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Tissue residue depletion kinetics and withdrawal time estimation of doxycycline in grass carp, *Ctenopharyngodon idella*, following multiple oral administrations

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ABSTRACT

This study aimed to determine the plasma and tissue residue depletion kinetics of doxycycline (DC) in grass carp (*Ctenopharyngodon idella*) after daily oral administrations at 20 mg/kg for 3 days, and to calculate the corresponding withdrawal times. Following drug administrations, samples of plasma, liver, kidney, gill and muscle + skin were collected at predetermined time points (0.25, 0.5, 1, 3, 5, 7, 14, 21, 28, 35, 42, 49 and 56 days) and analyzed for concentrations of DC using a LC-MS/MS method. The results showed that liver had the highest concentrations and the slowest depletion compared to other tissues, with detectable DC up to 49 days (58.9 ± 12.8 μg/kg). The WT 1.4 software and "reschem" package were used to calculate withdrawal times, and the results were similar. The results suggest a withdrawal time of 41 days for Europe and China and 50 days for Japan is needed for DC in grass carp after 3 daily oral administrations at 20 mg/kg. Overall, this study improves our understanding of the tissue residue depletion kinetics of DC in fish, and the results may help regulatory agencies to determine proper withdrawal periods based on different regulatory standards in different countries to ensure safety of aquatic food products.

1. Introduction

Fish contain high levels of proteins, unsaturated fatty acids and various vitamins; they are crucial sources of micronutrients and are easier to be digested and absorbed than other categories of meat by human intestine, thus fish play an important role for human health (Christopher et al., 2016). The unsaturated fatty acids in fish have been shown to be effective in lowering cholesterol levels in the human body to decrease the risk of hypertension and cardiovascular diseases (Larsson et al., 2011; Kirkhus et al., 2012; Chen et al., 2015). In order to keep healthy diet and adequate intake of high-quality nutrients, more and more people have chosen to eat fish. The Food and Agriculture Organization of the United Nations reported that the average amount of consumption per capita of fish has increased from 9.9 kg in 1960s to 19.2 kg in 2012 (FAO, 2014). The increasing demand of fish has boosted the rapid growth of aquaculture with efficient intensive cultivation methods. These new aquaculture systems not only bring a lot of economic benefits compared to traditional cultivation methods, but

Abbreviations: CCα, the decision limits; CCβ, the detection capability; DC, doxycycline; EMA, European Medicines Agency; FDA, United States Food and Drug administration; LC-MS/MS, liquid chromatography combined with triple quadrupole tandem mass spectrometry; LOD, the limit of detection; LOQ, limit of quantification; MRL, maximum residue limit; MS222, tricaine methanesulfonate; RSD, relative standard deviation; OTC, oxytetracycline; PBPK, physiologically based pharmacokinetic model; SPE, solid phase extraction; SRM, selective reaction monitoring; T1/2λz, the terminal elimination half-life; WT, withdrawal time

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also lead to fish stress and bacterial infections (Pulkkinen et al., 2010). The resulting fish infectious diseases can infect individuals in the confined culturing environments (Defoirdt et al., 2011). For the sake of treating disease outbreaks, many compounds have been used to treat infectious diseases in fish, including antibiotics, pesticides, antifungals, disinfectants and other chemicals (Cabello, 2006; Sapkota et al., 2008; Cañada-Cañada et al., 2009; Nunes et al., 2018). While these chemicals can effectively treat fish infectious diseases, they also accumulate in the fish body or are excreted into water environment, which will ultimately enter the human body through the food chain or drinking water. Additionally, drug-resistant bacteria in fish may be transferred to humans, thereby aggravating the already severe antimicrobial resistance problems in humans (Sapkota et al., 2008; Rosa et al., 2018). Therefore, it is important and necessary to closely monitor residue levels and calculate the proper withdrawal times of drugs in fish.

Doxycycline (DC) is an efficient and broad-spectrum drug of tetracycline class and is widely used in aquaculture against diseases resulting from Gram-positive and Gram-negative bacteria by inhibiting protein biosynthesis after binding to the 30 S ribosomal subunit in the bacterial cell (Shireman et al., 1976; Deng et al., 2014; Song et al., 2014; Liu et al., 2019). DC has been approved for use in yellowtail (*Seriola quinqueradiata*), red sea bream (*Pagrus major*), horse mackerel (*Trachurus trachurus*), tilapia (*Oreochromis niloticus*) and other bass species (*Lateolabrax japonicus*) in Japan, as well as for various aquatic species in India, China and Philippines (Arthur et al., 1996; Feng, 2010; Rico et al., 2012). Although DC has desirable therapeutic efficacy, it may also cause various side effects, including nausea, vomiting, abdominal pain, diarrhea and other gastrointestinal reactions (Pazzaglia et al., 2014; Affolter et al., 2017). In order to protect human health, many countries have established the maximum residue limit (MRL) for DC in various food products. In Europe, the MRLs of DC are 100 μg/kg in muscle, 300 μg/kg in fat and liver, and 600 μg/kg in kidney for bovine, porcine and poultry species, and 100 μg/kg in muscle + skin for finfish (EU, 2015). These MRL values in Europe are also adopted in China (MAA, 2017). In Japan, the MRL of 50 μg/kg of DC is used in muscle, fat, and liver for pigs and chickens, and in muscle + skin in Perciformes, including the bonito (*Katsuwonus pelamis*), horse mackerel (*Trachurus trachurus*), red sea bream (*Pagrus major*) and so on (JFCRF, 2006). Although the MRL of DC in fish meat has been stipulated, residue violations of DC in fish are still reported, in part, due to lack of data on the tissue residue depletion kinetics in order to determine the proper withdrawal time (WT) of DC in fish.

To calculate a WT of a drug, typically a tissue residue depletion study should be conducted first, and then the data are used to calculate the WT with a tolerance limit method using the WT 1.4 software adopted by European Medicines Agency (EMA) (Damte et al., 2012; EMA, 2018) or using the tolerance limit method (coded in the "reschem" R package) developed by US Food and Drug Administration (FDA) (FDA, 2018). WT 1.4 has been used extensively to calculate the WT of different drugs (Paschoal et al., 2012, 2013; Vardali et al., 2017; Rosa et al., 2018), but FDA's "reschem" package was released recently. Currently, there are no reports assessing the potential difference of the estimated WT using the WT 1.4 vs. the "reschem" package.

