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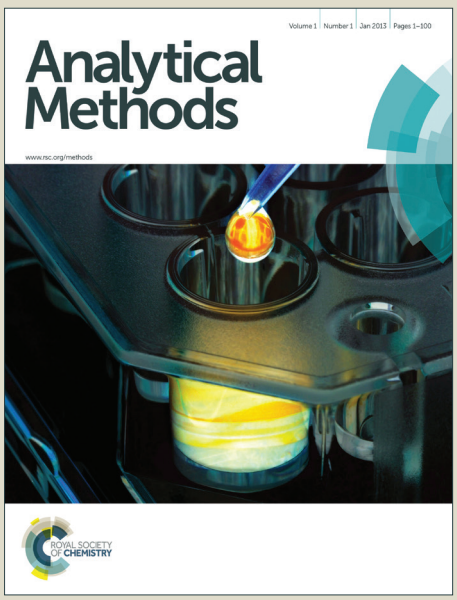

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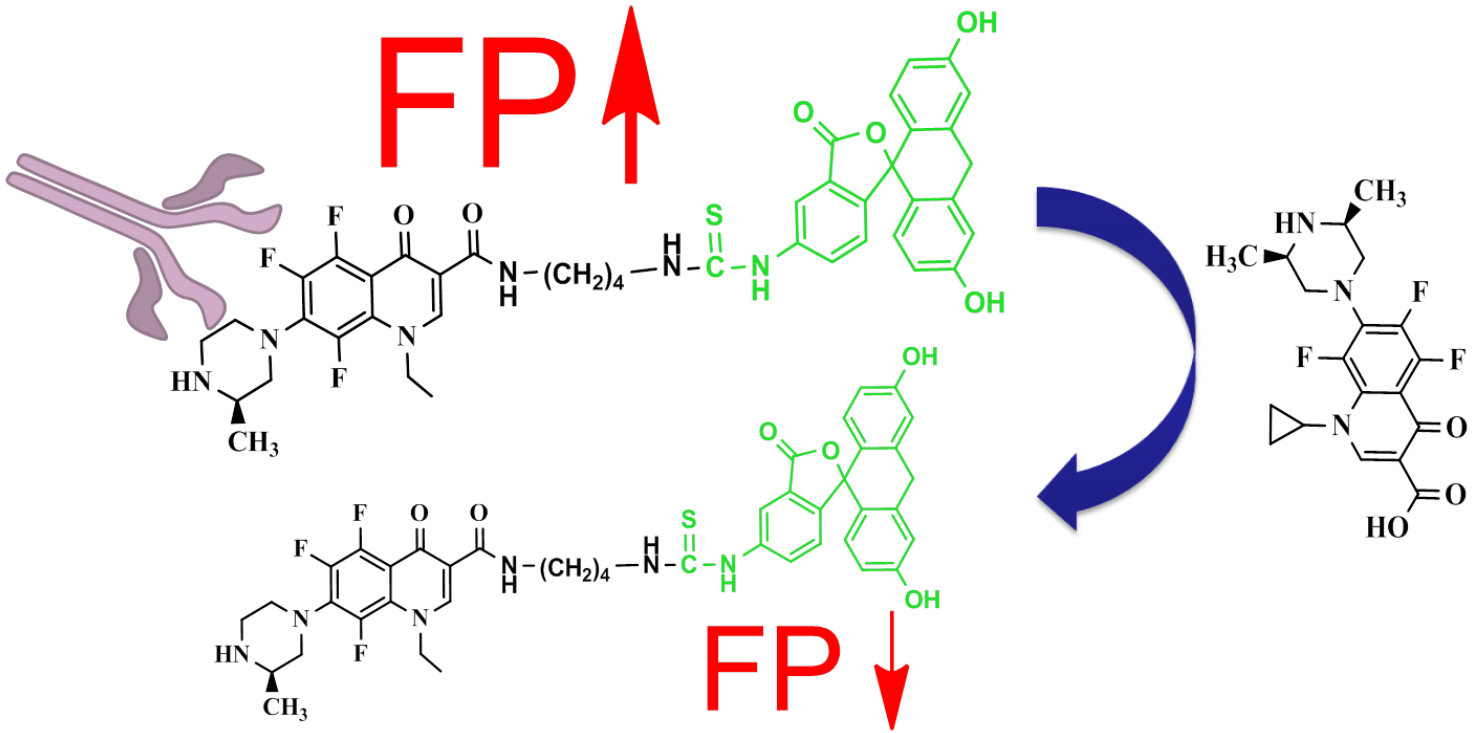
Rapid determination of orbifloxacin residue in milk by a optimized fluorescence polarization immunoassay (FPIA) based on a heterogeneous fluorescent tracer.



Development and Optimization of a Fluorescence Polarization Immunoassay for Orbifloxacin in Milk

Tiejun Mi,†[a] Xiao Liang,†[a] Long Ding,[a] Suxia Zhang,[a] Sergei A. Eremin,[b] Ross C. Beier,[c] Jianzhong Shen,[a] and Zhanhui Wang [a]*

[a] *Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, China Agricultural University, Beijing 100193, People's Republic of China*

[b] *Department of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119991, Russia*

[c] *Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2881 F&B Road, College Station, TX 77845-4988, USA*

†These authors contributed equally to this paper.

*Author to whom correspondence should be addressed.

Tel: 8610 - 6273 3088

Fax: 8610 - 6273 3088

E-mail: wangzhanhui@cau.edu.cn

Abstract A novel monoclonal antibody for orbifloxacin (ORB) was produced for the frist time and used to develop a homogeneous fluorescence polarization immunoassay (FPIA) for ORB determination in milk. Three coating antigens and six fluorescent tracers were prepared, and evaluated by ELISA and FPIA methods, respectively. Heterogeneous tracer, LOM-BDF, was selected to develop the FPIA, providing high sensitivity and stability. Moreover, a new optimization strategy for tracer and Ab concentration was proposed by integrating Z′ factor into checkerboard titration, and Z′ factor > 0.8 was considered as a prerequisite to ensure the robustness of quantitative FPIA. The optimized FPIA provided a detection limit of 3.9 ng mL^{-1} and IC_{50} of 24.5 ng mL^{-1} with an expected Z′ factor of 0.81. Saturated $(NH_4)_2SO_4$ precipitation was applied for milk sample pre-treatment due to high tolerance to elevated-ionic strength in the FPIA. Mean recoveries of fortified milk were ranged from 74.3 to 112 % at the adding levels of 10, 20 and 40 ng mL^{-1}. The results indicated that the developed FPIA was efficient and especially suitable for fast screening of ORB in milk.

