SUPPORTING INFORMATION

Accumulation–Excretion Characteristics of Enrofloxacin in Farmed Largemouth Bass (Micropterus salmoides)

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Text S1. Reagents and materials

Formic acid (FA), MeOH, and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). All of the other chemicals used were of analytical grade. Anhydrous sodium sulfate (analytical grade; Sinopharm Chemical Reagents, Shanghai, China) was heated at 650°C for 4 h, then cooled and kept in a sealed container until use. Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). HLB Pro solid-phase extraction (SPE) columns were provided by Shanghai Anpel Technology (Shanghai, China).

Text S2. Randomization and blinding

Fish were randomly allocated to nine tanks using a computer-generated random number sequence. The allocation sequence was generated by an investigator who was not involved in the subsequent drug administration or outcome assessment. To minimize potential confounding factors, the feeding order of the tanks was randomly rotated daily. The sampling order of the tanks was randomized daily. Water quality parameters were monitored and recorded daily for all tanks, and no significant differences were observed among groups.

Because personnel prepared the medicated feed by top-dressing ENR at two dose levels, they were aware of group allocation. However, all outcome assessments were performed by personnel blinded to group identity. Samples were labeled with tank codes and time points only. LC-MS/MS analysis was conducted in a randomized order by a blinded analyst. Histopathological sections were independently analyzed by two blinded pathologists. Antioxidant parameters (SOD, CAT, T-AOC, and MDA) were measured by a blinded technician. A blinded observer recorded growth measurements. Data analysis was performed by a statistician blinded to treatment allocation using the relevant software.

Text S3. Chromatographic conditions

The flow rate was 0.3 mL/min. Gradient elution was performed as follows: 0–1.1 min, 10% B; 1.1–3.5 min, 10% B to 30% B; 3.5–4.5 min, 30% B; 4.5–6.4 min, 30% B to 75% B; 6.4–6.5 min, 75% B to 95% B; 6.5–8.0 min, 95% B; 8.0–8.01 min, 95% B to 10% B; and 8.01–10.0 min, 10% B.

The injection volume was 5 μL.

Text S4. Quality control and method validation

Data acquisition and quantitative analysis were performed using Analyst software. Calibration curves were constructed at concentrations of 5, 10, 20, 50, 100, 200, and 400 ng/mL for each target analyte. Quantification was carried out using the internal standard method except for plasma samples (external standard method), and relative concentrations were calculated using peak area ratios. In preliminary experiments, samples with concentrations exceeding the upper limit of the linear range were reanalyzed after appropriate dilution and addition of an internal standard to ensure that the concentrations measured fell within the established linear range.

For both ENR and CIP, the limits of detection (LOD) and quantification (LOQ) were 2 μg/kg and 6 μg/kg in tissue samples, 10 μg/L and 20 μg/L in plasma, 0.2 μg/L and 0.4 μg/L in water, and 0.7 μg/kg and 1 μg/kg in feces, respectively. To ensure the reliability of the entire sample preparation procedure, quality control was performed using the standard addition method for both standard solutions and sample matrices. The recovery rate was 82.1% ± 8.4% for ENR and 86.4% ± 6.2% for CIP. The intra-day and inter-day relative standard deviations (RSDs) were 4.31% and 7.18%, respectively, and no interference was detected in any of the samples analyzed.

To verify that no background contamination was introduced during sample preparation and analysis, procedural blanks were prepared and analyzed alongside the actual samples.

Reagent blanks were prepared by replacing the sample with ultrapure water and subjecting them to the same extraction, purification, and instrumental analysis procedures as the actual samples. The reagent blanks were processed in parallel with each batch of samples.

Blank samples were prepared from the tissues, plasma, water, and feces of largemouth bass (*Micropterus salmoides*) in the blank group, and were subjected to the same extraction, purification, and instrumental analysis procedures as the experimental samples.

No enrofloxacin or ciprofloxacin was detected in either the reagent blanks or the sample blanks (Table S3). These results confirm that no background contamination was introduced during the sample preparation and analytical procedures, and that the measured concentrations of enrofloxacin and ciprofloxacin in the experimental samples were derived solely from the exposed fish.