The residue depletion kinetics of DC has been studied in cattle (Erdog Du et al., 2009; Addisalem Hunde et al., 2012), pigs (Croubels et al., 1998; Peeters et al., 2016), hens (Yoshimura et al., 1991; Gajda and Posyniak, 2015) and broilers (Anadon et al., 2012; Hsiao et al., 2016). Most of these existing studies did not calculate the WT based on their data except the study in pigs by Croubels et al. (1998). In addition, there is limited information on DC's tissue residue depletion in fish in the literature. Therefore, the objective of this study was to determine the tissue residue depletion kinetics of DC in grass carp after multiple oral administrations, and then calculate the WT using the WT 1.4 software and the FDA's "reschem" package. This study chose the grass carp because it is an important fish species that is extensively cultured and consumed in Asia with more than 5 million tons of production per year (CFFA, 2018).

2. Materials and methods

2.1. Analytical standards and reagents

Analytical standard DC (purity grade 98%) and DC-D_3 (purity grade 95%) were obtained from Dr. Ehrenstorfer GmbH. (Augsburg, Germany). The DC powder (purity grade 98%) used for oral administration was provided by Zhongbo Aquaculture Biotechnology Co. Ltd. (Wuhan, China). The anesthetic tricaine methanesulfonate (MS222) was purchased from Aibo Biotechnology Co. Ltd. (Wuhan, China). The HPLC-grade acetonitrile, methanol, water and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and J–T Baker (Philipsburg, PA, USA). Sodium dihydrogen phosphate, citric acid monohydrate and ethylenediaminetetraacetic acid disodium (EDTA-Na_2) were bought from Shanghai Guoyao Company (Shanghai, China). The 0.22-μm politetrafluoroetileno membranes, 1.5 mL vials and centrifugal tubes were purchased from Shanghai CNW Technologies (Shanghai, China).

2.2. Standard solutions

Standard stock solution of DC was prepared in methanol at a concentration 400 μg/mL. A 10 μg/mL working standard fortification solution was obtained through dilution of 0.5 mL stock solution to the corresponding volume with methanol. Afterwards, a 1 in 10 dilution of the standard was performed to get 1 μg/mL working standard solution. The labeled internal standard solution (DC-D_3) at 1 μg/mL was prepared with methanol. Stock and working standard solutions were prepared every 3 and 1 months, respectively, and were stored at −20 °C.

2.3. Blank samples

Grass carp (450.4 ± 52.6 g, 12 months of age, mixed genders) were obtained from the culture facility of Yangtze River Fisheries Research Institute (Wuhan, China) and acclimated for 14 days fed with drug-free feed in tanks (10 fish each tank; volume of each tank: 480 L). The drug-free feed was manufactured by the Nutritional Research Group in Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China. The feed contained 28.00% crude proteins, 7.06% crude fat, 15.00% crude fiber, 8.75% moisture, and 15.63% ash (Zhao et al., 2018). The relative water quality parameters were monitored daily and maintained at the following conditions: nitrite nitrogen concentrations < 0.072 mg/L, total ammonia nitrogen concentrations ≤0.74 mg/L, dissolved oxygen concentrations at 6.1–7.0 mg/L, and pH at 7.2 ± 0.2. The water temperature was controlled by aquarium heater and air-conditioner and maintained at 24 ± 0.5 °C. Oxygen was held close to saturation by bubbling air using air-stones. The blank samples of plasma and tissues (i.e., liver, kidney, muscle + skin and gill) were collected from fish without drug treatment and stored at −20 °C. These blank samples were used as the negative control to establish the LC-MS/MS (liquid chromatography-tandem mass spectrometry) determination method of DC.

2.4. Experimental design

The fish were arbitrarily divided into 12 groups with 10 fish each group and reared in tanks with flowing well water (26 L/min). A 20 mg/mL DC solution for oral administration was prepared by dissolving DC powder in pure water. When everything was ready, the fish were weighed and anaesthetized with MS222 solution (50 mg/L). The fish were stable enough for drug administration after about 0.5–1 min of using MS222. In line with other pharmacokinetic studies in fish, we used MS222 to mildly sedate and decrease stress of fish for the animal welfare purpose (Rigos et al., 2002a, b; Rigos et al., 2003; Rigos et al.,

2004; Hung et al., 2018; Sidhu et al., 2018), but the potential effect of MS222 on the observed pharmacokinetics remains to be investigated (Horsberg, 1994; Rairat et al., 2019; Yang et al., 2019b). The DC solution was administered orally to fish by inserting a plastic tube attached to a 1 mL micro–injector into the intestine at 20 mg/kg as these fish do not have a stomach. We chose oral gavage instead of the more commonly used commercial route of administration via medicated feed in order to dose the fish with the exact amount of DC according to the body weight. According to the stipulation of Compilation of National Standards for Veterinary Drugs in China (Feng, 2010), DC is approved to be used by oral administration at a dose of 20 mg/kg for 3–5 days in various cultured fish species in China (Feng, 2010). In our preliminary study, we found some fish succumbed to death after receiving oral gavage for four or more times, in part, due to possible injury and stress caused by repeated catching and oral gavage. Therefore, in order to be in line with the approved use of DC and for the purpose of animal welfare, the final dosing schedule of once daily for three consecutive days via oral gavage at 20 mg/kg was chosen for the present study. After oral administration, each fish was observed in a separate tank for regurgitation of DC. If DC solution was regurgitated, the fish was removed from the study and replaced.

After 3-day consecutive oral administrations, the blood and tissues (liver, kidney, muscle + skin and gill) were collected from six fish each time point at the following time points, including 0.25, 0.5, 1, 3, 5, 7, 14, 21, 28, 35, 42, 49 and 56 days. Each fish was only bled one time in the study, followed by tissue collection. A 2.5 mL heparinized syringe with 22 G needle was used to draw at least 2 mL blood samples from the tail vessels. Subsequently, the tissues of liver, kidney, muscle + skin and gill were also collected from each fish. The blood samples were centrifuged at 1500 g for 5 min at 4 °C, and plasma was transferred into a new tube. All samples were stored at −20 °C until analysis. All experimental protocols and procedure involving animals were approved by the Fish Ethics Committee of Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China.

