DOI: 10.1111/are.13439

ORIGINAL ARTICLE

WILEY Aquaculture Research

Depletion study and estimation of withdrawal periods for florfenicol and florfenicol amine in pacu (*Piaractus mesopotamicus*)

Tamires Valim Marques[1] | Jonas Augusto Rizzato Paschoal[2] | Rafael Simões Coelho Barone[3] | José Eurico Possebon Cyrino[3] | Susanne Rath[1]

[1]Institute of Chemistry, University of Campinas, Campinas, Brazil

[2]School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil

[3]Departamento de Zootecnia, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, Brazil

Correspondence
S Rath, Institute of Chemistry, Department of Analytical Chemistry, University of Campinas, Campinas, Brazil.
Email: Susanne.rath@gmail.com

Funding information
Fundação de Amparo do Estado de São Paulo, Grant/Award Number: 2013/09543-7

Abstract

The intensive production of farmed fish is at a global all-time high, and the control of bacteria proliferation in fish farms requires the frequent use of antimicrobials. This practice raises important environmental concerns related to the emergence of antimicrobial-resistant bacteria strains. Only a few antimicrobial drugs have been approved for use in aquaculture, one of which is florfenicol. This work studies the depletion and withdrawal period of florfenicol and its main metabolite, florfenicol amine, in pacu (*Piaractus mesopotamicus*), a neotropical characin widely farmed in the southern hemisphere. Juvenile pacu (average weight of 724 g) were stocked in a closed-loop laboratory system with controlled water temperature (25.8°C), and were fed for 10 consecutive days with a diet containing an intended dose of 10 mg/florfenicol per kg bw. Muscle and skin tissues were collected at 1, 3, 6, 8, 10, 12 and 16 days post-treatment, and florfenicol and florfenicol amine were quantified using a validated ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method. The limits of quantitation for florfenicol and florfenicol amine were 10 ng/g in muscle and 50 ng/g in skin. Considering a maximum residue limit of 1000 ng/g for the sum of florfenicol and florfenicol amine in muscle with skin in natural proportions a withdrawal period of 5 days (water temperature 25.8°C) or 129 degree days was calculated on the basis of the upper limit of the one-sided 95% confidence interval for the 99th percentile derived from the residue depletion study.

KEYWORDS
fish, florfenicol, pacu, *Piaractus mesopotamicus*, withdrawal period

1 | INTRODUCTION

Brazilian aquaculture has gained prominence in recent years, especially in the last 10 years, with 10% annual growth exceeding the global average of 6% (Kubitza, 2013). According to the Food and Agricultural Organization of the United Nations (FAO), Brazil could become one of the world's largest producers by 2030 (World Bank, 2013). Fish farmers are therefore moving towards the use of intensive systems that on one hand provide increased production, but on the other hand can favour disease outbreaks, necessitating the use of veterinary drugs, in particular antimicrobials, in the management of these systems.

The widespread use of antibiotics for the treatment of bacterial diseases has been linked to the emergence of antibiotic-resistant strains of *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella tarda*, *Edwardsiella ictaluri*, *Vibrio anguillarum*, *Vibrio salmonicida*, *Pasteurella piscicida* and *Yersinia ruckeri* (Canada-Canada, Munoz de la Pena & Espinosa-Mansilla, 2009). Multiple-antibiotic resistant strains

of *Aeromonas hydrophia* have been isolated from the warm freshwater fish species pacu *Piaractus mesopotamicus*) and tilapia (*Oreochromis niloticus*) (Belém-Costa & Cyrino, 2006).

Florfenicol, a broad-spectrum antimicrobial compound structurally related to chloramphenicol, is the only antimicrobial licensed for routine use in Brazilian fish farms as a feed additive (50% w/w florfenicol). The recommended oral dose is 10 mg florfenicol per kg of fish biomass, once daily for 10 consecutive days. Florfenicol has not been toxicologically evaluated by the Joint FAO/WHO Expert Committee on Food Additives. However, the European Agency for the Evaluation of Medicinal Products (Anonymous, 2001) has recommended a maximum residue limit (MRL) for finfish of 1000 μg/kg of the sum of florfenicol and its metabolites measured as florfenicol amine in muscle and skin in natural proportions. Despite the fact that this recommendation was based on a microbiologically acceptable daily intake of 3 μg/kg bw and on results of radiometric studies carried out at 5°C and 10°C using Atlantic salmon (*Salmo salar*) (Anonymous 2001) it has also been adopted for warm freshwater fish in Brazil.

Freshwater colossomid fish, particularly pacu (*P. mesopotamicus*), are widely farmed in many South American countries, with estimated annual production reaching 150,000 tons in Brazil alone (Fontes, Nikolik, Rasmussen & Ikeda, 2016). Pacu has a high carcass lipid content, which is a factor that can directly affect drug metabolism by the species (Paschoal, Quesada, Gonçalves, Cyrino & Reyes, 2013).

