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Application of a solid-phase fluorescence immunoassay to determine ampicillin residues in muscle tissue of olive flounder (*Paralichthys olivaceus*)

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Abstract : Parallux™, a solid-phase fluorescence immunoassay (SPFIA) developed for antibiotics residue detection in milk, was applied for analysis of fish muscle. The recommended therapeutic dose of ampicillin (100 mg/kg body weight, withdrawal period 7 days) was orally administered to a group of 25 olive flounders (*Paralichthys olivaceus*) for consecutive five days. Muscle was sampled after drug treatment 1st, 2nd, 3rd, 4th and 5th day. The concentration of ampicillin in muscle, determined by SPFIA, was compared with that of internal standard (10 ppb as ampicillin). The absorbance ratio of sample to internal standard (Bs/Bo) was employed as an index to determine the muscle residue in olive flounder. To investigate the recovery rate, the standard solutions were added to muscle samples to give final concentrations in muscle of 10 and 50 ng/m*l*. The recovery rates of all spiked samples were > 89% of the spiked value. Ampicillin was detected in muscle of fishes treated until the 3rd day of withdrawal period. The present study showed that the SPFIA can be easily adopted in predicting tissue residues for ampicillin in farmed fishes.

Key words : ampicillin, olive flounder, solid-phase fluorescence immunoassay

Ampicillin is a widely used β-lactam, semi-synthetic penicillin that has activity against both gram-positive and gram-negative organisms [9]. Although not approved for use in the United States and seldom used domestically, it is commonly used in Japan and Korea to control pasteurellosis in farmed fishes [5]. Ampicillin, like its congeners, may result in severe hypersensitivity reactions in some people [6]. Because an initial exposure to some form of penicillin is necessary to produce an eventual drug allergy, ingestion of ampicillin as a residue in seafood is of public health interest. The FDA has set a tolerance level of 0.01 ppm and a pre-slaughter withdrawal time in cattle of 6 days. 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 ampicillin [3]. To limit the spread of resistance, 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 to limiting the spreading of resistance, 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 [12].

The residue of ampicillin has been investigated in milk, animal tissues and fishes [2, 16, 19].

Traditional approaches for the detection of ampicillin residues include microbial inhibition tests, immunoassays, and chromatographic methods. Widely used microbial inhibition methods are relatively complicated, time consuming and non-specific to ampicillin [11]. However, chromatographic methods, such as HPLC, provide sensitivity and specificity for ampicillin, but certain disadvantages,

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for example, high price, requirement of special equipment, and sample extraction protocols by expert personnel, limit their suitability for the analysis of large numbers of samples [1, 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 [7, 8, 17]. As the consumption of meat and fish has continually been increasing, simple, sensitive, rapid and low-cost methods for detecting residues in those are needed for the detection of large numbers of samples [12].

In the present study, we applied solid-phase fluorescence immunoassay (SPFIA) to the detection of ampicillin in muscle of olive flounder, farmed fish. Fishes used in this study were 25 healthy olive flounders weighting an average of 350 ± 30 g with no previous history of antibiotic treatment. Samu ampicillin powder (100 g (activity)/kg, withdrawal period, 7days) was purchased from Samu Median Co. (Seoul, Korea). The IDEXX Parallux™ β-lactam assay cartridges were purchased from Korea Media Ltd. The recommended therapeutic dose of ampicillin (100 mg/kg body weight mixed feed) was orally administered to a group of 25 olive flounders (*Paralichthys olivaceus*) for consecutive five days. Muscles were sampled 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 Okerman *et al.* [14]. One gram of grinded muscle from each olive flounder was weighed in a 50 m*l* tube. Five m*l* 0.01 M sodium phosphate buffer (pH4.5, 1 L buffer solution was contained 4.969 g $Na_2H_2PO_4$ dibasic, 8.968g $NaH_2PO_4 \cdot H_2O$, and 3.894 g $Na_2S_2O_3 \cdot 5H_2O$) was added, and the contents were vigorously mixed for 15 min with a vortex. One m*l* 0.17 M H_2SO_4 and 1 m*l* 5% sodium tungstate solution were added and mixed, and centrifuged for 10 min at 30,000 × g. The upper solution moved into another 50 m*l* tube, and adjusted pH 8.1~8.2 with 5 M NaOH. The solution was filtered with Whatman GF/B filter. The filtrate was added 2 m*l* 20% NaCl, and loaded into tC18 cartridge. The cartridge was cleaned with 3 m*l* 20% NaCl and 3 m*l* distilled water. After dried for 5 min at the room temperature, ampicillin was extracted with 2 m*l* acetonitrile. After the elute was evaporated with nitrogen gas, the residue was dissolved with 0.5 m*l* 0.01 M sodium phosphate (pH 9.0). Add 75 μ*l* derivative reagent I (2.26 g benzoic anhydride in 50 m*l* distilled water) and left for 10 min on the room temperature, and continually added 425 μ*l* derivative reagent II (6.906 g 1,2,4-triazole and 5 m*l* 26 mM $HgCl_2$ in 50 m*l* distilled water, pH 9.0 ± 0.05), and reacted for 30 min at 65°C After the reaction, cooled with water, and filtered through 0.22 μm syringe filter (Nalgene, USA) and applied to HPLC apparatus.

