A Bridging Study for Oxytetracycline in the Edible Fillet of Rainbow Trout: Analysis by a Liquid Chromatographic Method and the Official Microbial Inhibition Assay

Oxytetracycline (OTC) is a drug approved by the U.S. Food and Drug Administration (FDA) to control certain diseases in salmonids and catfish. OTC is also a likely control agent for diseases of other fish species and for other diseases of salmonids and catfish not currently on the label. One requirement for FDA to extend and expand the approval of this antibacterial agent to other fish species is residue depletion studies. The current regulatory method for OTC in fish tissue, based on microbial inhibition, lacks sensitivity and specificity. To conduct residue depletion studies for OTC in fish with a liquid chromatographic method, a bridging study was required to determine its relationship with the official microbial inhibition assay. Triplicate samples of rainbow trout fillet tissue fortified with OTC at 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 ppm and fillet tissue with incurred OTC at approximately 0.75, 1.5, and 3.75 ppm were analyzed by high-performance liquid chromatography (HPLC) and the microbial inhibition assay. The results indicated that the 2 methods are essentially identical in the tested range, with mean coefficients of variation of 1.05% for the HPLC method and 3.94% for the microbial inhibition assay.

Oxytetracycline (OTC) is a drug approved to control ulcer, furunculosis, and bacterial hemorrhagic septicemia in salmonids cultured at or above 9°C, as well as bacterial hemorrhagic septicemia in catfish cultured at or above 16.7°C (1). It also has approved uses for marking salmonids in population assessment studies and for control of gaffkemia in lobsters (1). Other diseases in salmonids, catfish, and fish species not currently on the label may be effectively controlled with OTC. OTC may also be effective for diseases that occur below the approved temperature limits.

The extension and possibly the expansion of the label for OTC may require data on residue depletion in the species of fish to be included on the label. Currently, the official method for analysis of OTC is a microbiological inhibition assay (2). With the recent popularization of analytical methods such as liquid chromatography (LC), and the problems associated with potentially nonspecific microbiological methods, a chemical analysis of OTC is desirable. To conduct residue depletion studies for OTC in fish, a bridging study is required to determine the relationship between a recently validated LC method (3) and the official microbiological inhibition assay (2).

A validation package for the LC method to determine OTC in the edible fillet of fish (3) conducted at the Upper Midwest Environmental Sciences Center (UMESC) was reviewed by the Center for Veterinary Medicine (CVM), U.S. Food and Drug Administration (FDA), and found acceptable for use in a bridging study. The validation package contained the accuracy and precision of an analytical method for fortified control samples and the variability of recoveries from incurred tissues (3). Validation on fortified samples was conducted over a range that included the tolerance concentration of 100 ng/g (0.1 ppm) at the time of the study and the anticipated increase of the tolerance concentration to 2000 ng/g (2.0 ppm). The tolerance for OTC (total of all tetracyclines) in the edible fillet of fish was increased to 2.0 ppm in December 1996 (4).

The objective of this work was to conduct a bridging study to evaluate the relationship between an LC method and the official microbial inhibition assay for OTC in edible fish fillets. The LC analysis and generation of the spiked and incurred residues were conducted at UMESC, and the microbial inhibition assay was conducted at the FDA Denver District Laboratory.

Experimental

Apparatus, Reagents, and Solutions for the Microbiological Method

(a) Assay medium.—Antibiotic medium #8 (Becton Dickinson Microbiology Systems, Cockeysville, MD) or equivalent; prepare according to FDA or AOAC INTERNATIONAL methods (5, 6).
delivering 100 m
glass tanks or concrete raceways supplied with flowing well
held and reared to the appropriate size (300 to 700 g) in fiber-
cause they are among the most commonly treated fish species
Ennis, MT. Rainbow trout were chosen as test animals be-

gained as eyed eggs from Ennis National Fish Hatchery,

A U.S. Pharmacopeial Convention, Rockville, MD).

ce stock standard.—Accurately weigh 10.0 mg
OTC dihydrate standard into a 25 mL flask equipped with a
glass stopper and dissolve in sufficient 0.1N HCl to obtain a
total of 100 µg/mL stock solution (i.e., our specific lot of OTC had
a declared potency of 919 µg/mg; therefore 9.19 mL 0.1N
HCl was used). Prepare a 100 µg/mL solution in 1% pH 4.5
phosphate buffer from the 1000 µg/mL stock solution. Stan-
dards are stable under refrigeration in phosphate buffer for at
least 1 week.

bacillus cereus (ATCC 11778),—Difco (Detroit, MI)
spore suspension or equivalent; prepare according to AOAC
INTERNATIONAL methods (6).