Several studies have addressed the depletion of florfenicol residues in tissues of animals such as chicken (Anadón et al., 2008; Chang et al., 2010), swine (Li et al., 2006) and dogs (Park, Lim, Kim, Hwang & Yun, 2008). Published reports on the metabolism of florfenicol in animals in vivo are sparse. A study conducted in veal calves lead to conclude that most of the dosed florfenicol is excreted as the parent drug in urine. Besides florfenicol amine, minor urinary metabolites including florfenicol alcohol, florfenicol oxamic acid and monochloroflorfenicol were identified (Varma, Adams, Powers, Powers & Lamendola, 1986). Since florfenicol amine is the longest lived major metabolite in many species it could be used as the marker residue for withdrawal calculations (Sams, 1994).

In fish, the depletion of florfenicol has been evaluated in Atlantic salmon (Horsberg, Martinsen & Varma, 1994), Rainbow trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata* L.) (Di Salvo, Della Rocca, Terzetti & Malvisi, 2013), olive flounder (*Paralichthys olivaceus*) (Lim et al., 2010), channel catfish (*Ictalurus puncatus*) (Gaunt et al., 2012; Wrzesinski et al., 2006), Nile tilapia (*O. niloticus*) and hybrid tilapia (Feng & Jia, 2009; Feng, Jia & Li, 2008; Gaikowski et al., 2010), walleye (*Stizostedion vitreum*), and hybrid striped bass (*Morone saxatilis*) (Kosoff et al., 2009). The absorption, distribution, metabolism and excretion of ^{14}C-labelled florfenicol in Atlantic salmon was evaluated by Horsberg et al. (1994). The fish were treated with a single dose of 10 mg/kg bw of florfenicol. The fraction of the unchanged florfenicol in muscle decreased from about 90% (6 hr post dose) to about 20% (3 days post dose), while the fraction of florfenicol amine increased from approximately 7 to 70% during the same period. Other metabolites were detected, but were quantitatively of minor importance.

The aim of this study was to establish a residue depletion period for florfenicol and its main metabolite florfenicol amine in pacu, and to estimate the withdrawal period after treatment of fish using florfenicol-medicated feed, considering the MRL adopted by the European Union and Brazil.

2 | MATERIALS AND METHODS

2.1 | Chemicals

The following HPLC grade solvents and analytical grade reagents were used: acetonitrile (Panreac, Germany), methanol (Tedia, USA), formic acid (98%; Synth, Brazil), glacial acetic acid (99.7%; Synth, Brazil), anhydrous magnesium sulphate (98.0%; Vetec, Brazil) and sodium acetate trihydrate (99.5%; Sigma-Aldrich, Germany). Octadecyl (C_{18}) sorbent with particle sizes of 50 μm and 60 Å (Agilent, USA) was used in the sample preparation procedure. The standards used were florfenicol amine (99.8%; Fluka, Germany), florfenicol (98.0%; Fluka, China), florfenicol-d_3 (TRC, Canada) and thiamphenicol (97.5%; Sigma Aldrich, Germany). The water used to prepare the solutions was obtained from a Milli-Q system (Millipore, USA). Prior to the UPLC analyses, all the samples were filtered through 0.22 μm PVDF filters (Millipore, USA).

2.2 | Standard solutions

Stock standard solutions (1.0 mg/ml) of florfenicol, florfenicol amine and thiamphenicol were prepared based on purity of the standard materials in methanol. All solutions were stored at 4°C for a maximum of 6 months. Intermediate standard solutions (100 μg/ml) were prepared by dilution of the stock solutions in water:methanol (95:5 v/v). Working standard solutions were prepared daily at the concentration range from 5 to 30 μg/ml.

2.3 | Medicated feed

Florfenicol was incorporated in a commercial aquafeed (Nutripeixe; Purina do Brasil Ltda., Paulínia, São Paulo, Brazil). The feed composition is shown in Table 1. In this laboratory procedure, 10 g of FF-50 premix (50% w/w florfenicol; FAV Veterinary Pharmacology, Recife, Pernambuco, Brazil) was mixed into 55 ml of vegetable oil and evenly spread over the surface of a 70 L plastic bag, to which 5 kg of feed was added. The bag was agitated until the feed pellets were coated with a homogeneous oily film. The procedure was repeated three further times, yielding 20 kg of medicated feed. The final florfenicol concentration in the medicated aquafeed was determined by ultra-high performance liquid chromatography with photodiode array detection (UPLC-DAD).

2.4 | Animals and experimental design

One hundred and fifty male juvenile pacu (*P. mesopotamicus*), with average weight of approximately 724 g, were randomly stocked in