Keywords Fluorescence polarization immunoassay; Assay optimization; Monoclonal antibody; Orbifloxacin; Milk

1. Introduction

Quinolones (QNs) and the subset fluoroquinolones (FQs) represent a large group of synthetic antibiotics that are widely used for both the prevention and therapy of disease, not only in humans, but also in animal medicine. Some QNs including enrofloxacin (ENRO), danofloxacin (DANO), difloxacin (DIF), flumequine (FLU), marbofloxacin (MARB), orbifloxacin (ORB), oxolinic acid (OA) and sarafloxacin (SARA) are approved for veterinary practice in many countries.[1, 2] However, residues of these drugs in food could pose potential threats to human health; moreover, these residues may contribute to the development of antibiotic resistant pathogenic bacteria.[3, 4] With consideration of these potential hazards, in 2005 ENRO was withdrawn in the United States from use in poultry.[5] To minimize the risk of QNs exposure to humans *via* products from food-producing animals, maximum residue limits (MRLs) have been established for several QNs by a number of countries and organizations. Orbifloxacin, a third-generation fluoroquinolone developed exclusively for veterinary use, was approved in Japan for use in cattle and swine production for the treatment of gastrointestinal and respiratory infections, whereas in the United States it has been approved only for pets (dogs and cats).[1, 2, 6] MRL for ORB was set at 20 ng mL^{-1} (g^{-1}) in cattle and swine edible tissues and products, including milk.[7] It has been demonstrated that ORB transfers from the blood into the milk rapidly, and high-levels of ORB was observed in milk secretions.[8] Thus, it is necessary to develop reliable and accurate analytical methods for the determination of ORB residues in food animal products, especially milk.

The basis of many methods used for analysis of QNs is dominated by liquid chromatography coupled with fluorescence detection or mass spectrometry, that indicate excellent sensitivity and accuracy.[9, 10] However, these instrumental techniques are generally complicated, time-consuming, and expensive to use in routine screening programs. Immunoassay techniques like the enzyme-linked immunosorbent assay (ELISA) may be an effective and economical alternative to instrumental methods. However, ELISA is a heterogeneous solid-phase method, which requires a long time (more than 2 h) for the immunoreactions to reach equilibrium and for multiple-washing steps to separate the free and antibody-bound analyte. Fluorescence polarization immunoassay (FPIA) is a competitive homogeneous assay in solution phase based on differences in fluorescence polarization (FP) of the fluorescent-labeled analyte in the antibody-bound and non-bound fractions. In a homogeneous solution phase immunoassay, the immunoreaction can reach equilibrium in minutes or even seconds, and no separation or washing steps are required. Although FPIA is prone to interference by matrix and antibody cross-reactivity, the advantage in detection speed makes it more suitable for determining a large number of samples than the ELISA.[11] Multiple-FPIAs have been developed for the determination of food contaminants, the majority of which are for analysis of pesticides [12-15] and for mycotoxins [16-20]. Also, some veterinary drugs in food residue, including sulfonamides [21-26], maduramicin [27], and chloramphenicol [28] have been analyzed by FPIA.

In the current study, only two QNs, sparfloxacin (SPA) and lomefloxacin (LOM), which shared unique similarities with ORB in chemical structure, were cross-reactive with monoclonal antibody (MAb) against ORB, and selected to synthetize heterologous coating antigens for ELISA and fluorescent tracers for FPIA. Moreover, a new optimization strategy for tracer and Ab concentrations in FPIA was described based on Z′ factor and checkerboard titration. To our knowledge, this is the first report for production of MAb against ORB, synthesis of fluorescent tracers and development of a FPIA for ORB determination.

2. Materials and Methods

2.1 Chemicals and standards

Bovine serum albumin (BSA), Ovalbumin (OVA), fluorescein isothiocyanate (FITC) isomer I, *N*-hydroxysuccinimide (NHS), *N,N'*-dicyclohexylcarbodiimide (DCC) and Freund's complete and incomplete adjuvants were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyethylene glycol (PEG 2000) was purchased from Merck-Schuchardt OHG (Darmstadt, Germany). Cell culture media (DMEM) was obtained from Huamei (Beijing, P.R. China). Fetal calf serum and supplements were obtained from GIBCO BRL (Carlsbad, CA). The analytical standards of orbifloxacin, sparfloxacin, lomefloxacin, enoxacin (ENO), marbofloxacin, ofloxacin (OFL), danofloxacin mesylate, oxolinic acid and nalidixic acid (NAL) were obtained from Dr. Ehrenstorfer GmbH, (Ausburg, Germany). Ciprofloxacin hydrochloride (CIP), enrofloxacin, flumequine, norfloxacin (NOR),

pefloxacin methanesulfonate (PEF), sarafloxacin, and difloxacin were purchased from the China Institute of Veterinary Drug Control (Beijing, P.R. China). Common solvents and salts were analytical reagent grade and supplied by Beijing Reagent Corporation (Beijing, P.R. China).

Borate buffer (0.05 M, pH 8.0) with 0.1% sodium azide was used as the working buffer for all FPIA experiments. Individual stock standard solutions of the QNs (1 mg mL^{-1}) were prepared by dissolving 10 mg of each QN standard in 1 mL of 0.03% NaOH and diluted to a final volume of 10 mL with methanol. Aqueous standard solutions of the analytes in the range of 0.1 to 1000 ng mL^{-1} were prepared by dilution of the stock solution with borate buffer.

2.2 apparatus

Pre-coated silica gel 60G F_{254} glass plates (plate size: 10 × 10 cm; layer thickness: 0.15 ~ 0.2 mm, particle size: 2μm) for thin-layer chromatography (TLC) were purchased from Yantai XinDe Corporation (Shandong, P.R. China). Polystyrene microplates (96-well) for ELISA and black microplates (96-well) with a non-binding surface for FPIA were obtained from Corning Life Sciences (New York, NY, USA). A SpectraMax M5 microplate reader from Molecular Devices (Downingtown, PA, USA) was used to measure fluorescence polarization and optical density (OD) signal. High-performance liquid chromatography (HPLC) analysis were performed using a Waters 2695 Separations Module and a Waters 2475 Multi-Wavelength Fluorescence Detector equipped with a reverse-phase Symmetry C_{18} 250 mm × 4.6 mm column (Waters, Milford, MA, USA).

2.3 Synthesis of protein and fluorescent conjugates

The carboxylic group of ORB (20 mg, 50 μmol) was activated by gently stirring overnight at room temperature in 1mL of *N*,*N*-Dimethylformamide (DMF) containing 6 mg (60 μmol) of NHS and 12 mg (60 μmol) of DCC. The mixture was centrifuged at 1000 × g for 10 mins to remove precipitated dicyclohexylurea. The clear supernatant phase was collected and 900 μL of the supernatant was added drop-wise to 30 mg (0.5 μmol) BSA in 8 mL of sodium carbonate (0.01 M, pH 9.0). The reaction was stirred overnight at 4°C, and then dialyzed against 1000 mL of PBS (0.01 M, pH 7.4) for 3 days, which was changed twice a day. The immunogen, ORB-BSA, was diluted to 2 mg mL^{-1} with PBS and divided into aliquots and stored at –20°C until used. Three coating antigens (ORB-OVA, SPA-OVA and LOM-OVA) were prepared in the same way.