2.5. Sample preparation

The preparation procedure of samples was based on the method by Gajda et al. (2014) with some modifications. First, for the plasma samples after thawing at room temperature, 1 mL subsamples were pipetted into 10 mL polypropylene tubes, and then 10 μL internal standard solution was added into each tube. The samples were subjected to two sequential extractions using 5 mL of EDTA-Mcllvaine buffer (0.04 mol/L sodium dihydrogen phosphate, 0.06 mol/L citric acid monohydrate, and 0.1 mol/L EDTA-Na_2, pH = 4.0). During each extraction, the mixture was shaken for 2 min, and then centrifuged for 5 min at 5000 g at 4 °C. The resulting extractant was pipetted and pooled into another new tube, and then transferred to a polymeric SPE cartridge, which was prepared in advance with 3 mL methanol and 3 mL pure water. After the percolation of the whole solution, the columns were washed with 3 mL water. The DC was eluted with 5 mL methanol. The extracts were concentrated using nitrogen stream at 30 °C up to entirely dry. The dry residues were reconstituted by 1 mL of 5% methanol in water (0.1% formic acid) and filtered through 0.22 μm filters. A total of 10 μL sample was used for LC-MS/MS analysis. For the preparation of tissues samples, 2 g of muscle + skin and 1 g of liver, kidney and gill samples after homogenization and thawing were respectively extracted based on the method of plasma sample preparation described above.

2.6. LC-MS/MS analysis

In this study, all samples were analyzed by an LC–MS/MS system consisted of a triple quadrupole mass spectrometer, a LC binary pump and an auto-sampler (TSQ Quantum Access MAX, Thermo Fisher, USA). Data were obtained and processed through the Thermo Xcalibur software (Version 2.1.0). A Hypersil Golden (150 mm × 2.1 mm, 3 μm) with proper column temperature at 30 °C was used to separate the target chemical. The mobile phase included organic phase A (75% methanol and 25% acetonitrile containing 0.1% formic acid) and aqueous phase B (pure water containing 0.1% formic acid). The gradient elution procedure was performed from 10% A for 1 min, then increased up to 100% within 5 min, and remained at this proportion for 2 min, and subsequently returned to the initial percentage in 0.1 min. The flow rate was 0.2 mL/min and injection volume were 10 μL.

The target chemical was determined at positive mode using the heated electrospray ionization, and the ion source parameters were optimized by monitoring the MS/MS spectra of the analyte. The mode of selective reaction monitoring (SRM) was chosen to monitor corresponding protonated molecular ion for target analyte using a spray voltage of 3500 V, vaporizer temperature of 350 °C, ion transport tube temperature of 350 °C, sheath gas (high purity nitrogen) of 30 psi, auxiliary gas (high purity nitrogen) of 50 arb, collision gas (ultra-high purity argon) pressure of 1.50 mTorr, Q1 peak width of 0.70 amu, Q3 peak width of 0.50 amu, and a scan time of 0.1 s. SRM for DC was m/z 445 for 145 (qualitative) with a collision energy of 23 eV, and m/z 445 to 428 (quantitative) were set at 18 eV. SRM for DC-D_3 was m/z 448 to 431 with a collision energy of 17 eV.

2.7. Validation procedure

The developed determination method of DC in fish plasma, muscle + skin, liver, kidney, and gill was validated based on the guideline of the EU Commission Decision 2002/657/EC (EC, 2002). The specific parameters for validation of the method included the selectivity, linearity, precision, accuracy, the decision limits (CCα) and the detection capability (CCβ).

The limit of detection (LOD) was determined as the concentration that produced the area of the signal 3 times that of the baseline noise; whereas the concentration that produced the ratio of the area of the signal to the baseline noise of 10 was established as the limit of quantification (LOQ) (Zeng et al., 2019). The selectivity of the method was estimated in plasma, muscle + skin, liver, kidney and gill for detecting DC by comparing the LC-MS/MS behavior obtained from the blank samples (n = 10) and the samples fortified with DC standard solution. Possible exogenous and endogenous interferences in samples were avoided through the optimization of the LC separation and purification conditions. Due to the apparent matrix effect in plasma and various tissues for DC's determination, the matrix-matched curves of DC in plasma and tissues were determined by analyzing fortified blank samples with standard solutions at different concentrations of 5, 10, 20, 50, 100, 500, 2000 and 5000 μg/L. All curves were constructed by linear regression of the ratios of chromatographic peak area of the standards versus suitable internal standard with nominal concentrations. The correlation coefficients and the slope were calculated. Such calibration curves were obtained with each series of samples.

The accuracy of the method was assessed by the recovery rate which was determined through analyzing blank samples fortified in six replicates at the levels of 0.5 MRL, MRL and 1.5 MRL (MRL = 100 μg/kg). In order to evaluate the precision of the method, the repeatability values (intra-day precision) were analyzed as the coefficient of variation of measured concentrations of DC by analyzing samples fortified with standard of the analyst at three levels (0.5 MRL, MRL and 1.5 MRL) on the same day with the same instrument and by the same operator. The reproducibility (intermediate precision) results were obtained by determining samples spiked with target compounds using the identical method on three separate days with the same instrument and by the same operator.

The decision limits (CCα) were calculated by determining 20 blank samples spiked with DC at MRL. The detection capability (CCβ) was calculated as the decision limit plus 1.64 times the corresponding standard deviation.

2.8. Withdrawal time analysis

The WT of DC in plasma and all collected tissues were calculated using both the WT 1.4 software developed by EMA (EMA, 2018) and the "reschem" package developed by US FDA (FDA, 2018). The WT is typically calculated only in edible tissues, but in the present study the WT was calculated in all collected tissues and plasma in order to determine the target organ in which the tissue residue depletes the slowest in fish and in order to provide a more comprehensive comparison of the results among different tissues and between the WT 1.4 software and the "reschem" package. The first order rate constant (λ_z) associated with the terminal elimination phase was calculated from the slope of the apparent terminal phase at the last three sampling time points, while the elimination half-life ($T_{1/2\lambda z}$) was calculated as $0.693/\lambda_z$.

