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Antibacterial Activity of Oxytetracycline Hydrochloride, Environmentally-Protected or Not, after Feed-Pelleting and during Decay-Dispersion in Saline Water

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Abstract

Bivalent and trivalent cations quelate oxytetracyline (OTC), rendering it microbiologically inactive. Yet shrimp diseases are often treated with OTC in pelleted feeds. Attempts have been made to chemically protect the OTC concentration in shrimp feeds against degradation in brackish water and due to temperature. The antibacterial activity of two such environmentally-protected OTC preparations (premixes A and B) and one unprotected OTC premix (C) were tested (a) before and after pelleting the OTC premixes with the powdered feed ingredients and (b) during decay-dispersion of the OTC-medicated feed in brackish water. In all three treatments, the OTC concentration dropped by approximately half after pelleting (p<0.001). When pellets were placed in tanks containing water of 23-25°C, pH 7.6-7.8, and marine salt (40 g/l), a second order exponential decay of antibacterial activity followed, reaching 50% in 30 min. At 150 min, loss reached 80% in group A, 85% in group B, and 98% in group C, suggesting that only environmentally-protected OTC may, in some cases, reach the minimal therapeutic concentration necessary to treat *Vibrio* spp. diseases.

Introduction

The modern shrimp industry requires antibacterials to limit or prevent the outbreak and spread of bacterial diseases caused mainly by *Vibrio* and *Rickettsia* spp. (Lightner, 1993). In spite of being highly quelated by bi and trivalent cations such as Ca^{++} and Mg^{++} that are abundant in marine waters and pond sediments, the perhaps most widely used antibacterial drug is oxytetracycline (OTC), one of the few antibacterial agents approved for shrimp

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by the U.S. Food and Drugs Administration (http://www.fda.gov/cvm/drugsuseaqua.htm). Usually, OTC is added to pelleted feed as an ingredient or an external layer in concentrations that fluctuate 100-5000 mg/kg (Corliss et al., 1977; Wang et al., 2003).

While reasonable to assume that shrimp feeding on the treated pellets will receive an appropriate amount of OTC, to the best of our knowledge this assumption has not been proven. Shrimp eating habits do not comply with a linear approach. Species do not feed while molting (Hill and Wassenberg, 1992) or undergoing a disease (Lightner, 1993) and food intake may change when OTC is added to the feed. Further, OTC is degraded by sunlight, heat, and changes in pH, salinity, or pressure (Lunestad et al. 1995; Doi and Stoskopf, 2000), conditions to which pelleted shrimp feed is subjected during processing and before it reaches the stomach and gastrointestinal tract of the shrimp. It is therefore reasonable to question what fraction of the dose reaches the target species. Some pharmaceutical manufacturers incorporate polymer-covered OTC into feed-pellets to protect it from environmental influences. Neverthe-less, the efficacy of this practice remains to be evaluated.

The goal of this study was to assess the degree of OTC degradation during food processing and the influence of marine water on the decay-degradation of its antibacterial potency. These factors were compared in two environmentally-protected OTC premixes and one unprotected premix.

Materials and Methods

Feeds. Commercial 20% oxytetracycline hydrochloride (OTC) premixes, obtained from retailers, were added to antibacterial-free feed ingredients at a rate of 2000 µg/g (Table 1). Two of the premixes, Capsotetra® (Avimex, Mexico; A) and Ascotetra® (Avimex, Mexico; B), were polymer-covered and supposedly environmentally protected. The third premix, Terramix® (Pfizer, Mexico; C), was not protected. The powdered feed was pelleted at 82-145°C and a pressure of 620-740 psi (Cruz-Suarez et al., 2001) using Maxi-Bond® as a binder. Six batches of each premix were obtained from the retailers, used to prepare a batch of feed, and kept sealed under refrigeration for no more than seven days before antibacterial potency was determined.

Table 1. Composition of the diet as proposed by Tacon (1987ab).

