Multiresidue Determination of Sarafloxacin, Difloxacin, Norfloxacin, and Pefloxacin in Fish using an Enzyme-Linked Immunosorbent Assay

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Abstract

An indirect competitive ELISA (icELISA) method for multiresidue determination of Fluoroquinolones (FQs) residues in fish samples has been developed. For this purpose, Sarafloxacin (SAR) was employed to synthesize the immunogen and coating antigen through EDC conjugation method, and cell fusion technology was used to produce anti-SAR monoclonal antibody. Based on the square matrix titration, an icELISA method was established. The dynamic range for Sarafloxacin in assay buffer was from 0.004 to 18 ng/mL, with LOD and IC 50 value of 0.002 ng/mL and 0.32 ng/mL, respectively. After optimization, the physiological pH (7.4) was selected for the imunoassays, and this assay could tolerate up to 10% acetonitrile. The results of this assay showed a high cross-reactivity to Difloxacin (85.5%), Norfloxacin (61.7%), and Pefloxacin (34.8%). Under the 10-fold dilution in authentic fish samples, the regression curve equations for Sarafloxacin, Difloxacin, Norfloxacin and Pefloxacin were y = 1.0114x - 0.4003, R² = 0.9901; y = 0.9782x + 0.2754, R² = 0.9807; y = 0.9892x + 0.0489, R² = 0.9843; and y = 0.9797x + 0.8017, R² = 0.9844, respectively. The results suggest this immunoassay can be used for simultaneous detecting four kinds of FQs in fish samples.

1. Introduction

Infectious diseases are a serious problem for the livestock industries; therefore, various kinds of antibiotics and synthetic antibacterials are widely used for prevention and treatment. The fluoroquinolones (FQs) are the most important group of synthetic antibacterial, which are widely used in...
clinical practice because of their excellent antibacterial activity, wide spectrum of activity, and high degree of bioavailability [1]. However, due to the extensive use of FQs in the animal industry and aquaculture, residues of these drugs are a major concern, especially for those who are allergic to these drugs.

The most commonly used analytical technique for determination of FQs is HPLC with programmable fluorescence detection [2]. Confirmatory analysis using liquid chromatography-mass spectrometry (LC-MS) [3] and LC-MS/MS [4-5] have been reported. Capillary electrophoresis has also proved to be an effective separation tool for FQs [6]. Traditionally, microbiological inhibition tests are considered as multi-residue screening tests for antibiotics in milk, meat or other animal tissues [7]. However, they are not sensitive enough to perform the duty, and the progress of immunoassay development is regarded by the availability of antibodies with appropriate affinity and specificity characteristics. An earlier attempt for the development of a FQ generic immunoassay with polyclonal antibodies can detect nine FQs [8]. Recently, Huet et al. developed a generic immunoassay for a set of 11 FQs with IC₅₀ values ranging from 0.21 to 1.52 ng/mL [9]. But a generic immunoassay for FQs based on the use of a monoclonal antibody (mAb) has not been described.

In this paper we described the development of a generic ELISA for the detection of 4 FQs based on mAbs. The progress of mAbs production is depicted and the specific ELISAs to detect Sarafloxacin, Difloxacin, Norfloxacin and Pefloxacin are discussed. Limited performance data for each assay in fish muscle extracts are also presented.

2. Materials and Methods

2.1. Materials and equipments

Sarafloxacin, Difloxacin, Norfloxacin, and Pefloxacin were provided by Sigma (St. Louis, MO). SAR–BSA as immunogen and SAR-OVA as coating antigen were conjugated in our laboratory. FCA and FIA were obtained from Pierce. GaMIgG-HRP (whole molecule specific) was purchased from Sino-American Biotechnology Company (Shanghai, China). HAT and HT were obtained from Sigma-Aldrich (USA). RPMI-1640 with L-glutamine was obtained from Gibco. Polyethylene glycol 1500 (PEG 1500, 50%) was from Roche Diagnostics Corporation (Indianapolis, USA). A mouse monoclonal antibody isotyping kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). TMB, phenacetin, urea peroxide were obtained from Sigma Company. All other solvents and reagents were of analytical grade or higher, unless otherwise stated.

A spectrophotometric microtitre reader (MULTISKAN MK3, Thermo Company, USA) was used for absorbance measurements. A GS15R high speed refrigerated centrifuge were supplied by Thermo Company (USA). CO₂ incubator from RS-Biotech (Galaxy S+, UK) was used for cell cultivation. SW-CJ-2FD Superclean Bench was purchased from Suzhou purification equipment Co., Ltd (Suzhou, China). Inverted microscope (TS100-F, Nikon Company, Japan) was used for cell observation.

2.2. Immunization and cell fusion

Female mice (8-10 weeks old) were immunized with SAR-BSA conjugate by intraperitoneal injections. Doses consisted of an emulsion of 100 μL of PBS containing 100 μg of protein conjugate and 100 μL of FCA. Three subsequent doses were given every three weeks with FIA as emulsion. After a resting period of 4 weeks from the last injection, the selected mouse received a booster injection, 4 days before cell fusion.

