JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Preparation of Anti-gatifloxacin Antibody and Development of an Indirect Competitive Enzyme-Linked Immunosorbent Assay for the Detection of Gatifloxacin Residue in Milk

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A polyclonal anti-gatifloxacin antibody has been prepared, and an indirect competitive enzyme-linked immunosorbent assay (cELISA) was developed on the basis of the antibody prepared for the first time. The antibody shows high sensitivity with an IC_{50} value of 2.6 ppb and excellent specificity with only a minor cross-reaction with lomefloxacin (3.0%) among common (fluoro)quinolones evaluated in this study. The high specificity of the antibody was explained by the molecular structures of related drugs by comparison with published research. The cELISA test kit developed has a detection limit of 0.05 ppb and could be used as a screening method to detect and regulate illegal use of gatifloxacin in food and food products. The test kit was applied to the detection of milk samples spiked by gatifloxacin. The recovery rates were in the range of 86−106%, whereas the intra- and interassay coefficients of variation were <14.3 and <19.6%, respectively.

KEYWORDS: Gatifloxacin; drug residue; antibody; immunoassay; cross-reactivity

INTRODUCTION

(Fluoro)quinolones are the most important group of synthetic antibacterials used as medicine for humans and as veterinary drugs for animals to treat and prevent various infectious diseases since they were developed about 40 years ago. Because of their side effects and drug residues entering the food chain and contributing to bacterial resistance (*1*−*4*), more and more countries are setting maximum residue levels (MRLs) and withdrawal periods for (fluoro)quinolones. The MRLs of (fluoro)quinolones are set in the range of 30−1500 μg/kg (*5*, *6*) according to different food resources and different countries. 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). Gatifloxacin (GAT) is one of the most important (fluoro)quinolones and is used widely both as clinical medicine and as a veterinary drug in animal husbandry. According to an investigation in 2006, more than 170 firms were permitted to manufacture gatifloxacin in China. In recent years, however, more and more clinical studies have shown that gatifloxacin can cause glucose abnormality for patients (*7*, *8*). The U.S. FDA has asked drug sellers to add extra warnings for the potential risk related to the use of gatifloxacin. Bristol-Myers Squibb (BMS), the developer and seller of gatifloxacin (under the commercial name Tequin), even asked its distributor to stop selling the drug due to increasing lawsuits over side effects according to a report from New Times in May 2006.

To detect drug residues, traditionally, classical analytical methods such as chromatography, either gas or liquid, coupled to various detectors including UV absorbency, mass spectrometry, or fluorescence detection, are used (*9*−*16*). However, these methods require extensive sample preparation as well as highly trained individuals to operate sophisticated instruments and interpret complicated chromatogram or spectral results. Consequently, these traditional methods, although highly accurate, are time-consuming, costly, and generally not suitable for use in the field. A simple and rapid analytical method, thus, is required to serve as a screen to detect the presence of the drug. An 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 parts per billion range. So far, the (fluoro)quinolones that have been studied to develop immunoassays include enrofloxacin (*17*, *19*, *22*, *23*), sarafloxacin (*19*, *25*), ciprofloxacin (*19*−*21*), norfloxacin (*22*, *25*), nalidixic acid (*23*), flumequin (*23*, *24*), and pefloxacin (*26*) (**Figure 1**). There are no reports of the preparation of an antibody and the development of an immunoassay for gatifloxacin so far. By analyzing the structures of (fluoro)quinolones that have been studied to prepare corresponding antibodies, we found that most of these drugs, except for flumequine, have no substituent in position 8 (see **Figure 1** for the numbering of the quinolone ring). Flumequine is an exception, with a substituent located at position 8, but the research related to the anti-flumequine antibody has to do with

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10.1021/jf070978g CCC: $37.00
Published on Web 07/24/2007