Text S5 Detailed histopathological procedures

Dehydration was performed through a graded ethanol series (75% for 4 h, 85% for 2 h, 90% for 2 h, 95% for 1 h, and absolute ethanol I and II for 30 min each), followed by an ethanol–xylene mixture (5–10 min) and xylene I and II (5–10 min each). The samples were then infiltrated with paraffin in three baths (1 h each), embedded in paraffin wax, and sectioned at 4 μm. Before hematoxylin and eosin (H&E) staining, sections were deparaffinized and rehydrated at room temperature by sequential immersion in xylene I and II (20 min each), absolute ethanol I and II (10 min each), and 95%, 90%, 80%, and 70% ethanol (5 min each), followed by rinsing with distilled water. H&E staining was performed manually by sequential immersion: Harris hematoxylin for 3–8 min, rinsing with running water, differentiation with 1% acid alcohol, bluing with 0.6% ammonia water, and eosin staining for 1–3 min. Sections were then dehydrated through 95% ethanol I and II and absolute ethanol I and II (5 min each), cleared in xylene I and II (5 min each), and mounted with neutral resin.

Text S6. Semi-quantitative analysis

The weights required for calculating the histopathological index were based on the method reported by Bernet et al. [27], with appropriate modifications for the present study. The semi-quantitative histopathological assessment method was adapted from Costa et al. [28]. The histopathological index (I_h) was calculated using the weights (wj) and the scores (aj) assigned to individual histopathological alterations, according to the formula described by Almeida [29] and Geng et al. [30]. The weights (w) and scores (a) assigned to individual histopathological alterations are presented in Table S4 and Table S5, respectively.

$$I_h = \frac{\sum_1^j w_j a_j}{\sum_1^j M_j}$$

where I_h is the histopathological index; w_j is the weight assigned to the j-th histopathological alteration; a_j is the corresponding score assigned to that alteration; M_j is the maximum attributable value ($w_j \times a_j$) for the corresponding histopathological alteration.

Text S7. Detection ranges and validation information of the antioxidant assay kits

The detection ranges and validation information of the four commercial kits were as follows. For the CAT assay kit, the calibration curve was constructed using H_2O_2 standards over the range of 0–250 mM, with a linear regression equation of $y = 0.1413x - 0.0135$ ($R^2 = 0.9978$). For the SOD assay kit, the inhibition rate was maintained within 30–80% for all samples, and the assay was performed according to the manufacturer's instructions. For the T-AOC assay kit, the calibration curve was constructed using Trolox standards over the range of 0–3.6 μmol/mL, with a linear regression equation of $y = 0.0969x + 0.0045$ ($R^2 = 0.9998$). For the MDA assay kit, MDA content was calculated from $\Delta A = A_{532} - A_{600}$ according to the manufacturer's instructions.

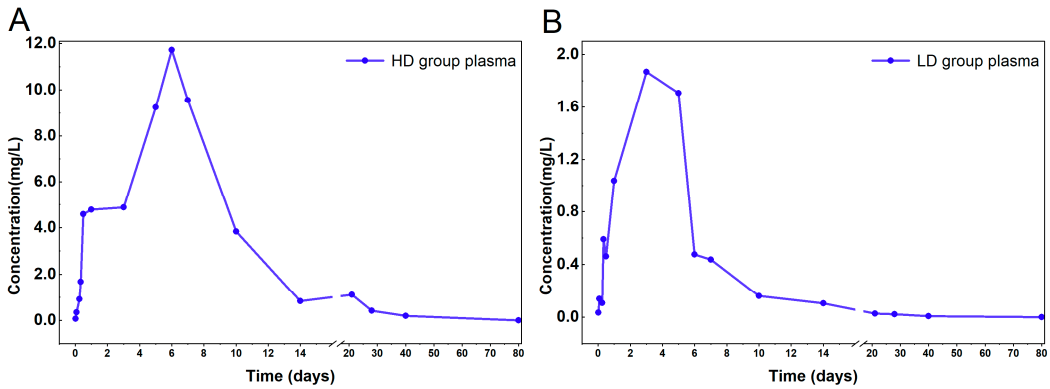


Figure S1. Time-concentration profiles of enrofloxacin and ciprofloxacin in the plasma of largemouth bass (*Micropterus salmoides*)