Three fluorescein derivatives, fluorescein thiocarbamyl ethylenediamine (EDF), fluorescein thiocarbamyl butanediamine (BDF) and fluorescein thiocarbamyl hexylenediamine (HDF) were synthesized from FITC as previously described.[29] ORB (4 mg, 10μmol) was activated with NHS and DCC as stated above and added to 4.8 mg (10 μmol) of EDF in 0.5 mL DMF. After stirring for 12 h at RT, a small portion of the reaction mixture was purified by TLC using methanol/AcOEt/NH_4OH (2:1:0.04, v/v/v) as the eluent. The major yellow band at R_f 0.5 was scraped from the plate and extracted with methanol (0.2 mL). Other tracers ORB-BDF, ORB-HDF, LOM-EDF, LOM-BDF and LOM-HDF were prepared by the same method (see Table 1). The tracer concentration was calculated according to the absorbance at a wavelength of

492 nm [28], and all the tracers were diluted with the borate buffer to get the routine working solution of 10nM, which the fluorescence intensity (FI) of tracers was about 10 times that of buffer background (2 ~ 3 RFU).[26]

2.4 MAb production

The procedures used for MAb production were similar to those described in our previous report.[23] Briefly, five 8-week old BALB/c mice were immunized with ORB-BSA at a dosage of 1 μg g^{-1} body weight in an equal volume of Freund's complete adjuvant. Booster injections were then given 2, 4, and 6 weeks later with the same dosage of immunogen emulsified with an equal volume of Freund's incomplete adjuvant. Blood was collected from the caudal vein of each mouse and antisera titers were determined before fusion. The mouse exhibiting the highest-titer and best sensitivity was sacrificed after the last immunization and the spleen was removed for hybridoma production. After fusion, the cells were selected using the selection medium. The growing hybridoma cells were screened for antibody production by ELISA in the absence and presence of 100 ng mL^{-1} ORB. The positive hybridomas were cloned by the limiting dilution method, and one stable clone was obtained. Ascites fluid of the positive hybridoma was collected and purified by $(NH_4)_2SO_4$ precipitation.

2.5 ELISA protocol

The ELISA approach was described as follows: The ELISA plate was coated with coating antigen (100 μL per well) in coating buffer (0.05 M carbonate buffer, pH 9.6) by incubation at 37 °C for 2 h. After washed with 300 μL per well of PBS (0.01 M,

pH7.4) with 0.05% Tween20 for 4 times, the plate was blocked by incubation with 300 μL per well of blocking buffer (PBS with 1.0% casein) for 1h and followed by another washing step. Then 50 μL per well of PBS in the absence or presence of standard was added, followed by adding 50 μL per well of diluted MAb. The plate was incubated for 30 mins at 37°C. After washing, 100 μL of diluted goat-anti-mouse IgG-HRP solution was added, and the plate was incubated for 30 mins at 37 °C again. Then, substrate solution (100 μL per well) was added after washing, and the reaction was stopped by 50 μL per well of 2 M H_2SO_4 after incubation at 37°C for 15 mins. Then the OD value was determined in dual-wavelength mode (450 nm for test and 630 nm for reference). The concentrations of MAb and coating antigen were optimized by checkerboard titration according to B_0 value of around 1.5 and sensitivity

2.6 FPIA protocol

The FPIA approach was described as follows: 50 μL per well of tracer solution was mixed with 50 μL per well of borate buffer in the absence or presence of standard in the microplate well. Subsequently, 50 μL per well of diluted MAb was added, and the mixtures were shaked for 10 s in the microplate reader. After a short incubation period (2min) at room temperature, FP value was measured at λ_{ex} = 485 nm, λ_{em} = 530 nm (emission cutoff = 515 nm, G factor = 1.0). And the blank control containing all assay elements except tracer was performed simultaneously for correcting polarization measurement by subtracting the background in assay buffer or sample matrix.

The antibody binding assay was performed by mixing tracer (10nM) with two-fold serially diluted MAb over the range of 1/200 to 1/102400. Then antibody titers (the dilution resulting in 50% tracer binding) for different tracers were obtained according to the respective dilution curves. The optimal pair of fluorescent tracer and antibody concentrations were selected by checkerboard titration according to appropriate Z′ factor [30] and sensitivity.

2.7 Curve fitting and cross-reactivity determination

The sigmoidal curve was used to fit both ELISA and FPIA data by OriginPro 7.0 (OriginLab Corp., Northampton, MA, USA). IC_{50} was the standard concentration at 50% of specific binding. The limit of detection (LOD) was defined as the standard concentration corresponding to the mean signal of 20 independent blank controls minus three times their standard deviation (SD). The limit of quantification (LOQ) was the standard concentration at IC_{80} and the dynamic range was defined as the standard concentration at the range of IC_{20} ~ IC_{80}. Cross-reactivity (CR) was calculated by both ELISA and FPIA methods according to the following equation:

$$CR\ (\%) = (IC_{50}\ \text{of ORB} / IC_{50}\ \text{of tested QNs}) \times 100 \qquad \text{Eq.1}$$

where IC_{50} values were obtained from calibration curves and CR was calculated using IC_{50} values in nM unit.

2.8 Effects of Physicochemical Conditions on Assay Performance

The effects of pH value, salt concentration, and organic solvent on assay performance were assessed by comparing δmP and IC_{50} parameters of the standard curves under various conditions. The ORB standard, tracer and MAb were dissolved

in buffers of different pH values, salt concentrations, and organic solvent concentrations, and were prepared as follows. The pH of a 50 mM borate buffer was about 9.4, and then the pH of the borate buffer was adjusted to different values (from pH 5 to 10) with concentrated HCl or 6 M NaOH. Buffers of different ionic strength (0 to 2.0 M) were prepared by adding solid NaCl to 50 mM borate buffer. Buffers with different organic solvents were comprised of 50 mM borate buffer containing methanol or acetonitrile in different proportions (0 to 20%).

