

## Comparison between Direct Competitive ELISA and LC-MS/MS Method for Detecting Sarafloxacin Residue in Poultry\*

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**Abstract**—Two different analytical methods for the detection of Sarafloxacin (SAR) residue in poultry have been developed, and the comparison was also performed. For this purpose, anti-SAR monoclonal antibody (mAb) was produced through cell fusion technology and SAR-HRP tracer was synthesized using EDC method. Based on the characteristics of mAbs and square matrix titration, a direct competitive ELISA (dcELISA) method was established. The working range in assay buffer was from 0.004 to 18 ng/mL, with LOD and IC<sub>50</sub> value of 0.002 ng/mL and 0.32 ng/mL, respectively. This assay showed negligible cross-reactivity (< 0.05%) to other compounds tested, showing highly specific. When applied in authentic poultry samples, no significant differences between the dcELISA and the confirmatory LC-MS/MS were observed. The correlation coefficient (R<sub>2</sub>) values were 0.9876 and 0.995 in chicken, 0.9959 and 0.9841 in duck, 0.9822 and 0.9952 in geese, for dcELISA and LC-MS/MS, respectively. The results suggest it was an advantage through coupling of dcELISA as screening method and LC-MS/MS as confirmatory method for detecting SAR residue in poultry.

**Keywords**- Direct competitive ELISA; Monoclonal antibody; Liquid chromatography tandem mass spectrometry; Sarafloxacin; Poultry

### I. INTRODUCTION

Fluoroquinolone antibiotics (FQs) are a group of broad-spectrum antibiotics used in veterinary and aquatic medicine, which are comparatively more effective against Gram-negative bacetria and some Gram-positive bacteria than quinolone antibiotics [1]. For example, Sarafloxacin (SAR) was specifically developed for veterinary use for the treatment of respiratory diseases and gastroenteritis in food-origin animals such as cattle, pigs, and poultry, as well as diseases in aquaculture [2]. However, with increased use, the residue of FQs entering the food chain may speed the antibiotic resistance of human pathogens and cause latent carcinogenicity [1]. It can also causes neurotoxin, renal function injuries, anaphylaxis, arthritis in young stock and other side effects [3]. In order to protect the health of consumers, maximum residue limits (MRLs) have been established for several FQs by many countries including the European Union (EU), and the China Ministry of Agriculture (no. 278.2003.5.22). For example, within the EU policy, the MRL has been set at 30 µg/kg for the sum of enrofloxacin and its active metabolite (ciprofloxacin) for muscle tissue [4].

Instrumental methods, including high performance liquid chromatography (HPLC) [5], liquid chromatography-mass spectrometry (LC-MS) [6-7] and LC-MS/MS [8-9], are the most current widely used methods for FQs residues detection. These methods are sensitive and highly specific, but they require expensive equipment, large volumes of solvents, and highly trained individuals for operating complicated instruments. Compared with physico-chemical methods, Enzyme-linked immunosorbent assay (ELISA) is rapid, simple, effective, and needs less sample preparation. Therefore, ELISA is very common as a biochemical and clinical analytical method, and is available for the detection of pesticides and veterinary. Numerous studies have been reported for the detection of FQs, including Ciprofloxacin [10], Enrofloxacin [4], Norfloxacin [11], and Pefloxacin [12].

The objective of this study was first to develop a direct competitive ELISA (dcELISA) based on high-affinity monoclonal antibody (mAb) for the determination of SAR in poultry. Secondary purposes included the measurement in real spiked samples and comparison between the established dcELISA method and the confirmatory LC-MS/MS technology.

### II. MATERIALS AND METHODS

#### A. Materials and equipments

Sarafloxacin, Enrofloxacin, Ciprofloxacin, Ofloxacin, Norfloxacin and Danofloxacin were provided by Sigma (St. Louis, MO). SAR-BSA as immunogen, SAR-OVA as coating antigen, and SAR-HRP as enzyme tracer were conjugated in our laboratory. FCA and FIA were obtained from Pierce. GaMlgG-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. CO<sub>2</sub> incubator from RS-Biotech (Galaxy S+, UK) was used for cell cultivation. Inverted microscope

(TS100-F, Nikon Company, Japan) was used for cell observation. The LC-MS/MS analysis were performed on a SURVEYOR liquid chromatograph (Thermo company, USA), equipped with a PDA Plus Detector (Thermo-Finnigan, USA) and a Thermo ODS-HYPERSIL column (3  $\mu$ m, 2.1  $\times$ 150 mm). Mass spectrometry was performed using a mass spectrometer equipped with a TurboIonSpray ESI source (LCQ Deca XP MAX, Finnigan Company, USA).

