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Pharmacokinetic Study of Florfenicol in Healthy and Vibriosis-infected *Pseudosciaena crocea* after Oral Administration

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Abstract The pharmacokinetics of florfenicol were studied in healthy and vibriosis-infected large yellow croaker (*Pseudosciaena crocea*) following administration of a single oral dose of 20 $mg \cdot kg^{-1}$ at 25±2°C. After oral administration, florfenicol levels in tissues (liver, kidney, muscle, serum, and skin) were analyzed using high-performance liquid chromatography. A two-compartment open model was used to describe the pharmacokinetics of florfenicol following oral administration. Compared to the healthy group, the absorption rate of vibriosis-infected fish significantly decreased, peak-time (T_{max}) delayed, maximum concentration (C_{max}) declined, total body clearance decreased, the elimination half-life ($T_{1/2\beta}$) was extended, and the area under the curve increased. These results indicate that a 20 $mg \cdot kg^{-1}$ oral dose of florfenicol administered once daily continuously for 4 or 5 days can be used for the treatment of *Vibrio alginolyticus* infection in large yellow croaker (*Pseudosciaena crocea*).

Keywords florfenicol · pharmacokinetics · *Pseudosciaena crocea* · *Vibrio alginolyticus*

Introduction

The large yellow croaker (*Pseudosciaena crocea*) is cultivated widely in East China. In Zhejiang, China, *P. crocea* is an economically important export product and its cage culturing accounts for 40% of the total export. However, bacterial disease is the most serious concern and a cause of high mortality in breeding because of the environmental changes they induce. Currently, diseases caused by *Vibrio alginolyticus* are a serious problem since they considerably damage the economy of the *P. crocea* industry. Currently, to reduce the losses incurred owing to diseases, antibiotics, as well as immunological and ecological prevention or control measures, are mainly adopted.

Florfenicol is the most commonly used antibacterial agent in aquaculture because of its significant activity, wide spectrum, and fewer side effects. Florfenicol, which is a synthetic drug with a chemical structure and spectrum of bactericidal activity similar to chloramphenicol, is devoid of plasmid-mediated resistance. Because of its high potency and safety in humans, florfenicol has been developed exclusively for veterinary medicine and is used extensively in fish farming as a chemotherapeutic agent in China. The use of this drug has positive effects; however, several hazards and side effects on both the fish and the environment are associated with its excessive use. These include immunosuppression, nephrotoxicity, decreased growth, increased incidence of resistant bacterial strains, and environmental problems such as drug residues in fish products. Therefore, its proper application is strongly recommended to avoid such problems. The knowledge of pharmacokinetics and residue depletion is important in order to minimize the human risk associated with drug residue and the environmental impact of the drugs.

A number of studies on the pharmacokinetic profile of florfenicol have been reported in aquaculture and livestock breeding (Samuelsen et al., 1998; Stefan et al., 2004; Gaunt et al., 2010). There are some reports of its pharmacokinetics determined in *Oncorhynchus mykiss, Piaractus brachypomus, P. crocea, Gadus morhua, Salmo salar, Acipenser baeri, Anguilla anguilla, Tilapia, Silurus asotus,* and Crucian carp (Horsberg et al., 1996; Lunden and Bylund, 2000; Samuelsen et al., 2003; Lewbart et al., 2005; Park et al., 2006; Feng and Jia, 2008; Bowser, 2009; Sun et al., 2010; Qin et al., 2010; Xie et al., 2012).

However, these studies were conducted in healthy animals, and there is very little information on the pharmacokinetics of the drug

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in infected or diseased fish. It is pertinent for there to be information available from studies of the pharmacokinetics of this widely use agent, in diseased fish. There are some data showing that the variations in biology (age, condition, and size), method (dosage and routes of administration), and environment (pH, ion content, and temperature) can cause a significant difference in the absorption, metabolism, and elimination of the same drug. Therefore, this present study was performed to investigate the pharmacokinetics including absorption and tissue disposition of florfenicol in vibriosis-infected *P. crocea* following oral administration. It is envisaged that these results will contribute pertinent information that would be helpful to further studies on the rational drug use and withdrawal period for formulation.