2.9 Sample preparation

Negative control milk (4 mL) was added into 10 mL polypropylene centrifuge tubes and fortified with the appropriate ORB standard solution. After mixing with a vortex mixer, the samples were allowed to stand for 30 mins in the dark at RT. An equal volume of saturated $(NH_4)_2SO_4$ solution was added to deproteinize the samples followed by mixing in a vortex mixer, and then sonicating for 5 mins. The mixtures were centrifuged at 7400 × g at 4°C for 10 mins. Some of the clear supernatant (500 µL) was filtered through a 0.45 µm filter and 50 µL of the filtrate was analyzed by FPIA. Additionally, 4 mL of supernatant was collected and the pH adjusted to 6.0 by the addition of HCl, followed by extracting with 4 mL of dichloromethane (CH_2Cl_2). The whole lower organic layer (around 4 mL) was transferred into an eppendorf tube and evaporated under a stream of nitrogen gas at 40°C. The residue was re-dissolved in 1.0 mL of the mobile phase and filtered through a 0.45 µm filter, 50 µL of the filtrate was subjected to HPLC analysis. HPLC method was modified based on the previous study.[8] The mobile phase consisted of a mixture of acetonitrile

and 0.5% trifluoroacetic acid solution (20:80, v/v). The HPLC system was operated isocratically at a flow rate of 0.8 mL min^{-1} and fluorescence detection was conducted at an excitation wavelength of 280 nm and an emission wavelength of 450 nm.

3. Results and discussion

3.1 Characterization and selection of tracers

In this study, three coating antigens (ORB-OVA, SPA-OVA and LOM-OVA) were prepared with respective cross-reactants (ORB, SPA and LOM), and evaluated in ELISA method. As observed in Fig. 1, the heterogenous coating antigen LOM-OVA exhibited the highest sensitivity in ELISA method. Then, LOM was selected to prepare heterogeneous fluorescent tracers to investigate the effect of tracer structure on the analytical characteristics of the FPIA. Three synthesized heterogeneous tracers which were different in the bridge length between LOM and fluorescein (two carbons length for LOM-EDF, four carbons length for LOM-BDF and six carbons length for LOM-HDF) were expected to improve assay sensitivity. And other three similar tracers, ORB-EDF, ORB-BDF and ORB-HDF were prepared as the homogeneous controls.

Six tracers were fristly characterized by the antibody binding assay. As the results shown in Fig. 2, antibody titers for LOM-EDF, LOM-BDF, LOM-HDF, ORB-EDF, ORB-BDF and ORB-HDF were 1/1600, 1/2400, 1/4000, 1/3000, 1/7000 and 1/8000, respectively. But two of these tracers (LOM-EDF, ORB-EDF) did not present the adequate increase in FP signal ($\delta mp<100$) after adding saturating amounts of

antibody. Other four tracers giving satisfactory binding (δmp>180) were subjected to a further identification by ESI-MS in positive ion mode, which indicated the *m*/*z* of 810.6 for LOM-BDF, 839.4 for LOM-HDF, 850.4 for ORB-BDF, 875.4 for ORB-HDF. Optimal tracer was selected according to assay sensitivity and stability that displayed in Fig. 3. The highest-sensitivity (lowest B/B_0) was obtained when using the heterogeneous tracer LOM-BDF with shorter linker (four carbons length). Generally, the antibody affinity for the hapten conjugate is higher than the analyte itself due to the homology of the hapten used in the immunogen and tracer.[31] In order to achieve the high sensitivity, the antibody affinity for the tracer should be of the same order of magnitude as for the analyte.[32] So, the tracers with structurally heterogeneous hapten or linker between hapten and fluorescein were considered to change the affinity with Ab. In agreement with previous reports [23, 31], the heterogeneous tracer LOM-BDF with shorter linker provided lower-relative affinity with antibody, resulting in higher-sensitivity in our work.

Moreover, FP signal was much more stable using the heterogeneous tracers during the incubation time over 60 mins compared with homogeneous tracers (Fig. 3). Only a short incubation time (1min) was required to reach interaction equilibrium, which proved to be more likely to develop a real "mix and read" assay. Thus, total time required for one 96-well microplate was about 15 mins for FPIA compared with more than 2 h for a conventional ELISA. So, LOM-BDF was selected as the optimal tracer in the following studies.

3.2 Z factor in FPIA

The Z factor (Eq.2) is a widely used statistical parameter to judge assay quality in high-throughput screening (HTS) assays, which indicates the ability to distinguish signals between sample and control.

$$Z = 1 - (3\sigma_s + 3\sigma_c)/|\mu_s - \mu_c| \quad \text{Eq.2}$$

Where μ_s and μ_c represent the average signal of the sample and the control, respectively. And σ_s and σ_c are the respective SDs of these values. $Z \geqq 0.5$ represents good separation of the distributions and indicates an excellent assay; $0 < Z < 0.5$ means moderate separation of the distributions and indicates a doable assay; $Z < 0$ is a sign of poor quality. Similarly, this factor can also serve as the parameter for the quality of the assay itself, which defined as Z' factor:

$$Z' = 1 - (3\sigma_{c+} + 3\sigma_{c-})/|\mu_{c+} - \mu_{c-}| \quad \text{Eq.3}$$

Where μ_{c+} and μ_{c-} represent the average signal of the positive and negative control, respectively. And σ_{c+} and σ_{c-} are the respective SD of these values.[30]

In order to apply Z factor in competitive FPIA for quantitative determination, these two factors were similarly defined as:

$$Z = 1 - 3 \times (SD_{max} + SD_x)/(mP_{max} - mP_x) \quad \text{Eq.4}$$

$$Z' = 1 - 3 \times (SD_{max} + SD_{min})/(mP_{max} - mP_{min}) \quad \text{Eq.5}$$

Where mP_{max} and mP_{min} are the observed mean FP signal for bound and free fluorescent tracer. Generally, mP_{max} and mP_{min} represent maximum (no inhibition) and minimum (complete inhibition) signal in standard curve; $mP_{max} - mP_{min}$, represents the assay window (δmP); mP_x is the observed mean FP value in the presence of

analyte at some concentration; SD_{max}, SD_{min} and SD_x are the respective SDs of these values.

Typically, a Z' factor $\geqq 0.5$ indicates a reliable assay in HTS. However, that may be not a suitable threshold value for quantitative determination in our opinion. If Z' factor = 0.5, $Z_{50} = 0$ (Z at IC_{50}), that inevitably resulted in poor quality ($Z<0$) for the concentration less than IC_{50}. On account of defining IC_{80} as LOQ, it was essential to keep $Z_{80}>0$ (Z at IC_{80}) in quantitative FPIA. As displayed in Fig. 4, $mP_{max} - mP_{80} >$ 6SD and δmP >30SD were achieved in the case of almost constant variance in FP value for the fixed tracer concentration (shown in Fig. 5). Consequently, $Z'>0.8$ was obtained and considered as a prerequisite to ensure the robustness of quantitative FPIA.