#### B. The direct competitive ELISA procedure

The dcELISA procedure was performed as follows, and microwell plates were coated with diluted antibodies of CBS at 100  $\mu$ L/well and incubated overnight at room temperature. Plates were washed three times with PBST and then non-specific antibody binding were blocked with 250  $\mu$ L/well of blocking buffer for 1 h at 37  $^{\circ}$ C. After washing three times, the competitive assays were performed by adding 50  $\mu$ L of diluted SAR standard or sample and 50  $\mu$ L of HRP-hapten conjugate in PBST to each well and incubating for 25 min at 37  $^{\circ}$ C. After another washing procedure, 60  $\mu$ L of TMB substrate solution was added to each well. The enzymatic reaction was stopped after 25 min by adding 100  $\mu$ L of 2 M sulfuric acid per well, and absorbance values were determined using a microplate reader.

#### C. Production of SAR mAb

Female mice (8-10 weeks old) were immunized with SAR-BSA conjugate by intraperitoneal injections. Doses consisted of an emulsion of 100  $\mu$ L of PBS containing 100  $\mu$ g of protein conjugate and 100  $\mu$ L 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.

Portions of the cell fusion procedures and cloning conditions were described previously by Chen et al. [13], with some modifications. Briefly, the splenocytes were isolated and fused with myeloma cells at a 10:1 ratio using PEG 1500 as the fusing agent, and then the fused cells (hybridomas) were distributed in 96-well culture plates supplemented with HAT medium containing 15% FBS with peritoneal macrophages as feeder cells from young BALB/c mice. 10-14 days after fusion, supernatants of hybridoma colonies were screened using a combination of noncompetitive and competitive indirect ELISA for the presence of significant SAR recognition activity. Selected hybridomas were subcloned by limiting dilution, and colonies of interest were expanded.

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  $^{\circ}$ C until use.

#### D. Preparation of dcELISA inhibition curve

Purification of mAb was achieved by saturated ammonium sulfate precipitation, and the class and subclass of the purified antibodies were determined by using a mouse

monoclonal antibody isotyping kit. Specificity was defined as the ability of structurally related chemicals to bind to the specific antibody and cross-reactivity (CR) was calculated as: (IC<sub>50</sub> of SAR)/(IC<sub>50</sub> of chemicals)  $\times$ 100. The lower the CR is, the higher the specificity of SAR mAb is. Based on the characteristics of mAbs and the results of checkerboard titrations, a dcELISA standard curve was developed. After optimization, suitable concentrations of SAR mAb and HRP tracer were used to pursue the sensitive IC<sub>50</sub> values.

#### E. Analysis of spiked poultry samples

The sample preparation procedure was followed Pearce et al. [14], with some modification. Briefly, homogenized chicken, duck, or geese muscle were weighed (2.0 g) into 50 mL tubes, and 9 mL of 0.1 M sodium hydroxide-acetonitrile (1:10) was added, then the mixture was agitated on a shaker for 10 min. The samples were centrifuged at 3000 rpm for 10 min at 15 $^{\circ}$ C, and the supernatants were submitted to solid-phase for clean-up process. The SPE C<sub>18</sub> cartridges (Dalian Sipore Co., Ltd., China) were consecutively conditioned with 5 mL of methanol and then 5 mL of deionized water at a flow rate of 0.3 mL/min. After loading with the aqueous extract solution, the cartridge was washed with 10 mL of the elution solution (*n*-hexane-ether [70:30, v/v]) at a flow rate of less than 0.5 mL/min. After centrifugation, the organic layer was removed under a stream of nitrogen in a water bath at 45  $^{\circ}$ C, and the extracts were redissolved in methanol for further analysis.

#### F. LC-MS/MS conditions

Analytes were separated using a mobile phase solution of 1% formic acid in water/acetonitrile/methanol (60:20:20, pH 2.5) at a constant temperature of 30 $^{\circ}$ C and a flow rate of 0.2 mL/min. Positive chemical ionization (PCI) mode was used and the relative collision energy was optimized to 36%. A pseudo-molecular ion [M + H]<sup>+</sup> was selected as the parent ion, and selected ion monitoring (SIM) was used for screening of individual analyte tested in this study. The corresponding fragment ions were used for identification and quantitation.

