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Application of Quantum Dot–Antibody Conjugates for Detection of Sulfamethazine Residue in Chicken Muscle Tissue

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A new indirect competitive fluorescence-linked immunosorbent assay (cFLISA) method for the detection of sulfamethazine (SM_2) in chicken muscle tissue was demonstrated using quantum dots (QDs) as the fluorescence label coupled with secondary antibody. The sensitivity of the cFLISA was compared with that of the HPLC method. The 50% inhibition value (IC_{50}) and the limit of detection (LOD) of the cFLISA were 7.7 and 1.0 ng/mL, respectively. When SM_2 was spiked at levels of 50, 100, and 200 ng/g, recoveries ranged from 80.6% to 117.4%, with a coefficient of variation of 6.9–9.6%. In the incurred sample analysis, the amounts of SM_2 quantified by cFLISA were similar to the results obtained by the HPLC method. This study shows that cFLISA could be used as a screening method in practice.

KEYWORDS: Quantum dots; fluorescence immunoassay; sulfamethazine; chicken muscle

INTRODUCTION

Current enhanced food safety and health concerns worldwide have made it an important issue to rapidly develop highly sensitive, nonisotopic biodetection assays. Therefore, it is highly desirable to develop new probes for biochemical assays. Recent development in nanotechnology has made it possible to obtain a new class of highly fluorescent homogeneous semiconductor nanocrystals termed "quantum dot" (*1*, *2*).

Quantum dots (QDs), such as CdSe–ZnS core shell nanocrystals, are somewhat spherical nanocrystals in the size range of 1–10 nm diameter (*3*). These QDs are a brand new class of luminescent inorganic fluorophores, which have several important advantages as compared with conventional fluorescent dyes (*4*). QDs have long-term photostability and high-quantum yield, narrow emission and broad excitation spectra which make it possible to excite a number of different QDs using a single excitation laser wavelength. Moreover, the emission color of QDs is tunable by changing the nanocrystal size and the type of core material used. Therefore, simultaneous multianalyte detection can be realized by using multicolor QDs. QDs have been successfully used for a variety of bioanalytical purposes, such as DNA hybridization detection (*5*), cellular labeling (*6*), and immunoassays. Direct, sandwich, or competition assay has been studied to detect protein toxins such as staphylococcal enterotoxin B (SEB) (*7*) and trinitrotoluene (TNT) (*8*). At the same time, the potential of QDs to address "multiplexing" was quickly realized. Goldman et al. (*9*) demonstrated simultaneous detection of cholera toxin, ricin, Shiga-like toxin 1, and SEB in a single well of a microtiter plate using four colors of QDs coated with antibodies.

Sulfamethazine (SM_2) is a commonly used sulfonamide especially in veterinary practice so as to control bacterial infections and to prevent the outbreak of diseases. As a consequence, residues of this drug and its metabolites may remain in food of animal origin. The hazard of SM_2 and its metabolites to people has been realized due to their potential toxic effects. To protect consumers from risks related to drug residues, the maximum residue limit (MRL) of SM_2 has been established by law. The European Community (EC) has adopted a MRL of 0.1 μg/g for sulfonamides in edible tissue (*10*). Conventional analysis of SM_2 or other sulfonamide residues was commonly carried out by analytical chemistry methods such as gas chromatographic–mass spectrometric (GC-MS) (*11*), liquid chromatography (LC) (*12*), liquid chromatography–mass spectrometry (LC/MS) (*13*), capillary electrophoresis (*14*), UV spectrometry (*15*), and microbial inhibition assays (*16*).

At present the application of QDs as a fluorescence label in the indirect competitive fluorescence-linked immunosorbent assay (cFLISA) method, especially for the analysis of veterinary drug residue, has not been reported. The purpose of this study was to develop a cFLISA method using QD secondary antibody conjugate to detect SM_2 residue in chicken muscle, and the determined results were confirmed by a HPLC method.

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MATERIALS AND METHODS

Reagents. SM_2 and the other analogues were purchased from Sigma Chemical Co. (St. Louis, MO). The monoclonal antibody against SM_2 was synthesized by Dr. He Jihong in our laboratory (*17*). The metabolite of SM_2 (N^4-acetylsulfamethazine) was synthesized in our laboratory. QD 655 goat anti-mouse IgG conjugate was purchased from Quantum Dot Corp. (Hayward, CA). Common solvents and salts were supplied by Beijing Regent Corp. (Beijing, P.R.C.). Coating buffer was 0.05 M carbonate buffer (pH 9.6). Washing buffer was phosphate-buffered saline (PBS) with 0.05% Tween 20. Blocking buffer was coating buffer with 0.5% casein. SM_2 and its analogues were prepared by dissolving known amounts of purified substances in methanol. The stock solution (1 mg/mL) was stored at −20 °C before being used for the preparation of standard solutions which were stored at 4 °C. The water used was prepared by the Milli-Q system (Millipore, Bedford, MA).

