

Leaching and Residual Kinetics of Oxytetracycline Incorporated Feed Treated to Juvenile Black Tiger Shrimp *Penaeus monodon* fabricious

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The leaching rate of oxytetracycline (OTC) incorporated medicated feed after immersion in water was high during the first 10 min. About 56% of loss of OTC from the medicated feed was observed after 2 h of immersion. The leaching rate was not uniform in the 10 h experiment period. Juvenile black tiger shrimp *Penaeus monodon* fed for 7 days with 50-100 mg/kg shrimp of OTC were examined for residual accumulation and depletion. A residue of 3.47 µg/g shrimp tissue was found after 1 day of post dosing. This concentration was estimated as 0.77% of the total OTC intake in the 7 days of experimental period. A substantial quantity of residue (1.56 µg/g) was observed up to 15 days of post-treatment. By the 20th day of treatment, the OTC concentration was found in trace quantity (0.42 µg/g) or below detectable limit.

Key words : Oxytetracycline, antibiotic-leaching, medicated feed, tiger shrimp, *penaeus monodon*, antibiotic-residue, residual kinetics

Indiscriminate use of antibiotics, especially unapproved drugs, in shrimp aquaculture can lead to serious environmental and public health problems due to the development of resistant bacterial strains and residual accumulation in the shrimp tissue (Selvin & Lipton, 2003). In India oxytetracycline (OTC) is one of the two major antibiotics frequently used to treat vibriosis and other bacterial diseases in the culture systems. The antibiotic is incorporated as surface-coatings in feed for oral treatment for bacterial diseases in the grow out phase of shrimp aquaculture (Park *et al.*, 1995). The level of loss during the period of shrimp feeding schedule (2 h) must be considered in order to get effective therapy. The studies on leaching rate of OTC incorporated medicated feed for shrimp aquaculture is scanty. The present study was taken up to evaluate level of uptake of OTC, and kinetics of the antibiotic residue in

shrimp tissue following medicated feed treatment for 7 days, and the leaching rate of OTC from the surface-coated feed.

Materials and Methods

Healthy, farm reared (30 DOC) juvenile black tiger shrimp *Penaeus monodon* obtained from an extensive type farm was acclimated in 1000 l capacity FRP tank for 15 d with constant aeration and daily water exchange. They were fed with pellet feed (C.P. Grower feed No.1) in three equal instalments per day and were maintained at a temperature of 30±2°C, pH 7.8±0.5, dissolved oxygen content 5.6±0.5 ppm. Commercial pellet shrimp grower feed No. 1 (C.P. feeds, Cochin) was used for preparing medicated feed for the experiments. The recommended dose (FDA, 1986) of OTC @ 50-55 mg/kg of shrimp was incorporated in the feed by spraying on the surface of the feed. The shrimps were fed at the rate of 3.2% of the shrimp body weight

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daily. Assuming 50% loss due to leaching and reduced feed intake during diseased condition (about 25%), the medicated feed was prepared with 4 g antibiotic per kg of shrimp feed so that the shrimp will consume 50 mg/kg of body weight. To prepare the medicated feed, 4g of OTC (as HCl, potency = 920 µg/mg, Himedia) was dissolved in 50 ml of 4% gelatin water and the mixture was sprayed on 1 kg of pellet feed using a TLC sprayer (Xu and Rogers, 1993). The sprayed medicated feed was dried in a hot air oven at 40° C.

The initial level of OTC in the medicated feed was analyzed by cylinder plate/agar diffusion method using *Bacillus cereus* var. *mycoides* (ATCC No. 11778) as sensitive organism (Clarke's, 1996, IP96, USP 1995). The feed was homogenized into powder in a mechanical grinder and sieved through a fine nylon mesh (1mm). Initially the OTC extraction solvent was determined on the basis of solubility and was completely soluble in N/10 hydrochloric acid (Clarke's 1986, IP 1996, USP 1995). One gram of OTC incorporated feed powder was dissolved in 25 ml of N/10 HCl and extracted by shaking for 1 h in a shaker at 100 rpm. The extracted sample was centrifuged at 4000 rpm for 15 min. The supernatant was removed and a second extraction was made with residue by adding another 25 ml of N/10 HCl. The combined extract was adjusted to pH 6.4 and serially diluted with phosphate buffer (pH 6.4) for OTC assay.