3.3 Tracer and antibody concentration

It is known that the tracer signal sets the sensitivity, low tracer concentration will result in the high sensitivity. In previous reports, tracer working solution was empirically set to its concentration exhibited about 10 times more FI signal than the buffer background.[26, 32] However, the precision of FP signal (SD), rather than the FP signal itself, is related to the tracer concentration, low tracer concentration also results in low precision in FP signal.[33] As the results shown in Fig. 5, almost constant SD of FP value ($SD_{max} \approx SD_{min} \approx SD$) was observed for the fixed tracer concentration, and precision decreased for the tracer concentration less than 10nM. It has been proved that the dilution of antibody corresponding to 50% tracer binding would provide best sensitivity.[23] However, the FP assay window (δmP) is very narrow and dependent on

the concentration of Ab, and low Ab concentration will not provide a good assay window for measurement.[34]

Therefore, it is necessary to establish a method for optimization of their concentrations simultaneously. One of the most effective methods was checkerboard titration, which has been widely applied in ELISA optimization. Moreover, Z′ factor was integrated into checkerboard titration, due to it incorporating the precision of FP signal and the assay window. As the results shown in Table. 2, 20 nM of LOM-BDF and 1/1600 of Ab dilution were selected as the optimal couple for the tracer and Ab concentrations on the basis of Z′ factor > 0.8 and higher sensitivity (lower B/B_0). 20 nM of LOM-BDF giving FI of ~50 RFU was approximately 20 times that of the background signal for borate buffer, and assay window was about 130 mP obtained at 1/1600 of Ab dilution, which was corresponding to 60% tracer binding. The optimal standard curve was shown in Fig. 4 with a IC_{50} of 24.5 ng mL^{-1}, LOD of 3.7 ng mL^{-1}, dynamic range of 7.3–90.7 ng mL^{-1} , R^2 of 0.997 and expected precision (Z′ = 0.81).

3.4 Cross-reactivity determination

The specificity of the ORB MAb was evaluated by determining the cross-reactivity with 15 other QNs (SPA, LOM, ENO, MARB, OFL, DANO, OA, NAL, ENRO, CIP, NOR, FLU, PEF, SARA and DIF) in both the ELISA and FPIA (Table 3). The MAb showed medium and less cross-reactivitiy with SPA and LOM, respectively, and showed negligible cross-reactivity with the other QNs. Based on the cross-reactivity results, some substituents of LOM, ORB and SPA may play important roles in the antibody recognition; when the CH_3-group is present at the R_3, or R_5, position of the

piperazine ring, and when the fluorine substituent is at the C_8 position of the quinolone nucleus, which are nonexistent structural features in other QNs (see Table 1).

3.5 Effects of Physicochemical Conditions on Assay Performance

3.5.1 pH effects

The relationship of the IC_{50} and δmP as a function of pH was shown in Fig. 6(a). The lowest IC_{50} and highest δmP were obtained at pH 8; whereas, δmP was significantly reduced and the IC_{50} was lower at pH 9 and 10. The results demonstrate that the assay performed optimally at pH 8, and did not function well at higher or lower pH values.

3.5.2. Ionic strength

The assay was tested in working buffer with salt concentrations ranging between 0 and 1.5 M, and the results are presented in Fig. 6(b). No negative effect on the IC_{50} or δmP was observed at salt concentrations ranging from 0 to 1.0 M. A 30% increase in the IC_{50} occurred as a result of the 1.5 M salt concentration, and δmP simultaneously increased by 10%. Therefore, elevated-ionic strength did not remarkablely affect the FPIA.

3.5.3 Organic solvent

The effects of methanol and acetonitrile were studied because these solvents are water-miscible and commonly used in sample extraction procedures. However, only 10% methanol or 2.5 % acetonitrile could be tolerated in current assay (Data not shown).

3.6 Analysis of spiked milk

A FPIA method was developed to determine ORB in milk. Milk is a very complex matrix consisting of different components (fats, proteins, various sugars, etc.), which can strongly interfere with the analytical determination of residues.[28] FPIA is susceptible to some of these components; therefore, milk protein removal is required. Since elevated-ionic strength almost does not affect the performance of our FPIA, a saturated solution of $(NH_4)_2SO_4$ was used for protein precipitation in the recovery study, which has proved efficient for removing protein from milk.[28] In order to confirm the recovery results, the samples were simultaneously analyzed by HPLC after a liquid–liquid extraction (LLE) clean-up step. The mean recoveries were 74.3 to 112 % with coefficient of variation (CV) ranging from 7.4 to 26.8 % at the adding levels of 0.5 ~ 2 MRLs (shown in Table 4), and improved recovery and coefficients of variation were observed by HPLC determination following the clean-up step. FPIA standard curves performed in both borate buffer and milk matrix were displayed in Fig.7, which suggests that the $(NH_4)_2SO_4$ precipitation step did not completely eliminate the matrix interference from milk; however, it was not only a sufficient pretreatment for screening assay, but a safe and environmental-friendly technology. Twenty four milk samples collected from the local markets were determined by these two methods, no positive results were found in these samples.

4. Conclusions

A rapid, simple, and sensitive FPIA for orbifloxacin determination in milk was developed for the first time. LOM-BDF with 4 carbon linkers was selected as optimal

fluorescent tracer due to its high sensitivity and short incubation time (1min). Total time required for measuring one 96-well microplate was about 15 mins for FPIA in comparison with more than 2 h for a conventional ELISA. Z′ factor>0.8 was considered as the prerequisite to ensure the robustness of quantitative FPIA, and the optimized FPIA showed satisfactory results for ORB analysis in milk using a simple and safe sample pre-treatment. The optimization strategy for tracer and Ab concentrations described in the present study could be beneficial in the development of FPIA for quantitative determination.

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Figure Captions

Fig. 1. The normalized ELISA calibration curves using three coating antigens: LOM-OVA (IC_{50} = 5.53 ng mL^{-1}), SPA-OVA (IC_{50} = 8.13 ng mL^{-1}) and ORB-OVA (IC_{50} = 14.8 ng mL^{-1}). Each point of the curve represents the mean Absorbance ± SD (n=3).

Fig. 2. Antibody binding curves for the anti-ORB MAb with six tracers: LOM-EDF, LOM-BDF, LOM-HDF, ORB-EDF, ORB-BDF and ORB-HDF with antibody titers of 1/1600, 1/2400, 1/4000, 1/3000, 1/7000 and 1/8000, respectively.

Fig. 3. Screening the optimal tracer by comparison of four tracers in sensitivity and stability by incubating the assay at room temperature over 60mins. Each point represents the mean of three replicates (n=3). The data was measured under 10 nM of tracers and respective antibody titers. $\delta mP = mP_{max} - mP_{min}$, represented the assay window. B/B_0 was calculated at the ORB concentration of 20 ng mL^{-1}.

Fig. 4. $Z' > 0.8$ was proposed as the prerequisite to ensure the robustness of FPIA according to $Z_{80} > 0$. Z_{80} was the Z factor obtained at IC_{80}; mP_{80} was the mean FP value at IC_{80} (LOQ).