3. Results

3.1. Method validation

In this study, the limit of detection (LOD) and limit of quantification (LOQ) of the analytical method were 2.5 and 5 μg/L or μg/kg, respectively in both plasma and tissues. The method had a high selectivity with no interferences, a good linearity (R) of > 0.995, and satisfactory recoveries (70.2–87.8%) (Table 1). The intra and inter-day precisions (CV%) were both lower than 10%. The decision limit (CCα) and the detection capability (CCβ) were from 108.0 to 122.0 μg/kg or μg/L. For samples with concentrations of DC in plasma and tissues over the upper limit of quantification in the initial measurement, the remaining samples were diluted with the corresponding blank plasma or tissue samples, and the measurement was repeated. The results showed that the method was suitable to be used to quantify DC's concentrations in plasma and various tissues.

3.2. WT estimation of DC using the WT 1.4 software and the "reschem" package

The concentrations of DC (mean ± SD) in plasma and tissues at different time points are listed in Table 2 and the residue depletion kinetic curves are shown in Fig. 1. The DC concentration at the first sampling time point of 0.25 day after the last oral administration was the highest both in plasma and tissues when compared to other sampling time points. Afterwards, the concentrations of DC were decreased sharply until day 7. On day 7, DC concentrations were below 1000 μg/kg or μg/L in muscle + skin, kidney, and plasma, but the concentrations were still above 2000 μg/kg in liver and gill. From 14 to 35 days, the depletion of DC in tissues was relatively slower than from 0.25 to 7 days. Additionally, the results showed that the highest concentrations were found in liver in comparison to other tissues and were still detectable up to 49 days (58.9 ± 12.8 μg/kg) when the concentrations were unquantifiable in other tissues. The terminal elimination half-lives were calculated as 6.25, 6.36, 5.14, 4.68 and 5.11 days in muscle + skin, liver, kidney, gill and plasma, respectively in grass carp exposed to DC at 20 mg/kg via oral gavage daily for 3 days.

Table 1
Accuracy and precision of the method for doxycycline in spiked muscle + skin, liver, kidney, gill and plasma of grass carp (*Ctenopharyngodon idella*).

Tissues	Spiked level (μg/kg or μg/L)	Recovery (%)	Within-day RSD (%)	Between-day RSD (%)
Plasma	50	82.2	4.2	5.2
	100	83.4	3.1	4.9
	150	87.8	2.8	3.9
Liver	50	70.2	3.7	5.3
	100	80.4	4.0	6.1
	150	79.9	4.5	7.2
Kidney	50	72.3	3.6	5.8
	100	81.9	2.1	4.2
	150	83.7	2.7	4.9
Gill	50	82.1	4.0	6.3
	100	85.9	3.5	5.7
	150	84.2	2.5	4.6
Muscle + skin	50	82.7	3.0	4.9
	100	85.2	4.4	7.3
	150	85.6	4.6	6.7

RSD, relative standard deviation.

The WT 1.4 and the "reschem" package were respectively used to calculate the WT of DC in muscle + skin, liver, kidney, gill and plasma. The calculated WT values based on different MRL values (100 μg/kg for China and EU vs. 50 μg/kg for Japan) are shown in Table 3. The WT 1.4 software can only analyze a dataset with maximally 7 time points. Therefore, in the present study only a part of each data set was selected for the analysis using WT 1.4. Specifically, we selected the time points of 1, 3, 5, 7, 14, 21 and 49 days for liver, of 1, 3, 5, 7, 14, 21 and 42 days for gill, kidney and muscle + skin, and of 1, 3, 5, 7, 14, 21 and 35 days for plasma because within these time ranges, the residue depletion kinetic profiles were properly captured and the lowest concentration for each tissue was lower than the MRL for the corresponding tissue. By setting the MRL as 100 μg/kg and considering a 95th percentile with a 95% confidence level for Europe and China (Yang et al., 2019a), the calculated WT times using the WT 1.4 were 40.54 days in muscle + skin, 55.28 days in liver, 41.21 days in kidney, 46.34 days in gill and 24.38 days in plasma, which were 41, 56, 42, 47 and 25 days, respectively after rounding to the next whole day (Fig. 2, Table 3).

Using US FDA's "reschem" package by setting the MRL as 100 μg/kg and considering a 95th percentile tolerance limit with a 95% confidence level, the calculated WTs were 41 days in muscle + skin, 56 days in liver, 42 days in kidney, 47 days in gill and 25 days in plasma, respectively (Fig. 3). These results showed that the calculated WT values for plasma and tissues were consistent between using the WT 1.4 and the "reschem" package in the present study. Thus, the WT estimation results using the WT 1.4 software and the FDA's "reschem" package had no considerable discrepancy based on the present datasets. In Japan, the MRL of DC in fish is 50 μg/kg and assuming the tolerance limit is the same as in Europe and China (i.e., 95th percentile), the calculated WTs were 50 days in muscle + skin, 62 days in liver, 48 days in kidney, 53 days in gill and 29 days in plasma (Table 3).

4. Discussion

Antibiotics are used in aquaculture in order to treat bacterial infectious diseases, increase fish production, and decrease economic loses, but their use could also result in drug residue violations in fish products and thus impact human food safety. In order to protect human health, regulatory authorities set up maximum residue limits (MRLs) and withdrawal periods of drugs used in food animals (FDA, 2010). MRL is an effective red line of protection to guarantee food safety. For the sake of ensuring drug residues not exceeding the MRLs in animal-derived food products, it is important to conduct studies to determine appropriate withdrawal times for different drugs at different therapeutic regimens. In this study, the tissue residue depletion kinetics of DC and withdrawal time of DC in grass carp after 3 daily oral administrations at 20 mg/kg were determined. The major findings were: (1) liver had the highest concentrations and the slowest depletion rate of DC among all collected tissues; and (2) the calculated WT values of DC were 41 days for Europe and China and 50 days for Japan in grass carp after 3 daily oral dosing at 20 mg/kg.