Tests to determine the rate of leaching of antibiotics from the medicated feed were conducted in three one-litre glass beakers, each containing 500 ml deionised water. One gram of medicated feed, enclosed in a nylon bag with 1 mm mesh was placed in each beaker. To ensure undisturbed release of antibiotic, the beaker was placed on a magnetic stirrer. The feedbag was carefully lowered without any shaking into the water for 10, 20, 30, 60, 120, 240, 360 and 600 min and then raised above the water column. Then the water was stirred and three water

samples (1 ml each) were collected. The level of antibiotic leaching from the medicated feed was determined by using modified cylinder plate double layer method (IP, 1996).

In the modified cylinder plate/agar diffusion method, the concentration of *Bacillus cereus* inoculum used for the assay was standardized first using 1 ml of different dilutions of 18 h fresh culture per 10 ml of antibiotic assay medium F (HiMedia), so that clearly defined zones of inhibition of suitable diameter are produced up to the least concentration of the antibiotic used for the assay. Initially the base layer was prepared with 1.5% agar at a thickness of 1 to 2 mm to avoid seepage of test compound through the bottom interface. Six sterile porcelain cylinders with outside diameter of 8 mm and length 10 mm were placed on the base layer about 60° apart before pouring the seed layer. Then the seed layer was prepared by adding a requisite quantity of the test organism to the medium at a temperature of 40° C and 50° C and immediately poured the inoculated medium on the top of base layer. After solidifying the seed layer the cylinders were carefully removed with a sterile forceps so that the uniform cavities were formed in the seed layer. Fifty micro liters each of standard or test compound were added in each well. Standard curves consisted of OTC concentrations of 0.05, 0.625, 1.25, 2.5 and 5 mg/ml with 2.5 mg/ml as the reference standard. Three alternate wells on each plate were filled with a reference standard and the remaining three were filled with the samples or standard. Inhibition zones were measured after 24 h of incubation at 30±2° C using a vernier caliper.

Prior to administering the drug, 100 healthy shrimp (average w = 2.75 g) were kept fasting for 24 h. The shrimp were treated with medicated feed @ 5% of the shrimp body weight per day at three equal installments for 7 d. Before applying the feed, the waste and unfed materials were

collected separately and dried at 40°C for evaluating the daily feed intake. Based on the daily feed intake, approximate daily antibiotic intake was also calculated.

On the day 1, 5, 10, 15 and 20 of post-treatment, 4 sets of 5 shrimp in each set were randomly harvested for the residual analysis of OTC. The harvested samples were immediately killed under ice and the shell and head peeled off. The pooled tissues were weighed and minced in a mortar. The minced tissue samples were transferred to the homogenizer tube and using 10 ml of N/10 HCl, the mortar and pestle was rinsed three times and added into the homogenizer tube. The tissue was homogenized thoroughly and the homogenate was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was collected and second and third extraction was made with 10 ml each of appropriate solvents. The combined extract was adjusted to pH 6.4 and diluted with KH_2PO_4 buffer for the OTC assay. The zones of inhibition of the shrimp homogenate tested were compared with zone of the reference concentration in the standard curve. The quantity of OTC residue in the shrimp tissue was calculated by using the following expression. The results were expressed in mg.

Inhibition zone of sample $\times$ wt. of std $\times$ dilution factors of sample

Inhibition zone of std $\times$ wt. of sample $\times$ dilution factors of std

The mean of OTC tissue concentration was plotted against time semi logarithmically. The elimination rate constant (b) was calculated from the slope of the line using the equation $b = \text{slope}/2.303$. The elimination half-life ($t_{1/2}$) of OTC for the tissue was calculated by $t_{1/2} = 0.693/b$ (Gibaldi & Perrier, 1975). The results are presented as mean $\pm$ SD of four samples.

