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Plasma and Tissue Depletion of Oxolinic Acid after Administration to Orange-Spotted Grouper (*Epinephelus coioides*), Snubnose Pompano (*Trachinotus blochii*) and Giant Seaperch (*Lates calcarifer*)

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Abstract

Pharmacokinetics and residues of oxolinic acid (OXO) in serum, muscle, liver, and kidney were measured in orange-spotted grouper *Epinephelus coioides*, snubnose pompano *Trachinotus blochii* and giant seaperch *Lates calcarifer*. Fish were treated with OXO after a single-dose trial (60 mg/kg) by oral gavages and repeat-doses of 60 mg/kg of body weight once daily in feed for five consecutive days. Analysis of OXO kinetic profiles, when determined by HPLC provided elimination half-life in muscle were 131, 26, and 85 h for 26-27 °C orange-spotted grouper, snubnose pompano, and giant seaperch, respectively. Depletion studies were conducted the time for OXO concentrations to fall below 0.1 µg/g (the current tolerance set by the European Medicines Agency (EMA) among fish species establishing an adequate depletion period). The mean tissue OXO concentration in the edible portion was below maximum residue level (MRL) of snubnose pompano at 6 d postdosing, for orange-spotted grouper and giant seaperch, at 10 d postdosing, respectively. When possible, depletion data were fitted to a one-compartment pharmacokinetic model. For all test species, the longest withdrawal period of 9 days in snubnose pompano and 15 days in orange-spotted grouper and giant seaperch were calculated because of a safety span (corresponds to 50% of the time point when at which residues fall below the MRL added to the depletion time). These results suggest withdrawal times with reference to human consumption of treated fish, to establish policy guidelines and basic principles regarding the use of OXO for fish-farming, and to fish-farmers for the proper handling to ensure safe fish that the consumer will not be at risk.

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Introduction

Mariculture has expanded because of technological advances associated with the intensification of cultural practices on the southwest coast of Taiwan. Unfortunately, aquatic animal disease outbreaks caused by pathogenic organisms often resulted in severe socioeconomic damage and ecological impacts on the aquaculture field. The rapidly growing grouper industry, for example, has undergone quite severe disease problems mainly caused by several *Vibrio* spp. (Feng et al., 2010). Most bacterial pathogens of fish are aerobic, gram-negative rods; epidemics of such bacterial diseases are frequent in high stocking densities of cultured fish. Predisposition to such outbreaks frequently is associated with poor water quality, organic loading of the aquatic environment, marked temperature changes, hypoxia, or other stressful conditions. In that respect, one of several strategies is the use of an antibacterial agent, which is economically acceptable therapeutic regimes should be carried out to prevent both infectious and noninfectious outbreaks (Ozturk and Altinok, 2014).

Owing to the low minimum inhibitory concentration (MIC) value for most susceptible fish pathogens and effective systemic distribution in fish when administered orally via medicated feed, oxolinic acid (OXO, a first-generation quinolone antibiotic) has been used extensively to treat systemic bacterial infections in fish, such as furunculosis, atypical furunculosis, classical vibriosis, cold-water vibriosis and yersiniosis (Samuelsen, 2006a). OXO showed resistance in 32.7% of the tested Aeromonas spp. Strains with MIC50 values below the reference breakpoint of susceptibility (≤ 0.12 µg/mL) and MIC90 of 16 µg/mL (Scarano et al., 2018), which increased the concentration of drug-enzyme-DNA cleavage complexes and converted them into cellular toxins (Franco and Drlica, 1988). Various countries have authorized the use of OXO for therapeutic in aquaculture due to its high bioavailability, relatively low toxicity and favorable and pharmacokinetics practice (Samuelsen, 2006a; Naeem et al., 2016). Concerning the extensive use of antibiotics in aquaculture has, in turn, resulted in the emergence of resistance in both foodborne and opportunistic human pathogens (Marshall and Levy, 2011), the effectiveness of antibiotics used in fish farming should be carefully monitored.