Available absorption and disposition studies of DC have demonstrated that DC is efficiently and rapidly absorbed by the intestine following oral administration and has a relatively longer plasma half-life (15–22 h) and more lipid-soluble than other analogues (EMA, 1996; Rigos et al., 2002a; Rigos et al., 2003; Yang et al., 2014; Yang et al., 2016). The present study suggests that upon absorption DC is extensively distributed in the body with the highest concentrations in

Table 2
The concentrations of doxycycline in muscle + skin, liver, kidney, gill and plasma of grass carp (*Ctenopharyngodon idella*) after daily oral administration at 20 mg/kg for 3 days.

Time (d)	Muscle + skin (μg/kg)	Liver(μg/kg)	Kidney(μg/kg)	Gill(μg/kg)	Plasma(μg/L)
0.25	3833.1 ± 684.5	54849.8 ± 6937.3	15039.2 ± 897.2	32172.9 ± 1257.4	5367.3 ± 586.0
0.5	3251.6 ± 229.0	53771.3 ± 1231.8	9058.8 ± 908.8	28729.3 ± 982.8	3293.3 ± 908.9
1.0	2071.2 ± 632.6	51492.5 ± 4032.6	5428.9 ± 739.4	18334.7 ± 667.6	3189 ± 143.2
3.0	1485.9 ± 113.2	31681.7 ± 1322.7	3278.1 ± 156.2	7895.1 ± 717.4	2508.3 ± 114.8
5.0	1015.8 ± 190.6	9687.0 ± 1578.8	1864.6 ± 164.5	5279.3 ± 325.1	1528.7 ± 68.6
7.0	854.1 ± 124.5	3596.5 ± 890.2	678.1 ± 98.5	2773.7 ± 199.7	854.2 ± 58.4
14.0	679.0 ± 78.9	1552.8 ± 127.4	437.6 ± 47.2	2031.8 ± 107.8	367.6 ± 33.2
21.0	348.1 ± 46.4	562.2 ± 48.3	332.9 ± 67.6	717.9 ± 54.2	71.5 ± 28.7
28.0	235.3 ± 22.4	503.9 ± 64.4	244.4 ± 40.6	682.4 ± 173.8	48.1 ± 8.3
35.0	187.2 ± 22.0	270.8 ± 49.6	61.3 ± 26.3	229.8 ± 84.1	10.7 ± 1.5
42.0	49.9 ± 5.1	185.8 ± 34.8	37.0 ± 7.1	85.8 ± 17.7	< LOQ
49.0	< LOQ	58.9 ± 12.8	< LOQ	< LOQ	< LOQ
56.0	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

< LOQ: below limit of quantification of 5 μg/kg or μg/L.

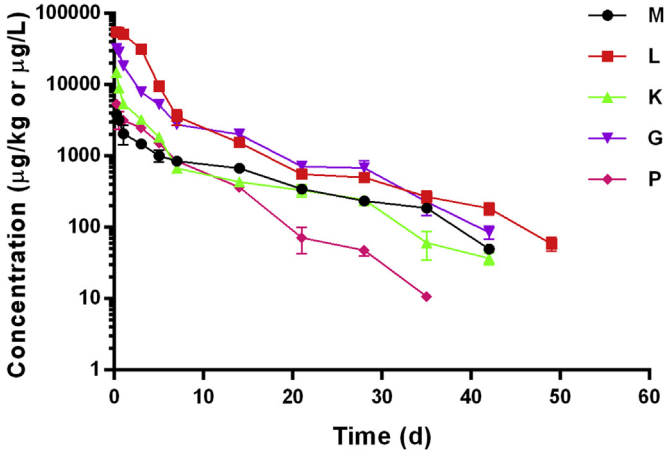

Fig. 1. Residue depletion kinetic curves of doxycycline in muscle + skin (M), liver (L), kidney (K), gill (G) and plasma (P) of grass carp (*Ctenopharyngodon idella*) after daily oral administration at 20 mg/kg for 3 days.

Table 3
Calculated withdrawal times of doxycycline in muscle + skin, liver, kidney, gill and plasma of grass carp (*Ctenopharyngodon idella*) after daily oral administrations at 20 mg/kg for 3 days based on different regulatory standards.

Tissues and plasma	Europe and China	Japan
Muscle + skin	41	50
Liver	56	62
Kidney	42	48
Gill	47	53
Plasma	25	29

Note: In Europe and China, the maximum residue limit (MRL) of doxycycline is 100 μg/kg in muscle + skin of fish. There are no MRL values for plasma and other tissues. As a result, the MRL of 100 μg/kg was used in the calculation of withdrawal times for plasma and other tissues. The withdrawal time was calculated using the WT 1.4 software with the tolerance limit of 95th percentile with a 95% confidence level.
In Japan, the MRL of doxycycline is 50 μg/kg in muscle + skin of fish. There were no MRL values for plasma and other tissues, and the MRL of 50 μg/kg was also used for plasma and other tissues. The withdrawal time was estimated by the WT 1.4 with 95th percentile with a 95% confidence level.

liver. This finding is similar to earlier studies in broiler chickens following oral administration at 10 mg/kg for 5 days (Gajda et al., 2014) and drinking water exposure at about 35–40 mg/kg for 7 days (Hsiao et al., 2016). It has been reported that DC has a high affinity for bile and exhibits cyclic discontinuous biliary excretion, which results in strong enterohepatic recycling of DC (Fabre et al., 1971; Pedersen and Miller, 1980). The enterohepatic recirculation of DC could, in part, contribute to the observed highest concentration of DC in the liver compared to other tissues in the present study.

Except the liver, our results showed that gill had higher concentrations of DC than kidney, plasma, and muscle + skin. At the first sampling time point of 0.25 day, the concentrations of DC in gill were about 8.4-fold of the concentrations in muscle + skin. After 42 days post dosing, the concentrations of DC in gill were still detectable and were higher than in muscle + skin and kidney. The function of gill in fish is equivalent to the organ of lungs in poultry and mammals. Fish have to take waters into the buccal cavity and expels it through the gill slit in order to obtain adequate oxygen. Previous studies have shown that DC is largely excreted in feces (Schach Von Wittenau and Twomey, 1971; EMA, 1996). Thus, the observed higher concentrations of DC up to 42 days post exposure could be, in part, because the fish excreted DC through feces, and DC in feces was re-dissolved in water, and then DC in water was further absorbed through the gill into the fish body.