### III. RESULTS AND DISCUSSIONS

#### A. Characterization of mAbs

Through cell fusion technology, a total of nine high-response hybridomas were observed, in which clone of S5-C8 was selected for further study. Using a mouse monoclonal antibody isotyping kit, the antibodies originated from S5-C8 were of the IgG<sub>1</sub> isotype with *k* light chain. And the protein concentrations were between 3.9-4.2 g/mL. In order to establish a sensitive dcELISA method, checkerboard titrations was employed to optimize the reagent concentrations. The results are presented in Fig. 1. As can be seen, with the dilution folds for SAR mAb and HRP tracer increasing, the sensitivity was decreased significantly. The optimum conditions of SAR mAb and HRP tracer were 1:20,000 and 1:500 dilution, respectively.

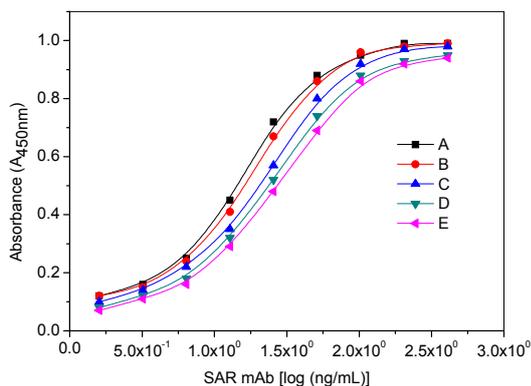


Figure 1. Determining optimal parameters for SAR mAb and HRP tracer by direct competitive ELISA. (A) MAb 1:20,000, SAR-HRP 1:500; (B) mAb 1:50,000, SAR-HRP 1:500; (C) mAb 1:50,000, SAR-HRP 1:500; (D) mAb 1:50,000, SAR-HRP 1:1000; (E) mAb 1:100,000, SAR-HRP 1:1000.

### B. Establishment of dcELISA standard curve

Based on the optimal reagent concentrations, a standard curve was obtained with the dcELISA format (Fig. 2). The dynamic range for the dcELISA was calculated as the concentration of the analyte providing a 20–80% inhibition rate ( $IC_{20}$ – $IC_{80}$  values) of the maximum signal, and the data ranged from 0.004 to 18 ng/mL. Sensitivity was evaluated using the  $IC_{50}$  values, which represented the concentration of SAR that produced 50% inhibition rate, and it was calculated as 0.32 ng/mL. The detection limit (LOD) of this assay, which represented a signal of 15% inhibition, was 0.002 ng/mL.

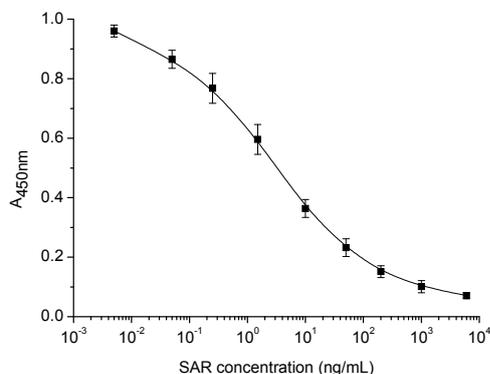


Figure 2. Optimized standard dcELISA inhibition curve based on clone of S5-C8 for SAR. Data were obtained by averaging three independent curves, each run in triplicate. Purified mAb was diluted 1:20,000 in PBS (pH 7.4), SAR-HRP was diluted 1:500 in assay buffer, SAR was prepared in PBS, containing 10% methanol.

### C. Specificity

Specificity is inherent to all immunoassays, which 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 cross-reactivity rate for each compound is presented in Fig. 3. Of all the cross-reacting analogues, this assay exhibited negligible cross-reactivities to compounds tested. It proves that the immunoassay is highly specific for SAR.

