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Withdrawal Time for Oxytetracycline in Red Tilapia Cultured in Freshwater

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Abstract

Oxytetracycline (OTC) was fed at a rate of 80 mg·kg^{-1} fish for 10 d to healthy red tilapia (average size 205 ± 44 g) cultured in freshwater; OTC was also given to red tilapia (average size 170 ± 41 g) in the form of a bath at 10 ppm for 4 d. After treatment the muscle of the fish (25 g of muscle taken along the lateral line) was analyzed by high-pressure liquid chromatography for OTC residues and the withdrawal time for OTC was calculated. The withdrawal time for OTC given orally for red tilapia cultured in freshwater at an average ambient temperature of 27.3 ± 0.5 °C was 17-18 d. Fish medicated by bath (10 ppm for 4 d) at an average ambient water temperature of 27.3 ± 0.7 °C had a withdrawal time of 12.5-16 d. The initial concentration of OTC residue in fish medicated orally was approximately seven times higher than those medicated through bath. Due to the low bioavailability, the therapeutic efficacy of bath treatment should be examined.

Introduction

Modification of the bacterial flora and the emergence of antibiotic-resistant bacteria in the gut of humans due to antibiotic residues in food have been of great concern to consumers. In many developing countries, the fear of antibiotic residues in fish has been enhanced in recent years because of the increasing production of fish and shellfish from aquaculture and the lack of regulation governing the use of antibiotics for farming aquatic animals in these countries. Possible hazards ranging from antibiotic residues in fish, development of bacterial resistance to drugs, environmental pollution and adverse effects on ecosystems, have received increasing attention in recent years (Austin 1985; Jacobsen and Berglind 1988; Bernoth 1991; Björklund et al. 1991).

To reduce the presence of antibiotic residues in farmed fish, farmers must be given advice on the withdrawal time needed when antibiotics have to be used in the culture. However, very little information is available on antibiotic withdrawal time for fish cultured in the tropics, although much work has been carried out in temperate countries. A lot of studies, for example, have been conducted in temperate countries on the pharmacokinetics for oxytetracycline (OTC), which is one of the most common antibiotics used in aquaculture.

Elimination of OTC in farmed fish is markedly temperature-dependent, and is faster at higher temperatures (Salte and Liestøl 1983; Jacobsen 1989;

Björklund and Bylund 1990). Salte and Liestøl (1983) reported a withholding period of 60 d for rainbow trout cultured in water temperature of above 10 °C and treated with 75 mg·kg^{-1} fish for not more than 10 consecutive d; for water temperature of 7-10 °C, a withholding period of 100 d was needed. Jacobsen (1989) recommended a withdrawal time of 90 d for rainbow trout cultured in water temperature below 6 °C, 70 d in water temperature of 6-12 °C, and 60 days above 12 °C; whilst Björklund and Bylund (1990) reported a withdrawal time of 92 d for rainbow trout cultured in water temperature at 5 °C, 48 d at 10 °C and 37 d at 16 °C. The results from Jacobsen (1989) were based on OTC residues from whole gutted fish, whereas that of Salte and Liestøl (1983) and Björklund and Bylund (1990) were from muscle. The United States Food and Drug Administration approved the use of OTC at 50 mg·kg^{-1} fish for 10 consecutive d when a withdrawal period of 21 d is observed (Plumb 1992). In Sweden, a withdrawal time of 30 d is required for OTC used in water temperature >9°C (Ackefors et al. 1990).

This paper attempts to establish the withdrawal time for OTC in red tilapia cultured in freshwater under ambient temperature in tropical conditions in the hatchery at the Fisheries Research Institute, Penang, Malaysia. The fish were medicated with OTC through feeding and immersion in a bath.

Materials and Methods

Experimental Conditions

Healthy red tilapia were obtained from the Freshwater Fish Culture Station in Jitra, Kedah. The fish were kept in freshwater in 2-tonne rectangular fiberglass tanks (300 x 100 x 80 cm) in the hatchery of the Fisheries Research Institute, Penang, under natural lighting of approximately 12 h light and 12 h darkness. The fish were placed under ambient water temperature which was recorded twice daily (morning and afternoon) using an LCD display Ama-digit ad 16th thermometer (Germany).