Based on the MRL of 100 μg/kg in finfish in China and EU (EU, 2015; MAA, 2017), the calculated WT values of DC were 41 days for muscle + skin, 56 days for liver, 42 days for kidney, 47 days for gill, and 25 days for plasma. These values are far longer than DC's analogues in other aquatic species. For example, the calculated withdrawal time was 0 day in fillet tissues of juvenile pike (*Esox lucius*) and walleye (*Stizostedion vitreum*) with mean weight under 200 g fed with oxytetracycline (OTC)-medicated diets (82.7 mg OTC-HCl/kg fish/day) for 10 consecutive days (Bernardy et al., 2003). Nogueira-Lima et al. (2006) reported that OTC was undetectable after 25 days in cultivated marine shrimp (*Penaeus vannamei* Boone, 1931) following treatment with medicated feed containing 4 g OTC/kg for 14 days and its WT value was less than 25 days. Paschoal et al. (2012) also estimated a WT of 6 days for OTC in tilapia (*Orechromis niloticus*) fillets following oral administration via feed at a dose of 80 mg/kg for 5 consecutive days. The difference in the withdrawal time between DC and OTC may be primarily because DC is more lipophilic and possesses better permeability through body fluids and tissues than other tetracyclines (Riviere and Papich, 2018). Its stronger lipophilic feature may lead to better bioavailability in animals and longer persistence in the body than OTC. A second reason could be due to the difference in the drug administration method. In the present study, DC could be absorbed more easily by the gastrointestinal tract because DC without mixing with feed was administered to fish (Purkins et al., 2003; Xu et al., 2018). Through the delivery of the drug by oral gavage, a bolus dose of DC entered the fish intestine directly, thus the delivered amount of DC was high compared to feeding DC in formulated rations. Additionally, oral gavage of DC prevented from treatment competition among the fish population and prevented from distributing the DC amount homogeneously into the

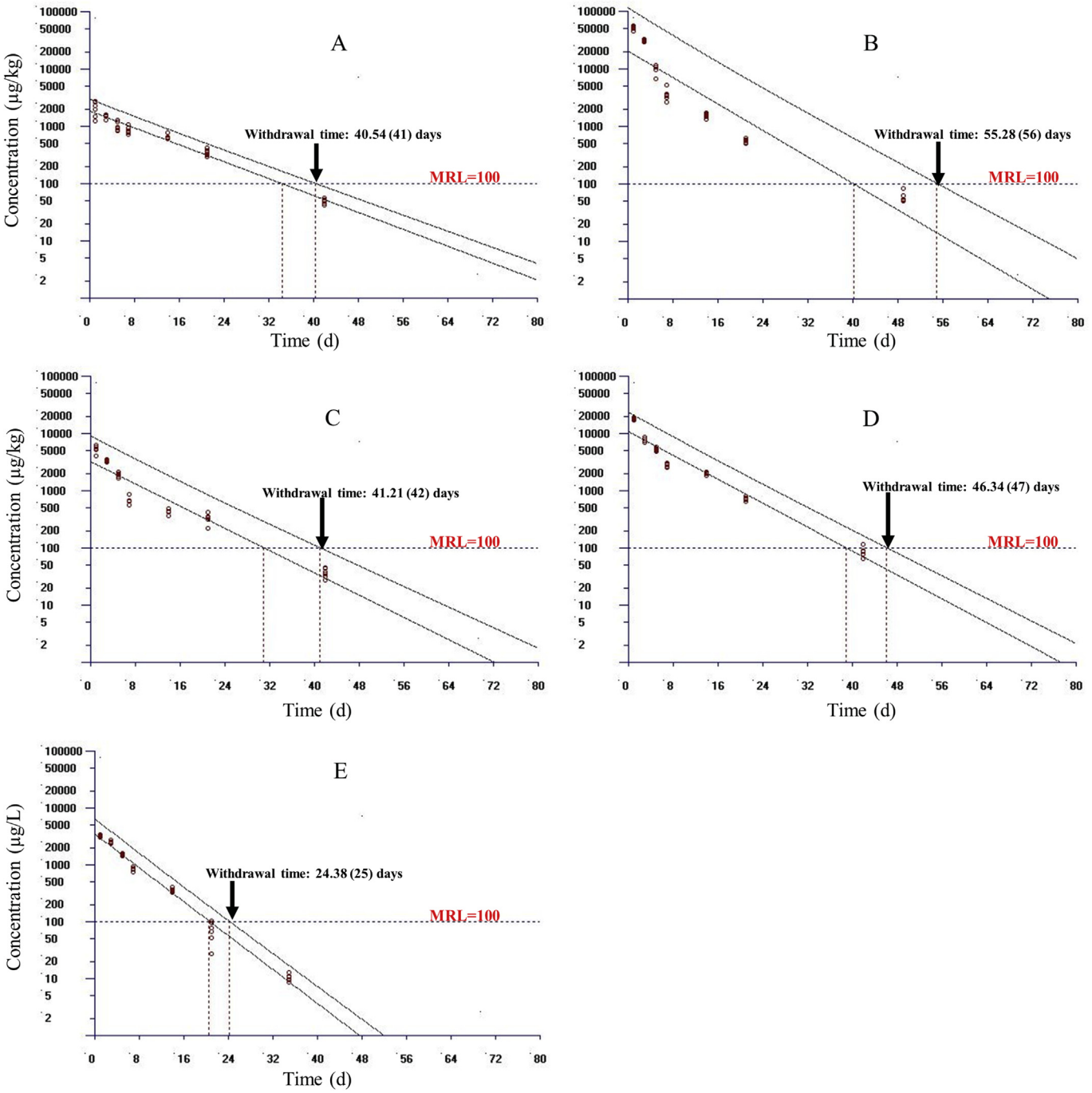

Fig. 2. Estimated withdrawal times for doxycycline in grass carp (*Ctenopharyngodon idella*) after oral administrations at 20 mg/kg for 3 days for Europe and China based on the EMA method using the WT 1.4 software (A for muscle + skin, B for liver, C for kidney, D for gill, and E for plasma). MRL: maximum residue limits for doxycycline from the European Medicines Agency (EMA, 2018). If the calculated withdrawal time was a fraction of a day, the estimated withdrawal time was rounded up to the next whole day shown in the parenthesis.