Pharmacokinetic profiles of OXO have been described in rainbow trout (*O. mykiss*) (Bjorklund and Bylund, 1991; Kleinow et al., 1994); channel catfish (*I. punctatus*)(Kleinow et al., 1994); orange-spotted grouper (*E. coioides*) (Guo and Liao, 1994); Atlantic halibut (*H. hippoglossus*) (Samuelsen and Ervik, 1999); corkwing wrasse (*S. melops*) (Samuelsen and Ervik, 2001); sea bass (*D. labrax*) (Rigos et al., 2002a); gilthead sea bream (*S. aurata*) (Rigos et al., 2002b); sharpsnout sea bream (*D. puntazzo*) (Rigos et al., 2004); turbot (*S. maximus*) (Samuelsen, 2003); cod (*G. morhua*) (Samuelsen et al., 2003; Samuelsen, 2006b); kuruma shrimp (*P. japonicus*) (Uno, 2004) and Atlantic salmon (*S. salar*) (Coyne et al., 2004). The orange-spotted grouper (*E. coioides*), snubnose pompano (*T. blochii*) and giant seaperch (*L. calcarifer*) are recognized as economically-important marine fish and abundantly cultured in Taiwan for domestic consumption and overseas export. These aspects were not studied previously on the snubnose pompano and giant seaperch. The pharmacokinetic properties of OXO studies are valuable tools for establishing optimal dosage regimes.

The present work aimed to obtain information on the half-lives and elimination time of OXO following single and multiple oral doses (for five consecutive days) in orange-spotted grouper, snubnose pompano and giant seaperch. The appropriate depletion period after administration is required to ensure that residues in edible tissues fall below established tolerance limits. The permitted limit, maximum residue limit (MRL), of OXO is 0.1 µg/g allowed by the European Union (EU) established for muscle with skin in natural proportions (EU, 2009). Therefore, these results may provide information on OXO distributions in muscle, liver, kidney and plasma and help to determine the OXO withdrawal period for these three-culture fish further to protect the health of consumers and guarantee export food safety.

Materials and Methods

Materials.

Oxoilinic acid, glucose, citric acid monohydrate, trisodium citrate dihydrate, phosphoric acid, sodium dihydrogen phosphate (NaH_2PO_4) and sodium dodecyl sulfate (SDS) were purchased from Sigma-aldrich (St. Louis, MO). Tricaine methanesulphonate (MS-222, anesthetic agent) was from Argent Chemical Laboratories (Redmond, WA); methanol and acetonitrile (HPLC grade) were from J.T. Baker (Philippsburg, NJ). Deionized water was purified using a Milli-Q lab water

purification system (Millipore, MA), and all the other chemicals were of analytical grade.

Chromatographic conditions.

For reversed phase chromatography analysis, a Dionex DX-600 chromatography system comprising of GS50 gradient pump, AS50 autosampler, temperature-controlled column compartment and RF-551 fluorescence detector (Shimadzu Co., Kyoto, Japan) equipped with PeakNet® 6 chromatography workstation (all from Dionex Co., CA) was used. Separation was performed on a Hypersil ODS C18 column (250 mm × 4.6 mm inner diameter, 5 µm particle size; Thermo Fisher Scientific, Waltham, MA). Column temperature was set to 35°C and the mobile phase of 50 mM NaH_2PO_4, pH 2.5 containing 0.1% SDS–acetonitrile (65 + 35, v/v) with 1 mL/min flow rate was used. The injection volume for all samples was 20 µL and eluted substances were carried out with fluorescence detection at 325 and 365 nm as excitation and emission wavelengths.

Fish.