Results and discussion

Using the microbiological method, the minimum detectable concentration (MDC) of

OTC in the standard was determined as 0.1 mg/ml (Table 1), which was equivalent to 0.42 mg/g of residue in the shrimp tissue. The zone size formed for the concentration 0.05 mg/ml was too small (8.5 mm) which is of 0.5 mm thickness around the cavity or no detectable zone. As the accuracy and sensitivity of the method completely relied on the density of bacterial inoculum, it was standardized prior to the residual analysis. The 10^1 dilution containing approximately 10^9 cfu/ml produced thin matted growth and clearly measurable zone of inhibition up to the MDC. In the present study, 4.46 g of OTC/kg of feed was chosen as the recommended treatment level. This level of OTC incorporated medicated feed will provide a concentration of 140 mg/kg of shrimp. As about 56% leaching of OTC from the medicated feed was observed in the initial period 2 h of feeding time, the estimated mean treatment level in the present study would contain about 62 mg/kg of shrimp body weight.

Table 1. Inoculum concentration for the OTC assay

Concentration of OTC ($\mu\text{g/ml}$)	Area of inhibition at various dilutions of inoculum		
	10^0 (10^{10} cells/ml)	10^1 (10^9 cells/ml)	10^2 (10^8 cells/ml)
10	+	22	22
5	-	19	20
2.5	-	17	18
1.25	-	14	15
0.5	-	11	10
0.1	-	9	8.5

The rate of daily feed intake and leaching rate were the two important factors for the correct quantification of percent of OTC remaining in the tissue as residue. The percent of daily feed intake of OTC is presented in Table 2. The daily feed intake was very low in the first two days and was ranged as 38.9% and 34.5% respectively. On the day three onwards, the intake of OTC medicated feed was increased to above 50%, which attained 76.0% on the day 7. Therefore

an average value of 58.0% feed intake was observed in the overall treatment period.

The leaching rate and concentration of antibiotics in the medicated feed is depicted in Fig 1. Loss of OTC from medicated feed was continuous throughout the immersion period of 600 min in water. It was found that 29.48% of OTC was leached in the first 10 min. The leaching rate of OTC drastically increased to 44.17% in 30 min and then decreased. Between 30 and 60 min, it was 1.79% (45.96% of total leaching) with a slight increase to the tune of 5.57% in the next 60 min (51.56% total leaching after 120 min). The leaching rate attained a steady state after 240 min of immersion (56.05%). The rate of leaching from 240 to 360 min and 360 to 600 min was 1.12% (57.17% and 58.29% respectively at 360 and 600 min).

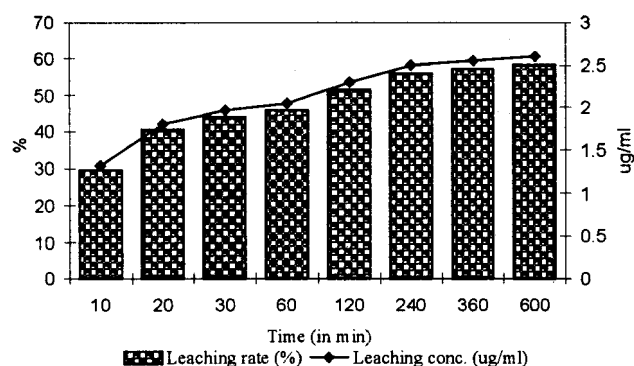


Fig. 1. Leaching rate and concentration of OTC in the environment

The level of residual antibiotics in the shrimp tissue in the first day of post-treatment was 3.37 $\mu\text{g/g}$ OTC (Table 3). A substantial quantity of residue was observed on the observation days 5, 10 and 15. On the 5th day, the quantity of OTC residue was 25 $\mu\text{g/g}$, followed by 1.76 and 1.56 $\mu\text{g/g}$ respectively for the days 10 and 15. The residue level of OTC reduced to 0.42 $\mu\text{g/g}$ or below the detectable limit on the day 20. The elimination rate of OTC in the shrimp tissue was 0.97 $\mu\text{g/g}$ on the first 5 days of post-treatment period. Between 5 and 10 days, a level of 0.74 $\mu\text{g/g}$ OTC was eliminated. The elimination rate was found