TABLE 1 Composition of the commercial feed used to prepare the medicated feed

Fattening Feed Nutripeixe Purina®	
Composition per 100 g	
Humidity (g)	13
Crude protein (g)	24
Ethereal extract (g)	4
Fibre (g)	10
Ashes (g)	14
Calcium (g)	2.5
Phosphorus (g)	1
Mineral enrichment	
Magnesium (mg)	700
Iron (mg)	100
Copper (mg)	15
Zinc (mg)	200
Manganese (mg)	30
Iodine (mg)	1
Selenium (mg)	0.3
Vitamin enrichment	
Vitamin A (UI)	9000
Vitamin D_3 (UI)	3000
Vitamin E (UI)	112
Vitamin K (UI)	7.5
Folic acid (mg)	7.5
Biotin (mg)	0.6
Colin (mg)	500
Niacin (mg)	112
Calcium pantothenate (mg)	37
Thiamine (mg)	22
Riboflavin (mg)	22
Piridoxina (mg)	22
Vitamin (B_{12}) (mcg)	26
Vitamin C (mg)	150

ten 0.8 m^3 plastic tanks (15 fish per tank), in a continuously aerated closed loop system, and allowed to acclimatize for 30 days at 25.8°C. Two daily meals (at 9 a.m. and 5 p.m.) were provided, using a commercial non-medicated feed. After acclimatization, the fish were pooled and 135 specimens were randomly selected, stocked in nine tanks and fed for 10 consecutive days with the medicated feed, at 9.7 mg florfenicol per kg bw. The remaining 15 fish, used as a control, were provided with non-medicated feed for 10 consecutive days. Two thirds of the total daily dose of florfenicol was administered in the first meal, with the remaining third provided in the second meal. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the University of Campinas, Brazil (protocol #2015-39).

After the treatment period, the fish were again fed the commercial non-medicated feed in two daily meals (1.5% bw, w/w). At 1, 3, 6, 8, 10 and 12 days, one tank was randomly chosen and 10 fish were collected, euthanized by immersion in a saturated solution of benzocaine (500 mg/L), and laparotomized for sampling of muscle and skin tissue. Samples were packed in identified plastic bags and stored frozen (at −18°C) for no longer than 45 days prior to analysis.

2.5 | Water quality parameters

Water temperature, pH, conductivity, dissolved oxygen and ammonia concentration were monitored during all phases of the trials. The values obtained were (average value ± standard deviation): temperature 25.8 ± 0.3°C; pH 7.6 ± 0.2; conductivity 4.3 ± 0.4 mS/cm; and dissolved oxygen 4.1 ± 0.5 mg/L. The total ammonium concentration was always lower than 0.5 mg/L.

2.6 | Analytical method and validation

Florfenicol in the feed was determined using UPLC-DAD. Florfenicol and florfenicol amine in the fish tissues were determined using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). A typical chromatogram using the selective reaction monitoring mode is shown in Figure 1.

2.7 | Florfenicol solid-liquid extraction from feed

A 3 g portion of homogenized medicated feed was weighed (to the nearest 0.1 mg) into a 50 ml Falcon tube, followed by addition of 150 μl of the thiamphenicol internal standard (20 mg/ml) and 10 mL of methanol. The mixture was vortexed for 1 min and centrifuged at 1,056 *g* for 5 min. The supernatant was collected and the extraction procedure was repeated. The supernatants were combined and an aliquot of 5 ml was transferred to a volumetric flask, with the volume made up to 50 ml with water. Finally, 1 ml of the diluted solution was filtered through a 0.22 μm PVDF membrane filter into a vial, and 3 μl was injected into the UPLC-DAD system.

2.8 | Extraction of florfenicol and florfenicol amine from fish muscle using QuEChERS

A small portion of fish muscle was homogenized using an Ultra-Turrax® and 3.0 g was weighed (to the nearest 0.1 mg) into a 50 ml polypropylene tube. The internal standard (florfenicol-d_3) was added to a concentration of 0.6 μg/g and the sample was vigorously vortexed for 15 s. A 10 ml aliquot of acetonitrile containing 1.0% (v/v) acetic acid was added and the tube was agitated for an additional 1 min. Magnesium sulphate (8.0 g) and sodium acetate (2.0 g) were added to the mixture, followed by vortexing for 1 min and centrifuging at 1,056 *g* for 5 min. A 5.0 ml aliquot of the supernatant was transferred to a polypropylene tube containing 750 mg of magnesium sulphate and 125 mg of C_{18}. The tube was immediately shaken vigorously for 1 min and centrifuged at 1,056 *g* for 5 min. The supernatant was removed using a flow of N_2, and the residue was