Water can be obtained as Petri dishes.—Plastic, sterile, 100 × 20 mm, Falcon series
(Becton Dickinson, Franklin Lakes, NJ), or equivalent.

Forced-air incubator.—Capable of maintaining 30°C ±
0.5°C.

Centrifuge.—High speed with a 50 mL centrifuge
head, Sorvall RC-5 super-speed centrifuge (Newton, CT).

50 mL polycarbonate centrifuge tubes.—Kimble
(Vineland, NJ), or equivalent.

Multiwrist action shaker.—Capable of speeds of 30 to
750 rpm, Lab-Line (Pittsburgh, PA).

Balance.—Top loading, capable of weighing a mini-
mum of 100 g, Mettler (Worthington, OH).

Analytical balance.—Capable of weighing a minimum
of 0.1 mg, Sartorius (Edgewood, NY).

Class A volumetric pipettes.—Volumes of 0.5, 1.0,
2.0, 3.0, and 10.0 mL.

Class A volumetric flasks.—50.0 and 100 mL capacities.

Microliter pipette with disposable tips.—Capable of
delivering 100 µL, Eppendorf (Westbury, NY).

Antibiotic zone reader.—Fisher-Lilly, Pittsburgh, PA.

Cylinder dispenser (dropper).—Arthur E. Farmer and
Son, Trenton, NJ.

Reagents, Solutions, and Apparatus for LC Method

Reagents, solutions, and apparatus used for the LC method
were previously described (3), except that OTC dihydrate was
a U.S. Pharmacopeial Convention (U.S. Pharmacopeial Conven-
tion). Standard solutions in LC mobile phase are stable at
room temperature for at least 1 week.

Test Animals

Rainbow trout (Oncorhynchus mykiss, Erwin strain) were
obtained as eyed eggs from Ennis National Fish Hatchery,
Ennis, MT. Rainbow trout were chosen as test animals be-

were not randomized as control or treated because the
sampling units were tissue homogenate samples, not the fish.

Fish were offered an appropriate size of Sterling Silver
Cup trout food (Murray Elevators, Murray, UT) daily during
weekdays at a rate appropriate to maintain growth, ca 1 to 2%
body weight/day for adult fish (9).

Generation of Fortified Edible Fillet

Control rainbow trout (n = 6) were euthanized by a blow to
the head, and the skin-on fillets were obtained. The skin-on
fillets were homogenized with dry ice (10), fortified with OTC
stock solutions (in LC solvent) to concentrations of 0.3, 0.6,
1.2, 2.4, 4.8, and 9.6 ppm OTC base, and mixed by hand for
15 min with a Teflon spatula to obtain a homogeneous sample.
At each fortification level, 75 g homogenized tissue was
mixed with 180 to 1440 µL of a 50 or 500 µg/mL stock solu-
tion to obtain the desired concentration. Quadruplicate
samples were weighed for analysis by the LC and microbiological
methods (6 fortification concentrations × 2 methods × quadru-
plicates = 48 samples total). Each assay (LC and microbiolog-
ical) was conducted in triplicate (one extra sample). Samples
were identified by a code so that analysts were not aware of
the intended fortification concentrations. Skin-on fillets ho-

Homogenized with dry ice but not fortified were used for validat-
ing the microbial inhibition assay.

Generation of Incurred Edible Fillet

Incurred tissues (0.75, 1.5, and 3.75 ppm) were generated at
UMESC by feeding fish with an OTC-medicated feed (Biodiet grower, 6%) commercially available from Bioproducts (Warrenton, OR). Twelve fish were fed for
10 days to receive an average of 100 mg/kg/day of OTC (nom-
inal concentration) in medicated feed. The number of fish was
more than necessary to generate the required incurred resi-
dues; previous experience has shown that fish will not feed if
too few were present. Single fish receiving 1 and 3 days of
medicated feed were removed from the treatment tank imme-
diately before the next day’s feeding to determine the approxi-
mate residue concentration in the skin-on fillet. The remaining
fish were euthanized ca 7 h after receiving medicated feed on
the 10th day. Skin-on fillets were obtained from individual
fish and were homogenized with dry ice (10). A preliminary
LC analysis of OTC in the skin-on fillets from individual fish
receiving medicated feed was conducted to determine approxi-
mate residue concentrations.