On the day of fusion, the mouse was sacrificed by cervical dislocation and the spleen was removed
aseptically. The splenocytes were isolated and fused with myeloma cells at a 10:1 ratio using PEG 1500 as the fusing agent. The fused cells were then distributed into 96-well culture plates, in which mouse peritoneal macrophages were prepared on the day before the fusion and were grown with the selective HAT medium. 10-14 days after fusion, supernatants of hybridoma colonies were recovered and screened using a combination of noncompetitive and competitive indirect ELISA. Well cultures showing significant SAR recognition activity were expanded and subcloned three times by limiting dilution. Stable antibody-producing clones were expanded with HT medium and cryopreserved in liquid nitrogen

2.3. Production and characterization of mAbs

A mature female BALB/c mouse was injected intraperitoneally (i.p.) with 0.5 mL of paraffin 10 days before receiving an i.p. injection of the positive hybridoma cells suspended in RPMI 1640 medium. Ascites fluid was collected 10 days after the injection and then stored at –20 °C until use. Purification of mAb was performed according to the modified caprylic acid ammonium sulphate precipitation (CAASP) method described before [10]. The protein content of the antibody was determined according to the following formula: protein concentration (mg/mL) = 1.45OD280 nm - 1.74OD260 nm, where OD value is the optical density. Measurement of monoclonal antibody affinity (Ka) was carried out according to the procedure described by Wang et al. The class and subclass of the isotypes of the purified antibody were determined by using a mouse monoclonal antibody isotyping kit.

2.4. Establishment and optimization of icELISA

An icELISA standard curve was developed based on the mAbs. Sensitivity was calculated using the IC50 values, which represented the concentration of SAR that produced 50% inhibition. The detection of limit (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition. The dynamic range was calculated as the concentration of the analyte providing a 20–80% inhibition rate (IC20–IC80 values) of the maximum signal. Specificity was expressed by the cross-reactivity, which was calculated as: (IC50 of SAR) / (IC50 of competitors) ×100.

It is commonly acknowledged that immunoassay performance is often affected by chemical parameters such as pH values and organic solvent concentration. The effects of these parameters were estimated by running standard curves under various conditions. The maximum absorbance (Amax, the absorbance value at zero concentration of SAR) and half-maximum inhibition concentration (IC50 values) were calculated, and the maximal Amax/IC50 ratio was chosen.

2.5. Sample preparation procedure

The sample preparation procedure was performed as a modification of that described by Wu et al. [10]. Briefly, 2 g of fish muscle homogenate was weighed into a glass centrifuge tube, and fortification samples were prepared at this point by adding various concentrations of standard solutions. 8 mL of hydrochloric (0.01 M) was added and the mixture was shaken for 2 min. After hydrolysis, the hydrolysates were extracted with 10 mL of ethyl acetate, and the mixture was centrifuged for 5 min at 3000 rpm. The ethyl acetate layer was removed by aspiration and discarded, and the supernatant hydrolysates were evaporated to dryness on a heating block at 40°C under nitrogen. The residues were dissolved in methanol-water (1:4, v/v) solutions, and used for ELISA detection.
3. Results and Discussions

3.1. Mabs production and characterization

Ten days following the fusion, growing hybridoma cell clones could be observed in many wells of the seeded 96-well plates. The fusion rate was about 75%. Supernatants of all wells were screened by simultaneous non-competitive and competitive assays. Finally, a total of five high-response hybridomas named S1-B2, S2-C6, S2-E7, S3-C5, and S3-E5 were obtained, and the mAbs from ascite liquids were purified and characterized. Using a mouse monoclonal antibody isotyping kit, all five antibodies were of the IgG1 isotype with k light chain. And the protein concentrations of all mAbs were between 3.2–4.6 g/mL. The affinity of an antibody for its corresponding antigen is crucial parameters affecting the performance of an immunoassay, and high-affinity antibodies can produce sensitive IC50 values. In our study, the affinity constant (Ka) of five selected hybridomas were between 4.6×109–2.8×1010 L/mol, in which S1-B2 was used for subsequent immunoassay development.

3.2. Establishment of the icELISA

The optimal reagent concentrations were determined when the maximum absorbance (A_max) was around 1.0, and the dose-response curve of inhibition ratio versus the SAR concentration pursued the lowest IC50 values. Based on the checkerboard titrations, a competitive curve was obtained with the icELISA format (Fig. 1). As can be seen, the optimum concentration of coating antigen was 0.2 μg/mL and pAb was 1:50,000 dilutions. This assay allowed the detection of SAR (20-80% inhibition of color development) from 0.004 to 18 ng/mL, with an IC50 value of 0.32 ng/mL. The limit of detection (LOD) of the assay was 0.002 ng/mL.