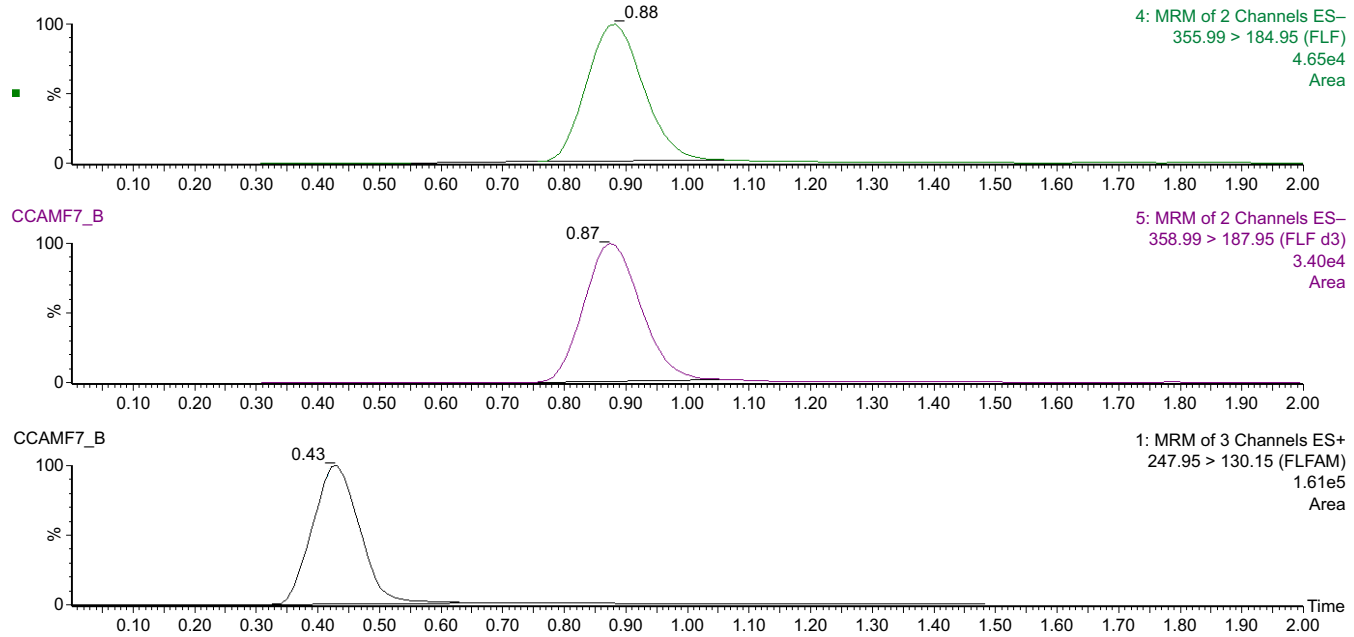

FIGURE 1 Selective reaction monitoring chromatogram of a fortified blank fish muscle sample fortified (at 1,000 ng/g) with florfenicol (green), florfenicol-d3 (purple) and florfenicol amine (black) [Colour figure can be viewed at wileyonlinelibrary.com]

resuspended in 750 μl of the mobile phase. The extract was then filtered through a 0.22 μm PVDF syringe filter before injection into the UPLC-MS/MS system.

2.9 | Extraction of florfenicol and florfenicol amine from fish skin

A random portion of skin was separated from the fish muscle and was homogenized using an Ultra-Turrax® and dry ice. A 2.0 g aliquot of the homogenate was weighed out (to the nearest 0.1 mg) and was transferred to a 50 ml polypropylene tube. The internal standard (florfenicol-d_3) was added to a concentration of 0.6 μg/g, and the sample was vigorously vortexed for 15 s. The preparation procedure was the same as that employed for the muscle samples, except that here the extract was resuspended in 500 μl of mobile phase. The extract was then filtered through a 0.22 μm PVDF syringe filter before injection into the UPLC-MS/MS system.

2.10 | Determination of florfenicol in the feed by UPLC-DAD

Florfenicol was determined using an Acquity UPLC™ liquid chromatograph (Waters, USA) equipped with a binary pump, an autosampler, a column manager, and a diode array detector. The chromatographic separation of florfenicol and the internal standard (thiamphenicol) was achieved using an Acquity UPLC™ BEH C_{18} column (2.1 × 50 mm; 1.7 μm) (Waters, Ireland) and a guard column containing the same stationary phase, kept at 40°C. The mobile phase was a mixture of water (eluent A) and methanol (eluent B), with the following gradient: A:B 80:20 v/v to A:B 40:60 v/v (0–0.5 min); A:B 40:60 v/v to A:B 80:20 v/v (0.5–2.0 min); and maintaining A:B 80:20 v/v (2–4.0 min). The flow rate and injection volume were 0.30 ml/min and 3 μl respectively. Florfenicol was quantified using a matrix-matched calibration curve constructed using blank feed fortified with florfenicol at concentrations of 0.4, 0.6, 1.0, 1.4 and 1.8 mg/g, with thiamphenicol as internal standard (1.0 mg/g). The method was validated and presented a linear range for florfenicol from 0.4 to 1.8 mg/g, linearity higher than 0.99, intra-day precision of 0.6% (n = 3), and accuracy of 105%.

2.11 | Determination of florfenicol and florfenicol amine in fish tissues by UPLC-MS/MS

Florfenicol and florfenicol amine were quantified and their identities were confirmed using an Acquity UPLC™ IClass system (Waters, USA) coupled to a Waters triple quadrupole mass spectrometer (Xevo TQD Zspray) equipped with an electrospray ionization (ESI) source operating in positive and negative mode. The conditions for the positive mode (ESI+) were: desolvation gas flow of 650 L/hr, capillary voltage of 3.5 kV, desolvation gas temperature of 500°C and source temperature of 150°C. The conditions for the negative mode (ESI-) were the same as described for ESI+, with the exception of the desolvation gas flow, which was 1000 L/hr. Table 2 shows the optimal collision energy and cone voltage values, together with the quantification and confirmation ions. Quantitation was carried out in selective reaction monitoring (SRM) mode, and all the analyses were carried out in duplicate. Separation of florfenicol, florfenicol amine and the internal standard (florfenicol-d_3) was achieved using an Acquity UPLC™ BEH C_{18} column (2.1 × 50 mm; 1.7 μm) (Waters, Ireland) and a guard column (5 × 2.1 mm) containing the same stationary phase, kept at 40°C. The mobile phase was a 65:35 (v/v) mixture of aqueous 0.1% formic acid and methanol containing

