Depletion of Veterinary Drugs Used in Aquaculture after Administration in Feed to Gilthead Seabream (Sparus aurata)

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ABSTRACT

This study was conducted to evaluate the depletion of residues of the antibiotics flumequine, oxytetracycline, sulfadiazine, trimethoprim, and oxolinic acid after in-feed administration to gilthead seabream. Fish were treated with the target antibiotics at doses of 30 mg/kg of body weight per day for 10 days at two seawater temperatures. Fish in each of five tanks were fed with a different medicated feed. After in-feed administration, five fish were randomly selected at different times, and antibiotic presence was analyzed in a mixture of muscle and skin. Antibiotic concentrations were determined through a validated analytical method based on liquid chromatography separation and mass spectrometry detection. Two trials were carried out with fish at different temperatures (14.0 and 19.5 °C). Depletion of antibiotics occurred more rapidly at the higher temperature. Elimination rates for all antibiotics assayed were high, which indicates that the withdrawal period for these antibiotics could be reduced. The results suggest that in gilthead seabream maintained at these two temperatures no detectable concentrations of the antibiotics used in this study will remain in edible tissues 35 days after treatment. For flumequine and oxolinic acid, the elimination time is shorter (4 and 20 days, respectively).

The three main sources of global fish production are the marine catches, inland catches, and aquaculture. Because of the economic importance of seafood consumers to the food industry, aquaculture is growing worldwide to meet market needs (11). However, the use of intensive fish farming techniques can lead to an increase in the use of antimicrobials for prevention and treatment of diseases (13). These compounds often are applied in high doses in feed (15) or in water (28). The administered dose depends on several factors such as the activity of the compound and environmental conditions such as light, temperature, or salinity. However, it is very difficult to predict how much of the drug is going to be consumed by healthy or unhealthy fish.

Aquaculture practices may affect the surrounding aquatic environment, damaging water, sediments, flora, and fauna, including threatened species (27). Among the chemicals used in aquaculture, special oversight should be given to veterinary drugs used to prevent and treat bacterial diseases. In the last few years, oxytetracycline and potentiated sulfonamides (sulfadiazine and trimethoprim) have been used widely to treat several diseases such as vibriosis and ulcerative diseases (19), although other broad-spectrum antibiotics such as oxolinic acid and flumequine also can be applied (27).

In aquaculture operations, antibiotics are mainly applied through medicated feed and enter the environment as a result of leaching from feces and uneaten treated feed (14). The use of antibiotics has significantly improved fish health and production efficiency in aquaculture operations (10). However, the extensive use of antibiotics as pharmaceuticals (2, 4) or growth promoters in the veterinary sector (5, 20, 29) can result in antibiotic residues in fish for human consumption (6), which could cause allergic reactions and increase the probability of development of antimicrobial resistance in resident microbial flora (18). Therefore, the residues of these antibiotics in fish for human consumption should be as low as possible to assure food safety. In this regard, both the European Union (EU) and the United States have regulated the use of veterinary drugs in aquaculture. However, no harmonization related to antimicrobial approval, maximum residue limits (MRLs), or tolerances in food fish have been defined worldwide. In the United States, the number of antimicrobials approved by the Food and Drug Administration (FDA) (31) is small compared with the number of compounds included in the EU Council Regulation 2377/90 (9). The EU has established MRLs for all antimicrobials assayed in the present study (50 μg/kg for trimethoprim, 100 μg/kg for oxytetracycline and sulfadiazine, and 600 μg/kg for oxolinic acid), whereas the only antimicrobial approved by the FDA is oxytetracycline, with a tolerance of 2 mg/kg.