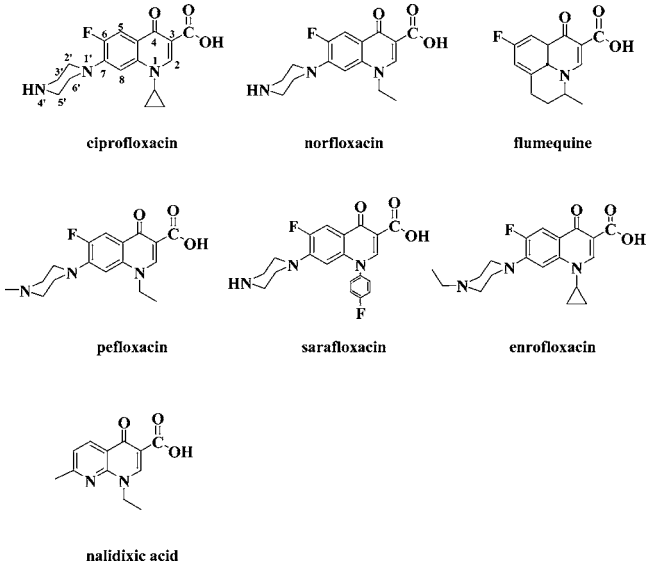

Figure 1. Structures of (fluoro)quinolones that have been studied to develop corresponding antibodies.

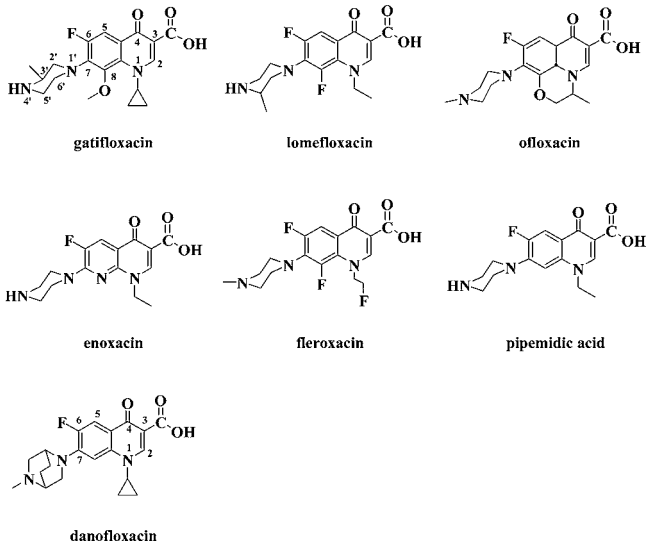

Figure 2. Structures of gatifloxacin and related (fluoro)quinolones evaluated in this study.

chicken egg yolk immunoglobulins IgY (*24*). Another study on flumequine antibody shows that the anti-flumequine antibody is highly specific (*23*). Gatifloxacin has a methyloxyl group located in position 8 (**Figure 2**). We are interested in knowing whether the antibodies of this type of (fluoro)quinolone have any unique properties. In fact, this study did show that the anti-gatifloxacin antibody has better specificity compared with other antibodies of (fluoro)quinolones that have no substituent in position 8. In this study, a high-quality polyclonal anti-galifloxacin antibody has been prepared, and a cELISA test kit based on the antibody was developed and applied to detect milk spiked by gatifloxacin. This is the first research to prepare an anti-gatifloxacin antibody and develop an immunoassay to detect residues of gatifloxacin in milk.

MATERIALS AND METHODS

Chemicals and Materials. Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants (cFA and iFA, respectively), and 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Hydroxysuccinimide (NHS) was provided by Cxbio Biotechnology Ltd. (Shanghai, China). Gatifloxacin, lomefloxacin, ofloxacin, ciprofloxacin, fleroxacin, enrofloxacin, enoxacin, norfloxacin, pefloxacin, sarafloxacin, danofloxacin, and pipemidic acid were obtained 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). Ethylenediamine dihydrochloride (EDA), dimethylformamide (DMF), hydrogen peroxide (30%), and other reagents used were provided by Guangmang Chemical Co. (Jinan, China).

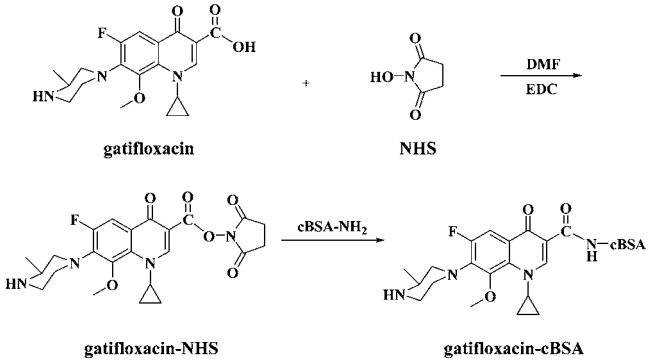

Figure 3. Procedure of immunogen preparation.