After receiving approval from the Institution Animal Care and Use Committee (IACUC) of National Taiwan University, healthy orange-spotted grouper (*E. coioides*, 28.1 ± 6.7 g), snubnose pompano (*T. blochii*, 150.6 ± 26.4 g) and giant seaperch (*L. calcarifer*, 424.7 ± 67.3 g) with no prior history of exposure to OXO were obtained from aquafarms in Fanliao (Pingtung, Taiwan). They were reared in cement pools of 10 tons (5.3×1.7×1.2 m), supplied with aerated seawater. Water quality parameters such at temperature (26-27 ºC), pH 7.4-7.5, and oxygen (7.2-7.4 mg/L) were frequently monitored. The fish were acclimatized for two weeks during which they were fed with antibiotic-free commercial fish feed and were starved for one day before administration of the drug.

Experimental design.

Fish were randomly placed into two groups, one for pharmacokinetics and the other for residue depletion analysis. Recommended volumes for administration via gavages for each fish species were presented in **Table 1**. OXO was dissolved in a small amount of 0.1 N NaOH, and the solution was diluted with isotonic sterile phosphate buffered saline (PBS). For pharmacokinetics evaluation, a single-dose trial was conducted using a 60 mg/kg dosage of orally administered OXO solution by gavages. All fish were manually restrained, as OXO was administered orally in accurate amounts, using a syringe placed in the mouth through the esophagus into the stomach; after each dose was administered, the mouths of fish were held closed for 1 min before being released back to the raising pool. After oral gavages, tissue samples of fish (n = 6) were sampled at 0.5, 1, 2, 4, 8, 16 hr, and 1, 2, 4, 6, 8, and 14 days to monitor the concentrations of OXO in selected tissues (serum, muscle, liver and kidney). To study the tissue residues of OXO, repeat-dose studies were conducted using 60 mg/kg dosages administered once daily in feed for five consecutive days. Then, samples (serum, muscle, liver and kidney; n = 6) were collected at 1, 2, 4, 6, 8, 10, 14, 21 and 28 days after the end of the experiment (**Figure 1**). Individual fish were lightly anesthetized with MS-222, and blood samples were collected from the caudal vein of each fish. Then, the fish was euthanized with an overdose of MS-222 (AVMA, 2013). Following the completion of tissue sampling, all samples were frozen and stored at -20 °C until being analyzed. The fish in the control set (no OXO added) were processed simultaneously.

Table 1 The amount required for the oral (gavage) administration of OXO was based on the fish weight, and the recommended volume for the stock solutions was 60 mg/kg body weight.

Stock 6 mg/mL		*Stock 30 mg/mL*			*Stock 60 mg/mL*
Fish weight (g)	*Volume (mL)*	*Fish weight (g)*	*Volume (mL)*	*Fish weight (g)*	*Volume (mL)*
Orange-spotted grouper		Snubnose pompano		Giant seaperch	
20	0.20	120	0.24	350	0.35
22	0.22	125	0.25	365	0.37
24	0.24	130	0.26	380	0.38
26	0.26	135	0.27	395	0.40
28	0.28	140	0.28	410	0.41
30	0.30	145	0.29	425	0.43
32	0.32	150	0.30	440	0.44
34	0.34	155	0.31	455	0.46
36	0.36	160	0.32	470	0.47
38	0.38	165	0.33	485	0.49
40	0.40	170	0.34	500	0.50

