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Application of a solid-phase fluorescence immunoassay to determine streptomycin residues in muscle tissue of olive flounder (*Paralichthys olivaceus)*, rockfish (*Sebastes schlegeli*), and red sea bream (*Pagrus major*)

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Abstact : Parallux (IDEXX Laboratories, USA), a solid-phase fluorescence immunoassay (SPFIA) developed for antibiotics residue detection in milk, was applied for analysis of antibiotics in muscle tissue of olive flounder (*Paralichthys olivaceus*), rockfish (*Sebastes schlegeli*), and red sea bream (*Pagrus major*). Fishes were dipped in streptomycin 20 g/ton water, the recommended therapeutic dose, for 3 consecutive days. Muscle was sampled after 1st, 2nd, 3rd, 4th and 5th day drug treatment. The concentration of streptomycin in muscle, determined by SPFIA, was compared with that of internal standard (200 ppb as streptomycin). The absorbance ratio of sample to internal standard (S/C) was employed as an index to determine the muscle residues in fishes. The standard solutions were added to muscle samples to give final concentrations in muscle of 0.2 and 0.5 mg/ml to investigate the recovery rate. The recovery rates of all spiked samples were > 84% of the spiked value. Streptomycin was detected in muscles of fishes treated after the 1st day of withdrawal period. The present study showed that the SPFIA can be easily adopted in predicting muscle tissue residues for streptomycin in farmed fishes.

Key words : olive flounder, red sea bream, rockfish, solid-phase fluorescence immunoassay, streptomycin

Streptomycin is an aminoglycoside antibiotics used as a veterinary drug for the treatment of gram-negative and gram-positive bacterial diseases. Susceptible strains include *Actinomyces bovis, Pasturella spp., Escherichia coli, Salmonella spp., Campylobacter fetus, Leptospira spp., and Brucella spp. Mycobacterium spp.* is also sensitive [1, 17, 24].

Mycobacterium marinum causes a chronic progressive fish disease, fish tuberculosis (TB), found in freshwater, saltwater, and brackish environments. Weight loss, non-healing open ulcers, a distended abdomen, loss of appetite, fin erosion, unusual coloration, pop-eye, spinal deformities, and listless behavior are all possible signs of infection. Unfortunately, it is also possible in an infected fish that will show no external signs and may die mysteriously. A post-mortem examination will reveal the telltale nodules on and in the internal organs; in particular, the kidney, spleen, and liver. Streptomycin has been reported with varying degrees of success to combat fish TB [3, 11].

The EPA has classified streptomycin as Toxicity class IV, relatively non-toxic. Products containing streptomycin bear the Signal Word “Caution” because of its potential to cause skin reactions [23]. Veterinary use of streptomycin is also regulated by the U.S Food and Drug Administration (FDA). A tolerance of zero has been established by FDA for residues of streptomycin in uncooked edible tissues of chickens, swine, and

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calves of 0.2 ppm in kidney and 0.5 ppm in other tissues [6]. However, no information is available regarding residues or withdrawal time in fish.

Intense usage of antibiotics has led to a wide distribution of antibiotic resistance among bacterial species, including resistance against streptomycin [20, 21]. To limit the spread of resistance against antibiotics, unnecessary dosing of antibiotics should be minimized. Control of usage in animal farming is possible by monitoring antibiotic residues in different biological samples. In addition, monitoring of residues also prevents the access of possible allergenic antibiotics into finished food products and ensures that the residues do not interfere with food production processes [16].

The residue of streptomycin has been investigated in milk, honey, and animal tissues [5, 24, 25].

Traditional approaches for the detection of streptomycin residues include microbiological methods, immunoassays, and chromatographic methods. Microbiological methods are not sensitive or specific enough [18]. Immunological assays are very sensitive and can be used as screening tests [4]. However, chromatographic methods, such as HPLC, generally provide sensitivity and specificity for antibiotics, but certain disadvantages, for example, high price, requirement of special equipment, and specific and complicate sample extraction protocols by expert personnel, and limitation on their suitability for the analysis of large numbers of samples [13-15].

Variety of immunoassay methods have been developed and adopted for detecting the generic groups of chemical residues in animal products. Immunoassay has become the most popular for chemical residue detection in food due to its sensitivity, simplicity and ability to screen large numbers of samples [12, 13, 19]. As the consumption of meat and fish has continually been increasing, simple, sensitive, rapid and low-cost methods for detecting chemical residues those are needed for the detection of large numbers of samples [12, 19, 22].