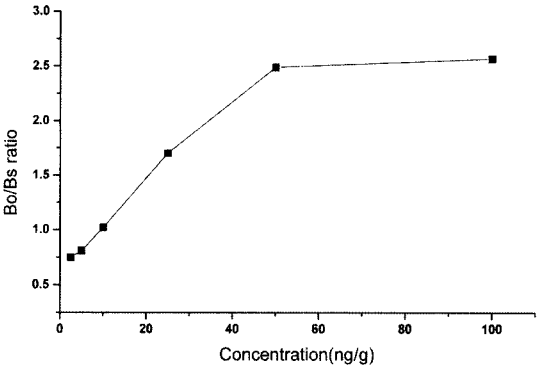

Fig. 1. Standard curve of ampicillin in muscle of olive flounder. Detection limit of ampicillin was calculated at 5 ng/g. Absorbance ratio obtained with blank tissue (Bo) and with muscle tissue spiked with ampicillin (Bs).

Stock solutions of 100 μg/m*l* of ampicillin (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 2.5, 5.0, 10, 25, 50 and 100 ng/m*l* working standard solutions. To get the recovery rate, the standard solutions were added to muscle samples to give final concentrations in muscle of 10 and 50 ng/m*l*. After blending, these samples were extracted as described above then analyzed in a blind fashion.

The standard curve of ampicillin was constructed to determine the detection limit of drug. As shown in Fig. 1, the detection limit of ampicillin was less than 5 ng/m*l* based on the Bo/Bs ratio of 0.8 in the assay system. The standard curve of ampicillin was shown linear regression between 5 and 50 ng/m*l* ($R^2 = 0.996$). Okerman *et al.* [14] determined different antibiotic residues in bovine and in porcine kidneys by solid-phase fluorescence immunoassay. The range of ratio from spiked samples spiked with 50 ng/m*l* ampicillin was 2.32-2.81. In our

Table 1. Recoveries of ampicillin in muscle of olive flounder with Solid-phase Fluorescence Immunoassay

Spiked concentration (ng/g)	Ratio* (Mean ± SD, n = 3)	Recovery (%)**
10	0.953 ± 0.037	93.4
50	2.275 ± 0.052	91.4

*Ratio obtained with blank muscle and with muscle spiked with ampicillin; **Recovery obtained from the formula, (absorbance of spiked muscle/absorbance of standard solution) × 100.

Table 2. Depletion profiles of ampicillin in flounder muscle during withdrawal period

Withdrawal (days)	No of Positive	Bs/Bo ratio (Mean ± SD)
1	5	2.825 ± 0.044
2	5	2.423 ± 0.036
3	5	1.021 ± 0.025
4	0	0.752 ± 0.054
5	0	0.648 ± 0.038

Bo; absorbance of negative control, Bs; absorbance of muscle sample.

study, the range of ratio for ampicillin was similar to that of study described above.

Recovery of 10 and 50 ng/m*l* of ampicillin spiked into non-treated muscle is shown in Table 1. All recoveries were more than 91% of the spiked value.

Han and Ko [4] studied the determination of ampicillin by High Performance Liquid Chromatography (HPLC), and investigated recovery rates after spiked at the concentration of 0.1 and 1.0 μg/m*l* of ampicillin in muscle tissues. The recovery rate for ampicillin was more than 91%. Tomoko and Masanobu [18] studied to analyze ampicillin residue in yellowtail tissue by HPLC, and examined recovery rates of ampicillin in yellowtail muscle. At the concentration of 0.1 and 0.2 μg/m*l*, recovery rates were 61.5% and 73.2%, respectively. In the research by Lambert *et al.* [10], pig muscle tissues were fortified with 0.04 and 0.2 mg/kg ampicillin, and the recoveries were 81.1 and 84.4%, respectively. Also, Wenhong *et al.* [21] reported that the recovery rates of ampicillin from catfish muscle fortified at 5, 10 and 20 ng/g levels, ranged from 89.9 to 95.2%. With the consideration of a kind of samples, the flounder muscle residue concentrations of ampicillin in our study were similar or little higher than those of studies described above. It was assumed that the different results of recovery rates were depended on experimental conditions and methods of sample extraction.

The analytical results of ampicillin in olive flounder muscle were shown in Table 2. All of ampicillin samples showed positive results (Bs/Bo ratio ≥ 10 ng/m*l*) after the 1st, 2nd and 3rd day of withdrawal. After the 4th day of withdrawal, all of muscle samples showed negative reaction (Bs/Bo ratio ≤ 0.1 μg/m*l*), and were believed to decrease under 5.0 ng/m*l*.

In the research by Verdon *et al* [20], incurred samples from a pig treated with ampicillin (20 mg/kg, body weight) were analyzed at the residue level of the drug in muscle tissue, and after 24 hr of drug treatment, the concentration of ampicillin in muscle was 0.102 mg/kg. Mohammed [13] investigated that kinetics of long-acting ampicillin injected intravenously at a dose of 4.166 mg/kg were determined in eight each of camels, sheep and goats. After 24 hr of administration, the concentration of plasma in camel was 0.157 mg/kg and that in sheep and goat was less than 0.01 mg/kg. With the consideration of the dosage and the route administered, the flounder muscle residue concentrations of ampicillin in our study were similar or little lower than those of studies described above.

According to our results, the applied methods can be adopted easily for use to screen ampicillin residue in muscle tissue of farmed fishes after minimal sample preparation. It is suggested that this method may be able to apply to screen for ampicillin in tissue of fishes 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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