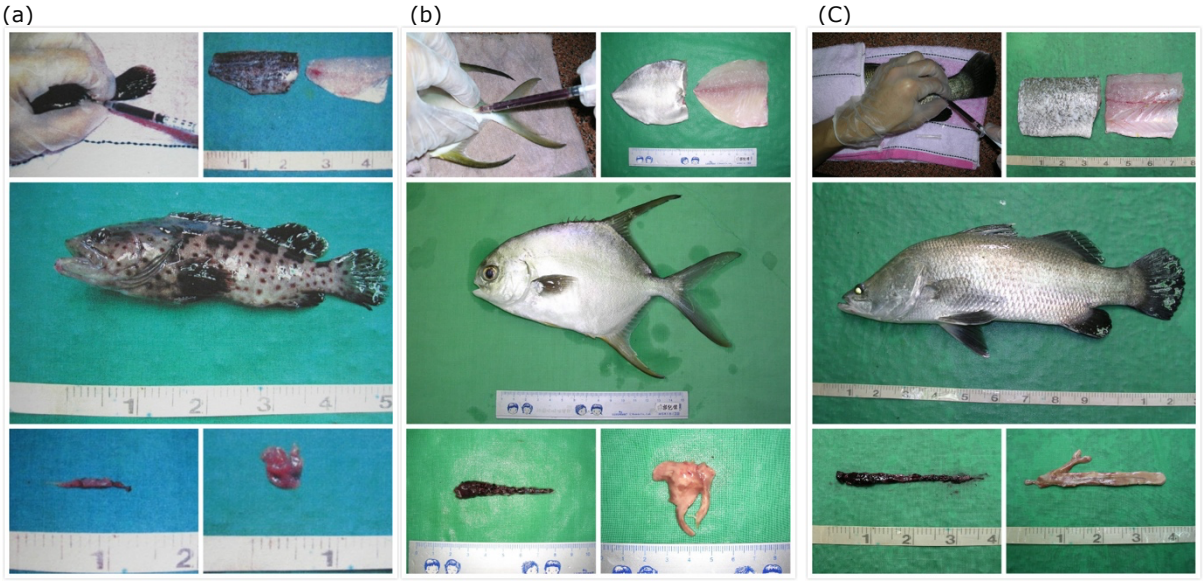

Figure 1 Photographs of (a) orange-spotted grouper, (b) snubnose pompano, and (c) giant seaperch, selected tissue and sample collection: serum (upper-left), muscle (upper-right), liver (lower-left) and kidney (lower-right).

Sample Preparation.

(A) Serum samples were diluted 1:1 (v/v) with 50 mM NaH_2PO_4 (pH 2.5), the preparation was then thoroughly mixed with acetonitrile (1:2, v/v). (B) A 1-g homogenate fish sample (muscle, liver and kidney) was well-mixed with 1 mL of a 50 mM NaH_2PO_4 at pH 2.5 in a centrifuge tube. Afterward, 4 mL of acetonitrile was then added and the mixture was thoroughly shaken by a vertical shaker (10 min), the resulting mixture (A or B) was then centrifuged (3,000 rpm, 10min). 200 μL of the upper clear solution was diluted with 50 mM NaH_2PO_4 (1:1, v/v), followed by vortex mixing (1 min) and centrifugation (12,000 rpm, 10min). The supernatant was filtered through a nylon centrifuge filter (0.45 μm). Aliquots of 20 μL were injected automatically on the HPLC column.

Quality control

The validated liquid chromatography method employing a fluorescence detector for the determination of OXO was modified from that described by Du et al., (2011). The parameters included were linearity, accuracy, precision, limit of detection and quantification. System repeatability was assessed by six replicate analyses of OXO at the concentration of 25, 50 and 100 ng/mL. The repeatability of sample application and measurement of peak area for OXO were expressed in terms of relative standard deviation (RSD). Method repeatability was determined from RSD value by repeating the assay six times on the same day for intraday precision. The intra- and inter-day variations for determination of OXO were carried out at three different concentration levels 25, 50 and 100 ng/mL. The accuracy of the methods was obtained by calculating the percent recovery (%) of the analyte recovered. In this case, blank tissue samples were spiked at 25, 50 and 100 ng/mL, then used to evaluate the extraction efficiency. The experiments were performed 3 times.

Results

Validation

Specificity of the analytical methods was determined using 20 blank samples, no interferences were detected for these samples on the analyte retention time for either serum, muscle, liver or kidney matrices. The following validation parameters were achieved: linearity of matrix-match calibration standard curves was higher than 0.99, the intra- and inter-day precision (CV%) were lower than 11.9 and 10.8%, respectively. The method showed satisfactory recoveries (83-99%) in all matrices and for all fortification levels. The limit of detection (LOD) was set at 3 ng/g with a signal-noise ratio greater than 3:1. The limit of quantification (LOQ) was estimated at 5 ng/g.

