Oxytetracycline Residues in Cultured White Shrimp Tissue by HPLC and a Microbial Receptor Assay

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ABSTRACT

The residue depletion of oxytetracycline (OTC) from muscle tissue of cultured shrimp (Penaeus vannamei) was evaluated. OTC was measured using high performance liquid chromatography (HPLC) and a microbial receptor assay. Samples were taken on days 7, 14 and 21 during the treatment and 23, 25 and 28 after the treatment. OTC was detectable in all groups after 7 days depletion time at concentrations of 0.28, 0.19 and 0.05 µg/g for diets containing 500, 250 and 100 mg/kg OTC, respectively. Results by the microbial receptor assay were in good agreement with those by HPLC.

Key Words: oxytetracycline, cultured shrimp, microbial receptor, Charm II, HPLC

INTRODUCTION

COMMERCIAL CULTURE OF SHRIMP HAS GROWN AND SOME ASIAN COUNTRIES ARE LEADERS IN THIS INDUSTRY (Irianto, 1997; Toyofuku, 1997). AS PRODUCTION GROWS, MORE CARE IS NEEDED TO KEEP FARMS IN GOOD SANITARY CONDITIONS. IN SOME COUNTRIES, SUCH AS JAPAN, TAIWAN AND CHINA, DISEASE HAS RESULTED IN PRODUCTION DROP, CAUSING IMPORTANT ECONOMIC LOSSES (Sano and Fukuda, 1987; Kwei, 1989; Anonymous, 1993b). THEREFORE, THE USE OF ANTIBIOTICS HAS BECOME AN IMPORTANT PART OF SHRIMP CULTURE TO REDUCE DISEASE PROBLEMS.

AMONG OTHER ANTIMICROBIALS, OXYTETRACYCLINE (OTC) IS WIDELY USED BECAUSE OF ITS LOW TOXICITY AND WIDE SPECTRUM ANTIMICROBIAL ACTIVITY AGAINST A WIDE RANGE OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA (Moretti et al., 1994). OXYTETRACYCLINE HAS PROVED TO BE A SUCCESSFUL PROPHYLACTIC AGAINST SPECIES OF VIBRIO AND IS USED BY PENAEID SHRIMP CULTIVATORS (Williams and Lightner, 1988; Carignan et al., 1993). SPECIAL CARE IS NEEDED IN THE USE OF THESE COMPOUNDS SINCE THEY LEAVE RESIDUES THAT MAY BE POTENTIAL CONTAMINANTS FOR HUMAN FOOD SUPPLY (Fong and Brooks, 1989). HOWEVER, THE TOLERANCE LEVELS FOR OTC IN WHITE SHRIMP HAVE NOT BEEN DETERMINED ACCURATELY, AND THESE ARE NEEDED TO ESTABLISH REGULATIONS FOR THE USE OF THIS ANTIBIOTIC IN COMMERCIAL CULTURES.

A FEW REPORTS HAVE BEEN PUBLISHED ABOUT WITHDRAWAL TIME OF ANTIMICROBIALS IN SHRIMP. Corliss (1979) found that after treatment Penaeus setiferus (1, 5 and 10 g OTC/kg of feed), must be without OTC for at least 10 days before being placed on the market to comply with the requirement of ≤0.1 µg/g in edible tissue. Higuera-Ciapara et al. (1991) administered OTC and sulfamethazine to juvenile Penaeus monodon at 250 mg/kg during 4 wk, finding residues of 150–200 µg OTC/kg in shrimp muscle.

Routine microbiological analysis for OTC and other antibiotics are time consuming, and detection limits are generally higher than those of chemical techniques. A rapid and sensitive method (Charm II test) based on a receptor assay developed for antimicrobials in milk (Charm and Chi, 1988) and adapted for tissues, feed and organo fluids (Anonymous, 1993a; Vázquez-Moreno et al., 1990) has been compared with HPLC for determination of sulfadimethoxine in salmon muscle, giving a correlation coefficient of 0.999 (Kitts et al., 1995). FOR QUANTITATIVE ANALYSIS OF ANTIBIOTICS, HPLC WITH FLUOROMETRIC OR SPECTROPHOTOMETRIC DETECTION IS THE MOST WIDELY USED METHOD (Kitts et al., 1995).