treated fish population. Thirdly, Paschoal et al. (2012) reported that the fish size also affected the depletion rate of tetracyclines, and that the younger and lighter fish had a higher metabolism rate for the given drug. Finally, the absorption and excretion rates of orally administered drugs in fish are also impacted depending on whether the fish has a stomach. Takii et al. (1997) evaluated the digestive and absorptive function by feeding test diets with an index substance of Cr_2O_3 in tiger puffer *Takifugu rubripes* that have no stomach and in red sea bream *Pagrus major* that have a stomach. In the tiger puffer, small amounts of fecal Cr_2O_3 were detected until 72 h post-feeding, while red sea bream excreted most of fecal Cr_2O_3 within 24 h after oral treatment with a peak at 8–12 h after feeding, indicating that tiger puffer processes digestion and absorption more slowly than red sea bream, in part, because tiger puffer do not have a stomach. As grass carp do not have a stomach, whether the present result can be extrapolated to other fish species that have a stomach remains to be investigated.

The calculated terminal elimination half-lives were 6.25, 6.36, 5.14, 4.68 and 5.11 days (150, 152.64, 123.36, 112.32, and 122.64 h) in muscle + skin, liver, kidney, gill and plasma, respectively in the present study. These values are greater than the recently reported terminal elimination half-lives for DC in grass carp after a single oral dose of 20 mg/kg at 24 °C, which were 24.22, 17.93, 37.56, 23.68, and 20.10 h

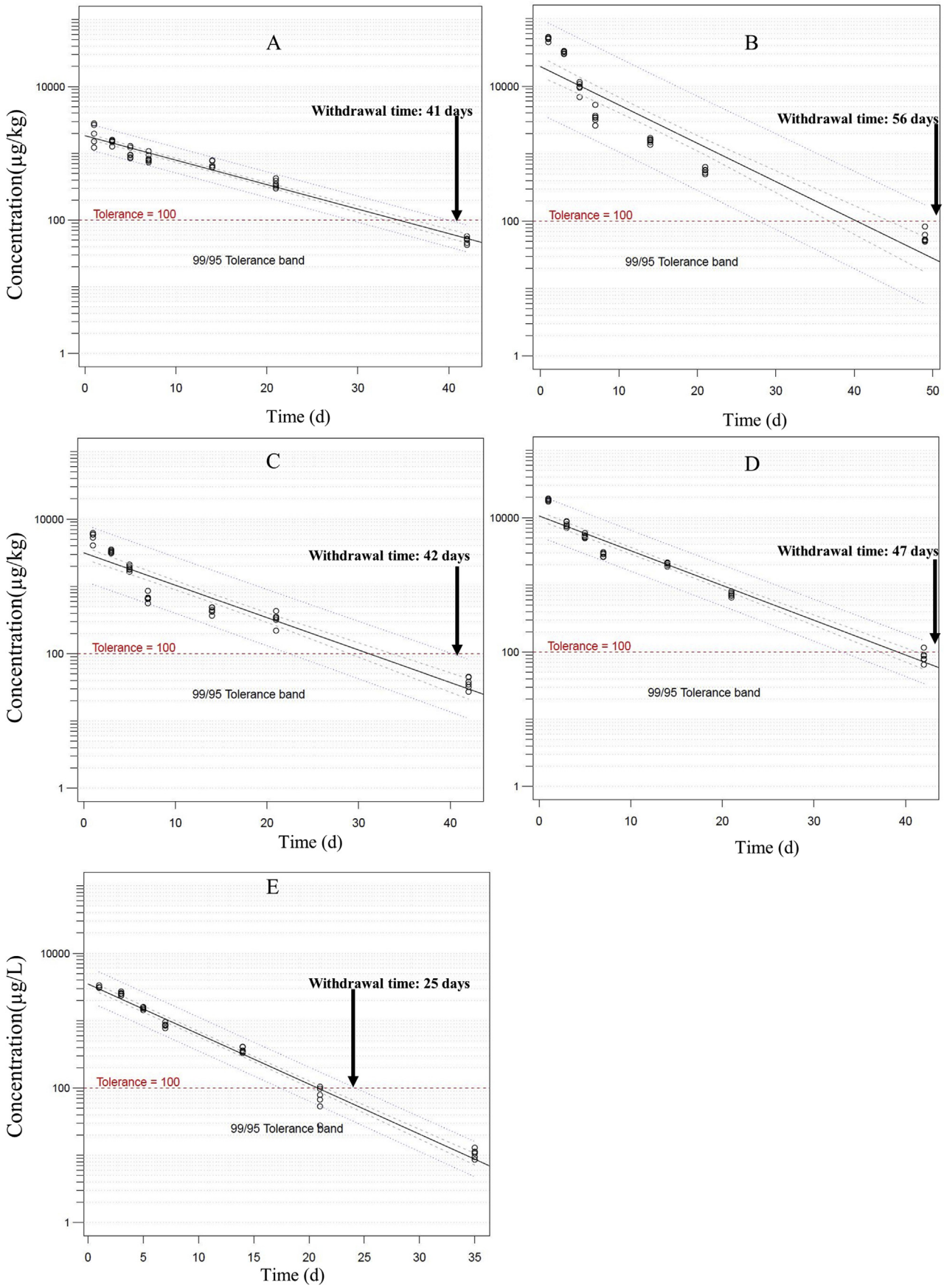

Fig. 3. Estimated withdrawal times for doxycycline in grass carp (*Ctenopharyngodon idella*) after oral administrations at 20 mg/kg for 3 days for Europe and China using FDA's tolerance limit method coded in the "rescheme" package (A for muscle + skin, B for liver, C for kidney, D for gill, and E for plasma). The withdrawal time was calculated based on the maximum residue limit (MRL or termed tolerance in the US) of 100 μg/kg for DC in fish plasma and tissues with a tolerance limit of 95th percentile with a 95% confidence level.

in muscle + skin, liver, kidney, gill and plasma, respectively (Xu et al., 2019). Note that in this earlier study (Xu et al., 2019), the terminal slopes were calculated using Phoenix's "Best Fit" calculation method to automatically select the best time points based on observed data collected from 0.08 to 144 h, where in the present study the terminal slopes were consistently calculated based on the last three quantifiable time points from 21 to 49 days. These results suggest that terminal elimination half-lives are highly dependent on the sampling time points, and could vary by several fold for lipophilic chemicals like DC depending on whether data are collected during the earlier kinetic phase or the very terminal kinetic phase.