Fig. 5. SD of the FP signal relatively depends on the tracer concentration (Fluorescence intensity). mP_{max} was the mean FP measured by mixing LOM-BDF with saturating amounts of antibody (1/100) ;mP_{min} was the mean FP for free tracer (LOM-BDF), SD_{max} and SD_{min} were the respective SDs. Each point represents the mean of eight replicates (n=8).

Fig. 6. Effect of a) pH and b) assay buffer salt concentration on the analytical characteristics of the ORB competitive standard curve. Each point represents the mean of three replicates.

Fig. 7. The normalized standard curves for the FPIA determination of ORB in borate buffer (IC_{50}= 24.5 ng mL^{-1}) and milk matrix (IC_{50}= 21.7 ng mL^{-1}). Each point of the curve represents the mean FP ± SD (n=3).

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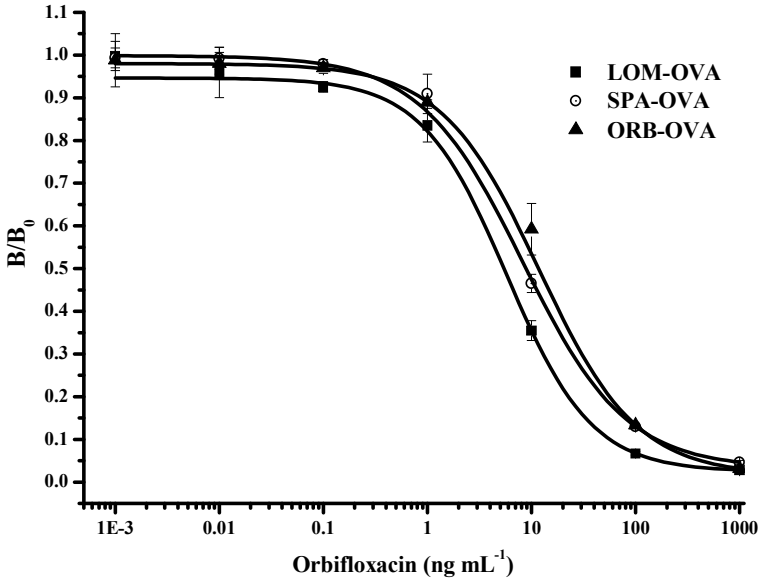

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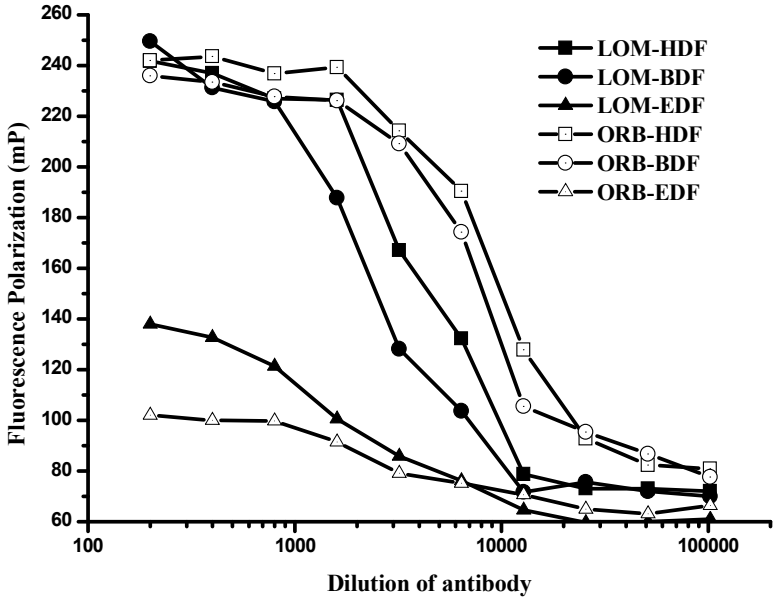

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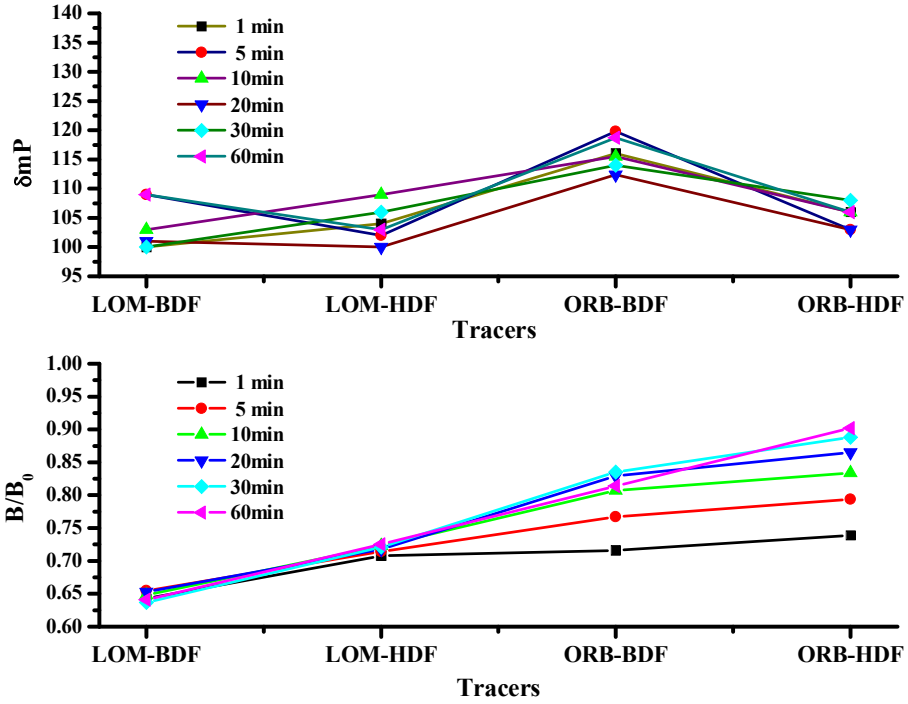