Materials and Methods

Fish. Healthy, large yellow croaker fish (weighing 250–300 g) were obtained from the Huangbidai Bay Cultured Fish Farm in Xiangshan Prefecture, China. Approximately 240 large yellow croakers were randomly divided into two groups including one consisting of healthy fish that received the drug orally while the fish in the other group were infected and received the drug orally. In each group, the fish were kept in tanks with recirculated water at a salinity of 28±1 ppt for about 1 month before treatment. The water temperature was 25±2°C, aeration was constant, and the pH of the water was 7.9±0.5. Almost all the fish were healthy and determined to be free of pathogens before drug administration. The fish were fed ad libitum with commercial pellets before and after drug administration.

Chemicals. The chemicals and chromatographic reagents used were high-performance liquid chromatography (HPLC)-grade acetonitrile, phosphoric acid, hexane, and triethylamine (Tedia, USA). Florfenicol of 99.5% purity were obtained from the China Institute of Veterinary Drug Control.

Oral administration. Florfenicol was mixed with the diet and orally administered through a catheter to the fish, which were anesthetized with eugenol (30 mg·L^{-1}). The dose of florfenicol was 20 mg·kg^{-1}.

The *V. alginolyticus* strain was identified using a molecular method and supplied from the aquaculture diseases Center for Disease Control of the Ningbo University (China), and diluted to a density of 10^{-7} colony forming units·mL^{-1} of the bacteria suspension with 0.85% sterile saline, after incubation at 28°C for 22 h. The fish were injected intraperitoneal with a 0.2 mL suspension of the *V. alginolyticus* strain, and then florfenicol was administered orally 24 h post-injection. No fish died in the florfenicol-administered group during the experimental period.

Blood and tissue sampling. Five fishes were sampled at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 24, 48, 72, 96, 120, 144, and 168 h after drug administration. The blood was sampled from the caudal vein, collected in Li-heparin vacuum blood tubes, and the hemolymph was obtained by centrifugation at 2500 rpm for 10 min. The muscle, skin, liver, and kidney were collected and stored frozen at −20°C until analyzed. Five untreated fishes (without drug) were sampled as a control group.

Analytical procedures. Florfenicol concentrations in the hemolymph and tissues of the large yellow croaker were simultaneously determined using HPLC analysis performed using an Agilent 1200 system (Agilent, USA) equipped with a dual pump, auto-injector, column temperature tank, and fluorescence detector.

Extraction and purification. Hemolymph: The hemolymph (1 mL) was added to 4 mL ethyl acetate, vortexed for 5 min, and then centrifuged at 6000 rpm for 10 min at 4°C. The supernatants were transferred to 20 mL tubes, and the extraction was repeated, and then the resultant supernatants were evaporated at 40°C in rotary flask evaporator. The clear supernatants were reconstituted with the mobile phase (1 mL) and *n*-hexane (2 mL), and the solution was transferred to a 10 mL centrifuged tube and centrifuged for 10 min at 6000 rpm. After filtration with 0.45 μm filters, the samples were ready for analysis.

Tissues: Samples (1 g) of the tissues (liver, kidney, muscle, and skin) were homogenized in 4 mL ethyl acetate, vortexed for 1 min, and then centrifuged for 10 min at 6000 rpm at 4°C. The clear supernatant was then transferred to a separatory funnel, and the extraction was repeated. The subnatant acetonitrile phase was evaporated at 40°C in a rotary flask. The residue was then reconstituted in the flask with the mobile phase (mL) and *n*-hexane (2 mL), transferred to a 10 mL centrifuge tube, and centrifuged for 10 min at 6000 rpm. After filtration with 0.45 μm filters, the samples were ready for analysis.

Chromatography. The florfenicol concentration in the hemolymph and tissues of the large yellow croaker were simultaneously determined using HPLC analysis performed with an Agilent 1200 system equipped with a dual pump, auto-injector, column temperature tank, and fluorescence detector. The column was a Diamonsil TM C-18 (150 mm×16 mm i.d., 5 μm) maintained at a temperature of 30±1°C using a column heater. The mobile phase was a mixture of acetonitrile and ultrapure water (27:73, v/v) adjusted to pH 7.0. The flow rate of the mobile phase was maintained at 1.0 mL·min^{-1}. The detection wavelength was set at 223 nm.

Pharmacokinetics analyses. The most common method of evaluating the pharmacokinetics of a drug is to assume that the drug concentration-time data can be described by one of several compartment models, and then fit the data to an equation consistent with the assumed model using a nonlinear least-squares regression. In our study, the pharmacokinetics parameters were calculated using the Practical Pharmacokinetics Program 3P97 (Mathpharmacology Committee, Chinese Academy of Pharmacology, China) on a personal computer to automatically determine the type of compartment model and pharmacokinetic parameters.