TABLE 2 Mass spectrometry conditions used for quantitation and identity confirmation

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Retention time (min)	Cone voltage (V)	Collision energy (V)
Florfenicol	355.99	184.95 335.5	0.88	30	20
Florfenicol-d3	358.99	187.95 338.5	0.88	30	20
Florfenicol amine	247.95	104.05 130.15	0.42	30	20

0.1% formic acid. The flow rate and injection volume were 0.35 ml/min and 2 μl respectively. Data were acquired using MassLynx 4.1 software (Waters, USA).

2.12 | Validation of the analytical methods for quantitation of florfenicol and florfenicol amine in fish muscles and skin

The methods were validated in-house, considering the following performance parameters: linearity and linear range, intra- and inter-day precisions, limit of detection (LOD), limit of quantitation (LOQ) and accuracy.

The linear range and linearity were obtained from matrix-matched calibration curves. For this purpose, blank fish muscle was fortified with florfenicol and florfenicol amine at eight concentration levels (0.025, 0.050, 0.100, 0.400, 0.600, 0.800, 1.000 and 2.500 μg/g) and skin was fortified at seven levels (0.050, 0.100, 0.400, 0.600, 0.800, 1,000 and 1.500 μg/g), both with the surrogate florfenicol-d_3 at a concentration of 0.6 μg/g. The linearities were expressed as the linear correlation coefficients (r).

Intra-day precision was evaluated from the results obtained on the same day for four replicates ($n = 4$), using blank fish muscle samples fortified at 0.050, 0.600 and 1.00 μg/g, and blank fish skin samples fortified at 0.050, 0.600 and 1.000 μg/g for each analyte. Florfenicol-d_3 was used as surrogate. For the inter-day evaluation, the analyses were repeated over two more days, in duplicate. All the precisions were expressed as the coefficient of variation.

The limits of detection (LOD) and quantitation (LOQ) were determined by analyses of blank fish tissues fortified with florfenicol and florfenicol amine in decreasing concentrations until signal-to-noise ratios of 3 and 10, respectively, were achieved.

The accuracy of the method was evaluated using a recovery test. For this purpose, blank fish muscle was fortified at 0.050, 0.600 and 1.00 μg/g, and skin was fortified at 0.050, 0.600 and 1.000 μg/g, using four replicates for each analyte.

2.13 | Determination of the withdrawal period

The statistical approach recommended by the European Agency for the Evaluation of Medicinal Products (Anonymous, 1995) was strictly followed to establish the withdrawal period for florfenicol in pacu (muscle with skin, in natural proportions). It was considered that the elimination phase of the drug from the tissues followed a one-compartment model. Results below the LOQ of the method were taken as half the LOQ. Data for which most values of the sum of florfenicol and florfenicol amine were below the LOD of the method were excluded.

The homogeneity of variances was evaluated using Cochran's test, and the normality of errors was assessed by plotting the ordered residuals against their cumulative frequency distribution on a normal probability scale. The withdrawal period was estimated from linear regression analysis of the log-transformed tissue concentrations and was determined as the time when the 99% upper one-sided tolerance limit (95% confidence level) was below the MRL.

3 | RESULTS

3.1 | Analytical methods

The sample preparation procedure required use of the QuEChERS approach (Anastassiades, Lehotay, Stajnbaher and Schenck (2003), which consists of an extraction step with acetonitrile followed by a subsequent liquid-liquid partitioning of residues by the addition of salts and subsequent clean-up steps. The procedures used for skin and muscle differed in terms of sample size, homogenization procedure and the volume of solvent used to re-suspend the residues prior to the UPLC-MS/MS analyses. Because of the inherent matrix effect, which can vary from sample to sample, quantitation of florfenicol and florfenicol amine were performed using matrix-matched calibration curves and with florfenicol-d_3 as surrogate. The validation parameter values (Table 3) were considered adequate for the intended purposes.

3.2 | Experimental design and establishment of the withdrawal period for florfenicol in *P. mesopotamicus*

Tissues (muscle and skin) were sampled at 1, 3, 6, 8, 10, 12 and 16 days post-treatment, but the samples collected on days 10, 12 and 16 were not analysed or included in the model, because the concentrations of florfenicol and florfenicol amine in the muscle tissue were almost below the LOQ of the method. Table 4 shows the concentrations of florfenicol and florfenicol amine determined in muscle and skin, as well as in muscle with skin in natural proportions.

The dispersion of the results obtained for each time point can be explained by the number of fish used in the experimental design, by biological differences (weight and size), and by the administration route used to provide the medication. For example, it is important to consider agonistic behaviour and competition for the feed during the administration of the drug in the feed.