![Fig.1. Optimized standard icELISA inhibition curve based on S1-B2 clone for SAR](image)

3.3. pH and organic solvents tolerance

To study the influence of pH on the assay characteristics, competitive curves were prepared using standards with pH values of 4.0, 5.0, 6.0, 7.4, 8.0 and 9.0. Fig. 2A presents the pH effects on the icELISA. Using the methods described above, these parameters of A_max and IC50 were considered, and the ratio of A_max/IC50 was used to estimate the optimum pH value. Although A_max values were lower at pH less than 5 and greater than 8, there were no significant fluctuations in the IC50 values between pH 6 and 8. This indicated that neutral assay buffer provides the best conditions for the binding of antibody and hapten, therefore the physiological pH (7.4) was selected for the immunoassays.

Acetonitrile was studied because it is water-miscible and often employed in sample extraction. The
effects of acetonitrile on the ELISA system was estimated by preparing standard curves containing varying amounts of organic solvent (0, 5, 10, 20, and 30% in PBS). Fig. 2B shows the normalized dose-response curves at various solvent concentrations. The results showed that the IC$_{50}$ and A$_{max}$ values of the immunoassay were varying obviously when increasing the amounts of organic solvents. It indicated that a reproducible inhibition curve could not be observed, only when the solvent was not higher than 10%. Therefore, to accurately determine the SAR, the acetonitrile in the assay buffer should be minimized.

3.4. Specificity

Specificity was evaluated by determination of the cross-reactivity values. Analytes that do not react with the antibody would produce absorbance near 100%; conversely, analytes that do react with the antibody would decrease in percentage of absorbance. The IC$_{50}$ value and cross-reactivity rate for each compound are presented. Of all the cross-reacting analogues, this assay exhibited a high cross-reactivity to Difloxacin (85.5%), Norfloxacin (61.7%), and Pefloxacin (34.8%). It proves that this immunoassay can simultaneously detect four kinds of veterinary FQs residues.

3.5. Multiresidue determination in fish samples

Under the 10-fold dilution programme, recovery studies were determined by spiking a pool of negative fish samples with different FQs concentrations based on the icELISA calibration curve. The concentration measured and concentration fortified was compared to validate the effectiveness of the developed immunoassay. The results are shown in Fig. 3.
It can be seen that the data spots were nearly distributed on both sides of the trendline, and the correlation coefficients ($R^2$) for Sarafloxacin, Difloxacin, Norfloxacin and Pefloxacin were 0.9901, 0.9807, 0.9843, and 0.9844, respectively. This indicates that an excellent correlation was found, and the results also suggest the veracity of the established icELISA method for detecting FQs residues in fish samples.

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References

[1] Simonin MA, Gegout-pottie P, Minn A, Gillet P, Netter P, Terlain B. Pefloxacin-Induced Achilles Tendon Toxicity in Rodents: Biochemical Changes in Proteoglycan Synthesis and Oxidative Damage to Collagen. *Antimicrob Agents Chemother* 2000; 44: 867–872.

[2] Zhao S, Jiang H, Li X, Mi T, Li C, Shen J. Simultaneous determination of trace levels of 10 quinolones in swine, chicken, and shrimp muscle tissues using HPLC with programmable fluorescence detection. *J Agric Food Chem* 2007; 55: 3829–3834.

[3] San Martín B, Cornejo J, Iragüen D, Hidalgo H, Anadón A. Depletion study of enrofloxacin and its metabolite ciprofloxacin in edible tissues and feathers of white leghorn hens by liquid chromatography coupled with tandem mass spectrometry. *J Food Prot* 2007; 70: 1952–1957.

[4] Hermo MP, Nemutlu E, Kir S, Barrón D, Barbosa J. Improved determination of quinolones in milk at their MRL levels using LC-UV, LC-MS and LC-MS/MS and validation in line with regulation 2002/657/EC. *Anal Chim Acta* 2008; 613: 98–107.

[5] Dufresne G, Fouquet A, Forsyth D, Tittlemier SA. Multiresidue determination of quinolone and fluoroquinolone antibiotics in fish and shrimp by liquid chromatography/tandem mass spectrometry. *J AOAC Int* 2007; 90: 604–612.

[6] Wang L, Wu X, Xie Z. Determination of enrofloxacin and its metabolite ciprofloxacin by high performance capillary electrophoresis with end-column amperometric detection. *J Sep Sci* 2005; 28: 1143–1148.

[7] Okerman L, De Wasch K, Van Hoof J. Detection of antibiotics in muscle tissue with microbiological inhibition tests: effects of the matrix. *Analyst* 1998; 123: 2361–2365.

[8] Bucknall S, Silverlight J, Coldham N, Thorne L, Jackman R. Antibodies to the quinolones and fluoroquinolones for the development of generic and specific immunoassays for detection of these residues in animal products. *Food Addit Contam* 2003; 20: 221–228.

[9] Huet A, Charlier C, Tittlemier S, Singh G, Benrejeb S, Delahaut P. Simultaneous determination of (fluoro)quinolone antibiotics in kidney, marine products, eggs, and muscle by enzyme-linked immunosorbent assay (ELISA). *J Agric Food Chem* 2006; 54: 2822–2827.

[10] Wu JE, Chang C, Ding WP, He DP. Determination of florfenicol amine residues in animal edible tissues by an indirect competitive ELISA. *J Agric Food Chem* 2008; 56: 8261–8267.