In the literatures, while there are few tissue residue depletion studies of DC in fish, there are some in poultry and swine. Croubels et al. (1998) reported that the WT was estimated to be 3 days in muscle of pigs following continuous drinking water exposure for 5 days at a dose of 10.5 mg/kg/day. In turkey, the WT was estimated to be 12 days in liver and 17 days in muscle after receiving drinking water exposure at the dose of 25 mg/kg for 4 days under field conditions (Croubelsa et al., 1998). In eggs, DC could be detected up to 12 days after drinking water exposure of laying hens for 5 days at the dose level of 10 mg/kg/day (Gajda and Posyniak, 2015). It was reported that DC's concentrations in egg white were decreased rapidly and a higher DC concentration existed in yolk than in egg white, which proved that DC's lipophilic property results in preferential distribution to yolk that has higher proportion of fat (Gajda and Posyniak, 2015). Additionally, Anadon et al. (2012) studied the WT of another analogue of DC, chlortetracycline, in fattening chickens after multiple oral doses at 60 mg/kg for 5 days. The results showed that the drug concentrations in liver and muscle were decreased to be below LOQ (1 μg/kg) at 3 and 5 days, respectively after cessation of medication, suggesting that at least 6-day WT was needed to ensure food safety. Compared to the reported WT values of DC or chlortetracycline in poultry or swine, the calculated WT value for DC in muscle of fish in this study is far longer than that in poultry and swine. This may be because poultry and mammals have faster elimination rates of DC than fish due to the fact that fish belong to heterothermic animals possessing lower efficiency of drug metabolic enzymes than that in poultry and mammals (Saito et al., 2001; Smith et al., 2010).

In this study, the WT 1.4 (EMA, 2018) and the "reschem" package (FDA, 2018) were employed to calculate the WT values in plasma and tissues. The WT 1.4 is relatively easy to use, and it only requires users to import a dataset with maximally 7 time points. The MRL (100 μg/kg) and the percentile of 95th or 99th with 95% of confidence can be set in the corresponding dialog. Users can simply click the button of "Analyze" to analyze the data, and then the calculated WT value and the residue depletion plot will be shown on the software interface. However, the "reschem" package is relatively difficult to use because the package is based on R language, which requires some familiarity of the R programming environment. The calculated WT values based on the present data using the two software tools were consistent (the same when rounding to the next whole day) for both plasma and tissues. However, another recent study from our group showed that the calculated WT values using WT 1.4 vs. "reschem" package could be different by 1–3 days depending on the number of animals per time point, the variability of the data, and the depletion rates of the drug in different tissues (Lin et al., 2019).

Although the WTs of DC were estimated by the present tissue residue depletion data in grass carp using the tolerance limit method, the results are limited to the species of grass carp and cannot be extrapolated to other species because tolerance limit method is a statistical method, not a physiologically-based mechanistic method. In this regard, multiple studies have shown that physiologically based pharmacokinetic (PBPK) models are a robust approach that can be used to predict tissue residues and withdrawal times of drugs in food animals, and can be extrapolated from one species to another by incorporating species-specific physiological information (Lin et al., 2016; Henri et al., 2017; Li et al., 2017; Yang et al., 2019a). The present tissue residue depletion data can be used help develop a PBPK model for DC in grass carp, which may be extrapolated to predict WTs in other fish species.

This study has some limitations. The number of animals sampled at each time point was 6 in this study, but the general recommendation from EMA is that a sufficient number of fish (15–20) be used to obtain at least 10 composite samples at each sampling time point for the tissue residue study (EMA, 2009). Thus, the present result should be considered as preliminary recommendation of the WTs of DC in fish in different countries. Future studies using a larger number of animals per time point that meet the EMA study guidelines are needed in order to provide a more conclusive recommendation. Also, the present study used a forced-administrated approach of oral gavage to deliver DC, rather than the more commonly used commercial route of administration for medicated feeds to fish in order to give a precise amount of the drug. In the actual practice, DC is administered via dietary exposure. Therefore, it is also necessary to conduct pharmacokinetic tissue residue depletion studies of DC in fish after dietary exposure and compare the potential differences in the calculated WTs between oral gavage and dietary exposure (Teles et al., 2016; Feng et al., 2019).

5. Conclusions

In the present study, the tissue residue depletion profiles of DC were determined in grass carp following daily oral administrations at a dose of 20 mg/kg for 3 days at 24 °C. The WT values in plasma and tissues were calculated using the WT 1.4 software and the "reschem" package based on different regulatory standards in different countries (EU vs. China vs. Japan). The results suggest that a WT of 41 days for Europe and China and 50 days for Japan is needed for DC at 20 mg/kg after 3 daily oral dosing in grass carp. Our analyses also suggest that the calculated WT values based on the same data using the WT 1.4 software vs. the FDA's "reschem" package are similar. Overall, the present study improves our understanding of the tissue residue depletion kinetics of DC after repeated oral exposure in fish, and the results provide insight into the regulatory decision on the proper withdrawal period of DC in fish under different regulatory standards for different countries to ensure fish-derived food safety.

Author contributions

Ning Xu and Xiaohui Ai conceived and designed the animal study. Ning Xu drafted the manuscript. Yu Fu and Xiaomei Zhang were responsible for rearing fish, collecting samples and analyzing the samples. Miao Li, Ning Xu, and Zhoumeng Lin contributed to the withdrawal time analysis using the "reschem" package and the WT 1.4 software. Zhoumeng Lin provided the facilities to conduct the analysis, designed the withdrawal time analysis plan, coordinated the project, and helped in writing the manuscript. All authors have read and approved the final manuscript.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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