The data were analysed considering the one-compartment elimination kinetics model, and the terminal phase elimination rate constants and the half-lives of florfenicol amine were calculated for muscle and skin. It was not possible to determine the kinetic

TABLE 3 Validation parameters for the determination of florfenicol (FFC) and florfenicol amine (FFA) in fish tissues

			Fish muscle		Fish skin	
Parameter		**Unit**	**FFC**	**FFA**	**FFC**	**FFA**
Linear range		ng/g	25–400	25–2500	50–1500	50–1500
Linearity (r)			0.996	0.998	0.993	0.989
Intra-day precision	50 ng/g	%	4	18	21	20
	600 ng/g	%	2	6	8	8
	1000 ng/g	%	2	5	5	10
Inter-day precision	50 ng/g	%	8	26	16	24
	600 ng/g	%	5	6	5	7
	1000 ng/g	%	4	6	4	10
Accuracy	50 ng/g	%	104	114	133	99
	600 ng/g	%	99	96	100	87
	1000 ng/g	%	100	102	101	93
LOD		ng/g	3	3	15	15
LOQ		ng/g	10	10	50	50

TABLE 4 Concentration of florfenicol and florfenicol amine in pacu tissues. Fish were fed at a daily dose of 9.7 mg/kg pc of florfenicol (medicated feed), for 10 consecutive days. Water temperature of 25.8°C. For each time post-dose 10 fish were sampled.

		Concentration (ng/g), Average (range)		
	Time post-dose (days)	**Florfenicol**	**Florfenicol amine**	**Florfenicol + florfenicol amine**
Fillet	1	37 (<LOQ–132)	735 (117–1508)	878 (117–1574)
	3	15 (11–20)	35 (22–58)	78 (27–246)
	6	7 (<LOQ–19)	11 (<LOQ–20)	15 (<LOQ–40)
	8	LOD	12 (<LOQ–22)	10 (<LOQ–19)
Skin	1	359 (157–862)	752 (223–1331)	1110 (404–2097)
	3	28 (<LOQ–53)	1197 (878–1669)	1225 (903–1722)
	6	<LOQ	680 (529–871)	705 (554–896)
	8	<LOQ	453 (116–1071)	478 (141–1096)
Fillet + skin	1			941 (194–1715)
	3			388 (267–645)
	6			201 (153–263)
	8			137 (48–310)

LOQ, limit of quantitation (10 ng/g for muscle and 50 ng/g for skin); LOD, limit of detection (3 ng/g for muscle and 15 ng/g for skin). The combined concentration of florfenicol and florfenicol amine in muscle + skin were calculated considering that the natural proportion of skin to fillet is 27% w/w.

parameters for florfenicol, because the concentrations after 1 day post-treatment were below the LOQ of the method. The kinetic parameters are summarized in Table 5. The depletion curves for florfenicol amine in pacu skin are shown in Figure 2.

Florfenicol is biotransformed to florfenicol amine fairly rapidly, and the metabolite has different half-lives for depletion in muscle and skin. The concentration of florfenicol amine in skin increased in the first three days post-treatment, and a terminal elimination phase was triggered immediately.

Figure 3 shows the plot of the $\log_e$-transformed tissue concentrations of florfenicol and florfenicol amine) in muscle with skin in natural proportions, together with the withdrawal period calculation. Data below the LOQ were set at half the LOQ (5 ng/g for muscle and 25 ng/g for skin). In our study, we only considered the parent compound and the main metabolite florfenicol amine for the calculations of the withdrawal period. Therefore, considering an MRL of 1000 ng/g for the sum of florfenicol and florfenicol amine in muscle with skin in natural proportions, a withdrawal period of 5 days

TABLE 5 Kinetic parameters of the terminal elimination phase of florfenicol amine in pacu muscle and skin

		Florfenicol amine			
	Time range (days)	**Ln C_0**	**β**	**$t_{1/2}$ (days)**	**R^2**
Muscle	1–6	6.14	–0.558	1.2	0.7249
Skin	3–8	7.78	–0.226	3.1	0.5806

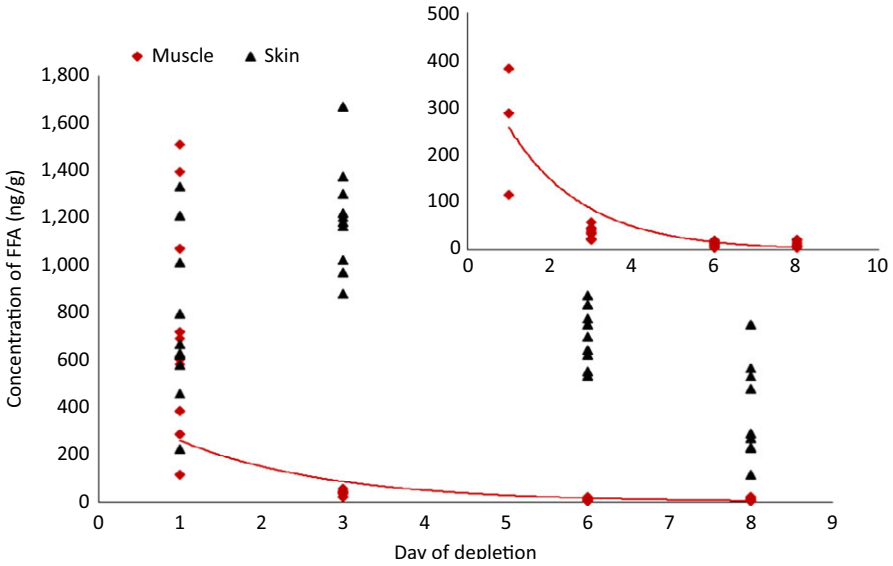

FIGURE 2 Florfenicol amine depletion curve in pacu skin and muscle [Colour figure can be viewed at wileyonlinelibrary.com]