Apparatus. The Costar brand opaque white polystyrene microtiter plates were purchased from Costar (Costar Inc., City, State). The instruments were a Cary Eclipse fluorescence spectrophotometer which was used to get the fluorescence spectra, a SpectraFluor Plus microtiter plate reader (Tecan Inc., City, State), and a centrifuge, Mikro 22R (Hettich Co., Kirchlengern, Germany). The high-performance liquid chromatograph (HLPC) system (Waters Co., City, State) was run with a diode array detector (Shimadzu, Kyoto, Japan).

Optical Characterization of the QD Conjugate. Fluorescence spectra of the QD conjugate were taken on a Cary Eclipse fluorescence spectrophotometer equipped with a 20 kW xenon discharge lamp as a light source. Spectra were typically taken at a scanning rate of 1200 nm/min with 20 nm excitation/emission slits and a 700 V photomultiplier tube voltage.

Fluoroimmunoassay. For the assay described below, measurements were done in triplicate, and mean fluorescence intensity values were zero corrected by subtracting the mean fluorescence intensity of the NSB (nonspecific binding) control. Fluorescence was measured on a SpectraFluor Plus microtiter plate reader (Tecan) with excitation/emission at 360 nm/655 nm and was analyzed quantitatively by Origin 7.0 software.

Wells of opaque white microtiter plates were coated overnight (4 °C) with 100 μL of the SM_2−OVA conjugate dissolved in 0.05 M $NaHCO_3$ (pH 9.6) at the optimal dilution and the same volume of buffer containing no antigen to control for nonspecific binding. After excess antigen or blank solutions were removed from the wells, the plates were washed with washing buffer. The excess binding sites were blocked with 200 μL of blocking buffer for 2 h at 37 °C. Subsequently, 50 μL of the samples (tissue extract or standard serial dilutions of SM_2 in PBS) together with 50 μL of the optimal antibody dilution was added to the wells (in triplicate). And then the plate was incubated for 1 h at 37 °C. Plates were washed three times with washing buffer. After washing, 100 μL of the QD secondary antibody conjugate (1:1000 dilutions in PBS) was applied to each well, and plates were then incubated for another 1 h at 37 °C. After incubation, unbound QD conjugates were discarded, and wells were washed three times with washing buffer. The plates were measured dry in a SpectraFluor Plus microtiter plate reader (Tecan). Standard curves were plotted as fluorescent intensity versus logarithm of analyte concentration. From the standard curves, we can obtain the 50% inhibition value (IC_{50}) and the limit of detection (LOD) defined as 10% inhibition.

Liquid Chromatography. An adaptation of previously reported procedures (*13*) was used for sample extraction and cleanup. A liquid chromatography unit (Waters) was equipped with a reversed-phase Supelco C18 (250 × 4.6 mm, 5 μm). The mobile phase components consisted of acetonitrile−water−acetic acid (25:75:0.05 v/v/v). The mobile flow was 1.0 mL/min at an ambient temperature. The detection wavelength was 265 nm. A 50 μL volume of the filtrate was injected into the HPLC system.

Sample Preparation for cFLISA. A 2 g homogenized chicken breast muscle was placed in a 50 mL glass centrifuge tube. The samples were spiked by adding the appropriate standard working solutions (50, 100, and 200 ng/g). These samples were extracted with 6 mL of acetonitrile−water mixture (84:16 v/v). The mixture was shaken for 30 min at room temperature at a middle speed (about 200*g*) and centrifuged at 3000*g* for 10 min. Then 3 mL of the supernatant was transferred into a plastic centrifuge tube and was mixed with 2 mL of PBS and 3 mL of acetic ester. The supernatant was evaporated in a 55 °C water bath under a stream of nitrogen, and the residual was dissolved in 1 mL of PBS (pH 7.2) and 1 mL of hexane. The mixture was shaken for 1 min before centrifugation for 5 min at 3000*g*. The upper organic solvent phase was discarded, and the lower aqueous phase was diluted for analysis by cFLISA.

Table 1. Specificity of MAb to SM_2 and Its Analogues

compound	cross-reactivity (%)
sulfamethazine	100
N^4-acetylsulfamethazine	87.6
sulfadimethoxine	<0.1
sulfamethoxazole	<0.1
sulfamonomethoxine	<0.1
sulfadiazine	<0.1
sulfathiazole	<0.1
sulfamethoxypyridazine	<0.1
sulfamerazine	16.2
sulfamethoxydiazine	<0.1

Incurred Sample Analysis. Thirty-six 8-week-old broilers (AA species; Zhengda Co., Beijing, P.R.C.) were randomly divided into three groups of 12 birds each and raised in a closed house; fresh feed and water were provided every day. The control group was given drug-free feed; one group was given feed with 200 mg/kg SM_2 (w/w), and the remaining group was given feed treated with 400 mg/kg SM_2 (w/w). The broilers were fed for 5 days consecutively. After 0, 3, 5, and 10 day withdrawal periods, one chicken of the 200 and 400 mg/kg groups was slaughtered. The chest muscle tissue samples were collected and stored at −70 °C for analysis by the cFLISA and HPLC methods.