When antibiotics are used, guidelines for specific doses and withdrawal periods must be followed to meet the
requirements established by the current legislation. Because fish are poikilotherms, their metabolic rate is determined by environmental temperatures, and withdrawal time is usually defined as the time at which the residues in all tissues of all observed animals have fallen below the established MRL. Therefore, withdrawal periods must be specified as degree-day values to standardize the obtained results. In the EU, Council Directive 2001/82 (10) indicates that unless the medicinal product used indicates a withdrawal period for the species concerned, a general withdrawal time of 500°C-days must be applied for off-label use of drugs in fish.

The aim of this study was to evaluate the elimination rates for oxytetracycline, sulfadiazine, trimethoprim, oxolinic acid, and flumequine in gilthead seabream (Sparus aurata). The withdrawal times for these antibiotics were evaluated at two water temperatures. Although similar studies have been published (16, 17, 21–23, 28), the results from the present study should contribute to minimizing consumer risk from antibiotic residues and the environmental impact of antibiotics used in aquaculture, bearing in mind that several classes of veterinary drugs have been evaluated. All previous reports concerning gilthead seabream were based on the depletion of a single target antibiotic: oxytetracycline (16, 23), flumequine (17, 30), or oxolinic acid (21, 22), and data were obtained via liquid chromatography (LC) using fluorescence (16, 17, 21, 22, 30) or UV detection (21). In the present study, the five target antibiotics were simultaneously evaluated in a mixture of skin and muscle through a validated analytical method that included LC coupled with single-quadrupole mass spectrometry (MS) (24).

**MATERIALS AND METHODS**

**Chemicals and reagents.** Oxytetracycline hydrochloride (purity > 98.5%), flumequine (purity > 99.0%), sulfadiazine (purity > 99.5%), trimethoprim (purity > 99.5%), and oxolinic acid (purity > 98.0%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Stock standard solutions of individual compounds (with concentrations ranging from 200 to 300 mg/liter) were prepared by dissolving approximately 100 mg of the powdered standard (accurately weighed) in 100 ml of high-performance (HP) LC grade methanol (Panreac, Barcelona, Spain). A multicompound working standard solution with 10 mg/liter concentrations of each compound was prepared by appropriate dilutions of the stock solutions with methanol and stored at −20°C in the dark. This solution was stable for 3 weeks, after which it was discarded and a fresh solution was made.

A 1 M citrate solution (pH 4) was prepared by dissolving citric acid (Panreac) in water and adjusting the pH with 1 M NaOH (Panreac). EDTA-Na₂ was obtained from Merck (Darmstadt, Germany), and acetonitrile (HPLC grade) was purchased from Panreac. Ultrapure water was obtained with a Milli-Q Gradient water system (Millipore, Bedford, MA). Other reagents were of analytical reagent grade.

Medicated and unmedicated feed products were obtained from Skretting (Burgos, Spain), and both commercial products had the same macronutrient composition (46% protein, 21% lipids, and 9.1% ash). The antibiotics concentration in feed was calculated to obtain an intake equivalent to a dose of 30 mg/kg of body weight.

**Experimental conditions: fish and medication.** One hundred twenty-five unmedicated gilthead seabreams (150 to 200 g each) were supplied by a local fish farm in Almería (Spain). The depletion of antibiotics was studied at seawater temperatures of 14 and 19.5°C. These temperatures were representative of conditions on the Mediterranean coast. Fish were brough to the laboratory in a container of aerated seawater. In the laboratory, fish were randomly transferred to five cylindrical tanks (25 fish per tank). The capacity of each tank was 1,000 liters, and each tank contained 950 liters of aerated seawater. Water was renewed daily with 200% of its volume and was continuously aerated with an air pump and maintained under natural conditions of photoperiod and temperature. Maximum and minimum temperatures were recorded throughout the experiment to establish the degree-day values. The variation in temperature depended on the season. In winter, the minimum fell to 11°C only twice; the usual variations were 13 to 15°C. In spring, the temperature fluctuated between 18 and 20°C with a peak of 21°C. Before the therapeutic treatment, fish were allowed to acclimatize for 5 days in the different tanks and were fed with unmedicated feed. After the adaptation period, therapeutic treatment started with in-feed antibiotic administration for 10 consecutive days. The fish in each tank were fed with a different medicated feed: flumequine (tank 1), oxytetracycline (tank 2), potentiated sulfadiazine (sulfadiazine and trimethoprim; tank 3), and oxolinic acid (tank 4). Fish in tank 5 were given unmedicated feed as a control. After 10 consecutive days of treatment, all fish were fed an unmedicated diet for the rest of the experiment. During the experimental period, fish were fed with a commercial diet (D4 Excel, Skretting), and the food intake levels were established following the recommendations of the company for each experimental temperature.

