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Development of an Immunoassay for Determination of Fluoroquinolones Pollutant in Environmental Water Samples*

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***Abstract*: An immunoassay method for determination of Fluoroquinolones (FQs) residues in water samples has been developed. For this purpose, NHS ester technology was employed to synthesize the immunogen and coating antigen of Sarafloxacin (SAR). SDS-PAGE, UV-visible spectra and Infrared spectra identification showed that the artificial antigen was conjugated successfully. Based on the square matrix titration, an icELISA method was established. The dynamic range in assay buffer was from 0.048 to 62.4 ng/mL, with LOD and IC_{50} value of 0.018 ng/mL and 2.5 ng/mL, respectively. This assay showed a high cross-reactivity to Difloxacin (86.7%), Norfloxacin (63.2%), and Pefloxacin (33.8%). When applied in authentic tap water, river water and fishing water samples, the recoveries for Sarafloxacin, Difloxacin, Norfloxacin and Pefloxacin were 89.5-119.5, 90.5-121.2, 92.1-118.6, and 88.2-116.8%, respectively, with coefficient of variation (CV) values all ＜10%. The results suggest this immunoassay can be used for simultaneous detecting Sarafloxacin, Difloxacin, Norfloxacin, and Pefloxacin residues in water samples.**

Keywords- Fluoroquinolones; Sarafloxacin; Artificial antigen; polyclonal antibody; Indirect competitive ELISA; Water

I. INTRODUCTION

Fluoroquinolones (FQs) are a large class of antibiotics that are widely used in fishery product and livestock husbandry. However, their residues may persist in animal body, resulting in the development of drug-resistant bacterial strains or allergies [1]. Further research demonstrate that these antibiotics and their metabolites may directly cause oxidative damages to cell membranes. Some FQs have also been suspected to affect the central nervous systems. For example, in human, they have been associated in some cases of severe disorders like headaches, dizziness or convulsions. In recent years, the residues of FQs in waters have also been found and confirmed in the U.S. [2], Europe [3], and China [4]. To assess the fate and risk of FQs in aquatic systems, different analytical methods have been developed.

In 2007, Lee et al. developed a LC-MS method with fluorescence detection for determination of ofloxacin, norfloxacin, and ciprofloxacin in sewage [5]; in 2010, Sun et al. established a LC analysis with solid-phase extraction to simultaneously detect malachite green, enrofloxacin and ciprofloxacin in fish farming water [6]. Yan et al. also reported an ultrasound-assisted HPLC method for determination of fluoroquinolones in pharmaceutical wastewater [7]. Other scholars have also developed different physico-chemical methods for determination of FQs residues in marine products [8-12]. However, they are all costly, need skilled personnel and lengthy sample preparation. The ideal assay for screening of FQs should be fast, simple, cost-effective, and enable measurement in small volume of liquids.

Our study aimed to prepare the artificial antigen of Sarafloxacin (SAR) and produce anti-SAR polyclonal antibody (pAb). On the basis, we developed an indirect competitive enzyme immunoassay (icELISA) to detect the FQs residues in circumstance waters. The analytical performance of this ELISA is reported and its applicability is discussed here.

II. MATERIALS AND METHODS

A. Materials and reagents

Sarafloxacin, Difloxacin, Norfloxacin, and Pefloxacin were provided by Sigma (St. Louis, MO). BSA, OVA and GaRIgG-HRP were purchased from Sino-American Biotechnology Company (Shanghai, China). FCA, FIA and EDC were obtained from Pierce. NHS was from Japan, MSDS available. O-(Carboxymethyl) hydroxylamine hemihydrochloride, Succinic anhydride, were supplied by Sigma while Dialysis bag was from Solarbio Company. 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), provided with a 450 nm filter, was used for absorbance measurements. Infrared spectra were acquired with an IR Spectrometer (TENSOR 27, Bruker Company, Germany). UV-visible spectra were obtained by using a DU800 Ultraviolet-visible Spectrophotometer (Beckman-Coulter Company, USA).

Female New Zealand white rabbits were obtained from the Laboratory Animal Center, Beijing Medical University, and raised under strictly controlled conditions in our laboratory chamber.