to be less (0.2 $\mu\text{g/g}$) between 10th and 15th of post-treatment. However on the 20th day, only trace amount of OTC was detected in one set of sample. The trend of bioavailability of OTC in the shrimp tissue to the estimated OTC intake (450 $\mu\text{g/g}$ tissue) indicated that 0.77% of OTC remained as residue in the shrimp tissue on the day 1 of post-treatment. This level gradually decreased to 0.09% and below the detection limit on the day 20 through 0.55% on the day 5, 0.39% on the day 10 and 0.34% on the day 15 of the post-treatment period. The estimated terminal elimination constant β was 0.43 and the corresponding elimination half-life $t_{1/2}$ was 1.59.

Table 2. Daily feed and estimated OTC intake of experimental shrimp (*P. monodon*)

Days	Daily feed intake (%)	Estimated daily OTC intake (mg/275 g of shrimp)
1	38.9	23.86
2	34.5	21.09
3	56.5	34.60
4	64.0	39.25
5	66.0	40.14
6	70.0	42.92
7	76.0	46.60

Takahashi *et al.*, (1985) indicated that a level of 50 to 200 mg of OTC/kg of body weight was effective against *Vibrio* sp. outbreaks in *P. japonicus* culture. The results of the leaching assay indicated that the leaching rate was much higher at the first 10 min and then it became gradual. About 56.0% loss was observed in the end of 2 h of immersion. This indicates that OTC leaching rate was not following a uniform pattern, with relatively greater losses occurring during the first few minutes of exposure in water than during subsequent period of immersion (Rigos *et al.*, 1999). Although it is possible to tackle the loss in the medicated feed by compensating the quantity during the feed preparation for the effective therapy, still the leaching will lead to adverse

environmental consequences. Thus, repeated antibiotic therapy may lead to specific bacterial resistance, which will reduce the efficiency of prophylactic antibacterial agents. According to Tendencia and Pena (2001), the incidence of *Vibrio* resistance in the shrimp farms was highest against OTC (4.3% of the total numbers of isolates) followed by furazolidone (1.6%), oxolinic acid (1.0%) and chloramphenicol (0.66%). Several studies have stressed the role of feed-derived antibiotics as a source of bacterial resistance Bjoerklund, *et al.*, 1990; Samuelsen *et al.*, 1992; Coyne *et al.*, 1994; Smith *et al.*, 1994, Kerry *et al.*, 1995). According to Twiddy & Reilly (1994), significant increase in antibiotic-resistant human pathogenic bacteria developed in aquaculture ponds where the antibiotics are routinely incorporated into feeds.

Table 3. Residual kinetics of OTC in shrimp tissue

Days	Average weight of shrimp tissue (g)	Residual OTC in shrimp tissue ($\mu\text{g/g}$)	% of total OTC remaining as tissue residue
1	6.33 $\pm$ 1.57	3.37 $\pm$ 0.15	0.77
5	5.87 $\pm$ 0.30	2.50 $\pm$ 0.63	0.55
10	4.66 $\pm$ 0.59	1.76 $\pm$ 0.25	0.39
15	3.95 $\pm$ 0.89	1.56 $\pm$ 0.14	0.34
20	4.4 $\pm$ 1.03	0.42*	0.09

*Value of one sample. Other three samples were negative
Values expressed as mean $\pm$ SD. N=4

In the present study, initially the feed intake was below the normal level. Low intake of medicated feed could be due to the non-palatability of antibiotics and possible indigestion of gelatin. Earlier reports have also shown that incorporation of antibiotics into medicated feeds reduced the feeding rates of shrimp and fishes. The case studies such as: OTC fed to brown shrimp *P. aztecus* and white shrimp *P. setiferus* (Corliss 1979); Romet-80 (specifically ormetoprim) fed channel catfish, *Ictalurus punctatus* (Poe and Wilson 1989, Robinson *et al.*, 1990), can be taken as examples.

