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# Residue Depletion of Florfenicol and Its Metabolite Florfenicol Amine in Swine Tissues after Intramuscular Administration

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A study of the tissue depletion of florfenicol (FF) administered intramuscularly twice to swine at a dose rate of 20 mg per kg of body weight at 24 h intervals was carried out. Forty healthy cross swine were treated with the FF injection formulation. Five treated animals were selected randomly to be sacrificed at 1, 3, 6, 9, 12, 14, 17, and 21 days withdrawal. FF and florfenicol amine (FFa) residue concentrations in muscle, liver, and kidney were determined using high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection at 225 nm. Liver samples showed the lowest FF and the highest FFa concentrations throughout the experiment period. However, the highest total concentrations of FF and FFa during the study were found in kidney, which indicated that kidney is the target tissue for FF. The sum of FF and FFa concentrations in all tissues analyzed was below the accepted maximum residue limits recommended by the Agriculture Ministry of People's Republic of China and the European Union at 8 days posttreatment.

**KEYWORDS:** Florfenicol; florfenicol amine; depletion; swine tissue; HPLC

## INTRODUCTION

Florfenicol (FF), [D-D-threo-3-fluoro-2-dichloroacetamido-1-(4-methylsulfonyl)-1-propanol], is a new semisynthetic member of the amphenicol family of antibiotics with a broad antibacterial spectrum. It was developed by Schering-Plough Corp. (United States) in the 1970s and marketed as a powder and injection solution for use in food animals. Although FF is a structural analogue of thiamphenicol, the antibacterial spectrum of activity is superior. It has greater activity not only against chloramphenicol-sensitive pathogens such as *Pasteurella multocida*, *Pasteurella hemeolytica*, and *Haemophilus somnus* but also against chloramphenicol-resistant strains of bacteria such as *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella typhimurium*, and *Proteus vulgaris* (1–4). Because of its effective antibacterial properties and low adverse effects, it has been widely used in food animals to prevent and treat infection with sensitive bacteria in pigs (5, 8), vibriosis in fish (6), and bovine respiratory disease (7, 8). FF has been approved to be used in swine and chicken in China since 1999 (9). At present, FF powder and injection solutions are usually used in clinical practice, and the recommended withdrawal periods are 20 and 14 days in swine and 5 and 28 days in chicken, respectively (10).

Reports have shown that FF is partly transformed into FF amine (FFa), FF oxamic acid, and FF alcohol in animal bodies after administration. Although the ratio of them is different in

different species, FFa is the largest in all metabolites in mostly food animals (11, 12, 14). Therefore, FFa is defined as one of the FF residue markers by many countries or organizations, and the maximum residue limits (MRLs) are shown in **Table 1** (13–15). There are many methods, such as high-performance liquid chromatography (HPLC) (16–19), liquid chromatography/mass spectrometry (20), gas chromatography (21), and gas chromatography/mass spectrometry (22), for the determination of FF and/or FFa in products from aquatic species, but few reports describe the simultaneous determination of FF and FFa in swine liver and kidney. The aim of the present work is to determine FF and its main metabolite FFa simultaneously in swine tissues and to study the depletion of FF in these tissues after intramuscular administration, to establish the withdrawal period.

## MATERIALS AND METHODS

**Reagents, Solvents, and Materials.** The FF injectable formulation (contains 300 mg mL<sup>-1</sup> FF) used in this study was provided by Shandong Mingfa Animal Medicine Co. Ltd. (Shandong Province, P. R. China). The FF and FFa standards (99 and 97.6%) were gifts from Schering-Plough Corp. (NJ). Acetone, dichloromethane, trichloromethane, triethylamine, trisodium phosphate 12-hydrate, hexane, and acetic acid were obtained from Beijing Chemical Reagent Co. (Beijing, P. R. China). Heptanesulphonate, methanol, and acetonitrile were purchased from Dikma Technology Inc. (Muskegon, MI). Water for HPLC analysis was Milli-Q filtered (Millipore, Bedford, MA). Solution A (0.02 M heptanesulphonate–0.025 M trisodiumphosphate) was made by dissolving 4.45 g of 1-heptanesulphonic acid sodium salt and 9.5 g of trisodium phosphate 12-hydrate in 750 mL of water, using acid phosphate (85%) to adjust the pH to 3.85, and water was added to

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**Table 1.** MRLs for FF + FFa in Swine Tissues (ng g<sup>-1</sup>)<sup>a</sup>

tissues	PRC	EU	USA
muscle	300	300	200
liver	2000	2000	2500
kidney	500	500	

<sup>a</sup> PRC, People's Republic of China; EU, European Union; and USA, United States of America.

make up the solution to 1 L. Solution B was methanol containing 0.1% triethylamine. The mobile phase was a mixture of solutions A and B at a ratio of 68:32. The eluted solution was prepared by the addition 10 mL of ammonium hydroxide and 10 mL of trichloromethane into 80 mL of acetonitrile, and it was mixed uniformly. Solid-phase extraction (SPE) cartridges (Oasis MCX 3 cm<sup>3</sup> 60 mg, Waters Corp., Ireland) were used to clean up the tissue samples.