Therefore, we investigated the possibility of Parallux (IDEXX Laboratories, USA) application to detect streptomycin in muscle of flounder, rockfish, and red sea bream. Fishes used in this study were 25 healthy olive flounders (average weight, 650 ± 50 g), 25 healthy rockfishes (average weight, 500 ± 35 g), and 25 healthy red sea breams (average weight, 630 ± 60 g), with no previous history of antibiotic treatment.

Strepsin (100 g (activity)/kg, withdrawal period, 5 days) was purchased from SF (Korea). The IDEXX Parallux (IDEXX Laboratories, USA) kit cartridges were purchased from Korea Media Ltd. Parallux (IDEXX Laboratories, USA) is a solid-phase fluorescence immunoassay (SPFIA)-based test, designed for milk analysis, which is very easy to perform and yields results within 5 min. Different types of test cartridges are available, each containing 4 capillary channels for detection of 1-4 different analytes. The test itself, including mixing unit for sample with the antibody, immunological reaction and reading, takes < 5 min [12, 13, 19]. The recommended therapeutic dose of streptomycin (20 g/ton water) was treated to 25 olive flounders, 25 rockfishes, and 25 red sea breams for 3 consecutive days using dipping administration. Muscles were sampled from all fishes during the withdrawal period on the 1st, 2 nd, 3 rd, 4 th, and 5 th day after administration of drug. The method of muscle sample extraction was modified from the protocol described by Haasnoot *et al.* [8]. To 1 g of homogenized muscle sample (weighed in a polypropylene extraction tube), 6 ml of a trichloroacetic acid solution (3% in water, pH 0.5) were added for deproteinization and mixed with a vortex for 1 min. The seraclear filter (Nieuwegein, The Netherlands) was pushed into the extraction tube and 880 μl of a 0.1 M phosphate buffer (pH 9.6) were added to 120 μl aliquots of the filtrate, and the whole was homogenized (equivalent to 0.02 g of muscle per ml of extract) of which 100 μl aliquots were pipette into the microtiter plates.

Stock solutions of 100 μg/ml of streptomycin (Sigma, USA) was prepared and stored at –20°C This standard solution was used for the preparation of both calibration solutions and fortified samples. Just before use, the stock solutions were diluted in muscle extracts from non treated fish, to prepare for a 0.025, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mg/kg working standard solutions. To get the recovery rate, the standard solutions were added to muscle samples to give a final concentration in muscle of 0.2 and 0.5 mg/kg. After blending, these samples were extracted as described above and then analyzed in a blind fashion.

The sample with Parallux (IDEXX Laboratories, USA) system was performed as manufacturer's instruction. After the wells in the reagent tray are filled with 100 μl sample, the contents are mixed with

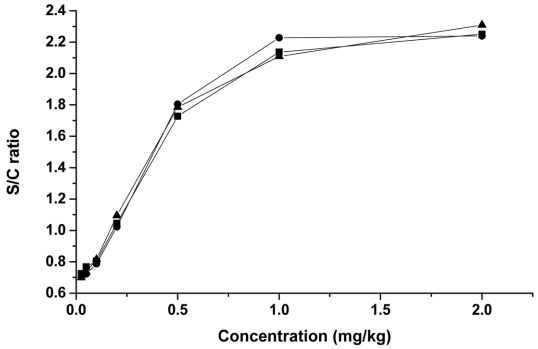

Fig. 1. Standard curve of streptomycin in muscle of olive flounder (▲), rockfish (●), and red sea bream (■). Detection limit of streptomycin was less than 0.2 mg/kg based on the S/C ratio of 1.0 in the assay system. S/C ratio is the ration of the sample inhibition value (S) to the cutoff inhibition value (C).

labeled antibodies already present in the wells; thereafter, the mixtures are allowed to react with the solid phase in the capillary tubes. When samples do not contain any analyte reacting with the antibodies, a large amount of labeled antibody remains free to bind to the solid phase. After the capillaries are washed and dried, a laser source excites the fluorescence, and the results is given as the ratio of the sample inhibition value/cutoff inhibition value (S/C), while the sample inhibition value = [1 – (sample signal/negative control signal)] × 100. The cutoff inhibition value is included in the bar code that must be scanned before each test. The S/C ratio > 1.0 is recorded as positive by the processor [12, 13, 19].

The standard curve of streptomycin was constructed to determine the detection limit of drug. As shown in Fig. 1, the detection limit of streptomycin was less than 0.2 mg/kg based on the S/C ratio of 0.8 in the assay system. The standard curve of streptomycin was shown linear regression between 0.05 and 0.5 mg/kg (olive flounder, R^2 = 0.999; rockfish, R^2 = 0.998; red sea bream, R^2 = 0.997). Okerman *et al.* [19] determined different antibiotic residues in bovine and in porcine kidneys by solid-phase fluorescence immunoassay. The range of ratio from spiked samples spiked with 1.0 mg/kg ceftiofur and 0.3 mg/kg oxytetracycline was 1.81-2.16 and 1.55-2.05, respectively. In our study, the ranges of ratio for streptomycin was similar to that of ceftiofur and little lower than that of oxytetracycline.

