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

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Abstract : Parallux, a solid-phase fluorescence immunoassay (SPFIA) developed for detection antibiotics residue 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 neomycin 140 mg/ton water, the recommended therapeutic dose, for 24 h. Muscle samples were obtained on 1st, 2nd, 3rd, 4th and 5th day after drug treatment. The concentration of neomycin in muscle was determined using an internal standard (100 ppb as neomycin). The absorbance ratio of sample to internal standard (S/C) was employed as an index to determine the muscle residues in fishes. To investigate the recovery rate, the standard solutions were added to muscle samples to give final concentrations in muscle of 0.2 and 0.5 mg/ml. The recovery rates of all spiked samples were > 85% of the spiked value. Neomycin was detected in muscles of fishes treated after the 1st day of withdrawal period. On the 2nd day after drug treatment, all muscle samples showed negative reaction (S/C ration ≤ 1.0). The present study showed that the SPFIA can be applied for predicting residues of neomycin in muscle tissues of farmed fishes.

Keywords : neomycin, olive flounder, red sea bream, rockfish, solid-phase fluorescence immunoassay

Neomycin is a widely-used broad spectrum water-soluble aminoglycoside antibiotic. It inhibits the growth of gram-positive bacteria such as *Staphylococcus aureus* and *Mycobacterium tuberculosis*, and gram-negative bacteria such as *E. coli*, *Enterobacter aerogenes*, *Klebsiella pneumonia*, and *Flavobacterium* (*F.*) *columnare* [1, 4, 10, 25]. *F. columnare* has been recognized as a worldwide pathogen of freshwater fish, and it is the etiological agent of columnaris disease which is characterized by gill necrosis, greyish white spots on the body, skin erosion, and fin rot. The lesions are characterized by the presence of long and thin rods that exhibit flexing movement and are able to form columns.

Neomycin has been reported with varying degrees of success to treat columaris disease [5, 7]. Neomycin is formulated, either alone or in combination with other antimicrobials such as lincomycin, penicillin, cephalosporins and some sulphonamides, for oral (including in-feed and medicated drinking water) administration, injection, intramammary infusion, and topical (including ocular) application. In aquaculture, neomycin is administered as a bath solution. Because the use of injectable formulations of neomycin is associated with ototoxicity (deafness in cattle) and nephrotoxicity, its use is generally limited to the treatment of serious gram-negative infections resistant to less toxic medications or as an alternative to costly medications. In some countries such as the USA, Canada and South Africa, injectable neomycin products are not authorized for use

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in food animals on account of such use being associated with a high risk of toxicity [20, 21].

Intense usage of antibiotics has led to a wide distribution of antibiotic resistance among bacterial species, including resistance against neomycin [3, 15]. 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 neomycin has been investigated in bovine milk and animal tissues [6, 22, 24]. Traditional approaches for the detection of neomycin residues include microbiological methods, immunoassays, and chromatographic methods. Microbiological methods are not sensitive or specific enough [17]. Immunological assays are very sensitive and can be used as screening tests [10]. However, chromatographic methods, such as high-performance liquid chromatography (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 [12-14].

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 [11, 12, 18]. 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 [11, 18]. Therefore, we investigated the possibility of Parallux™ application to detect neomycin in muscle of flounder, rockfish, and red sea bream.

Fishes used in this study were 25 healthy olive flounders (average weight, 630 ± 45 g), 25 healthy rockfishes (average weight, 470 ± 55 g), and 25 healthy red sea breams (average weight, 610 ± 50 g), with no previous history of antibiotic treatment.

Aqua Neosin (100 g (activity)/kg, withdrawal period, 5 days) was purchased from Dong Bang (Korea). The IDEXX Parallux kit cartridges were purchased from Korea Media Ltd. Parallux (IDEXX Laboratories, USA) is a solid-phase fluorescence immunoassay-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 [11, 12, 18]. The recommended therapeutic dose of neomycin (140 mg/ton water) was treated to 25 olive flounders, 25 rockfishes, and 25 red sea breams for 24 h using dipping administration. Muscle samples were obtained from all fishes during the withdrawal period on the 1st, 2nd, 3rd, 4th, and 5th day after administration of drug. The method of muscle sample extraction was modified from the protocol described by Haasnoot *et al.* [9]. 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 0.1M 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 neomycin (Sigma Chemical, 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 and 1.0 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 0.1 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 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 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

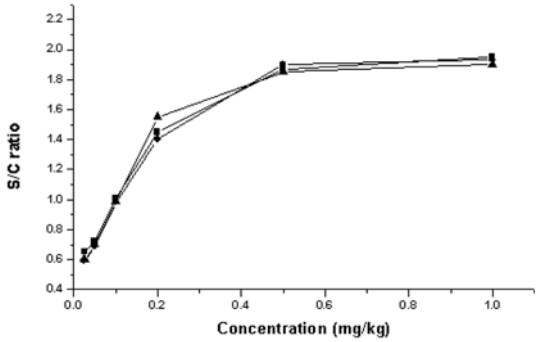

Fig. 1. Standard curve of neomycin in muscle of olive flounder (▲), rockfish (●), and red sea bream (■). Detection limit of neomycin was less than 0.1 mg/kg based on the S/C ratio of 1.0 in the assay system. S/C ratio is the ratio of the sample inhibition value (S) to the cutoff inhibition value (C).