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. Centrifugation was carried out with a refrigerated centrifuge (Biofuge Stratos, Heraeus). Protein dialyses were performed using dialysis tubes from Aibo Economic and Trade Co., Ltd. (Jinan, China). UV data were collected on a U-4100 spectrophotometer from Hitachi Co.

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 washing buffer (PBST) was a PBS buffer containing 0.05% (v/v) 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). To convert carboxylic acid groups in BSA into primary amino groups, BSA was treated with an excess of EDA. In this procedure, a solution of 1.5 g of BSA (22.5 μmol) and 84 mg of EDC (450 μmol) in 20 mL of PBS (0.1 M, pH 7.4) was added slowly into a solution of 27 mg of EDA (450 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 and other trashy substances for 3 days. The mixture was then dialyzed against ultrapure deionized water for another 3 days. 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 GAT−cBSA and Coating Antigen GAT−cOVA. The immunogen cBSA−gatifloxacin (GAT−cBSA) and the coating antigen cOVA−gatifloxacin (GAT−cOVA) were prepared according to a carbodiimide-modified active ester method described in the literature (*26*, *27*). In this procedure (**Figure 3**), 15.0 mg of gatifloxacin (40 μmol), 76.5 mg of EDC (400 μmol), and 22.5 mg of NHS (200 μmol) were added to 5.0 mL of DMF in order. The mixture solution was incubated for 24 h at room temperature without light. Then, 90.0 mg of cBSA (1.3 μmol) dissolved in 10 mL of PBS (0.1 M, pH 7.4) was added slowly under stirring. After that, the solution was incubated at room temperature for 3 h. Finally, the reaction mixture was dialyzed (mwco, 12000−14000 Da) under stirring against PBS

(0.1 M, pH 7.4) for 3 days with frequent changes of the PBS solution to remove the uncoupled free hapten and dialyzed under stirring against ultrapure deionized water for another 3 days. The solution was lyophilized, and the cBSA−gatifloxacin conjugate obtained was stored at −20 °C. A cOVA−gatifloxacin conjugate was prepared in a similar manner. UV was used to determine conjugation results.

Immunization of Rabbits. Two male New Zealand white rabbits were subcutaneously immunized at multiple sites in the back with cBSA−gatifloxacin conjugate. The initial immunization was subcutaneously injected with 0.5 mg of conjugate in 0.5 mL of NaCl (0.9%) and 0.5 mL of cFA. Subsequent booster injections [0.25 mg of conjugate in 0.5 mL of NaCl (0.9%) plus 0.5 mL of iFA] were performed 20 days later and then at 15 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 exsanguinated by heart puncture under general anesthesia 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 at 13000 rpm/min for 20 min. The crude serum obtained was purified by a 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 cELISA. The titer of the antibody was tested by indirect competitive ELISA (cELISA). The procedure is described below. The microplates were coated with coating antigen cOVA−gatifloxacin at 10 μg/mL (100 μL/well) by overnight incubation at 4 °C. Three washes with washing buffer were done between each step. The next day, plates were blocked with 250 μL/well of blocking buffer, followed by incubation for 1 h at room temperature. Then, the appropriate dilution of the antiserum was added, and the plates were incubated for 2 h at room temperature. The goat anti-rabbit IgG−HRP (1:3000, 50 μL/well) was added, followed by incubation for 2 h at room temperature. 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 (2 N HCl, 50 μL/well), and absorbances were measured at 492 nm. Preimmune withdrawal 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 cELISA. The checkerboard procedure was used to optimize the coating antigen and the primary antibody concentrations. To each well of a 96 well plate was added 100 μL of 10 μg/mL of cOVA−gatifloxacin solution in bicarbonate buffer (0.05 M, pH 9.6), and the mixture was incubated overnight at 4 °C. Three washes with washing buffer were done between each step. The plate was blocked with 250 μL/well of blocking buffer, followed by incubation for 1 h at room temperature. After the blocking solution was removed, 100 μL of primary antibody was added to each well followed by the addition of PBST buffer or competitor in PBST buffer, and the plate was incubated for 2 h. Then, the goat anti-rabbit IgG−HRP (1:3000, 50 μL/well) was added, followed by incubation for 2 h at room temperature. OPD substrate solution was added (50 μL/well), and the plate was incubated for another 30 min at room temperature. Color development was halted by adding stopping solution (2 N HCl, 50 μL/well), and absorbances were measured at 492 nm. Preimmune withdrawal serum was used as a negative control. The result was expressed in percent inhibition as follows: % inhibition = $\%B/B_0$, where B is the absorbance of the well containing competitor and B_0 is the absorbance of the well without competitor.