RESULTS AND DISCUSSION

Cross-Reactivity. Cross-reactivity of the compounds structurally related to SM_2 was investigated with the anti-SM_2 MAb using the cFLISA method. The cross-reactivity values were calculated by the equation:

$$\text{cross-reactivity (\%)} = \frac{IC_{50}\text{ of compound}}{IC_{50}\text{ of }SM_2} \times 100$$

Table 1 shows the cross-reactivity of 10 different sulfonamide analogues and the N^4-acetyl-SM_2 (main metabolite of SM_2). All analogues tested showed negligible cross-reaction, but the N^4-acetyl-SM_2 showed the highest cross-reactivity to the MAb. The result was similar to that of the ELISA method which was reported by Dr. He Jihong (*17*).

Fluorescent Features of QD Conjugate. A fluorescence spectrum of QD secondary antibody conjugate is shown in **Figure 1**. From the spectra we can see that the excitation spectra were broad and the emission bandwidth was narrow (with the full width at half-maximum of about 34 nm). The emission peak is 655 nm, which is in accordance with the data supplied by the Quantum Dot Corp. These advantages can minimize channel overlap and improve color discrimination and enable multiplexing without compensation or the use of multiple excitation filters.

Comparative Assay Sensitivity of cFLISA and HPLC. *cFLISA Method*. The sensitivity was investigated using SM_2 standard. The standard curves (**Figure 2**.) were obtained under optimal SM_2−OVA, antibody, and secondary antibody concentration. The IC_{50} and LOD for SM_2 were 7.7 and 1.0 ng/mL.

HPLC Method. The HPLC method can recognize SM_2 and N^4-acetyl-SM_2 simultaneously. The standard curves were constructed over a concentration range of 0.01−1 μg/mL for SM_2 and N^4-acetyl-SM_2. The linear correlation coefficients are 0.9995 and 0.9994, separately. The limit of detection (LOD), defined

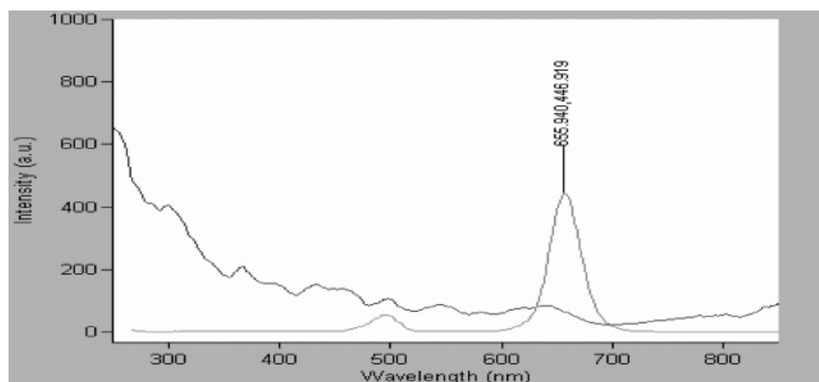

Figure 1. Absorption and emission spectra of QD conjugate. The emission peak is at 655 nm with full width at half-maximum of 34 nm.

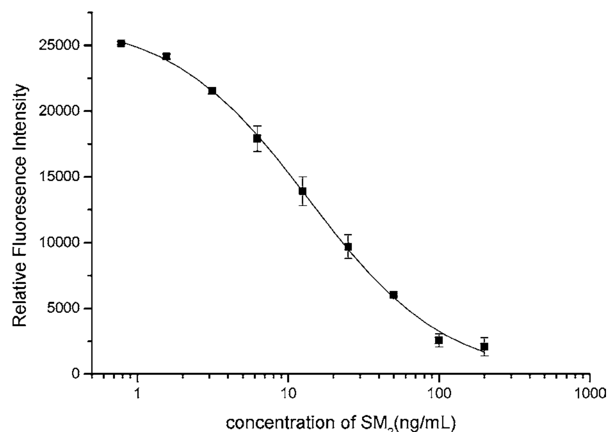

Figure 2. Standard curve of indirect competitive FLISA for sulfamethazine (SM_2) determination using monoclonal antibody and QD conjugate. Each point presents the mean of three replicates, and error bars represent standard deviation.