THEREFORE, THE OBJECTIVES OF THIS STUDY WERE TO QUANTIFY AND COMPARE OTC LEVELS IN MUSCLE OF SHRIMP THAT WERE FED DiETS THAT CONTAINED THIS ANTIBIOTIC BY CHARM II TEST AND SOLID-PHASE EXTRACTION-HPLC ULTRAVIOLET (UV) SPECTROMETRY AND TO DETERMINE THE WITHDRAWAL TIME REQUIRED FOR DIFFERENT OTC CONCENTRATIONS IN FEED.

MATERIALS & METHODS

Reagents and chemicals

WATER WAS PURIFIED BY DOUBLE DISTILLATION AND ION EXCHANGE DE-MINERALIZATION (CONDUCTIVITY <1 µS). OXYTETRACYCLINE HYDROCHLORIDE WAS PURCHASED FROM SIGMA (ST. LOUIS, MO). SOLVENTS WERE HPLC GRADE FROM COMMERCIAL SOURCES. ALL OTHER REAGENTS WERE ANALYTICAL GRADE (SIGMA).

STOCK SOLUTIONS OF OTC WERE PREPARED FRESH DAILY IN HPLC-GRADE METHANOL AND DILUTED TO DESIRED LEVELS IN METHANOL IMMEDIATELY BEFORE USE. THESE SOLUTIONS WERE PROTECTED FROM DIRECT SUN AND ARTIFICIAL LIGHT THROUGHOUT ANALYSIS. REAGENTS USED FOR THE CHARM II TEST WERE OBTAINED FROM CHARM SCIENCES INC. (MALEDEN, MA) AND STORED AT –20°C.

Feed

THE DIET INGREDIENTS WERE PROVIDED BY THE DEPT. OF AQUACULTURE, UNIV. OF SONORA. THE DIET (0% ANTIBIOTIC) WAS FORMULATED FROM PURIFIED INGREDIENTS (37.3% wheat flour, 15.3% soyaflour, 19.6% fish flour, 14.7% shrimp flour, 1.9% soluble fish protein, 1.9% fish oil, 0.9% sojaoil, 1.4% vitamin premix, 3% mineral premix, 2% sodium alginate, 2% sodium hexametaphosphate). THREE MEDICATED DIETS CONTAINING 100, 250 AND 500 mg OTC/kg of feed were prepared and an OTC free diet served as a control. Antibiotic was mixed in powdered form with all ingredients of the diet and the mixture was pelleted. Diets were dried 24 h at 60°C. PROXIMATE COMPOSITION OF THE RESULTING DRY FEED WAS DETERMINED BY AOAC (1990) METHODS.

Bioassay

NINETY-SIX ADULT SHRIMP (Penaeus vannamei) WITH MEAN BODY WEIGHT 17 g WERE RANDOMLY DIVIDED INTO 12 AQUARIUMS WITH EIGHT INDIVIDUALS EACH. ADULT SHRIMP WERE USED SINCE THEY ARE THE FINAL STAGE BEFORE HUMAN CONSUMPTION. THE ORGANISMS WERE ALLOWED TO ACCLIMATE IN AQUARIUM FOR SEVEN DAYS. SEA WATER WAS SAND-FILTERED AND ULTRAVIOLET RADIATED BEFORE USE. WATER TEMPERATURE RANGED FROM 18°C TO 24°C DURING THE EXPERIMENT, AND FLUORESCENT ILLUMINATION WAS FOR 12 h.