Standard Curve Generation. The cOVA−gatifloxacin (10 μg/mL) was used as a coating antigen, and indirect cELISA was performed as described above. The selected antiserum at 1:8000 dilution was utilized as primary antibody and co-incubated with gatifloxacin. The standard calibration curve with final gatifloxacin concentrations of 0.5, 1.0, 5.0, 10, and 50 ng/mL was run in PBST (see Supporting Information).

Matrix Effects Determination. The milk sample was defatted by centrifugation at 4 °C (10000*g*, 30 min) and was artificially contaminated by adding gatifloxacin standard solution in PBST. Competitive curves with final gatifloxacin concentrations of 0.01, 0.1, 0.5, 1.0, 5.0, 10, 50, 100, and 1000 ng/mL were run in PBST and in various dilutions (1:5, 1:10, and 1:20) of the defatted milk with PBST to determine the matrix effect of milk (**Figure 4**). IC_{50} and B_0 values from each diluted curve were obtained by comparing with IC_{50} and B_0 values generated from the PBST buffer solution.

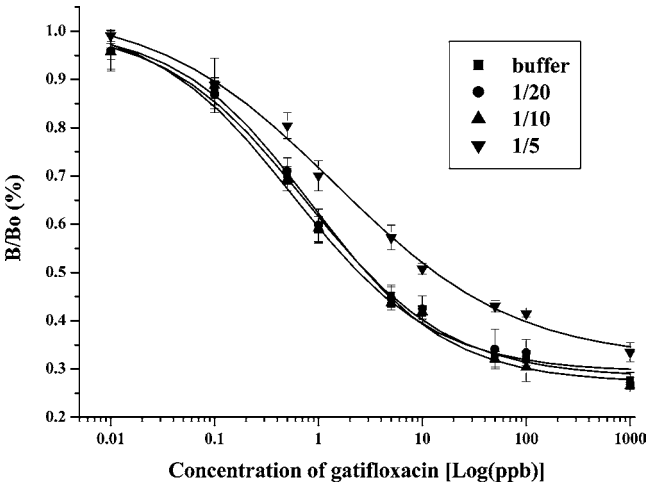

Figure 4. Competitive curves of gatifloxacin in PBST (■) and in various dilutions of 1:5 (▼), 1:10 (▲), and 1:20 (●) of the defatted milk solutions in PBST.

RESULTS AND DISCUSSION

Hapten Conjugation. As a small molecule with a molecular mass of 375.4, gatifloxacin is not able to elicit the immune response of an animal to produce the anti-gatifloxacin antibody. It has to be conjugated with a carrier protein to stimulate the immune response of an animal to produce the anti-gatifloxacin antibody. Among protein carriers, BSA and OVA are two of the most commonly used ones, and, usually, they give satisfactory results. BSA was treated with an excess of EDA as described previously (*17*, *18*) to convert carboxylic acid groups into primary amine groups to prepare cBSA. The cBSA prepared has the advantage over BSA (*23*) that more primary amino groups become available on cBSA to couple with functional groups, such as carboxylic groups, on hapten. Moreover, the use of cationized carrier proteins can minimize cross-linking and increase their p*I* values to generate more immune responses as compared to their native forms (*27*).