Results

Standard curve, limit of detection, average recovery rate and accuracy. The linear regression equation was Y - 57.35138X-

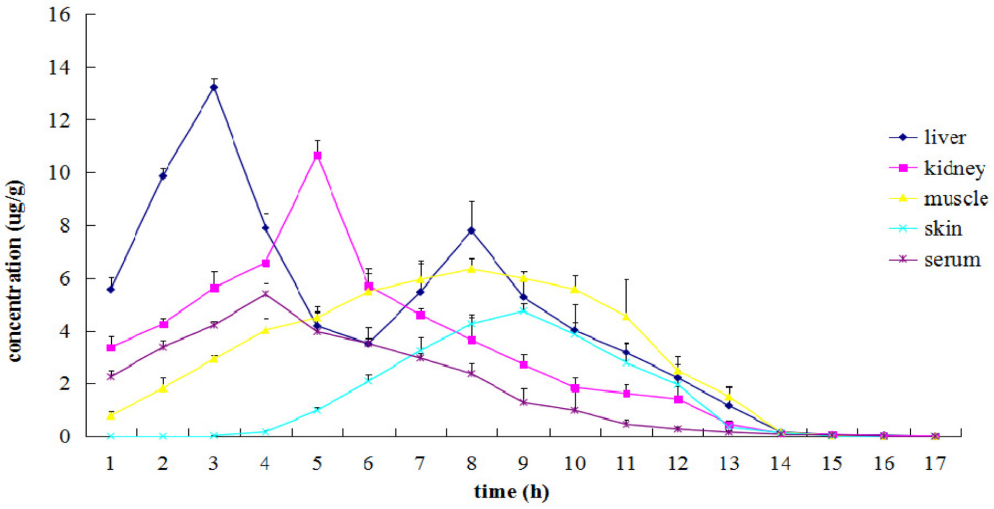

Fig. 1 Florfenicol concentrations in main tissues from heathy *Pseudosciaena crocea* after oral administration.

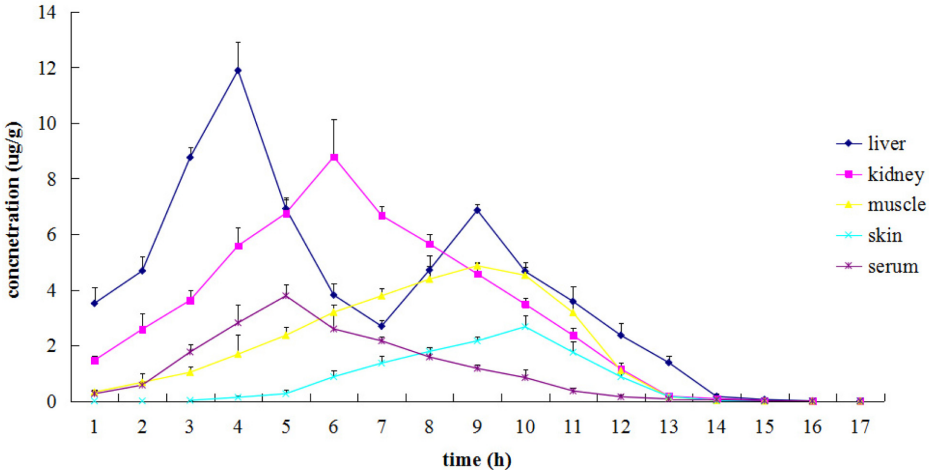

Fig. 2 Florfenicol concentrations in main tissues from vibriosis-infected *Pseudosciaena crocea* after oral administration.

2.49223, r - 0.99996. The limit of quantitation and limit of detection were 0.045 and 0.025 μg·mL^{-1}, respectively. The average rates of drug recovery from the liver, kidney, muscle, skin, and serum were 89.4±1.11, 92.6±0.55, 95.1±1.36, 88.2±0.51, and 88.2±0.51%, respectively. It can be seen that the recovery of florfenicol in the main tissues was more than 87%. The intra-day and inter-day precision of florfenicol in the main tissues from *P. crocea* were within 5%. These results both showed that the method used to detect florfenicol had the advantage of high accuracy and stability.