TREATMENTS WERE CARRIED OUT FOR 21 DAYS, FEEDING THE SHRIMP WITH THE CORRESPONDING DIET AND HELD FOR A CLEARING PERIOD OF SEVEN DAYS. AMOUNT OF FOOD WAS CALCULATED AS 3% OF TOTAL BIOMASS INTO EACH AQUARIUM, AND IT WAS EQUATED TO CONSUMPTION TO DIMINISH WASTE. THE DAILY PORTION OF DIET WAS DIVIDED IN THREE DOSES EVERY 8 h, TO AVOID
OTC lixiviation as much as possible. One shrimp from each aquarium was sampled at days seven, 14, 21, 23, 25 and 28. Samples were frozen immediately at –20°C and kept until analysis. They were weighed before freezing. Survival was monitored during the experimental period, and dead animals were removed daily. Three replicates were used for each treatment group.

**Sample preparation**

Samples were kept at –20°C until analysis. Weight was determined for whole shrimp and muscle after thawing. A factor was determined to correct for weight loss by the thawing process. The shrimp muscle was homogenized in a blender and divided into two portions: one for the Charm II test and the other for HPLC analysis. Individuals were analyzed separately.

**HPLC analysis**

The procedure by Long et al. (1990) was used for analysis of OTC, adding some minimal modifications. Samples (0.5 g) were accurately weighed into a glass mortar. Octa-decylsilyl derivatized silica (C18) (2 g), 0.05 g of disodium ethylenediaminetetraacetate (EDTA) and 0.05 g of oxalic acid were added. Sample was allowed to stand for 1 min. Then, the mixture was gently homogenized with the pestle. The paste was placed into a syringe with a filter paper disc in the tip to form a column. Column matrix was compressed to 4.5 ml and two volumes of 8 ml of hexane were passed through. OTC was eluted with two portions of 8 ml of acetonitrile-methanol (1:1 v/v), containing 0.06% of BHA and BHT. Extract was evaporated to dryness in a water bath at 40°C under air flow. Residue was diluted with 1 ml of mobile phase and sonicated 45 min until suspension. Solution was centrifuged 10 min at 17,000×g. Supernatant was filtered through a 0.45 µm nylon membrane.

Analysis was performed on a liquid chromatograph (Varian 9010, Sunnyvale, CA) equipped with an ultraviolet-visible detector (Varian 9050) at 365 nm. The injected sample was eluted with an isocratic mobile phase consisting of 0.02 M aqueous oxalic acid: acetonitrile:methanol (70:27.5:2.5, v/v/v) at a flow rate of 1 mL/min, in a reverse-phase octadecylsilyl C18 derivatized silica (5 µm, 4.6 × 250 mm) column (Alltech Associates, Inc., Deerfield, IL). OTC in the sample was identified by comparison of retention time with that of the standard. All samples were assayed in duplicate.

Standard curves were elaborated by plotting peak areas of standard OTC against its concentration. Standard curves were defined by linear regression analysis using concentrations of 0.05, 0.1, 0.2, 0.4, 0.8, 1.5, 2, and 3 µg/mL.

**Charm II test**

Oxytetracycline in the shrimp muscle was determined by Charm II test (Anonymous, 1993a). The tetracycline competitive assay consisted of an antibody bound to a microbial cell which functioned as the receptor, the tagged tetracycline and tetracycline from the sample competed to bind on the receptor. Antibodies were selected for their multiple specificity within the family (Scheemaker, 1996). Analysis included both negative and positive controls.

**Assay validation**

To evaluate the accuracy and precision of the assay procedures, samples were prepared with OTC added at different concentrations. The ratio of the amount of OTC extracted from spiked muscle to the amount that had been added was used to estimate recovery.

**Statistical analysis**

A 4×6 factorial arrangement with a general linear model was utilized to analyze the bioassay data. Dose levels and feeding periods were the tested factors, and the concentration of OTC in muscle was the response variable. The SAS® computational program (SAS Institute Inc., 1991) was used to test the proposed model. Results from the Charm II test and the HPLC procedure were analyzed separately.