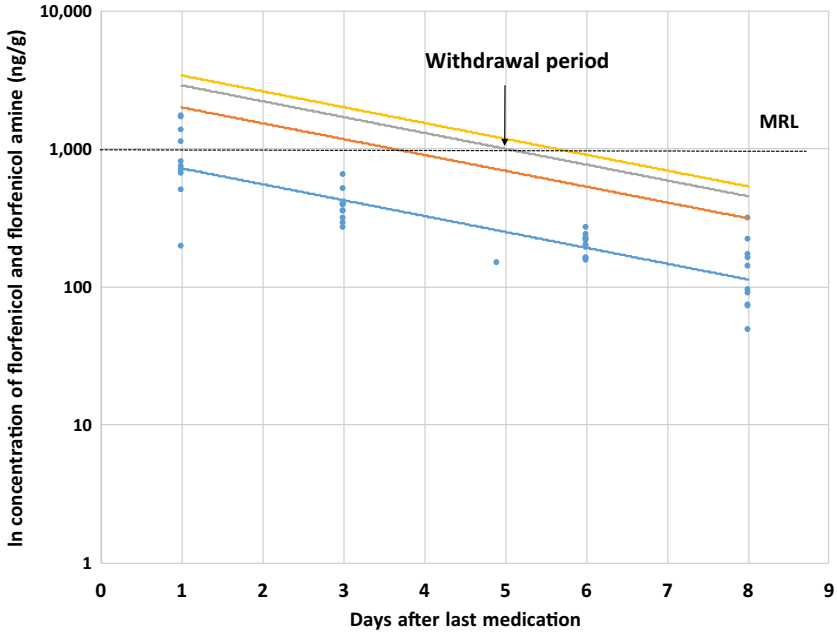

FIGURE 3 Log_e-transformed tissue concentrations of the marker residues (FFC + FFA) in muscle with skin in natural proportion. (Blue line – regression line; orange line – upper limit of the one-sided 95% confidence interval over the 95th percentile, grey line – upper limit of the one-sided 95% confidence interval over the 99th percentile and yellow line – upper limit of the one-sided 99% confidence interval over the 99th percentile) [Colour figure can be viewed at wileyonlinelibrary.com]

(water temperature of 25.8°C) or 129° days was calculated on the basis of the upper limit of the one-sided 95% confidence interval for the 99th percentile derived from the residue depletion study.

4 | DISCUSSION

The UPLC-MS/MS method developed for the determination of florfenicol and florfenicol amine in fish tissues enabled quantification of the analytes at concentrations of up to 10 ng/g in muscle and 50 ng/g in skin, with adequate accuracy and precision. The method could be deemed suitable for depletion studies and for monitoring of this antimicrobial and its metabolite in fish, with satisfactory selectivity. It is worth emphasizing that the methods reported in the literature for use in residue depletion studies are all based on high performance liquid chromatography with ultraviolet detection, which lacks selectivity. Some studies included an acid hydrolysis step to convert florfenicol and related metabolites to florfenicol amine (Di Salvo et al., 2013; Gaikowski et al., 2010; Kosoff et al., 2009 and Wrzesinski et al., 2006). It is also important to highlight that the use of the surrogate florfenicol-d_3 improved the quality of the data for florfenicol, since this internal standard corrected for the matrix effects associated with the use of mass spectrometry. For florfenicol amine no surrogate is commercially available. However, both analytes were quantified in the samples using the matrix-matched calibration curve, which minimizes the matrix effect.

Florfenicol is currently the sole antimicrobial drug with a formulation intended for applications involving fish and fish farming. It is available as a premix that can be either dispersed in vegetable oil and added to feed pellets (as a coating) or incorporated in the feed

mixture before pelleting. The coating process recommended by the manufacturer leads to non-homogeneous medicated feeds. This is an important factor to be considered in depletion studies. In this work, an alternative procedure for coating of the feed is proposed, which enabled the preparation of 20 kg of medicated feed with satisfactory homogeneity (coefficient of variation <5%).

The depletion curve for florfenicol and the metabolite florfenicol amine in muscle and skin in natural proportions is shown in Figure 2. The variability at the concentrations determined for each post-dose time could have been caused by competition between the fish during feeding. Depletion of the antimicrobial in the fish muscle was much faster (at least three times faster) than in the skin.