From the structure (**Figure 2**), it can be seen that gatifloxacin contains a carboxylic acid group and a secondary amino group. We can, thus, prepare the immunogen and coating antigen by the linkage of carboxylic acid group with the amino group of the carrier protein or by the linkage of the secondary amino group of gatifloxacin with the carboxylic acid group of the carrier protein. In this study, we chose the carboxylic acid group of gatifloxacin as the site to link to carrier protein in order to expose the structural part representing the feature of gatifloxacin toward the outside to increase the specificity of the antibody. The carbodiimide-modified active ester method was used to prepare immunogen. The procedure of synthesis of the immunogen is given in **Figure 3**. UV spectrometry was used to determine the effectiveness of the conjugation reaction. The absorbance results as shown in **Figure 5** clearly give the evidence supporting successful conjugation. The absorbance for cBSA−gatifloxacin (290, 330 nm) gives a red-shifted peak at 290 nm compared with the 285 nm peak for gatifloxacin (285, 331 nm), indicating the conjugation process results in a lower

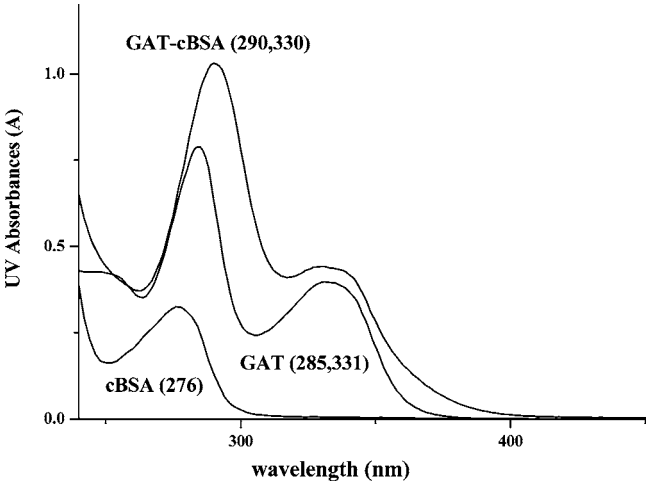

Figure 5. UV absorbances for GAT–cBSA, GAT, and cBSA.

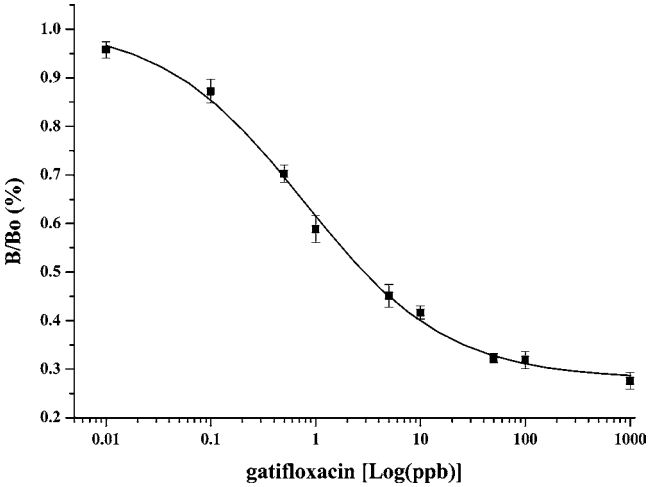

Figure 6. Competitive curve of anti-gatifloxacin antibody with gatifloxacin as competitor in PBST.

energy in electron shift. The coating antigen cOVA−gatifloxacin gives a UV pattern similar to that for cBSA−gatifloxacin.

Characterization of the Antibody. The titer of the antibody was determined by indirect cELISA as >1024000 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 that is twice that of the background.

The inhibition in an indirect cELISA is affected by the strength of interaction between the antiserum and the coating antigen. The antibody was evaluated for its ability to bind to gatifloxacin and other (fluoro)quinolones by cELISA. To determine the specificity of the antibody prepared, cross-reactivity studies were carried out by an indirect competitive ELISA by adding various free competitors at different concentrations (from 0.01 to 1000 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 gatifloxacin. The anti-gatifloxacin antibody shows excellent sensitivity, with an IC_{50} value of 2.6 ppb and high specificity (**Table 1**).