Recoveries of 0.2 and 0.5 mg/kg of streptomycin spiked into non-treated muscles are shown in Table 1.

Table 1. Recoveries of streptomycin in muscle of fishes with SFFIA

Fishes	Spiked concentration (mg/kg)	S/C ratio[a] (Mean ± SD, n = 5)	Recovery[b] (%)
P. olivaceus	0.2	0.894 ± 0.034	85.6
	0.5	1.538 ± 0.056	89.0
S. schlegeli	0.1	0.908 ± 0.041	88.7
	0.5	1.609 ± 0.039	89.1
P. major	0.1	0.922 ± 0.047	84.2
	0.5	1.625 ± 0.051	91.0

[a] S/C ratio is the ration of the sample inhibition value (S) to the cutoff inhibition value (C).
[b] Recovery obtained from the formula, (S/C ratio of spiked muscle/S/C ratio of standard solution) × 100.

All recoveries were more than 84.2% of the spiked value.

Bruijnsvoort *et al.* [24] studied the determination of streptomycin by liquid chromatography with tandem mass spectrometry (LC-MS/MS), and investigated recovery rates after spiked at the concentration of 10 μg/kg of streptomycin in milk and honey. The recoveries for streptomycin in milk and honey were 81 and 92%, respectively. In the research by Bogialli *et al.* [2], a sample of bovine milk was spiked with streptomycin 0.2 mg/kg, and the recovery rate was 75%. Horie *et al.* [10] studied to determine streptomycin in swine and bovine muscle by liquid chromatography/ mass spectrometry, and examined recovery rates of streptomycin from swine and bovine muscle fortified at 0.2 mg/kg were 73.2-82.6%. Edder *et al.* [4] studied the determination of streptomycin residues in food by solid-phase extraction and liquid chromatography with post-column derivatization and fluorometric detection, and investigated recovery rates after spiked with 1.0 mg/kg streptomycin in honey, crude milk, meat, and liver. Recovery rate ranges from 80-90% in these studies.

With the consideration of a kind of samples, the fish muscle residue concentrations of streptomycin in our study were similar or little higher than those of other studies described above. It was assumed that the different results of recovery rates were dependent on experimental conditions and methods of sample extraction.

The analytical results of streptomycin in fishes muscle were shown in Table 2. All of streptomycin

Table 2. Depletion profiles of streptomycin in muscle of fishes during withdrawal period

Fish	Withdrawal (days)	No of Positive	S/C ratio[a] (Mean ± SD)
P. olivaceus	1	0	0.773 ± 0.054
	2	0	0.726 ± 0.045
	3	0	0.699 ± 0.037
S. schlegeli	1	0	0.735 ± 0.047
	2	0	0.702 ± 0.042
	3	0	0.676 ± 0.039
P. major	1	0	0.788 ± 0.051
	2	0	0.735 ± 0.047
	3	0	0.706 ± 0.046

[a] S/C ratio is the ration of the sample inhibition value (S) to the cutoff inhibition value (C).

samples showed negative results (S/C ratio ≤ 1.0) after the 1st day of withdrawal, and were believed to decrease under 0.2 mg/kg.

Habrda and Malicova [9] investigated that streptomycin concentrations were determined in various calf tissues after intramuscularly administration of streptomycin in the dose of l g. After 24 h of administration, the concentration of streptomycin in skeletal muscle was not detected. In the research of Friedlander and Stephany [7], lactating cows and sheep received an intramuscular administration of streptomycin at a dose of 10 mg/kg body weight once daily for three consecutive days, and the concentration of streptomycin in milk 24 h after administration was 0.09 and 0.19 mg/kg, respectively. After 48 h of administration, the concentration of streptomycin in cow milk was less than 0.05 mg/kg and that in sheep milk was 0.07 mg/kg.

With the consideration of the species, the dosage and the route administered, the fish muscle residue concentrations of streptomycin in our study were similar or little lower than those of other studies described above.

According to our results, the applied methods can be adopted easily for use to screen streptomycin residue in muscle tissue of farmed fishes after minimal sample preparation. It is suggested that this method can be able to apply for screening of streptomycin in tissues of fish especially on the place of shipment or on fish farm. If the inspected fishes show positive results, these could be banned from shipping until retest results become negative before they are forwarded.

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