Pharmacokinetics

OXO concentrations in serum, muscle, liver and kidney samples were quantified via a lineal regression analysis of the calibration curves on their respective fortified matrices. To this end, we considered those curves that presented an $R^2 \geq 0.99$. The mean concentrations of OXO versus time in tissues of fish after oral dose were calculated and the relevant pharmacokinetics values of OXO were obtained, in orange-spotted grouper, snubnose pompano and giant seaperch following administration of a single oral dose of 60 mg/kg, by running the ADAPT 5 software and were summarized in **Table 2**. The high-dose OXO was used in fish to monitor safety (at the 2 dose levels for therapeutic purposes (Ellingsen et al., 2002) within the trial., Results showed that OXO was rapidly absorbed following oral administration and declined much faster in snubnose pompano than those in grouper and giant seaperch.

Table 2 Concentration-time curve equations and pharmacokinetics values following single oral administration of OXO at 60 mg/kg body weight.

Tissues	*Equation*	r^2	*Elimination half-life (h)*	T_{max} *(h)*	C_{max} *(µg/g)*
Orange-spotted grouper (*E. coioides*)					
Serum	$C = 278.04\ e^{-0.0051t}$	0.9586	136	4	0.59
Muscle	$C = 1915.4\ e^{-0.0053t}$	0.7906	131	4	1.76
Liver	$C = 1606.8\ e^{-0.0045t}$	0.9673	158	4	2.18
Kidney	$C = 2340.6\ e^{-0.0063t}$	0.8088	110	4	3.61
Snubnose pompano (*T. blochii*)					
Serum	$C = 46.207\ e^{-0.0137t}$	0.8263	51	0.5	0.43
Muscle	$C = 174.00\ e^{-0.0269t}$	0.8452	26	0.5	1.43
Liver	$C = 434.34\ e^{-0.0154t}$	0.8115	45	0.5	1.42
Kidney	$C = 888.06\ e^{-0.0141t}$	0.8368	49	0.5	3.14
Giant seaperch (*L. calcarifer*)					
Serum	$C = 137.86\ e^{-0.0039t}$	0.6230	178	1	1.55
Muscle	$C = 417.18\ e^{-0.0082t}$	0.8934	85	1	2.55
Liver	$C = 846.33\ e^{-0.0065t}$	0.8750	107	1	8.18
Kidney	$C = 2129.8\ e^{-0.0062t}$	0.8336	112	1	4.73

Depletion studies

Table 3 presents the average OXO concentrations for different matrices, and for each time point after treatment on five consecutive days at a daily dose of 60 mg/kg, decreased over time after the initial increase. These data indicated that the concentrations of OXO in the tissues were the highest at day one and declined in the following days, we determined that on day 6 (snubnose pompano), and day 10 (orange-spotted grouper as well as giant seaperch), respectively, the residues in all tissues were less than the MRL value at 0.1 µg/g set by the EU (2009). The highest residue concentration occurred in liver and kidney at all depletion times.

Table 3 Concentrations (mean ± SD) of OXO (ng/g) in tissues of *E. coioides*, *T. blochii* and *L. calcarifer* following multiple dosing (60 mg/kg/day, for 5 days, n = 6/group) at 26-27 °C.

