendiamine; DMF, *N*,*N*-dimethyl formamide; SAS, saturated ammonium sulfate; cFA, complete Freund's adjuvant; iFA, incomplete Freund's adjuvant.

Supporting Information Available: UV spectra for cBSA, pefloxacin, and cBSA−eda-pefloxacin conjugate and standard curve prepared in buffer for calibration. This material is available free of charge via the Internet at http://pubs.acs.org.

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