Table 2. Accuracy and Precision of SM_2 in Fortified Muscle Tissues by cFLISA and HPLC ($n = 5$)

	cFLISA		HPLC	
fortified (ng/g)	recovery (%)	CV (%)	recovery (%)	CV (%)
200	80.6	9.6	94.8	5.7
100	99.0	6.9	88.4	4.1
50	117.4	7.0	84.7	7.4

as the lowest concentration of SM_2 that can be reliably detected, is 10 ng/mL at a signal to noise (S/N) ratio of >3. The N^4-acetyl-SM_2 has the same value.

Fortification. The accuracy and precision of the method were checked by the recovery experiment. Known amounts of SM_2 were added into control chicken muscle tissue at levels of 50, 100, and 200 ng/g. Five replicates for each concentration were determined by the cFLISA and HPLC methods. Because various substances existing in complex matrices can affect the antigen–antibody interaction, the extract was diluted to correct for matrix interferences. The statistical data for the recoveries of SM_2 in chicken tissues were shown in **Table 2**. Average recoveries of SM_2 for chicken muscle tissue at 50, 100, and 200 ng/mL ranged from 80.6% to 117.4% by cFLISA and from 84.7% to 94.8% by HPLC. The recovery of cFLISA was reversely related to the drug concentration (decreased recovery with increased concentration) while the HPLC had the opposite trend. This seems that the new method has a larger potential of interference, and thus at a lower concentration, the recovery was higher. In comparison with cFLISA, HPLC is a stable method which can be used to quantify accurately the content of the samples. The results show that the recoveries (>80%) and coefficients of variation (CV) (<10%) were satisfied. The MRL for SM_2 on edible tissues is 100 ng/mL, so the cFLISA can be applied as screening methods for SM_2 residue in chicken tissues. The positive results can be further detected by the HPLC method.

Table 3. Results from Analysis of SM_2 in Incurred Tissues by cFLISA and HPLC ($n = 3$)

group	withdrawal time (days)	determined (ng/g)	
		cFLISA	HPLC
control	0	ND[a]	ND
200 mg/kg	0	538.1 ± 71.7	501.2 ± 85.7
400 mg/kg	0	1859.7 ± 95.9	1630.5 ± 270.4
200 mg/kg	3	21.9 ± 1.7	14.6 ± 2.3
400 mg/kg	3	41.7 ± 1.5	29.8 ± 5.7
200 mg/kg	5	8.2 ± 0.7	ND
400 mg/kg	5	13.4 ± 1.1	ND
200 mg/kg	10	ND	ND
400 mg/kg	10	ND	ND

[a] ND = not determined.

Comparison of the cFLISA and HPLC Methods for Analysis of SM_2 in the Incurred Sample. To verify the cFLISA method, sample chickens were given feed with different concentrations of SM_2, and the amounts of SM_2 in chicken tissues were measured. The cFLISA method is usually used as a screening method; therefore, the positive results in incurred samples must be validated by the chromatographic method. From the results (**Table 3**) we can see that the residue levels were decreasing and dropped rapidly after 3 days. After 5 days, the residues were not detected by HPLC; after 10 days, the residues were not detected by cFLISA or HPLC. SM_2 may undergo N^4-acetylation and deamination or form N^4-glucosides, N^4-glucuronides, diconjugates of N^4-acetylated metabolites, and other uncharacterized metabolites (*18*). The HPLC method can detect the parent SM_2 and N^4-acetyl-SM_2; therefore, the amounts of the residues we detected by HPLC should include SM_2 and N^4-acetyl-SM_2. The N^4-acetyl-SM_2 should also be detected by cFLISA because of its high cross-reactivity to the MAb. As seen in **Table 3**, the cFLISA results showed a systemically higher concentration than the HPLC. This seemed to be due to the interference of the method and the cross-reactivity of the MAb. There are no significant differences between the amounts of SM_2 quantified by both methods.

CONCLUSION

A method of fluorescence-linked imunosorbent assay with quantum dots–antibody conjugates was developed to detect SM_2 in both fortified and incurred chicken muscle tissue samples. The results obtained from incurred samples were also confirmed

by the HPLC method, which indicated that the cFLISA used for the detection of SM_2 and its metabolite residue in chicken muscle is feasible.

ABBREVIATIONS USED

cFLISA, indirect competitive fluorescence-linked immunosorbent assay; QDs, quantum dots; CV, coefficient of variation; GC-MS, gas chromatographic−mass spectrometric; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibition value; LOD, limit of detection; LC, liquid chromatography; LC/MS, liquid chromatography−mass spectrometry; MRL, maximum residue limit; MAb, monoclonal antibody; NSB, nonspecific binding; ND, not determined; PBS, phosphate-buffered saline; SEB, staphylococcal enterotoxin B; SM_2, sulfamethazine; TNT, trinitrotoluene; UV, ultraviolet detection.

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