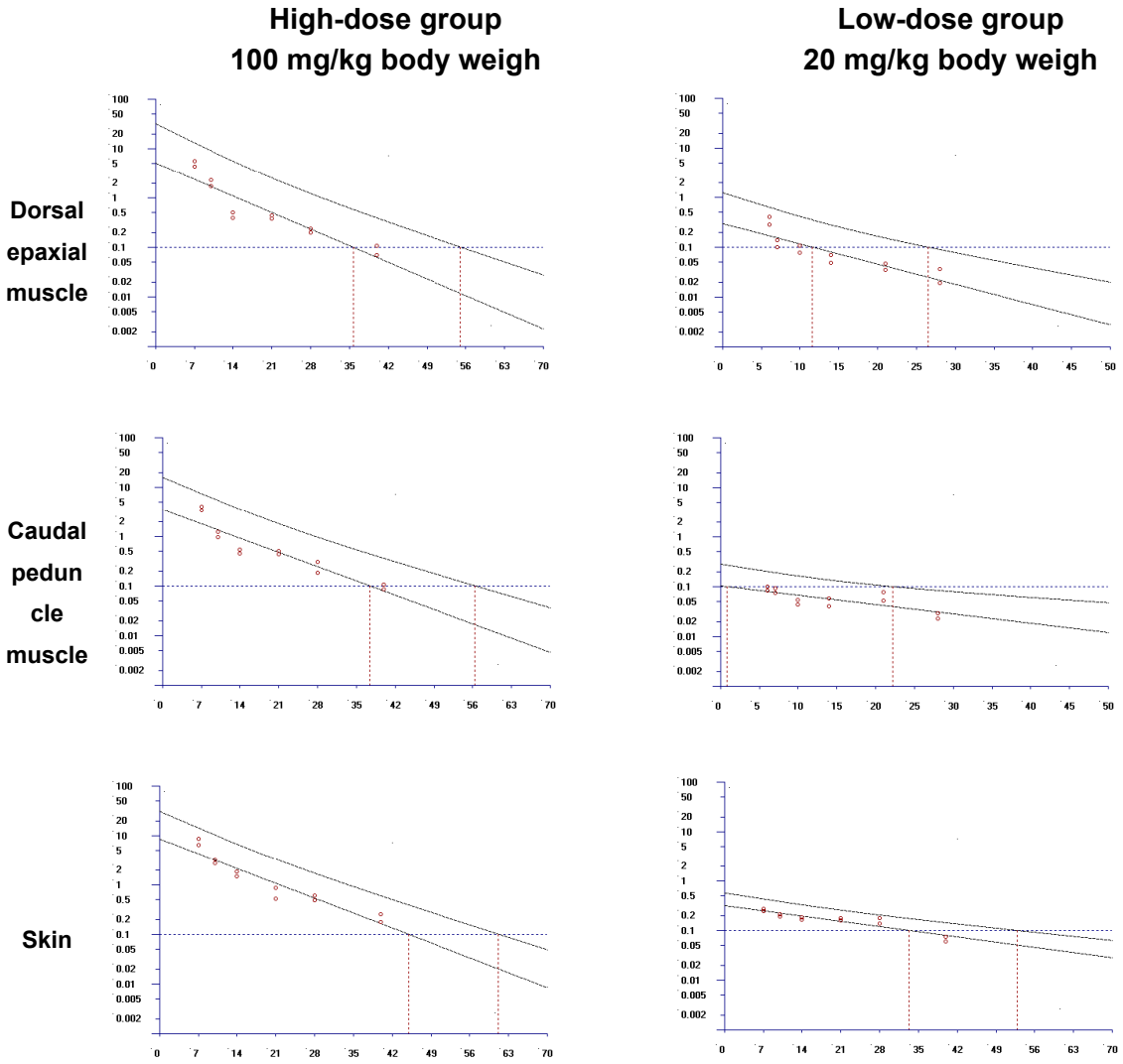


Figure S2. Withdrawal period models of enrofloxacin and its metabolite ciprofloxacin in three edible tissues of largemouth bass (*Micropterus salmoides*)

Table S1. Water quality parameters during the acclimation and exposure periods in each treatment group (Mean ± SD)

Period	Treatment	Temperature (°C)	DO (mg/L)	pH	Ammonia Nitrogen (mg/L)
Acclimation	CON	14.1±0.0	6.7±0.0	8.25±0.02	0.41±0.05
Acclimation	LD	14.6±0.1	7.2±0.2	8.22±0.02	0.39±0.07
Acclimation	HD	14.5±0.1	7.2±0.0	8.24±0.03	0.43±0.05
Exposure	CON	15.6±0.7	7.0±0.4	8.18±0.04	0.40±0.03
Exposure	LD	15.2±0.8	7.3±0.8	8.19±0.03	0.42±0.03
Exposure	HD	15.1±0.5	7.0±0.2	8.22±0.01	0.44±0.04