Drug Administration

ORALLY

The fish (205 $\pm$ 44 g) were acclimatized for a week and starved 2 d before the experiment. They were fed a commercial fish feed, CPP-TP-1 (Central Pangan Pertiwi, Indonesia) with OTC (oxytetracycline dihydrate - Sigma Chemical Co., St. Louis, USA) incorporated by mixing a ratio of 100 g pellet feed to 100 ml distilled water to which OTC was dissolved. The dough was extruded through a meat mincer and cut into pellets and fed moist. The OTC dosage was 80 mg·kg^{-1} fish incorporated into 1% body weight of feed. Feeding was carried out twice a day for 10 d. At this low feeding rate, normally all the feeds were consumed by the fish. The tanks were cleaned with an approximately

80% water change carried out daily. After the medication period, four to six fish were sampled each time for OTC at 0, 2, 4, 6, 8 and 10 d after treatment. The fish analyzed at 0 d were collected 2 h after the last medicated feed. OTC analyses were carried out immediately after filleting the fish at the appointed treatment interval.

BATH

The fish (170 ± 41 g) were given a bath of OTC (oxytetracycline dihydrate, Sigma Chemical Co., St. Louis, USA) at 10 ppm for 4 d; during this period no water change was carried out. Since OTC in solution degrades in the presence of light, the concentration of OTC in the bath would be less than 10 ppm at the end of the experiment. However, initial and final concentration of OTC in the bath was not measured. The fish were fed at a daily rate of 1% body weight with CPP-TP-1 feed. After medication, four fish were used each time for OTC analysis at 0, 3, 6, 9 and 12 d after treatment. The analyses on 0 d were made immediately after the 4-d bath treatment. OTC analyses were carried out immediately after filleting the fish at the appointed treatment interval.

Chemical Analysis

CHEMICALS

Methanol, dichloromethane, acetonitrile (all of HPLC grade), petroleum benzine extra pure DAB (b.p. 40-60 °C) and hydrochloric acid fuming 37% GR were obtained from Merck (Darmstadt, Germany); oxalic acid (Analar Grade) and sodium hydroxide pellets (Analar Grade, minimum assay 99%) from BDH (Poole, England). OTC was obtained from Sigma Chemical Co., St. Louis, USA.

APPARATUS

A high performance liquid chromatography (HPLC) pump Model Waters 600E (Millipore Corporation, USA) connected to an autosampler and injector Model Waters 715 Ultra WISPS, a UV detector Model Waters 486 and a Waters Maxima 825 chromatography software and workstation were used. The column used was a Novapak steel column (3.9 x 150 mm with spherical packing material of 4 um) which was connected to a Guard-Pak ™ μ Bondapak ™ C_{18} HPLC precolumn insert.

SAMPLE PREPARATION

OTC extraction and recovery were adapted from the method described by Moats (1986). A 25 g sample of muscle tissue taken from the lateral line region behind the operculum of the fish was homogenized for 10 minutes and acidi-

fied with 75 ml 1N hydrochloric acid. Eight ml of the homogenate was deproteinized with 32 ml of acetonitrile. After standing for 5 minutes, the supernatant was decanted and a liquid-liquid extraction using 20 ml dichloromethane and 20 ml petroleum benzine was carried out and the OTC portion partitioned in the aqueous layer. Recovery experiments in which known amounts of OTC stock solution were added to muscle tissue from untreated red tilapia and allowed to stand for 30 minutes were also carried out.

CHROMATOGRAPHY

Using the autoinjector, 50 ml of the aqueous extract was injected into the column. The mobile phase (pH 4.01) used comprised of methanol, acetonitrile and 0.01 M aqueous oxalic acid (pH 3.5 adjusted with 6N NaOH) in the following ratio, 1:1.5:7.5. The mobile phase was filtered using an FH 0.5 mm millipore filter and further degassed with a stream of helium (He) at 100 $ml \cdot min^{-1}$ for 15 minutes. A small stream of He (20 $ml \cdot min^{-1}$) was passed through the mobile phase throughout the analyses. The detection of OTC was made at a wavelength of 360 nm, a temperature of 30 °C, a flow rate of 1.0 $ml \cdot min^{-1}$, a pressure of about 1,200-1,400 psi and a detector sensitivity of 0.01 AUF.