Residual accumulation of OTC in the shrimp tissue on the first day of post-treatment was 3.37 $\mu\text{g/g}$ and this value exceeded the MIC of OTC required for 13 bacterial isolates taken from diseased shrimp (Mohny, *et al.*, 1992). In the present study, residual OTC in the shrimp tissue was eliminated to 0.1 mg/ml levels or 0.42 $\mu\text{g/g}$ MDC on the day 20 of post treatment. Mohny *et al.*, (1997) reported that residual OTC in juvenile blue shrimp *P. stylirostris* fed with medicated feed for 14 days was detectable on the day 3 (0.55 $\mu\text{g/g}$) and it reached beyond MDC on the day 5. This result is not agreeable to the present study and it may be due to the solvent system used for the extraction. In the present study, the extraction solvent was selected on the basis of solubility of OTC as per Clarke's (1986). Namdari *et al.* (1996) reported the residual kinetics of OTC in chinook salmon and coho salmon fed with OTC incorporated medicated feed at a rate of 75 mg/kg fish for 21 days. The residue accumulated in the chinook salmon acclimated at 9°C and 15°C. fell below detectable limit (0.05 mg/g) on the days 50 and 41 respectively. The results clearly indicated that 4.48 $\mu\text{g/g}$ and 4.35 mg/g respectively of OTC residue was observed on the day of post-dosing and this higher level of muscle tissue residue was drastically reduced to 2 $\mu\text{g/g}$ and 2.33 $\mu\text{g/g}$ respectively on the day 15 of post-dosing of chinook salmon acclimated at 9°C and 15°C. These findings are similar to the present observation of residual kinetics in shrimp tissue.

The results obtained from Japanese oyster *Crassostrea gigas* and blue mussel *Mytilus edulis* treated with OTC and oxolinic acid (OA) at the rate of 0.15mg/l (i.e. equivalent to 0.9 to 16mg/kg) and 1.5mg/l (0.7 to 1.9mg/kg) for 10 d, indicated that the OTC, in a large part, is bound to ions and organic molecules in seawater (Pouliquen *et al.*, 1996). The binding capacity of OTC to minerals and organic compounds in bivalve tissue was stronger than OA and therefore it was eliminated more slowly from the

bivalve tissue than OA. It may be the reason for slower elimination rate observed in the present study. Because of its high binding capacity, the risk of leached OTC in the environment was also lower than that of CAP.

The level of OTC in the shrimp tissue at post-dosing elimination period in the present study was similar to the residue noted by Corliss (1979) with 21 d exposure with 10,000 mg OTC/kg feed. However the author reported that no residue was found after 3 days at 1000 mg OTC/kg feed and depletion after 3 to 14 days at 5000-mg/kg feeds. The elimination time expected for 5000 mg/kg and 10,000 mg/kg in the present study is longer than that obtained from the data of Corliss (1979). Even the least residue formed on the 20th day post-dosing in the present study was higher to the USFDA's limit for commonly sold seafood (0.1 mg/g-Capnone *et al.*, 1996). The health risks associated with ingesting food containing antibiotic residues are highly controversial (DuPort & Steele, 1987; Yndestad, 1992). However the level exceeding the maximum acceptable tissue residue levels defined by public health authorities merits further attention.

Residues in products can be controlled by following recommended withdrawal time (Reilly *et al.*, 1998). Considering the safety and the residual level, it could be inferred that the shrimp should be harvested at least 25 days of post-treatment.

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