Fillet tissue from fish receiving medicated feed for 3 days
was used to generate incurred residues at 0.75 ppm. The fillet
from a single fish was used to generate the incurred residues
at 1.5 and 3.75 ppm. Incurred tissue was blended with con-

management of medicated feed was conducted to determine approxi-
imate residue concentrations.
conducted in triplicate (one extra sample). Samples were coded so that analysts were not aware of the intended incurred concentrations.

**Microbial Inhibition Assay**

Analyses of edible fillets of rainbow trout by the official microbiological method were conducted at the FDA Denver District Laboratory with some adaptations to the methods outlined in the “Green Book” (5, 11–13). Fortified and incurred samples prepared at UMESC were shipped frozen and on “blue” ice by overnight mail to the sample custodian at the Denver laboratory. Samples were kept frozen (≤–70°C) until analyses and processed within a week of receipt. At the request of CVM, the stability of OTC in samples was not determined as part of the study.

(a) **Standard curve.**—Use the 100 µg/mL OTC stock standard to obtain standard curve concentrations of 3.2, 1.6, 0.8, 0.4, 0.2, and 0.1 µg/mL. Prepare individual centrifuge tubes containing 10 g homogenized skin-on fillets of trout and the appropriate amount of 1% phosphate buffer at pH 4.5 to yield a final volume of 20 mL (antibiotic solution and buffer). This mixture yields a final dilution factor of 3 (antibiotic solution, buffer, and tissue), and theoretical OTC values of 9.6, 4.8, 2.4, 1.2, 0.6, and 0.3 ppm, respectively, in muscle.

(b) **Extraction of samples for standard curves.**—Place each centrifuge tube containing the fortified muscle for the standard curve, unknown sample, or negative control tissue with the 1% phosphate buffer at pH 4.5 on a wrist shaker for 15 min. Centrifuge for 15 min at 5000 rpm, and analyze the supernatant.

(c) **Preparation of plates and calculations.**—Sterilize antibiotic medium #8 at 121°C for 15 min and cool to ca 55°C. Pipet a 6 mL agar base layer into each Petri dish with a sterile 25 mL glass pipet, distribute evenly, and allow to harden for ca 30 min. Add a 4.0 mL agar layer seeded with *B. cereus* spore suspension to each plate. (The optimum concentration of the spore suspension to give a zone diameter of 18.0 ± 1.0 mm was determined in a preliminary analysis. With a Difco spore suspension, a 0.1 mL inoculum/100 mL agar produces sharp zones of inhibition of ca 17.5 mm with the reference concentration of 0.4 µg/mL solution.) Use a total of 3 plates for each curve point, negative control sample, and fortified sample. Fill 3 alternating cylinders/plate with the reference standard concentration of 0.4 µg/mL and the remaining 3 wells with either sample, fortified sample, or sample to generate standard curve. Incubate the plates at 30°C for 16–18 h and read the zones of inhibition to the nearest 0.1 mm with a Fisher-Lilly zone reader. Perform linear regression analysis with a computer spreadsheet program for a nonlinear plate assay curve (14).

(d) **Intralaboratory method validation.**—The method was validated by 2 analysts over a 3-day period (2). Each analyst prepared fresh standard curves, fortified samples (in triplicate), and controls daily. The calibration curve was obtained with extracts from homogenates of fortified skin-on fillets (control tissue). The calibration concentrations were 3.2, 1.6, 0.8, 0.4, 0.2, and 0.1 µg/mL, which were equivalent to OTC concentrations in muscle of 9.6, 4.8, 2.4, 1.2, 0.6, and 0.3 ppm, respectively. Homogenates of skin-on fillets (control tissue) were fortified at 1.00, 2.00, and 4.00 ppm and analyzed in triplicate by each analyst over the 3-day period.

**LC Method**

LC analyses were conducted at UMESC by a previously described method (3). The fillet homogenate was extracted with McIlvane/EDTA buffer, and the supernatant was deproteinized with trichloracetic acid and cleaned up on a phenyl solid-phase extraction column. Extracts were analyzed for OTC by reversed-phase LC with absorbance detection at
Table 2. Mean oxytetracycline (OTC) concentrations in fortified and incurred samples of skin-on homogenate of rainbow trout