The standard curves were prepared using OTC hydrochloride (88.7% purity). The stock (100 ppm) was prepared by dissolving the OTC in methanol and the working solution was prepared by diluting the stock solution with the mobile phase.

The peak integrations and concentrations (peak areas) were calculated using the Waters Maxima 825 chromatography software and workstation.

Withdrawal Time Calculation

Two methods were used to determine withdrawal time for OTC. The method described by Salte and Liestøl (1983) involves the calculation of the linear regression from the natural logarithm of the drug residue concentration against time. The 95% confidence limits for the linear equation were calculated using the method described by Steel and Torrie (1980). The withdrawal time was determined by the intersection between the detection limit and the 95% upper confidence limit. The method described by Jacobsen (1989) is based on the calculation of the time taken for the OTC level in the fish tissues to reach 1/5 the detection limit.

Results

Detection Limit and Recovery

The detection limit of OTC was 0.07 $ug \cdot g^{-1}$ and the % recovery was 83±8 % (coefficient of variation, n=4). The HPLC response for OTC over the

range of 0.01-2 ppm OTC standard solutions was linear with a coefficient of determination, (r^2) = 0.998 ± 0.002. The retention time of OTC was 2.69 ± 0.10 minutes.

Oral Medication

Table 1 shows the OTC concentration in the muscle of red tilapia following oral medication. There is considerable variation in the OTC concentration between fish especially immediately after treatment, at 0 d. A steep drop in the OTC concentration in muscle was observed 2 d after treatment, and 10 d following treatment, two fish out of six had OTC levels below the detection limit.

Table 1. OTC concentration in muscle of red tilapia following oral medication (80 $mg \cdot kg^{-1}$ fish for 10 d).

Days after treatment	No. of fish analyzed	Average size (g ± SD)	OTC ($ug \cdot g^{-1}$ ±SD)
0[1]	5	224.5 ± 47.9	3.47 ± 1.12
2	6	223.0 ± 23.4	0.63 ± 0.04
4	6	198.9 ± 38.2	0.90 ± 0.13
6	6	Not measured	0.21 ± 0.06
8	4	172.2 ± 25.4	0.25 ± 0.04
10	6[2]	189.1 ± 44.6	0.22 ± 0.12

[1] Two hours after last feeding with medicated pellets.
[2] Six fish were analyzed but two fish had OTC levels below the detection limit.Therefore mean OTC concentration was calculated from four fish.

The withdrawal time for OTC in red tilapia medicated orally and kept at a water temperature of 27.3 ± 0.5 °C was 17 d using the method of Salte and Liestøl (1983) and 18 d using the method of Jacobsen (1989). The linear regression of OTC muscle concentration against time has an equation of Y = 0.62 - 0.27X, and a coefficient of determination (r^2) of 0.713.

Bath

Table 2 shows the OTC concentration in the muscle of red tilapia medicated by bath. Less variability was seen in the muscle OTC concentration when compared to the concentration in fish medicated orally. OTC concentration was detected in only two out of four fish, 6 d after treatment, one out of four fish after 9 d, and 12 d after treatment, all the four fish analyzed had OTC levels below detection limits.

The withdrawal time for OTC concentration in red tilapia medicated through a 10 ppm bath for 4 d is 12.5 d using the method of Salte and Liestøl (1983), and 16 d using the method of Jacobsen (1989). The plot of ln concentration against time gave a linear regression of Y = - 0.63 - 0.23X with a coefficient of determination (r^2) of 0.879.

Table 2. OTC concentration in muscle of red tilapia following treatment by bath (10 ppm for 4 d).

Days after treatment	No. of fish analyzed	Average size (g ± SD)	OTC ($ug \cdot g^{-1}$ ± SD)
0[1]	4	167.0 ± 64.1	0.52 ± 0.12
3	4	187.5 ± 29.5	0.31 ±0.08
6	4[2]	182.5 ± 25.7	0.11 ± 0
9	4[3]	114.5 ± 22.5	0.07
12	4[4]	171.0 ± 35.8	Not detected

[1] Fish analyzed immediately after 48 h.
[2] Four fish analyzed but two had levels below the detection limit. Therefore mean OTC concentration calculated from two fish.
[3] OTC level in three fish below detection limit.
[4] All fish had levels below the detection limit.