Distribution of florfenicol in the main tissues of *P. Crocea*. Fig. 1 shows the florfenicol concentration in the main tissues from healthy *P. crocea* after oral administration, Fig. 2 shows the concentration in the main tissues from vibriosis-infected *P. crocea* after oral administration. The results revealed that the metabolic determination of florfenicol concentrations in main tissues from healthy and vibriosis-infected *P. crocea* after oral administration were similar. At 0.25 h after oral administration, the presence of drug was detected in the main tissues of both groups except in the skin. The order of magnitude of the florfenicol concentration in the tissues was the liver > kidney > serum > muscle while the drug was detected in the skin 1 h later. Moreover, from the two figures, the peak time of drugs in the main tissues in order of magnitude was the liver > serum > kidney > muscle > skin; peak concentration was the liver > kidney > muscle > serum > skin, and concentration in the liver showed double peaks. Compared to the healthy *P. crocea,* the peak time and concentration of infected group delayed or reduced.

Pharmacokinetic characteristics. According to the 3P97, the two-compartment, open, first-order absorption model can be used to describe the pharmacokinetic characteristics of the drug in this study.

Table 1 shows the concentration-time curve equations of florfenicol in healthy and disease *P. crocea.* Table 2 shows the pharmacokinetic parameters of florfenicol in healthy and diseased *P. crocea.* The pharmacokinetic characteristics of the drug in the liver ($T_{1/2\beta}$, CLs, Vd), muscle ($T_{1/2Ka}$, T_{peak}, C_{max}, CLs, Vd), and

Table 1 Concentration-time curve equations of florfenicol in healthy and disease *Pseudosciaena crocea*

			D	
Tissues	Equation	R^2	Equation	R^2
Liver	$C_{liver}=43.637001e^{-1.472205t}+6.109747e^{-0.030646t}-49.746748e^{-2.184108t}$	0.98	$C_{liver}=160.013977e^{-0.772405t}+5.274416e^{-0.018345t}-165.288393e^{-0.855736t}$	0.96
Kidney	$C_{kidney}=22.606833e^{-0.218225t}+1.770690e^{-0.016401t}-24.377523e^{-0.524946t}$	0.97	$C_{kidney}=13.068055e^{-0.178906t}+7.208973e^{-0.053333t}-20.277028e^{-0.345717t}$	0.98
Muscle	$C_{muscle}=305.880620e^{-0.087076t}+504.614500e^{-0.083990t}-810.495120e^{-0.087280t}$	0.98	$C_{muscle}=48.838001e^{-0.117136t}+27.641232e^{-0.067346t}-76.479233e^{-0.111388t}$	0.98
Skin	$C_{skin}=8.103964e^{-0.089445t}+20.049276e^{-0.053342t}-28.153240e^{-0.092564t}$	0.95	$C_{skin}=4.383391e^{-0.295082t}+12.590437e^{-0.059891t}-16.973828e^{-0.117499t}$	0.95
Serum	$C_{serum}=7.647265e^{-0.142467t}+0.35439\ 2e^{-0.020476t}-8.001657e^{-1.050621t}$	0.96	$C_{serum}=9.439323e^{-0.202728t}+0.608547e^{-0.030241t}-10.047870e^{-0.454548t}$	0.96

Table 2 Pharmacokinetic parameters of florfenicol in healthy and diseased *Pseudosciaena crocea*

Parameters	Liver		Kidney		Muscle		Serum		Skin	
	H	D	H	D	H	D	H	D	H	D
A (μg/mL)	43.63	160.01	22.61	13.07	305.88	48.84	7.65	9.44	8.10	4.38
B (μg/mL)	6.11	5.27	1.77	7.21	504.62	27.64	0.35	0.61	20.05	12.59
α (/h)	1.47	0.77	0.22	0.18	0.09	0.12	0.14	0.20	0.09	0.30
β (/h)	0.03	0.02	0.02	0.05	0.08	0.07	0.02	0.03	0.05	0.06
Ka (/h)	2.18	0.86	0.53	0.35	0.09	0.11	1.05	0.46	0.09	0.12
K_{10} (/h)	0.10	0.07	0.09	0.08	0.08	0.06	0.11	0.13	0.05	0.01
K_{12} (/h)	0.95	0.52	0.11	0.03	0.00	0.01	0.03	0.06	0.00	3.51
K_{21} (/h)	0.46	0.21	0.04	0.12	0.09	0.13	0.03	0.05	0.09	3.15
$T_{1/2Ka}$ (h)	0.32	0.81	1.32	2.01	7.94	6.22	0.66	1.53	7.49	5.90
$T_{1/2\hat{a}}$ (h)	0.47	0.90	3.18	3.87	7.96	5.92	4.87	3.42	7.75	2.35
$T_{1/2\hat{a}}$ (h)	22.62	37.78	42.26	12.99	8.25	10.29	33.85	22.92	12.99	11.57
T_{peak} (h)	0.72	1.57	3.04	4.96	11.64	12.22	2.26	3.36	13.98	16.34
C_{max} (μg/mL)	10.77	9.59	8.39	7.26	7.39	4.20	5.14	3.15	4.11	2.28
AUC (μg/mL · h)	206.23	301.52	165.12	149.56	234.64	140.70	63.37	44.58	162.31	80.62
CLs (L/kg · h)	0.097	0.07	0.12	0.13	0.09	0.14	0.32	0.45	0.12	0.25
Vd (L/kg)	0.99	0.96	1.34	1.61	1.01	2.38	2.87	3.45	2.28	4.24