Time (days) [1]	*Concentrations (ng/g)*			
	Serum	*Muscle*	*Liver*	*Kidney*
Orange-spotted grouper (*E. coioides*)				
1	86.4 ± 8.4	205.1 ± 26	232.4 ± 15.1	1949.7 ± 41
2	82.2 ± 2.2	174.7 ± 11	265.0 ± 5.4	323.5 ± 9.3
4	43.6 ± 8.5	138.7 ± 8.9	280.2 ± 6.6	374.4 ± 5.1
6	43.7 ± 6.4	70.2 ± 14.1	250.3 ± 3.2	164.7 ± 3.4
8	21.4 ± 3.8	69.8 ± 13.4	189.1 ± 8.4	110.8 ± 8.1
10	16.4 ± 1.1	44.4 ± 5.0	94.2 ± 5.4	92.5 ± 4.2
14	15.2 ± 3.9	42.4 ± 5.1	82.8 ± 2.2	72.2 ± 4.7
21	14.3 ± 1.4	24.4 ± 3.0	29.0 ± 1.4	11.4 ± 4.9
28	2.5 ± 0.8	14.1 ± 3.9	19.4 ± 4.0	11.3 ± 3.1
Snubnose pompano (*T. blochii*)				
1	26.3 ± 2.3	106.9 ± 16	120.1 ± 17	210.5 ± 25
2	21.0 ± 7.2	94.1 ± 7.1	95.0 ± 9.5	192.1 ± 21
4	19.9 ± 7.4	37.0 ± 3.3	70.0 ± 8.1	130.4 ± 8.9
6	12.7 ± 6.7	18.0 ± 4.6	52.4 ± 4.3	88.4 ± 9.2
8	12.4 ± 4.9	8.6 ± 0.7	34.2 ± 3.8	44.9 ± 7.7
10	5.0 ± 3.6	4.5 ± 0.8	29.7 ± 5.6	39.2 ± 7.2
14	2.8 ± 2.5	4.6 ± 0.7	11.8 ± 6.7	24.2 ± 2.5
21	1.2 ± 0.4	3.4 ± 0.7	5.5 ± 2.5	13.4 ± 6.6
28	0.5 ± 0.4	2.7 ± 0.3	7.0 ± 1.3	6.3 ± 0.7
Giant seaperch (*L. calcarifer*)				
1	50.1 ± 7.4	268.9 ± 59	485.1 ± 69	1034.3 ± 30
2	31.7 ± 5.0	185.2 ± 12	279.7 ± 16	496.1 ± 58
4	31.0 ± 8.3	69.2 ± 8.0	199.4 ± 11	378.1 ± 31
6	26.2 ± 7.9	41.8 ± 8.4	155.2 ± 7.3	215.5 ± 15
8	24.0 ± 9.0	30.7 ± 9.0	116.4 ± 8.5	146.7 ± 11
10	23.5 ± 5.0	22.8 ± 8.4	94.5 ± 4.9	91.6 ± 7.2
14	20.9 ± 7.7	20.4 ± 6.6	72.1 ± 4.2	64.3 ± 7.4
21	16.1 ± 4.1	20.1 ± 6.3	29.1 ± 7.6	46.7 ± 6.8
28	15.5 ± 6.3	15.5 ± 5.7	21.1 ± 5.4	21.2 ± 9.4

[1] Day 1 is the first day after the final dose.

Discussion

The usual oral dosage of OXO for veterinary use is 12-20 mg/kg for five to ten days (EMA, 2005). Interestingly, it also acts as a dopamine reuptake inhibitor and has stimulant effects in mice (Garcia de Mateos-Verchere et al., 1998). Relatively high concentrations (2 times the recommended level in "Guidelines for the Use of Drugs in Animals" set by the Council of Agriculture (COA), Taiwan) used in the current study prevent the incorrect and excessive dosage of OXO in fish farming. A one-compartment pharmacokinetic model can describe the time course of OXO concentrations in the three species after oral administration (60 mg/kg). In serums, the elimination half-life of OXO in giant seaperch (178 h) was longer than that found in snubnose pompano (51 h) and orange-spotted grouper (136 h). While in muscle tissues, the elimination half-life values were 131, 26 and 85 h in giant seaperch, snubnose pompano and orange-spotted grouper, respectively. The residual OXO was rapidly eliminated in snubnose pompano than that of the two species. No depletion studies have previously been performed with snubnose pompano or giant seaperch to which OXO was administered through multiple-dose regimens. Studies on the pharmacokinetics of OXO in a variety of fish species treated by the oral route at doses ranging from 10 to 75 mg/kg depending on several factors such as illness severity, pathology, size of the individuals, water conditions (Bjorklund and Bylund, 1991; Guo and Liao, 1994; Samuelsen and Ervik, 1999; Rigos et al., 2004; Samuelsen et al., 2003; Samuelsen, 2006b) (i.e., salinity (Hustvedt and Salte, 1991) or temperature (Bjorklund et al., 1992), and food intake.