B. Artificial antigen synthesis

The immunogen of SAR-BSA was prepared using the NHS ester method. The hapten (10 mg) was dissolved in DMF (2 mL) with 10% HCl (0.01 M). After the solution was cooled with 4 ℃ methanol, 20 mg NHS and 40 mg EDC were added to the hapten solution, and the mixture was

stirred at room temperature overnight. 66 mg BSA was dissolved in 2 mL of phosphate buffer (pH 7.4) and added dropwise to the active NHS solution with continuous stirring and further stirred at room temperature for 4 h. The hapten-BSA were dialyzed against PBS (pH 7.4) for 6 d with repeated changes of the PBS solution to remove the unconjugated hapten, and then characterized by SDS-PAGE, UV-visible spectra and Infrared spectra identification. A SAR-OVA conjugate was prepared according the same way.

C. *Production of SAR pAb*

New Zealand white rabbits were subcutaneously immunized with SAR-BSA conjugate to produce the pAb. FCA was employed in the first immunization and FIA was used in the subsequent boost injections. Rabbits were immunized every 30 days with 500 μg of immunogen, and blood samples from the marginal vein of the ear were taken for ELISA identification. Ten days after the final boost, all rabbits were exsanguinated by heart puncture and the serum was separated from blood cells by storing at 4 ℃ overnight. Purification of pAb was performed according to the modified caprylic acid ammonium sulphate precipition (CAASP) method described before [13].

D. *Development of icELISA standard curve*

Indirect competitive ELISA (icELISA) was employed to determine the sensitivity and specificity, using a procedure described below. The microplates were coated with coating antigen SAR-OVA (100 μL/well) by overnight incubation at 4 ℃. Plates were washed with PBST three times and and unbound active sites were blocked with 250 μL/well of blocking buffer, followed by incubation for 2 h at room temperature. Then, 50 μL per well of analyte in PBS containing 10% methanol and 50 μL per well of purified antibody were added. All samples were run in triplicate wells. After another washing procedure, GaRIgG-HRP (1:1000, 50 μL/well) was added, followed by incubation for 25 min at 37 ℃. The plates were washed, and 60 μL/well substrate solution was added to present the absorbance values.

Based on the optimized concentrations protocol, an icELISA method was developed. Sensitivity was evaluated according to the inhibition rate, and the data were calculated using the IC_{50} values, which represented the concentration of SAR that produced 50% inhibition of antiserum binding to the hapten conjugate. The detection of limit (LOD) was defined as the lowest concentration that exhibits a signal of 15% inhibition [14]. The dynamic range for the icELISA was calculated as the concentration of the analyte providing a 20–80% inhibition rate (IC_{20}–IC_{80} values) of the maximum signal. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody. The cross-reactivity was calculated as: (IC_{50} of SAR)/ (IC_{50} of competitors) ×100.

E. *Spiking in authentic water samples*

For use in the ELISA performance evaluation, three different matrices (tap water, river water and fishing water) were chosen according to established MRLs criteria, or as not allowed substances. Stock solutions were prepared in PBS containing 10% of methanol. The collected water samples from river or fishing lake were immediately shaken vigorously for 1min and then centrifuged at 4000 rpm for 15 min. The supernatant was transferred into a 50-mL beaker for spiking analysis. The tap water was detected without any pretreatment. The recoveries were calculated by interpolation of the mean absorbance values on a standard curve constructed by icELISA, and accuracy was expressed as the recovery data of the estimated concentration.

III. RESULTS AND DISCUSSIONS

A. *Immunogen and coating antigen synthesis*

SAR with a molecular weight of 385.36, is too small to be immunogenic, and must be conjugated to a carrier protein to elicit an immune response. Among protein carriers, BSA and OVA are two of the most preferred ones which were used for synthesizing immunogen and coating antigen respectively. The synthetic pathways for complete antigens are presented in Fig. 1.

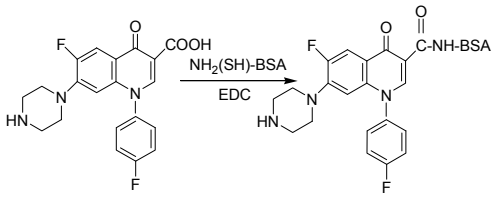

Figure 1. Synthesis procedure for SAR immunogen through NHS ester method.

B. *Identification and characterization of SAR artificial antigen*

a) *SDS-PAGE identification*

The results are shown in Fig. 2. As can be seen, the electrophoretic velocity of BSA was faster than that of SAR-BSA, which meaned that the molecular weight of SAR-BSA was bigger, also showed the conjugating method was successful.