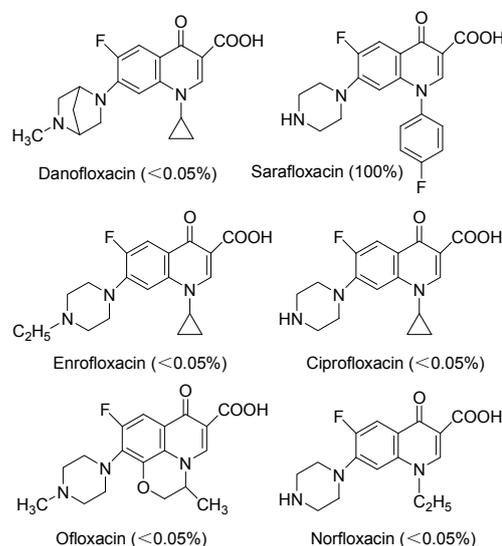
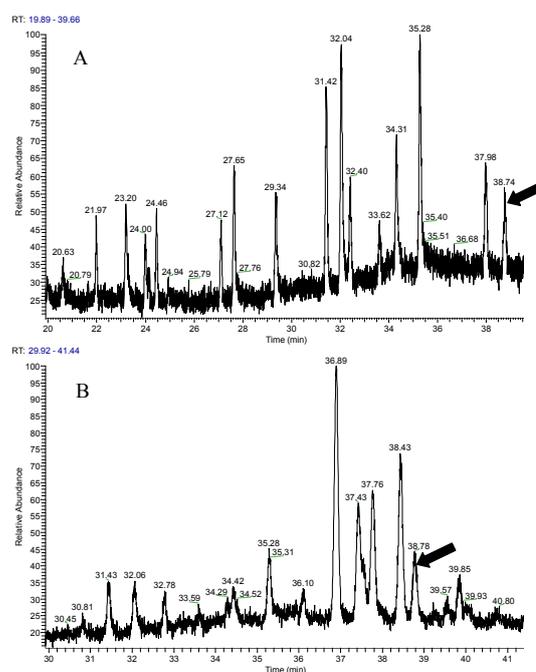


Figure 3. Cross-reactivity of functional related analogues in the SAR immunoassay.

### D. LC-MS/MS detection

Three spiked specimens collected from the poultry were employed to validate the established dcELISA method. The SIM spectra of spiked samples are shown in Fig. 4.



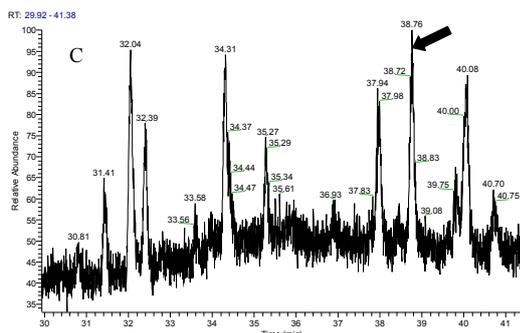


Figure 4. SIM total ions chromatograms of spiked samples. (A) chicken muscle; (B) duck muscle; (C) geese muscle.

### E. Comparison studies

Comparison between two different analytical techniques, LC-MS/MS and dcELISA, are shown in **Fig. 5**.

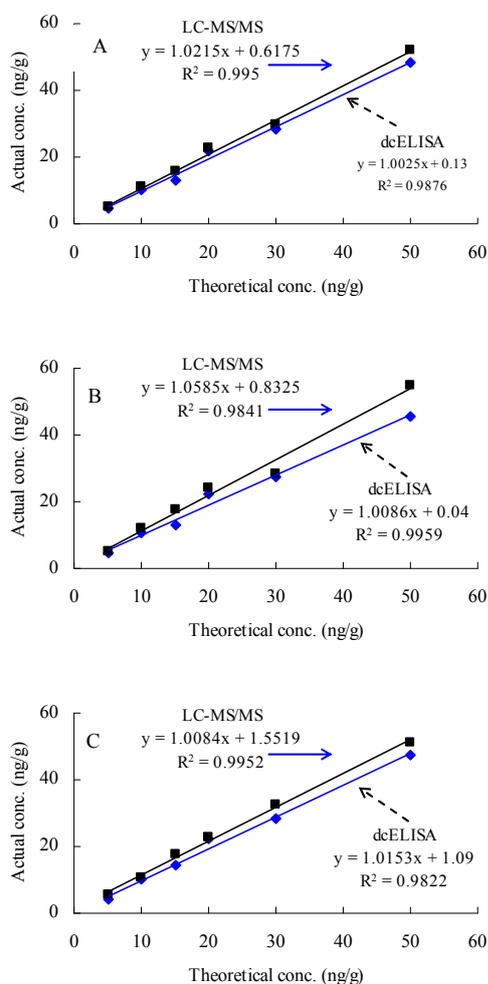


Figure 5. Correlations between concentration spiked and concentration determined by LC-MS/MS and dcELISA method in poultry samples

fortified with different concentrations of SAR. (A) In chicken muscle; (B) in duck muscle; (C) in geese muscle.

Regardless of the matrix, the statistical analysis of concentration spiked and concentration determined by LC-MS/MS and dcELISA showed no significant differences. The results proved that the coupling of dcELISA as screening method and LC-MS/MS as confirmatory method was an advantage for detecting SAR residue in poultry.

### ACKNOWLEDGMENT

This work was supported by Henan Innovation Project for University Prominent Research Talents (2010HASTIT026).

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