H: healthy; D: diseased; A and B: intercepts of the two phases; α and β: the rate constants of the distribution and elimination phases; K_a: absorption rate constant; K_{10}: the elimination rate constant from the central compartment; K_{12}: the first-order rate constant of transporting from central to periphery compartment; K_{21}: the first-order rate constant of transporting from periphery to central compartment; $T_{1/2\alpha}$ and $T_{1/2\beta}$: half-lives of the two phases; $T_{1/2Ka}$: half-lives of the absorption; C_{max}: the peak concentration of a drug after administration; $T_{peak:}$ time to reach the maximum concentration; AUC: the area under the curve to infinity; CLs: total body clearance; Vd: volume of distribution at steady-state

serum (Ka, $T_{1/2Ka}$, $T_{1/2\beta}$, T_{peak}, Vd) of the infected group were significantly different (p <0.05) from those of the healthy group while their liver (Ka, $T_{1/2Ka}$, T_{peak}, C_{max}, AUC), muscle (Ka, $T_{1/2\beta}$, AUC), and serum (AUC, CLs) values were significantly altered (p <0.01).

Discussion

The pharmacokinetics data was best described by a two-compartment open model following bolus injection; florfenicol was eliminated by a first-order process in large yellow croaker (*P. crocea*). In Atlantic salmon, the pharmacokinetics of florfenicol after oral administration had also been analyzed by a two-compartment model (Bernt, 1993). In contrast, Fangke (2006) used a one-compartment model to describe the elimination of florfenicol in Crucian carp. The differential pharmacokinetics of florfenicol could be attributed to variations in fish species, size of fish, water temperature, or experimental design.

This is the first study to investigate the pharmacokinetic parameters of florfenicol in healthy and *V. alginolyticus*-infected *P. crocea*. When administered orally, florfenicol is absorbed rapidly and distributed extensively in *P. crocea*. This study indicated that different tissues have differential rates of absorption (liver > kidney > serum > muscle > skin). This is probably because the liver has digestive and metabolic functions and therefore, the drug enters first into the liver before it reaches the muscle, kidney, and other tissues. This was consistent with the results reported in previous studies (Elema, 1996; Bowser, 2009; Sun et al., 2010). The drug concentration in the liver declined, eventually peaking

after 10 h oral administration owing to enterohepatic circulation. This phenomenon was also reported in rainbow trout and sea bass (Bjorklund et al., 1992; Intorre et al., 2000). After drug concentrations in the liver peaked, its distribution and levels in other tissues, especially the kidneys, increased. This result indicated that the kidney is the main site for florfenicol enrichment and degradation. Serum and muscle concentration-time curves were similar; time of peak drug concentrations in serum was shorter than that in muscle; however, the peak serum concentration was lower than that of muscle. This observation was in agreement with the results of the pharmacokinetic study in codfish (Samuelsen et al., 2003). Florfenicol could not diffuse into the bloodstream owing to poor water solubility; hence, florfenicol concentration in blood is lower than its concentration in other tissues (Elemam, 1995). Low levels of florfenicol could be detected in the skin after 1 h of administration owing to poor skin cell membrane permeability and reduced amount of drug in blood circulation, which subsequently reduced the rate and efficiency of drug metabolism (Bernt, 1993).