<table>
<thead>
<tr>
<th>Nominal concentration of fortified (F) or incurred (I) samples, ppm</th>
<th>OTC, ppm (CV, %)</th>
<th>By the LC method</th>
<th>Microbiological inhibition assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 (F)</td>
<td>0.28 (0.36)</td>
<td>0.28 (7.35)</td>
<td></td>
</tr>
<tr>
<td>0.6 (F)</td>
<td>0.56 (2.07)</td>
<td>0.56 (2.71)</td>
<td></td>
</tr>
<tr>
<td>1.2 (F)</td>
<td>1.14 (0.88)</td>
<td>1.15 (4.93)</td>
<td></td>
</tr>
<tr>
<td>2.4 (F)</td>
<td>2.30 (1.22)</td>
<td>2.37 (5.98)</td>
<td></td>
</tr>
<tr>
<td>4.8 (F)</td>
<td>4.45 (1.13)</td>
<td>4.37 (3.95)</td>
<td></td>
</tr>
<tr>
<td>9.6 (F)</td>
<td>9.24 (0.53)</td>
<td>9.92 (2.95)</td>
<td></td>
</tr>
<tr>
<td>0.75 (I)</td>
<td>0.74 (0.62)</td>
<td>0.69 (2.20)</td>
<td></td>
</tr>
<tr>
<td>1.5 (I)</td>
<td>1.59 (0.50)</td>
<td>1.43 (3.89)</td>
<td></td>
</tr>
<tr>
<td>3.75 (I)</td>
<td>3.94 (2.14)</td>
<td>3.89 (1.48)</td>
<td></td>
</tr>
</tbody>
</table>

355 nm. Samples were kept frozen (≤70°C) until analyses and processed within a week.

(a) Validation of method with type of OTC salt.—Because previously published results were obtained with OTC hydrochloride (3), a preliminary determination of accuracy and precision of the method with fortification using OTC dihydrate was done. Homogenized edible fillets were fortified with 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 ppm OTC base as the dihydrate salt before the bridging study to determine the effect of salt type on the LC extraction (n = 4 at each concentration).

(b) Validation for generation of homogeneous fortified samples for LC and microbiological methods.—The accuracy and precision of the LC method were previously developed with individually fortified edible fish fillet samples (3). To minimize variability that could result with fortifying individual samples, single batches of skin-on fillet at each fortification level were generated for all samples used in the LC and microbiological methods. Approximately 75 g trout homogenate was fortified and mixed by hand for 15 min with a teflon spatula. These were the same samples used to compare recoveries of OTC dihydrate and hydrochloride salts. Homogeneity of mixing was assessed from the coefficients of variation (CVs) of replicate LC analyses.

Results and Discussion

Method Validations

The mean day-to-day results of the microbiological method are shown in Table 1. Both analysts produced standard curves with coefficients of determination (r²) of at least 0.999, with limits of detection ranging from 0.17 to 0.47 ppm, and levels of quantitation of 0.29 to 0.69 ppm depending on the analyst, day of analysis, and fortification level. The results indicated that the microbiological method can be performed satisfactorily at fortification levels between 1.00 and 4.00 ppm (2). By contrast, Meinertz et al. (3) reported limits of quantitation for the LC method for different species of fish from 6 to 22 ppb, indicating approximately a 10-fold difference in sensitivity.

Preliminary extractions of control fillet homogenates fortified with OTC dihydrate were conducted to determine an average extraction efficiency. Recoveries ranged from 81.6 to 86.9% at the various fortified concentrations, with an average of 83.4%. By comparison, Meinertz et al. (3) reported an average recovery of 85.8% for homogenates of edible fillet fortified with OTC hydrochloride (range, 83.2 to 89.1%). Thus the salt of OTC (hydrochloride versus dihydrate) used to fortify fillet tissue does not appear to affect extraction efficiency. Relative standard deviations for the preliminary extractions at the test concentrations were 2% or less, compared with an average of 2.7% obtained by Meinertz et al. (3), indicating that a homogeneous sample was obtained for the bridging study.

The measured concentrations in the fortified (0.3 to 9.6 ppm) and the incurred (0.75 to 3.75 ppm) tissue homogenates are reported in Table 2. In all cases, the measured concentrations approximated the expected concentrations for incurred and fortified samples. On the basis of the essentially identical results, a CVM official suggested that “For regulatory purposes and for generating pivotal tissue residue depletion data, the LC method can be used in place of the microbiological assay provided the performing laboratory can meet the performance criteria established in the bridging study” (15).

Conclusion

This bridging study demonstrated that the official microbiological method and the LC method for quantitation of OTC residues in rainbow trout edible fillet between 0.3 and 9.6 ppm for fortified and between 0.75 and 3.75 ppm for incurred tissues produce essentially identical results.

Acknowledgments

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