For each single sample, the difference between results obtained by Charm II test and HPLC was calculated as follows:

\[ d = \text{result by Charm} - \text{result by HPLC}. \]

That difference was used as the paired test parameter. A distribution histogram of frequencies was made to test the distribution of “d” data. The t-test was used to find significant differences between methods.

**RESULTS & DISCUSSION**

The calibration curves for OTC by the HPLC method showed a linear response (\( r^2 = 0.999 \)), over a concentration range of 0.05 to 3.0 µg/mL. Charm II test also gave a linear response within a range of 0.1–0.3 µg/mL (data not shown). The typical chromatograms of an OTC standard (3.2 µg/mL) and a shrimp sample with 3.2 µg/g of added OTC were compared (Fig. 1). Retention time for OTC was 4.5 min on the liquid chromatography system. No interfering peaks were found in the control or OTC-treated shrimp muscle.

During the bioassay, the mortality rate in all groups was 5–15%. Corliss (1979) reported a higher mortality rate (20–30%) in cultured juvenile shrimp (Penaeus setiferus) fed with OTC at concentrations from 1 to 10 g/kg of feed for 3 wk. In our surveys the surviving shrimp seemed healthy and vigorous when handled, thus indicating that food consumption had been sufficient to maintain normal activity.

The recovery of OTC from shrimp muscle determined by spiking untreated tissues with 0.05, 0.10, 0.2 and 0.32 µg/g ranged from 95–110% using the HPLC method. These values were higher than 82.0±4.8% reported by Long et al. (1990). For the Charm II test procedure the mean recovery was 86.5%. The detection limit for OTC using shrimp extract was 0.1 µg/g in the Charm II test, while the HPLC method showed a lower detection limit of 0.05 µg/g.

Residues of OTC in shrimp tissues in all the different treatments...
were compared (Fig. 2). High levels of OTC were found on the first days of treatment and these levels had a tendency to decrease rapidly as soon as the antibiotic treatment was suspended. Three weeks after starting the medication, the maximum concentrations of OTC were found in the group fed with 500 mg/kg, which gave 1.86 µg/g in the HPLC method and 1.78 µg/g in the Charm II test, and decreased to 0.28 µg/g and 0.25 µg/g as determined by HPLC and Charm II, respectively, at the end of the experiment.

Since each shrimp was analyzed separately, not as a bulk, variation among organisms was high by Charm and HPLC.

Both values exceeded the 0.1 µg/g tolerance limit established for salmonids, catfish and lobsters by the U.S. Food & Drug Administration (FDA, 1988), only the group fed 100 mg/kg had OTC residues below that limit. The levels we measured were higher than those reported previously for other shrimp species fed with higher concentrations of OTC. Corliss (1979) performed trials measuring the uptake of OTC in *Penaeus setiferus* at three doses (1, 5, and 10 g/kg) monitoring resulting levels in shrimp using the microbiological plate diffusion method with *Bacillus cereus*. Corliss (1979) measured withdrawal times of antibiotic after feeding had ceased. Residues were undetectable within three days for the 1 g/kg group and within 2 wk for groups fed with 5 and 10 g/kg. Higuera-Ciapara et al. (1991) fed black tiger shrimp *P. monodon* with 250 mg/kg of OTC during 4 wk. Residues of OTC at the end of the trial were 150–200 µg/kg in the shrimp.

Statistical analysis showed that the concentration of OTC in shrimp muscle was influenced (p<0.0001) by dose level and feeding period. The residual concentration of OTC was above the 0.1 µg/g limit set for salmonids, catfish and lobsters. That suggested a waiting period of at least seven days between the antibiotic treatment and harvesting of the animals (*Penaeus vannamei*) medicated with 100 mg/kg (Table 1). For OTC treatments higher than 100 mg/kg the required withdrawal time must be greater than seven days and needs to be determined.

The t-test used on “d” data showed that difference between methods was not significant (p<0.01). This supports the conclusion that the Charm II test is a valid alternative to the HPLC method in measuring OTC in shrimp muscle tissue. This test is rapid, sensitive and can be run by personnel with a minimum of training.

### REFERENCES