Several studies have reported the pharmacokinetics of florfenicol and florfenicol amine in fish tissues and plasma after florfenicol administration (Di Salvo et al., 2013; Feng & Jia, 2009; Feng et al., 2008; Gaikowski et al., 2010; Gaunt et al., 2012; Wrzesinski et al., 2006). However, no reports were found concerning the pharmacokinetics or depletion of florfenicol in pacu, with most studies having been performed using Nile tilapia.

The findings demonstrated that the depletion of residues of florfenicol and its metabolite florfenicol amine in pacu, after oral administration of 10 mg/kg bw for 10 consecutive days, was very similar to the results reported for other fish. Radio-depletion studies with Atlantic salmon kept at 8.5–11.5°C indicated that around 90% of the radioactive compounds were removed and that the main metabolite was florfenicol amine, with over 80% of the radioactivity corresponding to florfenicol and florfenicol amine. Other metabolites were detected, but were quantitatively of minor importance (Horsberg et al., 1994). Analogous behaviour is expected for pacu, since the muscle and skin compositions are similar, and the non-extractable residues are likely to be of the same magnitude. Moreover, it is expected that the metabolism follow the same pathway and that florfenicol amine is the main metabolite. Therefore, in the present work, only florfenicol and florfenicol amine were considered for the calculations of the withdrawal period. Considering an MRL of 1000 ng/g for the sum of florfenicol and florfenicol amine in muscle and skin in natural proportions, a withdrawal period of 5 days (129° days) is recommended for pacu.

The average concentrations of florfenicol in the muscle and skin of pacu 24 h after administration of the final dose of florfenicol were 37 and 359 ng/g respectively. The average values obtained for the main metabolite, florfenicol amine, were 735 and 752 ng/g, respectively, for muscle and skin. In a study reported by EMEA (Anonymous, 2001), in which Atlantic salmon kept at 10°C were fed with florfenicol at a dose of 10 mg/kg bw for 10 consecutive days, florfenicol concentrations 24 h after the end of treatment were 1800 ng/g in muscle and 690 ng/g in skin, while florfenicol amine concentrations of 7270 and 6350 ng/g were found in muscle and skin respectively.

Residues of florfenicol and its metabolites measured as florfenicol amine were evaluated in catfish (*Ictalurus punctatus*) after a daily oral dose in the range from 8.1 to 9.3 mg/kg bw for 12 consecutive days (Wrzesinski et al., 2006). At a water temperature of 20.7°C, a withdrawal period of 4 days was recommended.

Florfenicol amine was determined after acid-catalysed hydrolysis in the skin-on muscle of Nile and hybrid tilapias (Gaikowski et al., 2010). Florfenicol was administered to the fish at a daily dose of 15 mg/kg bw for 12 consecutive days, with the water temperature ranging from 25.8 to 27.0°C, and a withdrawal period of 7 days was calculated. The longer withdrawal period reported in the present work, compared with the previous studies, was probably associated with the larger dose and longer medication period used.

In a study of the pharmacokinetics of florfenicol in tilapia by analysis of the kidney, liver, bile, gill and skin-on muscle tissues, it was reported that florfenicol and its metabolites showed longer half-lives in the kidney, liver and gill, with the longest half-life in bile, indicating possible hepato-biliary, renal and branchial elimination (Feng et al., 2008). In other work, similar results were reported for salmon (Horsberg et al., 1994), and it was demonstrated that florfenicol and its metabolites were mainly excreted by the kidney and biliary organs.

Residues of florfenicol and its metabolites measured as florfenicol amine were assessed in sea bream (*Sparus aurata* L.) after a daily oral dose of florfenicol at 10 mg/kg bw for 10 consecutive days (Di Salvo et al., 2013). The average fish weight was 150 g and the water temperature was 27.4 ± 1.9°C. Under these conditions, a withdrawal period of 3.97 days was recommended.

The kinetics of florfenicol residues were determined in three species of fish (Nile tilapia, walleye and hybrid striped bass) after administration of florfenicol in medicated feed at 10 mg/kg bw for 10 consecutive days at different temperatures: 30 and 25°C for Nile tilapia and 25 and 20 °C for walleye and hybrid striped bass (Kosoff et al., 2009). The authors reported faster florfenicol elimination kinetics at higher temperatures.

In the present study, carried out at 25.8°C, the elimination half-lives of florfenicol in pacu were 1.2 and 3.1 days in muscle and skin, respectively, corroborating the fast elimination of florfenicol reported in the literature for other fish species, as well as the slower elimination in skin, compared with muscle. It appears that there are no great differences in the depletion rates of florfenicol among different fish species, with water temperature being the most important parameter influencing elimination of the drug from the tissues. The water temperature used in this work realistically simulated real conditions in fish farms in tropical and temperate regions of the southern hemisphere. The results reported here provide information important for the intensive farming of neotropical species, particularly pacu, which is a fish attracting increased commercial interest.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant #2013/09543-7), CAPES and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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How to cite this article: Marques TV, Paschoal JAR, Barone RSC, Cyrino JEP, Rath S. Depletion study and estimation of withdrawal periods for florfenicol and florfenicol amine in pacu (*Piaractus mesopotamicus*). *Aquac Res*. 2018;49:111–119. https://doi.org/10.1111/are.13439