The proportionality constant relating drug concentration in the serum to the amount of drug in the body has been termed the apparent volume of distribution (V_d). The V_d of florfenicol in serum of Korean catfish (Park, 2006) and Atlantic salmon (Martinsen and Horsberg, 1995) was 1.09 and 1.12 L kg^{-1}, respectively. In the present study, the V_d of florfenicol in healthy and infected *P. crocea* serum was 2.87 and 3.45 L kg^{-1}, respectively, most likely owing to the difference in the route of administration and species. It was found that V_d of florfenicol in fishes was large thereby improving its bioavailability and antibacterial effect.

Elimination half-life ($T_{1/2\beta}$) and total body clearance (CL) are important parameters used to characterize drug disposition. $T_{1/2\beta}$ of florfenicol was estimated to be 33.85 h in healthy *P. crocea* and 22.92 h in vibriosis-infected fish after oral administration. These were similar to the half-life of European eel (18.39 h; Xie et al., 2012), but were longer than that of tilapia (10.03 h; Feng and Jia, 2009), Atlantic salmon (12.2 h; Horsberg et al., 1996), and chicken (1.73 h; Shen and Wu, 2002). These differences indicated that the elimination rate of florfenicol might be influenced by factors such as species, increased emunctory function, and physiological mechanism. However, the specific reasons warrant further investigation. The CL of florfenicol in cod, kio carp, and chicken were 0.02, 0.07, and 0.73 L $kg^{-1}\cdot h^{-1}$, respectively (Shen and Wu, 2002; Samuelsen et al., 2003; Yanong and Cuds, 2005). This experiment showed that the CL of florfenicol in healthy and infected *P. crocea* serum were 0.32 L $kg^{-1}\cdot h^{-1}$ and 0.45 L $kg^{-1}\cdot h^{-1}$, respectively. The CL of florfenicol was not high in many animals. Ho et al. (1999) reported that the difference in clearance rates was mainly influenced by drug chemical structure; replacing the -OH of florfenicol by -F decreases its conjugation with glucuronic acid, thereby delaying drug excretion.

The area under curve (AUC) represents the extent of drug absorption and determines bioavailability. The AUC of florfenicol for tilapia serum and muscle were 9.45 and 695.39 μg $mL^{-1}\cdot h^{-1}$, respectively (Feng and Jia, 2009). The AUC of florfenicol in healthy and infected *P. crocea* were 234.64 and 140.70 μg $mL^{-1}\cdot h^{-1}$ for muscle, 63.37 and 44.58 μg $mL^{-1}\cdot h^{-1}$ for serum. The difference may be owing to the difference in species, route of administration, and exposure temperature. AUC for serum was less than the AUCs for other tissues, indicating that florfenicol could be distributed to the deeper tissues thereby exerting its effects even on the deep animal tissue infection.

Pharmacokinetic data can be used to design treatment regimens and predict their possible clinical outcomes. Frequently, the efficacy of an antimicrobial drug is related to its capacity to reach and maintain adequate concentration at the site of infection. Although there are no published data concerning the antibacterial activity of florfenicol against *P. crocea*, previous studies showed that MIC of florfenicol for many bacterial pathogens are in the range of 0.25-2 μg mL^{-1}, for example, *Pasteurella piscicida* (0.004-0.6 μg mL^{-1}, Fukui et al., 1987; Kim et al., 1993); *V. anguillarum* (0.2-0.8 μg mL^{-1}, Samuelsen et al., 2003); *E. ictaluri* (0.25 μg mL^{-1}, Mc et al., 2003); *Aeromonas salmonicida* (0.25-1.6 μg mL^{-1}, Inglis et al., 1991; Grant and Laidler, 1993); *A. hydrophila* (0.4 μg mL^{-1}, Ho et al., 2000); *Edwardsiella tarda* (0.4-1.6 μg mL^{-1}, Fukui et al., 1987). Blood drug concentration above MIC can achieve ideal therapeutic effect; the dosing interval was usually similar to $T_{1/2\beta}$. The experiment revealed that after a dose of 20 mg kg^{-1} florfenicol was administered, the blood drug concentration of *P. crocea* was above 0.3 μg mL^{-1}, at 48 h after administration. $T_{1/2\beta}$ of florfenicol in *P. crocea* was more than 24 h. In conclusion, since this study demonstrated that florfenicol was absorbed rapidly, distributed extensively, and eliminated quickly in *P. crocea*, the 20 mg kg^{-1} dose of florfenicol could be orally administered once daily for 4 or 5days for the treatment of *Pseudosciaena crocea*.

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