For (fluoro)quinolone molecules, there are two potential sites that can be used to link with carrier proteins. One is the carboxylic acid group, and the other is the secondary amino group located in the piperazinyl moiety (there are no secondary amino groups in ofloxacin, fleroxacin, enrofloxacin, pefloxacin, danofloxacin, and pipemidic acid, see **Figures 1** and **2**). The

Table 1. IC_{50} Values and Cross-Reactivity of Anti-gatifloxacin toward Selected (Fluoro)quinolones

compound	IC_{50}[a] (ng/mL)	cross-reactivity[b] (%)
gatifloxacin	2.6	100
lomefloxacin	88	3.0
ciprofloxacin	727	0.4
ofloxacin	942	0.3
enrofloxacin	>1000	<1
sarafloxacin	>1000	<1
flerfloxacin	>1000	<1
pipemidic acid	>1000	<1
pefloxacin	>1000	<1
norfloxacin	>1000	<1
enoxacin	>1000	<1
danofloxacin	>1000	<1

[a] IC_{50} values were the competitor concentrations at which the absorbance value was decreased by half as compared to the absorbance value of no competitor. Data represent three separate experiments run on three different days. [b] Cross-reactivity was determined by comparing the concentration of analyte required to produce a $B/B_0 = 50\%$. Results are expressed as a percentage relative to the figure for gatifloxacin.

immunogen used to prepare the anti-gatifloxacin antibody in this research was synthesized by the linkage of the carboxylic acid group of gatifloxacin with the amino group of the carrier protein (**Figure 3**). In this linkage, the farthest group of gatifloxain from the linking point is the piperazinyl moiety. The antibody shows high affinity toward gatifloxacin, but only minor cross-reactivities with lomefloxacin (3.0%), ciprofloxacin (0.36%), and ofloxacin (0.3%), and no detectable cross-reactivities with the others shown in **Figures 1** and **2**. Bucknall et al. (*23*) used 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 the 4-quinolone carboxylic acid moiety, the common part for (fluoro)quinolone drugs (**Figures 1** and **2**), as the immunodominant area. Consequently, the antibody obtained shows fairly high cross-reactivity with many other (fluoro)quinolones. Bucknall et al. also prepared the other three antibodies of (fuoro)quinolones, enrofloxacin, flumequin, and nalidixic acid, 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 (*23*) among common (fluoro)quinolones. 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 the farthest from the attachment site of the hapten to the carrier protein (*28*, *29*). From a structural point of view, the three (fluoro)quinolones selected in Bucknall's research have certain special features. The farthest groups from the attachment site of the hapten to the carrier protein are very special compared with other common (fluoro)quinolones. There is no piperazinyl moiety in flumequin and nalidixic acid. For enrofloxacin, which is different from most other (fluoro)-quinolones, there is an ethyl group connected with the nitrogen atom of the piperazinyl moiety, instead of a methyl or hydrogen atom as in most other drugs. These special structural features also contribute to high specificities of the antibodies.

Lu et al. (*26*) reported research on preparing an antibody against pefloxacin. The immunogen was prepared by the linkage of the carboxylic acid group of pefloxacin with the amino group of a carrier protein. In this linkage, the farthest group of pefloxain from the linking point is the piperazinyl moiety. Because these drugs have methyl or ethyl groups connected to their piperazine moieties through a nitrogen atom

(**Figures 1** and **2**), fleroxacin, enrofloxacin, and ofloxacin show high cross-reactivities with the anti-pefloxacin antibody (*26*). This research tells us that the farthest group such as methyl or ethyl connected with the piperazine ring is the most important structural factor to determine the cross-reactivity for a (fluoro)-quinolone toward pefloxacin antibody. For anti-gatifloxacin antibody prepared in this study, there is a methyl group located in position 3′ (see **Figure 2** for numbering of the molecule). This methyl group is special for gatifloxacin among the (fluoro)-quinolones listed in **Figures 1** and **2**. We believe this unique structural feature leads to very high specificity of the anti-gatifloxacin antibody. Compared with the structures of (fluoro)-quinolones that have been studied to prepare corresponding antibodies (**Figure 1**), a substituent located at position 8 is an obvious structural feature for gatifloxacin. This feature is believed to contribute to the high specificity of the anti-gatifloxacin antibody. Flumequine is the only compound in **Figure 1** having a substituent in position 8; its antibody, anti-flumequine, shows high specificity (*24*). It can be understood from these arguments why anti-gatifloxacin antibody prepared by linking carboxylic acid with an amino group in carrier proteins shows such excellent specificity. Some other research, however, has demonstrated that the substituents located in quinolone ring also, more or less, influence the cross-reactivity of antibody. Huet et al. (*25*) 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 (**Figures 1 and 2**), is the most important structural factor to determine the cross-reactivity for a (fluoro)-quinolone toward sarafloxacin antibody or norfloxacin antibody. Their results of cross-reactivity study did not show significant difference whether a molecule has an ethyl or a methyl or no group connected to the piperazinyl moiety.