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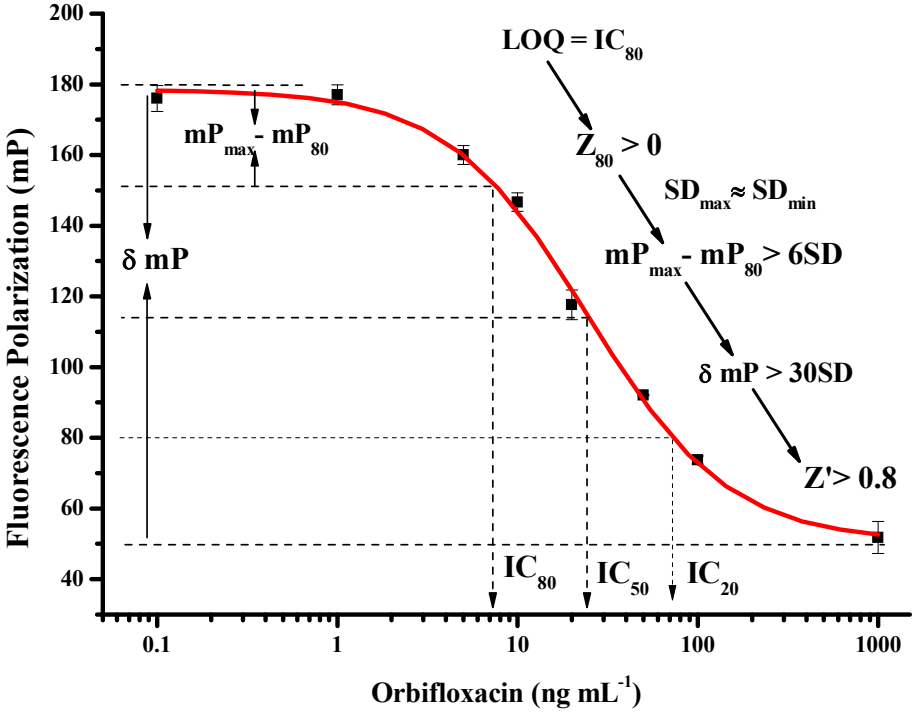

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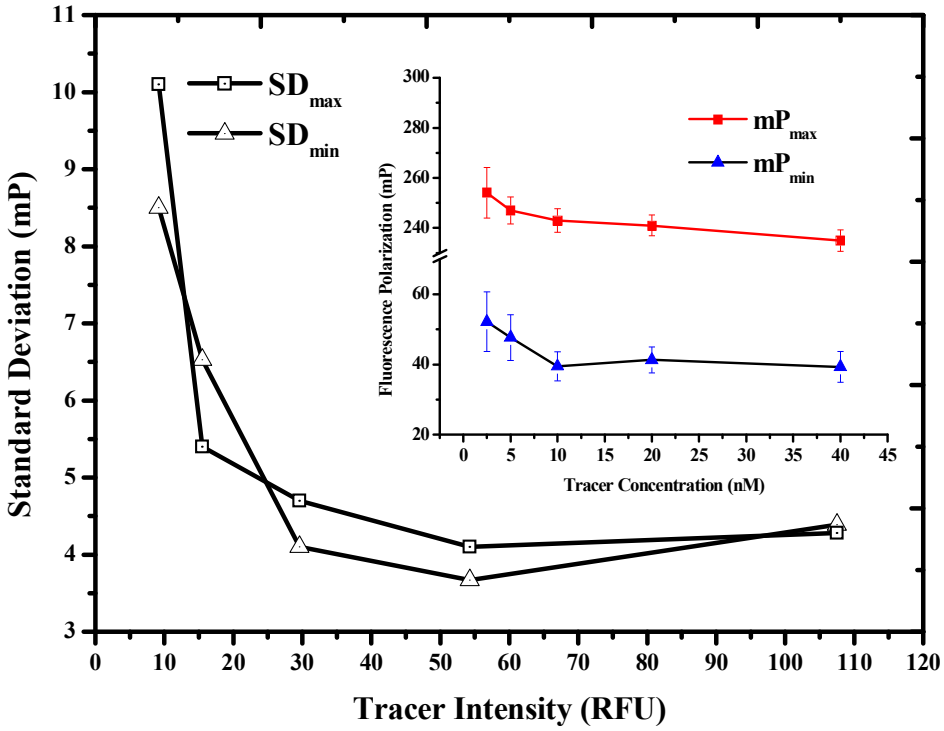

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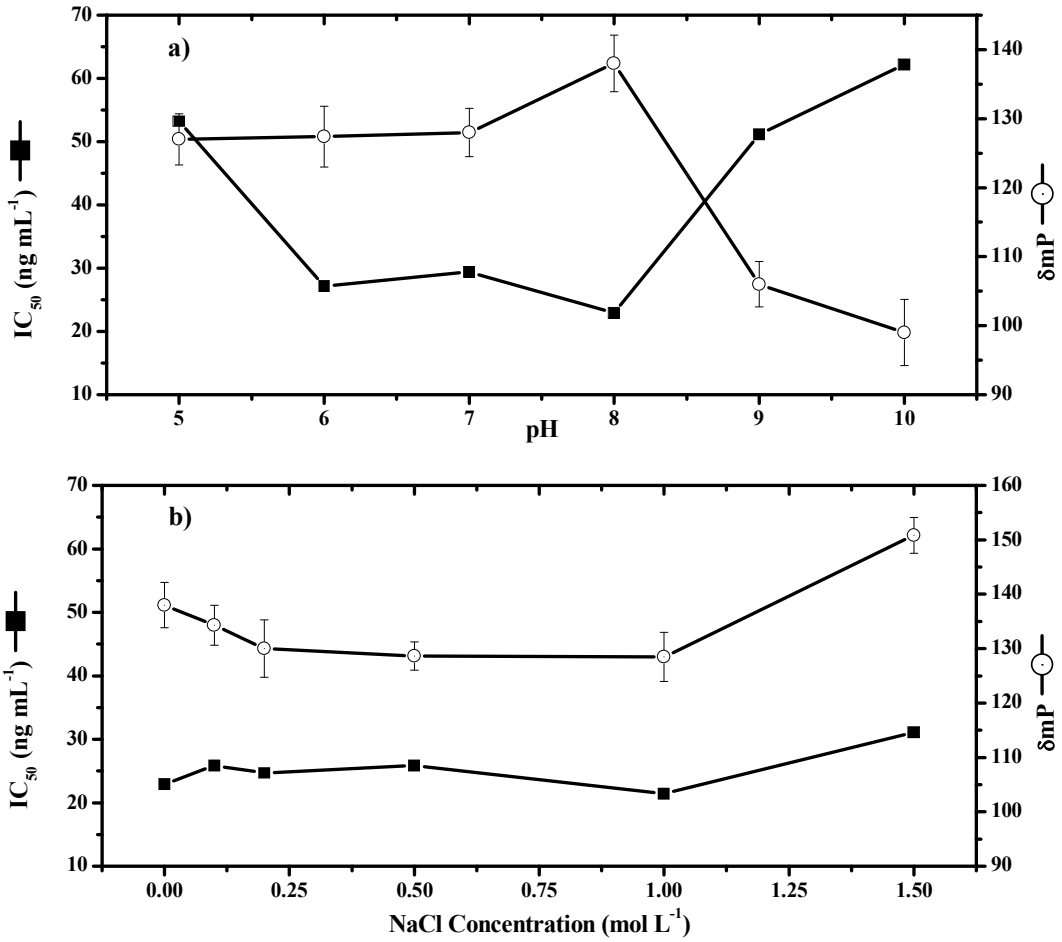
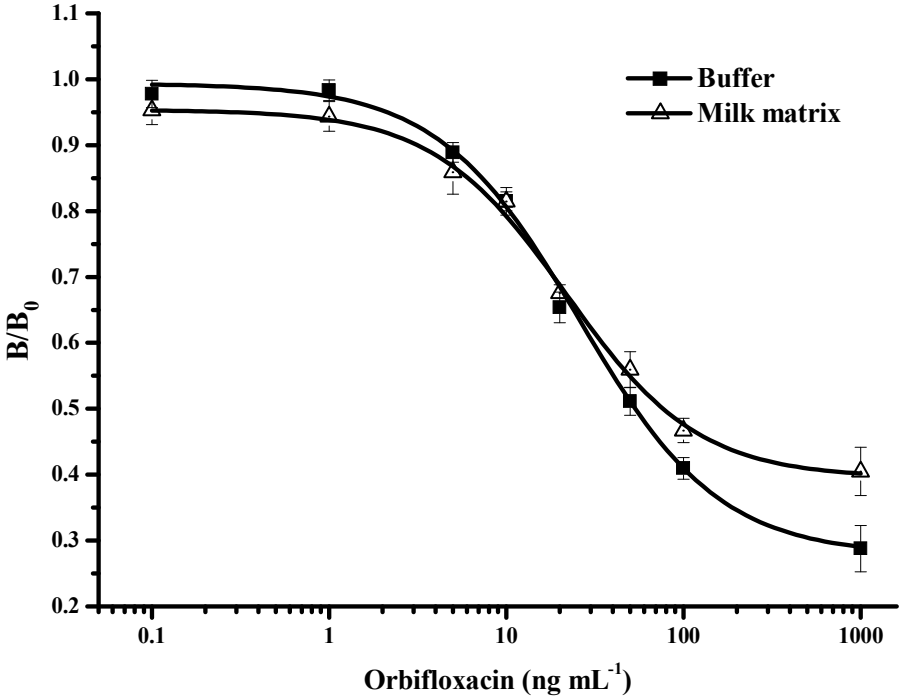