Ingredient	*%*
Fish meal	34.8
Soybean oil cake	30.0
Wheat meal	30.2
Shrimp meal	0.8
Fish oil	1.17
Lecithin	0.5
Vitamin mix[1]	0.5
Vitamin C-StayC®	0.05
Lysine	0.5
Methionine	0.5
Binder[2]	0.8

[1] Vitamin mixture: 4,000 IU/g vitamin A; 24,000 mg/kg B1; 16,000 mg/kg B2; 30,000 mg/kg calcium d-pantothenate; 30,000 mg/kg B6; 80 mg/kg B12; 60,000 mg/kg C; 16,000 mg/kg K3; 3,200 IU/g D3; 60,000 mg/kg E; 400 mg/kg H; 20,000 mg/kg niacin; 4,000 mg/kg folic acid

[2] Maxi-Bond® (urea formaldehyde/calcium sulfate)

Antibacterial potency. The antibacterial potency of the OTC was tested before and after mixing it into the powdered feed and after pelleting the mixtures. Potency was determined in six replicates per batch (i.e., 324 analyses).

Decay-dispersion. To assess the influence of salinity on OTC degradation, 40 g/l Coralife® Salt (Energy Savers Unlimited) was used to mimic brackish water in standard Mexican ponds. Temperature was kept at 23-25°C with a thermostat (LED 200 Watts Dymax), pH was approximately 7.6-7.8 (Conductronic pH20), and aeration was continuously provided at 6.79-6.56 mg/l. The pel-

leted feeds (six replicates per batch of premix) were placed in 40-l glass containers and left to interact with the water for 2.5 h. Samples of sedimented pellets (5 g) were taken from each water container after 1, 5, 30, 60, 90, 120, and 150 min (an additional 756 analyses). Samples were dried at 25°C for 8 h, then placed in a vacuum dryer for 3 h, and kept in air-tight containers at -20°C until analyzed.

Extraction of oxytetracycline. OTC was extracted as described by Martinez and Shimoda (1988) as follows. Samples were thawed at room temperature, 5 g were separated, vortexed for 20 min with 25 ml of McBuffer A (614.5 ml 0.10 M citric acid, 385.5 ml 0.20 M disodium hydrogen phosphate, and 0.372 g Na_2EDTA to pH 2), then centrifuged for 15 min at 4000 x *g*. The supernatant was collected and the sediment extracted with 25 ml of McBuffer B (614.5 ml 0.10 M citric acid, 385.5 ml 0.20 M disodium hydrogen phosphate, and 37.2 g Na_2EDTA to pH 4.5). The two pooled samples of supernatant extractions were put into an Erlenmeyer flask, nitrogen was injected with a pointed tube, and the extractions were dried in a water bath for approximately 45 min. The resulting powder was resuspended in 50 ml of de-ionized sterile water and centrifuged for 15 min at 2500 x *g* to obtain a clear supernatant that was filtered through Millipore nylon membranes (0.45 µm). Antibacterial activity, expressed as OTC concentration, was assessed in 100-µl samples of the supernatant.

Oxytetracycline concentration. OTC concentrations were determined by modified agar diffusion analysis as described by Bennett et al. (1966) with *Bacillus cereus* (ATCC 11778) grown on Mueller-Hinton agar (Bioxon®) as the test organism. Drug concentrations were determined using linear regression analysis to compare diameters of zones of inhibition with those of various dilutions of the standard prepared in distilled sterile water. The intra-assay coefficient of variance was <4.6 and inter-assay error <4.9. The analytical assay was linear over a range of concentrations of 0.391-500 µg/ml with a recovery of 99.64±0.569% (n = 16) and a correlation coefficient of 0.995±2.1.

Statistical analysis. Data regarding the effects of pelleting were entered into an SAS-statistical package (SAS System V. 8.8 2004, Institute Inc.) and mean differences in antibacterial activity, expressed as OTC concentration, were compared using ANOVA. Losses of antibacterial activity with time (decay-dispersion), again expressed as OTC concentration, were compared by repeated measurements using mixed model analysis through ANOVA.

Results

OTC contents in premixes and powdered feed before pelleting did not significantly differ ($p<0.001$) between treatments (Table 2). However, after pelleting, the content in C was significantly lower than in B ($p = 0.0086$), i.e., 11.01% lower and 48.9% less than expected.

After exposure to 2.5 h in water, pellets underwent a sharp, constant, and well-defined drop in antibacterial activity (Table 3). At 30 min, more than half the concentration had been lost in all treatments. Later, the slopes of premixes A and B differed from the slope of premix C (Fig. 1) although regression analysis in all three cases best fitted second order exponential curves ($r^2>0.99$ in all cases). After 150 min, OTC concentrations in pellets prepared with premixes A and B were statistically higher than in pellets prepared with premix C ($p<0.001$). Nevertheless, the OTC loss in all groups was considerable, reaching final losses of 80% in group A, 85% in group B, and 98% loss in group C.