Discussion

The calculation of withdrawal time is dependent on the sensitivity of the analytical methods used, and therefore comparison of withdrawal times from various laboratories may encounter problems. The method used in this study gives a detection limit of 0.07 $ug \cdot g^{-1}$, and this sensitivity is comparable to those reported by other authors using the HPLC technique. Jacobsen (1989) reported a detection limit of 0.05 $mg \cdot kg^{-1}$ in the muscle of rainbow trout, Ueno et al. (1989) a limit of 0.05 ppm in the muscle of rainbow trout, Björklund et al. (1991) a limit of 0.05 $ug \cdot g^{-1}$ in muscle of rainbow trout, Carignan et al., (1993) a limit of 0.05 $ug \cdot g^{-1}$ in muscle of salmon, and Reimer and Young (1990) a level of 0.08 ppm in salmon muscle tissue. Nordlander et al. (1987), however, reported a much lower detection limit at 0.005 $ug \cdot g^{-1}$ in the muscle of rainbow trout, and Rogstad et al. (1988) a limit of 5 $ng \cdot g^{-1}$ for fish muscle. Microbial assay techniques for the detection of OTC are generally more sensitive than HPLC methods. Salte (1982) reported a limit of 0.02 $ug \cdot g^{-1}$ in muscle of rainbow trout, Salte and Liestøl (1983) a limit of 0.04 $ug \cdot g^{-1}$ in muscle of rainbow trout, and Strasdine and McBride (1979) a limit of 0.01 $ug \cdot l^{-1}$ in serum of sockeye salmon. Although microbial assays are more sensitive than HPLC techniques, they lack specificity for regulatory purposes (Oka and Uno 1984; Nordlander et al. 1987; Carignan et al. 1993). Ueno et al. (1989) reported the standard OTC values provided for the Settlement of the Residue Analysis (Norin-Suisan Sho, Tikusan Kyoku : Shiryotenkabutu no Hyokakijun ni motozuku Shiken no Tebiki, Tokyo, 1980, pp. 65-67) for meeting the requirement of OTC analysis to have >70% recovery, <10% coefficient of variation and <0.1 ppm detection limit. Hence the OTC analyses carried out in this study provided values that met the above requirements.

The amount of OTC recommended by extension workers to farmers in Malaysia is 1 $g \cdot kg^{-1}$ feed for fish in the grow-out phase weighing 200-600 g. Based on a feed consumption of 5% body weight, the medicated fish would consume OTC at a rate of 50 $mg \cdot kg^{-1}$ fish. However, fish infected with diseases normally have a poor appetite, and the feeding rate could be even less than 2% body weight. Assuming that fish farmers used the recommended dosage given

by extension workers, the initial OTC residue in farmed fish would probably be lower and the withdrawal time could differ from the values in this experiment.

The initial concentration of OTC residue in fish medicated orally is about seven times higher than those medicated through bath. Strasdine and McBride (1979) reported that there was no uptake of OTC in adult sockeye salmon after a bath treatment of 5 $mg \cdot l^{-1}$ for 2 h. Grondel et al. (1987) reported that the minimum inhibitory OTC concentration (MIC) for common fish pathogens varies widely for different pathogens, with values of 0.25-8 $ug \cdot l^{-1}$. Due to the low bioavailability in the bath administration, the therapeutic efficacy of such treatment should be examined.

This study also shows that withdrawal time for OTC was longer when red tilapia were medicated orally compared to those medicated through bath. However, fish given oral medication were slightly bigger than those given bath treatment, and this may have contributed to the difference in pharmacokinetic behavior. Normally OTC medication is given orally to fish in farms in Malaysia, and a period of 21 d is usually recommended by fisheries extension workers. This recommendation appears sound, since this study shows that a withdrawal time of 17-18 d was required when red tilapia were cultured in water temperature of 27.3 $\pm$ 0.5 °C and medicated orally at a dosage of 80 $mg \cdot kg^{-1}$ fish for 10 d.

Differences in pharmacokinetic behavior of OTC in different fish species have been reported (Grondel et al. 1986, 1989), and the response of different fish species to OTC may vary resulting in different withdrawal times. Hence, withdrawal time for other species commonly cultured in Malaysia such as sea perch (*Lates calcarifer*), grouper (*Epinephelus* spp.) and penaeid prawns (*Penaeus monodon* and *P. merguiensis*) should also be studied.

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