The dosage (60 mg/kg per day for 5 days) was selected to monitor their safety and revealed no observable side effects. The moderate interindividual variation noted in the serum and tissue

concentrations of OXO in this study may be reflective of absorption, metabolic, and elimination differences between individuals. Results showed that OXO persisted for a more extended period and at higher concentrations in tissues such as liver and kidney. Overall, even at such a high dosage, the mean tissue OXO concentration in the edible portions was below the established maximum residue level (0.1 μg/g) by day 6 (snubnose pompano) and day 10 (orange-spotted grouper as well as giant seaperch) after treatments. OXO exhibited similar depletion behaviors as reported for orange-spotted grouper (Guo and Liao, 1994). Also, the pharmacokinetics of OXO is affected by differences in fish species, drug administration (through medicated feed versus by gavage) and culture and experimental conditions (Samuelsen et al., 1992; Rigos et al., 2003; Gonzalez et al., 2010).

Characteristically, OXO is a weak acid and tends to complex, especially with magnesium, the pH and the concentration of divalent cations in the intestine of the fish are essential factors affecting absorption (Schuchardt and Hahn, 2017). The bioavailability of orally administered OXO is therefore reduced in seawater than from freshwater (Kleinow et al., 1994; Rigos et al., 2002b). There was a significantly lower terminal elimination half-life in seawater than in freshwater. In contrast, the difference was mainly due to the higher total clearance in seawater, i.e., the sum of all elimination processes caused by differences in the rate of elimination, metabolism, or both in the fish (Hustvedt and Salte, 1991). Renal excretion is the primary route of elimination for most quinolones, both glomerular filtration and tubular secretion are involved. It has also been demonstrated that the urine flow rate in salmonids is dramatically reduced in seawater compared to freshwater (Hunn, 1982). Therefore, renal excretion of the drug was not considered the rate-limiting step in the elimination of OXO; and about 50% of OXO in the plasma at therapeutic concentrations is unbound that can freely interact with the secretory system in the nephrons (Benjaminsen and Hustvedt, 1986). Another major pathway for excretion of quinolone in fish is via the bile into the intestine. In contrast, this process is also found to be more rapid in seawater than in freshwater (Samuelsen, 2006a).

Our results agree with the general trend described by the studies mentioned above, temperature increase induces an increase in the metabolic and excretory rates of the fish. The moderate elimination at 26-27 ºC in our studies suggests a dosage regime with medication as well as a reasonable withholding period following medication. The present study showed that the distribution of OXO from serum to snubnose pompano tissues is faster and that elimination is more rapid in serum than in tissues. In order to account for the high variability of the residue data, it is recommended to determine the withdrawal period at the time point where the concentrations of residues in edible tissues fell below the respective MRL. Besides, the estimation of a safety margin, which is intended to compensate for inter-animal variability, should be calculated from half of tissue depletion time can be seen as appropriate (EMA, 2018). In this study, the residue depletion of OXO was investigated in three-culture fish following multiple oral gavage (60 mg/kg for 5-day) at 26-27 °C. A withdrawal period of 15 days was calculated as the sum of 10-day depletion time and 5-day safety span for orange-spotted grouper and giant seaperch, and result in a feed withdrawal period of about 9 days for snubnose pompano. As a result, the depletion behavior we have detailed could, therefore, aid the design and implementation of policies of antimicrobial surveillance, and they should be used cautiously and under veterinary medicine guidance.

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