Fig.6.



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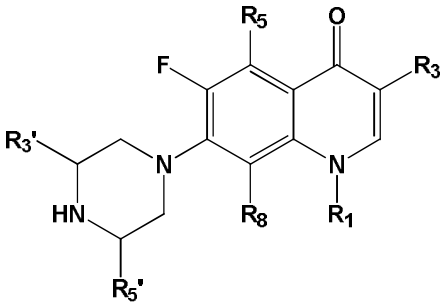
506 Fig.7.

Table 1. Chemical Structures of Fluoroquinolone Drugs, Hapten−Protein Conjugates (Immunogen and coating antigens), and Fluorescent tracers.



Compound	Substituents					
	$R_{3'}$	$R_{5'}$	R_1	R_5	R_8	R_3
FQs						
ORB	CH_3	CH_3	△	F	F	COOH
SPA	CH_3	CH_3	△	NH_2	F	COOH
LOM	H	CH_3	C_2H_5	H	F	COOH
Immunogen						
ORB-BSA	CH_3	CH_3	△	F	F	CO-NH-BSA
Coating antigens						
ORB-OVA	CH_3	CH_3	△	F	F	CO-NH-OVA
SPA-OVA	CH_3	CH_3	△	NH_2	F	CO-NH-OVA
LOM-OVA	H	CH_3	C_2H_5	H	F	CO-NH-OVA
Fluorescent tracers						
ORB-EDF	CH_3	CH_3	△	F	F	CO-NH-$(CH_2)_2$-NH-FITC
ORB-BDF	CH_3	CH_3	△	F	F	CO-NH-$(CH_2)_4$-NH-FITC
ORB-HDF	CH_3	CH_3	△	F	F	CO-NH-$(CH_2)_6$-NH-FITC
LOM-EDF	CH_3	CH_3	C_2H_5	H	F	CO-NH-$(CH_2)_2$-NH-FITC
LOM-BDF	CH_3	CH_3	C_2H_5	H	F	CO-NH-$(CH_2)_4$-NH-FITC
LOM-HDF	CH_3	CH_3	C_2H_5	H	F	CO-NH-$(CH_2)_6$-NH-FITC

△: cyclopropyl, ORB: Orbifloxacin, SPA:Sparfloxacin, LOM: Lomefloxacin, BSA: Bovine serum albumin, OVA: ovalbumin, EDF: Fluorescein thiocarbamyl ethylenediamine, BDF: Fluorescein thiocarbamyl butanediamine, HDF: Fluorescein thiocarbamyl hexylenediamine

Table 2. Determination of optimal concentrations for tracer LOM-BDF and antibody using checkerboard titration in FPIA.

Ab dilution	Parameter	LOM-BDF concentration (nM)			
		10	20	40	80
400	B/B_0	0.967	0.900	0.843	0.748
	Z'	0.86	0.85	0.86	0.85
800	B/B_0	0.904	0.809	0.696	0.780
	Z'	0.84	0.84	0.83	0.74
1600	B/B_0	0.740	0.619	0.727	0.852
	Z'	0.82	0.81	0.67	0.53
3200	B/B_0	0.588	0.670	0.784	0.921
	Z'	0.63	0.58	0.27	-0.33

B/B_0 was calculated at the ORB concentration of 20 ng mL^{-1}.

516 Table 3. Cross-reactivity of several structurally related analogues in the ELISA and FPIA

Analyte	ELISA[a]			FPIA[b]		
	IC_{50} (ng mL^{-1})	IC_{50} (nM)	Cross-reactivity (%)	IC_{50} (ng mL^{-1})	IC_{50} (nM)	Cross-reactivity (%)
ORB	5.94	15.0	100	22.4	56.7	100
SPA	10.5	25.5	58.9	27.8	70.8	80.1
LOM	106.7	275.1	5.45	197	507.9	11.2
Other QNs	$>10^5$	$>10^5$	<0.01	$>10^5$	$>10^5$	<0.01

517 [a] LOM-OVA was the coating antigen for the ELISA

518 [b] LOM-BDF was the fluorescent tracer for the FPIA

519 Table 4. Recoveries of ORB from milk by FPIA and HPLC (n = 4)

ORB	FPIA			HPLC		
Added ($ng\ mL^{-1}$)	Found ($ng\ mL^{-1}$)	Recovery (%)	CV (%)	Found ($ng\ mL^{-1}$)	Recovery (%)	CV (%)
10	11.2 ± 3.0	112	26.8	9.1 ± 1.2	91.0	13.2
20	16.6 ± 2.4	83.0	14.4	17.2 ± 1.5	86.0	8.7
40	29.7 ± 2.2	74.3	7.4	32.5 ± 2.5	81.3	7.7

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