Discussion

Before pelleting, OTC concentrations in the powdered feed did not differ between treatments. However, the process of pelleting reduced antibacterial activity, expressed as OTC concentration, by almost half (44% in A, 38% in B, and 49% in C). The statistically significant difference between groups B and C suggests that the environmental protection actually reduced degradation of the active principle. However, almost 1000 µg/g of OTC were lost during pellet processing; therefore, further work is needed to obtain efficient protection.

Table 2. Concentration of oxytetracycline (OTC) in three premixes and feeds before and after pelleting

	*OTC premix**						
		A		*B*		*C*	
	Expected OTC content according to product label	*Actual content*	*% of expected*	*Actual content*	*% of expected*	*Actual content*	*% of expected*
In premix (mg/g)	200	198.17±4.44[a]	99.09±2.22	198.80±2.75[a]	99.40±1.38	196.79±6.31[a]	98.40±3.16
In feed before pelleting (µg/g)	2000	1949.10±94.22[a]	97.46±4.71	1960.21±87.43[a]	98.01±4.37	1992.65±199.30[a]	99.63±9.97
In feed after pelleting (µg/g)	2000	1111.40±66.99[ab]	55.57±3.35	1242.45±335.95[b]	62.12±16.80	1022.20±39.80[a]	51.11±1.99

Values within a row with different superscripts differ significantly (p<0.05). In all groups, there was a significant (p<0.001) reduction in antibacterial activity after pelleting.

* A and B premixes were polymer-coated as protection against environmental factors. C was not protected.

Table 3. Decay-dispersion (mean±SD) of three oxytetracycline hydrochloride (OTC) pelleted-medicated feeds, placed in glass tanks containing water with 40 g/l salinity, 23-25°C, and pH 7.6-7.8.

Time	*OTC premix*					
(min)	*A*		*B*		*C*	
	Concentration (µg/g)	*Loss (%)*	*Concentration (µg/g)*	*Loss (%)*	*Concentration (µg/g)*	*Loss (%)*
Initial	1111.40±66.99[ab]	44.43	1242.45±335.95[b]	37.88	1022.20±39.80[a]	48.89
1	1088.42±100.12[a]	45.58	1228.29±4.62[b]	38.59	778.06±29.20[c]	61.10
5	1013.39±43.02[a]	49.33	781.26±30.73[b]	60.94	627.07±32.68[c]	68.65
30	542.71±31.93[a]	72.86	498.04±17.48[ab]	75.10	448.72±50.69[b]	77.56
60	396.73±16.92[a]	80.16	398.67±5.48[a]	80.07	139.23±26.80[b]	93.04
90	392.37±21.53[a]	80.38	331.34±14.79[a]	83.43	92.64±14.02[b]	95.37
120	392.32±21.80[a]	80.38	352.57±4.60[a]	82.37	55.48±13.68[b]	97.23
150	401.41±12.00[a]	79.93	306.12±7.91[b]	84.69	37.52±10.94[c]	98.12

Values within a row with no common superscript differ significantly (p<0.05)