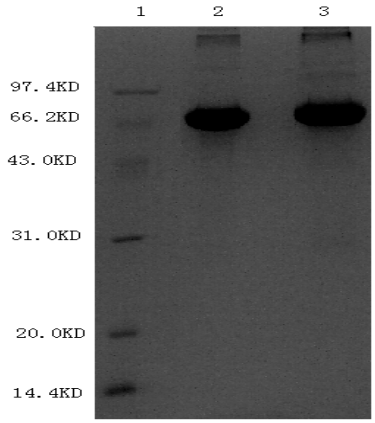

Figure 2. Identification of SAR-BSA conjugation by SDS-PAGE. Note: 1. Mark, 2. BSA, 3 SAR-BSA.

b) IR identification

Fig. 3 shows the IR spectra of artificial antigen. The infrared absorption bands were extremely similar in 2 500~3 200 cm^{-1} and 1660~1 500 cm^{-1} between BSA and SAR-BSA, which were the characteristic absorption peak of amino acid in BSA, showed the BSA character in SAR artificial antigen. As compared to SAR-BSA, a strong sharp absorption peak at 3200 cm^{-1} which attributed to carboxyl group, was appeared only in SAR, proved the conjugation had been successful.

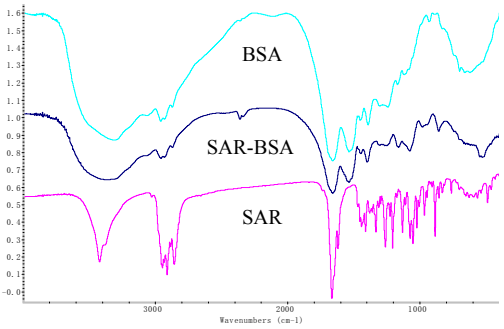

Figure 3. The IR spectra of BSA, SAR and SAR-BSA.

c) UV-vis spectrogram

UV-vis spectrum for SAR-BSA, SAR, and BSA are presented in Fig. 4. The absorbance for SAR-BSA (248, 274, and 283 nm) gave a significant shifted peak at 274 nm compared with the 271 nm peak for SAR (247, 271, and 281 nm), which indicated the SAR was successfully conjugated with BSA. The coating antigen of SAR-OVA gave a UV pattern similar to that of immunogen.

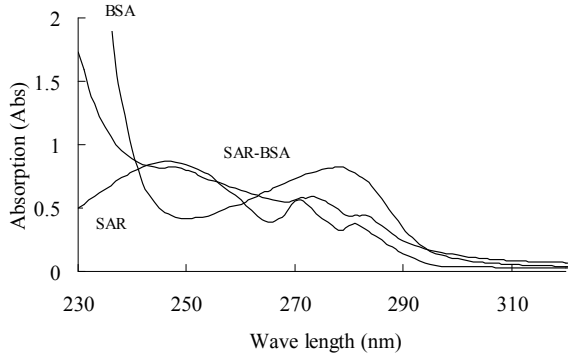

Figure 4. UV-vis spectrum of the artificial antigen of SAR, BSA and SAR-BSA.

C. *Establishment of the indirect competitive ELISA*

Checkerboard titrations were performed, taking into account the optimal dilutions. 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 logarithm of SAR concentration pursued the lowest IC_{50} values. From the checkerboard assays, five representative standard inhibition curves are shown in Fig. 5. Based on the results, a competitive curve was obtained with the icELISA format (Fig. 6). As can be seen, the optimum concentration of coating antigen was 0.5 μg/mL and pAb was 1:10,000 dilutions. This assay allowed the detection of SAR (20-80% inhibition of color development) from 0.048 to 62.4 ng/mL, with an IC_{50} value of 2.5 ng/mL. The limit of detection (LOD) of the assay was 0.018 ng/mL.

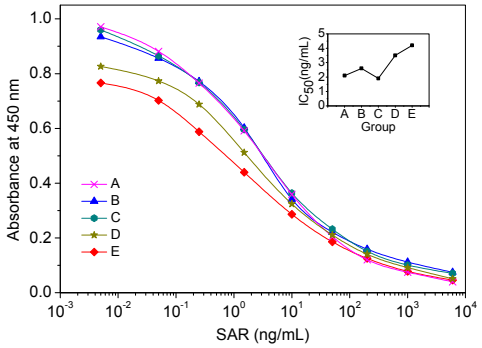

Figure 5. Determining optimal parameter of coating antigen and SAR pAb by indirect competitive ELISA. (A) coating antigen 1 μg/mL, pAb 1:5000; (B) coating antigen 2 μg/mL, pAb 1:10,000; (C) coating antigen 0.5 μg/mL, pAb 1:10,000; (D) coating antigen 0.2 μg/mL, pAb 1:10,000; (E) coating antigen 0.2 μg/mL, pAb 1:20,000.