**Experimental conditions: sampling.** The experimental protocol involved five sample collections for each antibiotic. The first samples were collected the day after the medication was finished, and four additional samples were subsequently collected. Sampling was based on the elimination times established following the recommendations indicated by the company based on a withdrawal time established for each antibiotic in the veterinary literature: 6 days for oxolinic acid, 2 days for flumequine, and 500°C-day for tetracycline and sulfonamide.

Groups of five fish for each antibiotic were randomly sampled at various days after the last administration of medicated feed. Fish were sampled on days 1, 2, 3, and 4 for flumequine, on days 1, 3, 6, and 9 for oxolinic acid, and on days 1, 7, 20, and 34 for oxytetracycline, sulfadiazine, and trimethoprim.

For antibiotic residue analysis, five fish from each tank were killed following the guidelines of Royal Decree 1201/2005 (12) concerning use of animals for experiments, and the dorsal muscle was removed and stored frozen (−20°C) until analysis.

**Experimental conditions: analytical procedures.** The applied analytical procedure has been detailed previously (24). A 1-g fish sample (muscle plus skin) was mixed with 10 ml of acetonitrile, 1 ml of 1 M citric acid (pH 4.0), and 0.5 ml of 0.5 M EDTA-Na₂ and homogenized with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) for 3 min. The sample was centrifuged at 2,390 × g for 10 min, and the supernatant was evaporated to dryness under a gentle stream of nitrogen. The obtained residue was redissolved in 1 ml of mobile phase, injecting 20 μl into the LC system.

Chromatographic analyses were carried out using an HPLC system (Waters, Millford, MA). A C₁₈ column (150 by 2.00 mm inside diameter by 5 μm; Waters) was used for all separations, and the column temperature was set at 30°C. The elution of the compounds was performed using an aqueous solution of 0.1%
formic acid (eluent A) and methanol (eluent B) at a flow rate of 0.30 ml/min, applying the following gradient profile: 90% eluent A for 3 min, decrease eluent A linearly to 45% over 9 min and maintain at 45% for 3 min, decrease eluent A linearly to 10% over 3 min and maintain at 10% for 5 min, and restore eluent A to 90% over 2 min and maintain at 90% for 5 min.

The HPLC system was coupled to a ZQ 2000 single quadrupole mass spectrometer (Waters-Micromass, Manchester, UK) equipped with an electrospray source working in positive mode. Data acquisition was performed with MassLynx 4.0 software (Waters). The ionization source parameters were capillary voltage of 3.5 kV, source temperature of 120°C, desolvation temperature of 350°C, flow rate for desolvation of 350 liters/h, and flow rate for cone gas of 50 liters/h from a nitrogen generator (Claind, Lenno, Italy).

Quality control. To ensure the quality of results when the proposed method was applied in routine analyses, internal quality controls were used. A blank extract was used to identify false-positive results obtained through contamination from the extraction process, instruments, or chemicals and to identify possible matrix interferences. Another blank sample was spiked at 50 mg/kg and used to evaluate the extraction efficiency. A calibration curve was prepared daily from the blank matrix extract.