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 [11, 12, 18].

The standard curve of neomycin was constructed to determine the detection limit of drug. As shown in Fig. 1, the detection limit of neomycin was less than 0.1 mg/kg based on the S/C ratio of 1.0 in the assay system. The standard curve of neomycin was shown linear regression between 0.05 and 0.2 mg/kg (olive flounder, $R^2 = 0.999$; rockfish, $R^2 = 0.996$; red sea bream, $R^2 = 0.997$). Okerman *et al*. [18] determined different antibiotic residues in bovine and in porcine kidneys by solid-phase fluorescence immunoassay. The range of ratio from spiked samples spiked with 0.1 mg/ kg cephapirin and 0.3 mg/kg tetracycline was 1.80-1.96 and 1.77-1.93, respectively. In our study, the ranges of ratio for neomycin were similar to that of tetracycline and lower than that of cephapirin.

Recoveries of 0.1 and 0.5 mg/kg of neomycin spiked into non-treated muscles are shown in Table 1. All recoveries were more than 85% of the spiked value.

Posyniak *et al*. [19] studied the determination of neomycin by HPLC, and investigated recovery rates after spiked at the concentration of 0.5 mg/kg of neomycin in porcine kidney. The recovery of neomycin in porcine kidney was 83.7%. In the research by Shaikh *et al*. [23], the recovery rate of neomycin from

Table 1. Recoveries of neomycin in muscle of fishes with solid-phase fluorescence immunoassay

Fishes	Spiked concentration (mg/kg)	S/C ratio* (Mean ± SD, n = 3)	Recovery† (%)
Paralichthys olivaceus	0.1	0.985 ± 0.045	87.1
	0.5	1.568 ± 0.061	89.0
Sebastes schlegeli	0.1	0.938 ± 0.047	85.2
	0.5	1.709 ± 0.052	94.4
Pagrus major	0.1	0.991 ± 0.038	91.2
	0.5	1.633 ± 0.049	90.2

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

Table 2. Depletion profiles of neomycin in fish muscles during withdrawal period

Fish	Withdrawal (days)	No of Positive	S/C ratio* (Mean ± SD)
Paralichthys olivaceus	1	5	1.109 ± 0.032
	2	0	0.614 ± 0.041
	3	0	0.499 ± 0.032
Sebastes schlegeli	1	5	1.095 ± 0.052
	2	0	0.607 ± 0.043
	3	0	0.479 ± 0.037
Pagrus major	1	5	1.218 ± 0.042
	2	0	0.622 ± 0.051
	3	0	0.504 ± 0.048

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

animal kidney tissues spiked at 1-30 μg/kg was 96%. Serrano and Silva [22] studied to determine neomycin in bovine milk by micellar electrokinetic chromatography with laser-induced fluorescence detection, and examined the recovery rate of neomycin from bovine milk spiked at 0.1 mg/kg was 93.3%.

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

The analytical results of neomycin in fishes muscle were shown in Table 2. All of neomycin samples showed positive results (S/C ratio 1.0) after 24 h of

withdrawal. At the 2nd day of withdrawal, all of samples showed negative reaction (S/C ratio 1.0), and were believed to decrease under 0.1 mg/kg.

Askbacher and Feil [2] investigated that neomycin concentrations were determined in various calf tissues after orally administration of neomycin in the dose of 30 mg/kg body weight. After the 4th day of administration, the concentration of neomycin in skeletal muscle was 0.016 mg/kg. In the research of Deluyker [6], lactating cows received intramammary infusions of 100 mg neomycin and 330 mg lincomycin in each mammary quarter, and the concentration of neomycin in milk 24 h after administration was 4.92 μg/kg. In the residue depletion studies in cattle, swine, and sheep, respectively, 15.4 mg of neomycin per kg of body weight was given to the animals as a single daily dose over fourteen days. The concentration of neomycin in all samples was less than 0.5 mg/kg (the limit of quantification) at one day after the last administration of the drug [8].

With the consideration of the species, the dosage and the route administered, the fish muscle residue concentrations of neomycin 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 neomycin 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 neomycin 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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