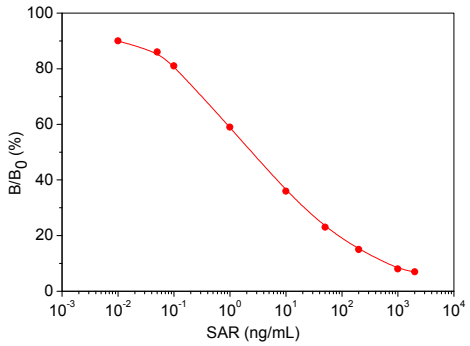

Figure 6. Optimized standard icELISA inhibition curve for SAR. Data were obtained by averaging three independent curves, each run in triplicate. SAR-OVA (0.5 μg/mL) as coating antigen was prepared in CBS (pH 9.6); purified anti-serum produced by SAR-BSA as immunogen was diluted 1:10,000 in PBS (pH 7.4); SAR was prepared in PBS, containing 10% methanol; GaRIgG-HRP was diluted 1:1000 in incubation buffer.

D. *Specificity*

Specificity was evaluated by determination of the cross-reactivity based on the IC_{50} 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 in Table 1. Of all the cross-reacting analogues, this assay exhibited a high cross-reactivity to Difloxacin (86.7%), Norfloxacin (63.2%), and Pefloxacin (33.8%). It proves that

this immunoassay can simultaneous detect four kinds of veterinary FQs residues.

TABLE 1 CROSS-REACTIVITIES OF RELATED FUNCTIONAL ANALOGUES IN THE SAR IMMUNOASSAY.

analogues	IC_{50} (ng/mL)	CR (%)
Sarafloxacin	2.5	100
Difloxacin	2.88	86.7
Norfloxacin	3.96	63.2
Pefloxacin	7.4	33.8

E. Application in real water samples

FQs are widely used in fish farming for prevention infectious diseases; in consequence, they are often accumulated in different water samples. In our study, tap water, river water and fishing water were tested for FQs residues. For spiking experiments, three different FQs concentrations (low 5 ng/mL, medium 20 ng/mL, high 50 ng/mL) based on the icELISA calibration curve were checked, and the results are shown in table 2.

TABLE 2. RECOVERY DATA OF WATER SAMPLES FORTIFIED WITH DIFFERENT CONCENTRATIONS OF FQS (N = 6)

FQs	Fortified levels (ng/mL)	Recovery ± CV (%)		
		Tap water	River water	Fishing water
Sarafloxacin	5	96.5 ± 8.6	101.2 ± 5.5	113.8 ± 7.3
	20	93.2 ± 7.1	103.8 ± 4.3	119.5 ± 6.6
	50	89.5 ± 9.2	108.4 ± 5.2	110.8 ± 5.3
Difloxacin	5	97.3 ± 8.4	107.2 ± 4.6	111.7 ± 7.8
	20	94.5 ± 7.6	105.6 ± 2.5	112.1 ± 4.2
	50	90.5 ± 6.8	108.8 ± 6.6	121.2 ± 6.8
Norfloxacin	5	92.1 ± 4.6	98.7 ± 6.8	118.6 ± 9.2
	20	96.3 ± 7.2	103.1 ± 7.0	108.5 ± 7.7
	50	93.2 ± 2.9	107.2 ± 4.9	113.9 ± 7.9
Pefloxacin	5	97.4 ± 8.4	105.2 ± 5.8	115.6 ± 9.2
	20	93.5 ± 4.9	108.7 ± 7.3	116.8 ± 7.4
	50	88.2 ± 4.8	96.2 ± 4.6	108.7 ± 5.4

It can be seen that the mean recoveries for Sarafloxacin, Difloxacin, Norfloxacin and Pefloxacin were 89.5-119.5, 90.5-121.2, 92.1-118.6, and 88.2-116.8%, respectively. The results showed that higher recoveries were obtained in fishing water than other water samples, indicating the pollution degree. Excellent CV values (＜10%) also indicate that this established icELISA can be used in water matrices for determination of FQs residues.

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