RESULTS AND DISCUSSION

Five drugs, i.e., sulfadiazine, trimethoprim, oxytetracycline, flumequine, and oxolinic acid, from different classes of antibiotics were evaluated in this study. Figure 1 shows the structure of the selected antibiotics. The analytical method used was developed previously (24) and is based on LC-MS methodology. Figure 2 shows a representative LC-MS chromatogram of a fish blank sample spiked at 25 µg/kg. Table 1 provides the limits of detection (LODs), limits of quantification (LOQs), and MRLs established by the EU (9).

To evaluate the influence of water temperature on the elimination rate of the selected antibiotics, this study was carried out at two temperatures, 14.0 and 19.5°C, considered representative of conditions in winter and spring, respectively, on the Mediterranean coast.

All the antibiotics were given at the same rate of 30 mg/kg of body weight per day. The dosage ranges for antibiotics used for therapeutic purposes in fish are very large and depend on several factors such as pathology, illness severity, water conditions, weight of fish, and food intake (3, 7). In this study, the fish were healthy, and the aim of this work was to compare the antibiotic elimination rate at two temperatures. The dosage was selected to minimize the influence of different levels of antibiotic ingestion and administration time (10 days for every antibiotic used). Thus, the doses administered were in the therapeutic range for each antibiotic.

Calculation of withdrawal time and elimination half-life of antibiotics. To evaluate withdrawal time and elimination half-life of the antibiotics, a first-order kinetics model was used applying the following expression, $C(t) = C_0e^{-\beta t}$, where $C(t)$ is the concentration (micrograms per kilogram) at time $t$ (day), $C_0$ is the initial concentration, and $\beta$ is the elimination rate constant (per day). To evaluate the withdrawal time, the method described by Salte and Liestøl (26) was applied, which involves the calculation of the linear regression from the logarithm of the drug residue against time, estimating the elimination half-life ($t_{1/2}$) as $0.693/\beta$. 

FIGURE 1. Molecular structure of the selected antibiotics.
Table 2 shows the mean concentrations of flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid for five replicates at each sampling day for both temperatures assayed. These concentrations were obtained after treatment was finished, and a mixture of muscle plus skin was analyzed. The mean concentrations of flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid were 55, 22, 304, 268, and 62 \( \mu \)g/kg, respectively, on day 1 after treatment when fish were kept in seawater at 14°C. For the last samples tested (testing ended on different days after treatment had finished), antibiotic concentrations were below the LODs, except for oxolinic acid, which was detected at 23 \( \mu \)g/kg.

However, for fish kept in seawater at 19.5°C, the mean concentrations of flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid were 11, 32, 109, 249, and 8 \( \mu \)g/kg, respectively, on day 1 after treatment. For the last samples tested (testing ended on different days after treatment had finished), antibiotic concentrations were below the LODs, except for trimethoprim, which was detected at 11 \( \mu \)g/kg.

Elimination curve equations are shown in Figure 3. Determination coefficients higher than 0.95 were obtained for all cases when the experimental data were adjusted to \( C(t) = C_0 e^{-bt} \). Half-lives \( (t_{1/2}) \) were calculated through the corresponding values of the elimination rate constant \( (b) \) and the obtained values were 1.73, 12.79, 7.44, 1.67, and 6.11 days for flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid, respectively, when fish were kept in seawater at 14°C. However, for fish in seawater at 19.5°C, the half-lives of flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid were 1.61, 8.94, 3.66, 1.26, and 3.74 days, respectively.

The theoretical withdrawal periods at both temperatures, i.e., when antibiotic concentrations would be below the MRLs and LODs, are indicated in Table 3. All antibiotics concentrations in fish were lower than the established MRLs on the first day after treatment (day 1) except those of oxytetracycline and sulfadiazine, which fell below their MRLs on days 12 and 2, respectively, when the seawater temperature was 14°C and on days 1 and 2, respectively, when the seawater temperature was 19.5°C. Flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid fell below their LODs on days 5, 31, 47, 9, and 19, respectively, when the seawater temperature was 19.5°C and on days 1, 27, 17, 7, and 1, respectively, at 19.5°C. The reduced theoretical withdrawal times at higher temperatures were 5, 31, 47, 9, and 19 days, respectively, for flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid.