Table S2. Mortality of largemouth bass in each treatment group during the experimental period

Experimental day	Number of deaths								
	CON			LD			HD		
	1	2	3	1	2	3	1	2	3
Day 0-9	0	0	0	0	0	0	0	0	0
Day 10	0	0	1	0	0	0	0	0	0
Day 11-27	0	0	0	0	0	0	0	0	0
Day 28	1	0	0	0	0	0	0	0	0
Day 29-42	0	0	0	0	0	0	0	0	0
Day 43	0	0	0	0	0	0	0	1	0
Day 44-80	0	0	0	0	0	0	0	0	0
Mortality (%)	5	0	5	0	0	0	0	5	0

Note: Fish euthanized for scheduled sampling were not included in the mortality records.

Table S3. Results of enrofloxacin and ciprofloxacin in reagent and sample blanks

Blank type	Sample No.	ENR	CIP
Reagent blank	1	ND	ND
	2	ND	ND
	3	ND	ND
Sample blank	1	ND	ND
	2	ND	ND
	3	ND	ND
plasma blank	1	ND	ND
	2	ND	ND
	3	ND	ND
Water blank	1	ND	ND
	2	ND	ND
	3	ND	ND
fecal blank	1	ND	ND
	2	ND	ND
	3	ND	ND

Note: ND = not detected

Table S4. The assigned histopathological weights (w)

Tissue	Histopathological alteration	Reaction pattern	w [27]
liver	Vacuolar degeneration	Regressive changes	1
	Hemocyte infiltration	Inflammation	2
	Necrosis	Regressive changes	3
	Nuclear alterations and atrophy	Regressive changes	2
	Disorganization of hepatic cords	Regressive changes	1
kidney	Morphological alterations or necrosis of renal tubular epithelial cells	Regressive changes	3
	Alterations in renal tubular lumina	Regressive changes	1
	Proportion of necrotic/apoptotic cells	Regressive changes	3
	Interstitial inflammatory cell infiltration	Inflammation	2
	Structural abnormalities of the interstitial tissue	Regressive changes	1
spleen	Alterations in lymphoid tissue architecture	Regressive changes	2
	Decreased or increased numbers of lymphocytes	Regressive changes	1
	Necrosis	Regressive changes	3
	Hemorrhage or hyperemia	Circulatory disturbance	2

Note: The CON group showed no histopathological alterations and was assigned a condition index of 0.

Table S5 Semiquantitative scoring criteria for histopathological lesions

Area affected (%)	a [31]
0	0
1 - 25	1
25 - 50	2
51 - 75	3
75 - 100	4

Note: The score (a) was assigned based on the percentage of the microscopic field affected by the histopathological alteration.

Table S6. Relative proportions of ciprofloxacin of the combined enrofloxacin and ciprofloxacin residues in dorsal epaxial muscle, caudal peduncle muscle, skin, feces, and water samples (Mean ± SD, n = 3)

Sample type	Sampling time	HD			LD		
		ENR (μg/kg) /(μg/L)	CIP (μg/kg) /(μg/L)	CIP (%)	ENR (μg/kg) /(μg/L)	CIP (μg/kg) /(μg/L)	CIP (%)
Dorsal epaxial muscle	3d	6974.9±498.4	160.4±13.8	2.3	562.3±53.5	17.2±1.6	3.0
	5d	6737.8±56.6	113.6±8.6	1.7	402.0±29.4	15.05±1.2	3.6
	6d	5683.7±475.7	95.0±21.7	1.6	330.5±33.8	11.3±1.1	3.3