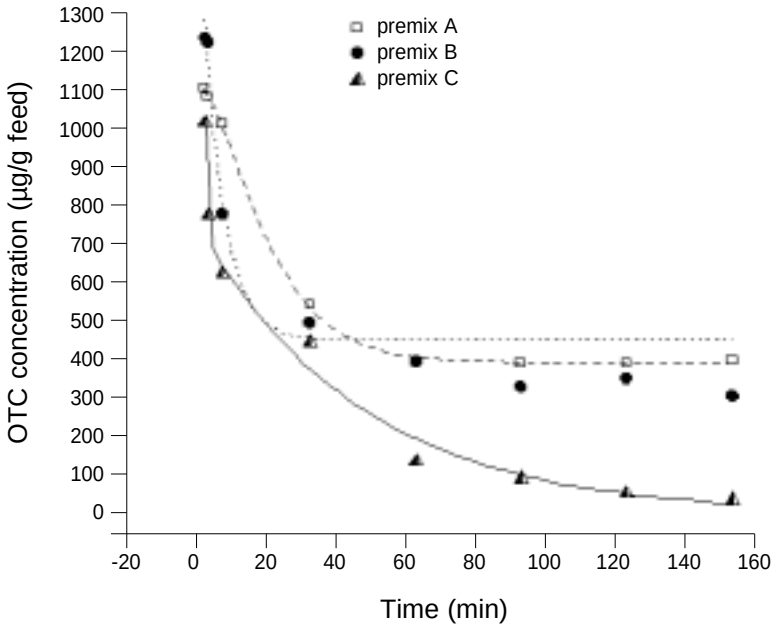

Fig. 1. Decay-dispersion of oxytetracycline (OTC) in brackish water, presented as second order exponential decay (r>0.99) of antibacterial activity (expressed as concentration) in pelleted shrimp feeds prepared with environmentally-protected oxytetracycline premix A or B or unprotected premix C.

Because OTC is highly chelated by calcium and magnesium ions, our findings could have been predicted and agree with those of Lunestad and Goksoyr (1990) and Doi and Stoskopf (2000) who showed that OTC is more prone to be chelated with bi and trivalent cations when subjected to environmental factors such as high temperature (>43°C), high salinity, alkaline pH, or lamp light (Lunestad et al., 1995). In particular, Mohney et al. (1997) highlighted temperature as the most degrading factor; standard pelleting usually requires temperatures above 60°C.

In our study, OTC losses due to pelleting were much more pronounced than reported by Frelier (1995) who obtained losses ranging 10-40%. Using unprotected OTC in extruded pellets, Fernandez-Gonzalez et al. (2002) obtained a 30% loss of concentration, lower than values obtained in our study even in the environmentally-protected treatments (38-49%). Hence, the polymer-protective outermost layer of the OTC particles in premixes A and B can be regarded as having little effectiveness in preventing degradation when feed is pelleted, although differences among authors can be attributed to analytical methods used in each case.

A well-defined decay-dispersion process, with a second order exponential OTC decay, was obtained in all three treatments. Although slight protection was obtained with premixes A and B, OTC concentrations in treatments A and B after 150 min reveal a protective effect of only 15-20% compared to the unprotected treatment C. The resulting loss of OTC concentration means that shrimp in group A that ingest softened pellets from the bottom of a pond after 150 min will receive a dose of 400 µg OTC/g feed rather than 2000 µg/g, as

expected. Disregarding palatability, a 7-g shrimp in optimum conditions may have a daily food ingestion equivalent to 4.4% of its body weight (Jory, 1995). Such a shrimp would consume 123.2 µg OTC per day, equivalent to 17.6 mg/kg body mass, probably enough to cover but not surpass the minimum inhibitory concentration (MIC) for most *Vibrio spp*. microorganisms (0.1-12.5 µg/ml; Takahashi et al., 1985). The OTC concentration in unprotected treatment C was noticeably lower and the final dose for a 7-g shrimp ingesting softened pellets after 150 min would be only 11.6 µg OTC per day, equivalent to 1.7 mg/kg biomass. More importantly, the food ingestion of shrimp infected by *Vibrio* drops to only 0-1% their body weight (Lightner, 1993). In such a case, the total OTC dose of a 7-g shrimp consuming environmentally-protected OTC would be only 4 mg/kg body mass which is unlikely to meet the required MIC. The above assumptions need revision as *Penaeus* spp. have varying eating habits (Sedgwick, 1979; Nunes and Parsons, 2000).

Early detection of *Vibrio* infection requires a relatively simple laboratory procedure (Lightner, 1993). At early stages of infection, food consumption is not drastically hampered. Therefore medication with OTC is possible, but only if environmentally-protected OTC is used to manufacture feed pellets. Better protection of this drug should be sought because a slight reduction in feed consumption or more rapid decay and dispersion may result in inadequate levels of OTC in these animals. This is a pharmaceutical task that should take into account not only the stability of OTC but also the palatability of the outermost layer of the OTC particles.

In short, standard pellet processing of shrimp feeds containing OTC premixes reduces the antibacterial activity of this drug by approximately half, whether the premix is environmentally-protected or not. Further and rapid decrease in antibacterial activity occurs when OTC-containing feed pellets are placed in brackish water. The loss of antibacterial activity of environmentally-protected OTC occurs at a slightly slower rate than of non-protected OTC. This difference may be important when treating diseased *Penaeus*, depending on the progress of the disease and the eating habits of the species.

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