Matrix Effects and Variation Determination for Milk Sample. It has been known that various substances existing in complex biological systems can effect antigen−antibody interaction in immunoassays (*30*). To reduce matrix effect, we can either clean up the sample or dilute the sample solution. The second method was applied in this study because it is easier to perform. Three different dilute concentrations were used to applied cELISA to compare the competitive curves obtained with the result in PBST buffer (**Figure 4**). It can be seen from the results that when dilution of defatted milk increased from 1:5 to 1:20, the absorbance gradually approached the PBST buffer values, indicating that as dilution increases, the matrix effect decreases gradually. The IC_{50} values with gatifloxacin as the competitor were 13.99, 3.01, and 2.98 ng/mL when dilution factors were 1:5, 1:10, and 1:20, respectively, compared with 2.60 ng/mL in PBST. Although the IC_{50} values between 1:10 dilution and PBST buffer show minor differences, indicating a certain level of matrix effect, a 1:10 dilution should be good enough to be used to perform cELISA in a milk system to measure inter- and intra-assay variations. The limit of detection (LOD), estimated as the concentration of gatifloxacin giving a 10% inhibition of the maximum absorbance, was determined to be 0.05 ppb. The standard curve (see Supporting Information) was prepared by using gatifloxacin concentrations of 0.5, 1.0, 2.5, 5.0, and 10 ng/mL in PBST. The curve obtained shows good linearity with R^2 equal to 0.9758. Using this standard curve, a milk system was used as a real system to test the applicability of the cELISA test kit developed (**Table 2**). The different concentrations of 0.5, 1.0, 2.5, 5.0, and 10 ppb of gatifloxacin were spiked into milk samples, and the recovery rates and coefficients of variation were measured. The coefficients of variation were below 14.3% for intra-assay and below 19.6% for interassay. Recovery rates were within 20% of theoretical values, indicating acceptable accuracy.

Table 2. Inter- and Intra-assay Variation of Raw Milk Spiked with Gatifloxacin

	interassay[a]				intra-assay[b]			
level (ppb)	*n*	measured (ppb)	recovery (%)	CV (%)	*n*	measured (ppb)	recovery (%)	CV (%)
0.5	5	0.43 ± 0.09	87	19.6	5	0.44 ± 0.03	88	5.9
1	5	0.95 ± 0.06	95	6.6	5	0.86 ± 0.06	86	7.2
2.5	5	2.52 ± 0.28	101	11.2	5	2.47 ± 0.35	98	14.3
5	5	5.19 ± 0.91	104	17.5	5	5.14 ± 0.38	103	7.4
10	5	10.55 ± 1.20	106	11.4	5	10.40 ± 0.71	104	6.8

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

In summary, we have prepared a high-quality polyclonal anti-gatifloxacin antibody and developed an indirect cELISA to detect residues of gatifloxacin in milk for the first time. The antibody shows high sensitivity with an IC_{50} value of 2.6 ppb and specificity with almost no cross-reactivity toward the common (fluoro)quinolones. The cELISA test kit has a limit of detection of 0.05 ppb and is suitable for use as a rapid screening method for regulation purposes. The applicability of the cELISA kit developed has been tested to detect milk samples spiked by gatifloxacin, and satisfactory results have been obtained.

ABBREVIATIONS USED

MRL, maximum residue level; BSA, bovine serum albumin; GAT, gatifloxacin; OVA, ovalbumin; GAT−cBSA, conjugate of gatifloxacin and cBSA; cBSA, cationized BSA; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; LOD, limit of detection; IC_{50}, concentration at 50% inhibition; Da, unit of molecular mass, dalton; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Tween 20; OPD, *o*-phenylenediamine; DMF, *N*,*N*-dimethylformamide; SAS, saturated ammonium sulfate; cFA, complete Freund's adjuvant; iFA, incomplete Freund's adjuvant.

Supporting Information Available: Standard calibration curve for gatifloxacin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review April 4, 2007. Revised manuscript received June 11, 2007. Accepted June 13, 2007. This research was supported by National Natural Science Foundation of China (No. 20675048).

JF070978G