	7d	4883.5±502.7	118.4±12.8	2.4	107.9±15.8	11.5±1.1	9.6
	10d	1928.4±203.3	75.1±7.1	3.8	77.3±9.6	14.3±1.4	15.6
	14d	422.6±38.12	26.7±7.3	5.9	53.46±7.6	6.6±1.0	11.0
Caudal peduncle muscle	3d	4595.9±305.9	93.5±8.6	2.0	1037.0±84.0	21.1±3.1	2.0
	5d	7537.9±582.8	142.8±11.7	1.9	116.1±11.8	13.8±1.7	10.6
	6d	4561.3±400.4	95.8±7.8	2.1	81.05±7.7	11.2±1.6	12.2
	7d	3665.8±147.4	104.3±10.6	2.8	74.04±7.6	11.2±1.5	13.1
	10d	1020.5±133.0	77.4±6.7	7.1	40.46±5.2	9.0±1.2	18.3
	14d	469.5±49.5	26.8±4.7	5.4	40.41±4.7	9.5±1.2	19.0
Skin	3d	7747.3±614.5	113.6±10.8	1.4	684.7±65.1	16.0±1.7	2.3
	5d	10728.2±857.0	201.4±18.3	1.8	860.73±76.	14.9±1.6	1.7
	6d	9232.7±357.3	88.9±7.6	1.0	278.9±24.1	11.7±1.2	4.0
	7d	7414.6±584.3	115.6±10.5	1.5	248.5±22.1	11.1±1.6	4.3
	10d	2902.1±265.9	93.70±7.4	3.1	186.9±13.4	15.0±1.4	7.4
	14d	1568.0±134.4	76.2±7.7	4.6	166.9±12.1	8.8±0.9	5.0
	21d	684.9±65.4	10.6±1.8	1.5	166.7±14.9	2.2±0.3	1.3
	28d	544.8±47.5	8.0±1.2	1.5	153.8±9.6	3.0±0.4	1.9
Feces	2d	38.6±4.3	1.2±0.3	3.0	4.0±0.4	ND	-
	3d	148.2±15.8	4.6±0.6	3.0	5.9±0.7	ND	-
	4d	140.1±16.8	12.5±1.9	8.2	3.1±0.3	ND	-
	5d	58.4±9.1	8.5±1.3	12.7	2.3±0.3	ND	-
	6d	72.8±9.7	9.9±1.6	11.9	1.0±0.1	ND	-
	7d	32.1±4.2	6.0±1.0	15.8	1.6±0.2	ND	-
	8d	17.8±2.1	3.2±0.4	15.3	1.0±0.1	ND	-
	9d	12.7±2.0	1.74±0.	12.1	1.9±0.3	ND	-
	10d	12.9±1.9	2.5±0.5	3.0	0.9±0.1	ND	-
Waters	3d	0.9±0.1	ND	-	ND	ND	-
	4d	0.6±0.1	ND	-	0.2±0.0	ND	-
	5d	0.7±0.1	ND	-	0.2±0.0	ND	-
	6d	0.2±0.1	ND	-	ND	ND	-
	7d	0.2±0.0	ND	-	ND	ND	-

Note: ND = not detected; - = not applicable.

Table S7. Pharmacokinetic parameters of enrofloxacin and its metabolite ciprofloxacin in the plasma and six tissues of largemouth bass (*Micropterus salmoides*) following oral administration of enrofloxacin at two dosage levels.

Dose	Parameter	Plasma	Absorption-related organs			Excretion-related organs		
			Gill	Stomach	Intestine	Liver	Kidney	Spleen
HD group	T1/2β（h）	182.09	366.90	259.99	269.71	214.71	161.35	199.23
	Tmax（h）	144	72	120	120	72	72	120
	Cmax（mg/L）/（mg/kg）	11.72	13.01	22.58	16.14	10.69	20.25	20.72
	$AUC_{0-\infty}$（h*mg/L）/（h*mg/kg）	2286.45	2009.09	3146.83	2256.52	1935.35	2765.01	3717.77
	MRT（h）	234.68	242.59	256.71	200.68	196.24	161.29	228.05
	CL/F（L/h/kg）/（kg /h/kg）	0.04	0.05	0.03	0.04	0.05	0.04	0.03
	Vd/F（L/kg）/（kg /kg）	11.49	26.35	11.92	17.24	16.01	8.42	7.73
	λz（1/h）	0.0038	0.0019	0.0027	0.0026	0.0032	0.0043	0.0035
LD group	T1/2β（h）	155.41	277.66	222.23	113.46	123.69	117.72	251.84
	Tmax（h）	72	72	72	72	72	72	72
	Cmax（mg/L）/（mg/kg）	1.87	0.62	0.78	1.80	1.79	1.54	1.31
	$AUC_{0-\infty}$（h*mg/L）/（h*mg/kg）	259.92	102.77	154.23	155.76	167.43	164.10	382.61
	MRT（h）	137.79	313.3	279.69	169.69	175.42	172.83	471.86
	CL/F（L/h/kg）/（kg /h/kg）	0.08	0.19	0.13	0.13	0.12	0.12	0.05
	Vd/F（L/kg）/（kg /kg）	17.25	77.95	41.58	21.02	21.31	20.70	18.99
	λz（1/h）	0.0045	0.0025	0.0031	0.0061	0.0056	0.0059	0.0028