### Table 1

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>LOD (( \mu )g/kg)</th>
<th>LOQ (( \mu )g/kg)</th>
<th>MRL (( \mu )g/kg)</th>
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<td>16</td>
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<td>100</td>
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<td>100</td>
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<td>Trimethoprim</td>
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TABLE 2. Mean concentrations of the selected antibiotics at two seawater temperatures (14 and 19.5°C)

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<th>Sampling day</th>
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<th>Trimethoprim</th>
<th>Oxytetracycline</th>
<th>Sulfadiazine</th>
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<td>14°C</td>
<td>19.5°C</td>
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<td>11 (9.3)</td>
<td>22 (6.8)</td>
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<td>3</td>
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<td>&lt;8</td>
<td>32 (2.8)</td>
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<td>133 (3.4)</td>
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</table>

a Day after the last administration of medicated food.

b n = 5. RSD, relative standard deviation.

FIGURE 3. Mean concentrations of flumequine (a), oxolinic acid (b), oxytetracycline (c), sulfadiazine (d), and trimethoprim (e) in muscle and skin of gilthead seabream at seawater temperatures of 14°C (——) and 19.5°C (— —). The MRLs established by the European Union for each antibiotic also is plotted.
temperatures were calculated from the same doses at both temperatures.

Comparison of the results. The elimination rates of antibiotics depend on several environmental factors such as light, salinity, or seawater temperature. To evaluate the influence of seawater temperature on fish metabolism and consequently on the elimination rate of drugs, the time parameter also was expressed as degree-days, which were calculated by multiplying the mean daily water temperature (in degrees Celsius) by the total number of measured days. Figure 3 indicates that depletion was not similar at both temperatures, and degradation or elimination of antibiotics occurs more rapidly at higher temperatures (1, 25). Other researchers (8) have suggested that elimination rates can vary by 10% when seawater temperature changes 1° C. In the present study, the mean concentrations of antibiotics on day 1 after treatment (Table 2) were much lower at 19.5°C than those observed at 14°C, except for trimethoprim, which was the most persistent at both temperatures. These data support previous results that indicated that microbial degradation contributes more to the disappearance of trimethoprim than do environmental factors such as temperature (15).

The elimination rate constants (β) of flumequine, trimethoprim, oxytetracycline, sulfadiazine, and oxolinic acid were 0.399, 0.054, 0.093, 0.414, and 0.114, respectively, at 14°C and 0.428, 0.078, 0.189, 0.551, and 0.186, respectively, at 19.5°C, i.e., they were all lower at 14°C. These findings support previous results (26) that indicated that β is highly influenced by water temperature. Elimination half-lives also were lower at 19.5°C in all cases.

For the withdrawal period, the results indicate that this time period should be shorter in fish kept at 19.5°C than in fish kept at 14°C. All antibiotics except for oxytetracycline and sulfadiazine had postmedication concentrations lower than the established MRLs at both temperatures. This finding may be associated with the high concentrations of tetracycline and sulfadiazine detected when treatment was finished, suggesting that longer time is needed to eliminate these antibiotics. At 14°C, oxytetracycline and sulfadiazine concentrations fell below their MRLs on days 12 and 2, respectively, after finishing treatment, whereas antibiotic levels fell below their MRLs on days 1 and 2, respectively, at 19.5°C.

No antibiotics were detectable 34 days after treatment, although for flumequine, this period was shorter (4 days), indicating that fish without traces of some antibiotics can be obtained before the established withdrawal period.

The present study provides preliminary data supporting more prudent use of selected antibiotics in gilthead seabream and suggesting a possible reduced withdrawal time after treatment.

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