**Standards.** A stock solution of 1 mg mL<sup>-1</sup> was prepared by dissolving 100 mg of each FF and FFa standard in 100 mL of acetonitrile. The working standard solutions of 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 µg mL<sup>-1</sup> were prepared in 0.01 M Na<sub>2</sub>HPO<sub>4</sub>-methanol (80:20, v/v).

**Animal Treatment.** The experiments were conducted in 45 healthy Chester White-Yorkshire cross-breed swine that weighed 50–60 kg, were 3–4 months old, and were kept in individual metabolic cages in a closed room. During acclimatization for 3 weeks and the subsequent treatment periods, they were fed drug-free assorted feed ad libitum with free access to water. The health of the animals was monitored by a veterinarian. Forty animals were weighed and treated with FF injected intramuscularly at 20 mg kg<sup>-1</sup> of body weight on the right side of the neck. The second injection was given 24 h after the first injection. Five animals were kept as untreated controls. Five animals of the treated group were randomly sampled at 1, 3, 6, 9, 12, 14, 17, and 21 days after the last injection. Samples of muscle, liver, and kidney were collected from each animal and stored at -20 °C until they were processed. The untreated animals were sacrificed on day 21 to obtain blank tissues.

**Sample Preparation.** Swine tissues (muscle, liver, and kidney) were minced and homogenized in the homogenizer for 1 min. An amount of 5.0 g of homogenate swine tissue was accurately weighed into a 50 mL polypropylene centrifuge tube. A volume of 2.0 mL of water and 8.0 mL of acetone was added into the tube, and the mixture was homogenized and centrifuged for 5 min at 3000g. The supernatant was removed into a 100 mL polypropylene centrifuge tube, and the tissue in the tube was extracted again following the above-mentioned procedure. The two extracts were combined into a tube, and 20 mL of dichloromethane was added. The tube was capped and vortexed. After centrifugation for 5 min, the lower organic layer was transferred into another 100 mL flask. The upper aqueous layer was extracted again using 8 mL of acetone and 7 mL of dichloromethane. The extracts were combined into a flask. Ten milliliters of acetonitrile was added to the tissue pellet to repeat extraction a third time. The mixture was mixed vigorously for 1 min by a vortex mixer and centrifuged as before. The supernatants were combined into the flask, and 1.5 mL of 0.1% acetic acid (v/v) and 3 mL of isopropanol alcohol were added into the flask. Then, the extracts were evaporated to about 1.5 mL at 50 °C. Another 1.5 mL of 0.1% acetic acid was added, and the mixture was mixed uniformly. Five milliliters of *n*-hexane was added into the flask for defatting, and the hexane layer was discarded. The defatting step was repeated more than two times with an additional 5 mL of hexane each time. The remaining aqueous extract was subjected to SPE cleanup.

Before use, the MCX cartridge, placed in a vacuum manifold system, was preconditioned with 3 mL of methanol and then 3 mL of water. After the loaded aqueous extract was drained through the cartridge by applying a vacuum, the cartridge was washed with 3.0 mL of water. The MCX cartridge was dried for at least 1 min. FFa and FF were eluted with 6.0 mL of eluting solution. The collected elute was evaporated to dryness under a nitrogen stream in a 50 °C water bath and then reconstituted in 1 mL of 0.01 M Na<sub>2</sub>HPO<sub>4</sub>-methanol (80:20,

**Table 2.** Recoveries of FF Fortified in Swine Tissues (*n* = 5)

samples	added (ng g <sup>-1</sup> )	interday		intraday	
		average recovery (%)	CV (%)	average recovery (%)	CV (%)
muscle	100	81.7	2.1	80.3	7.3
	500	85.4	4.5	85.5	7.6
	2000	84.8	7.2	84.5	6.1
liver	100	76.1	5.8	75.2	7.7
	500	82.3	5.7	82.4	9.2
	2000	84.5	2.9	81.3	8.5
kidney	100	80.1	4.9	80.2	9.7
	500	87.9	3.1	83.6	8.2
	2000	86.5	5.9	83.8	9.5

**Table 3.** Recoveries of FFa Fortified in Swine Tissues (*n* = 5)

samples	added (ng g <sup>-1</sup> )	interday		intraday	
		average recovery (%)	CV (%)	average recovery (%)	CV (%)
muscle	100	74.2	7.9	72.4	9.1
	500	74.7	7.8	79.2	6.5
	2000	83.8	4.3	83.3	8.7
liver	100	74.1	5.4	73.8	7.7
	500	76.2	5.9	75.3	6.3
	2000	79.7	4.0	76.6	9.3
kidney	100	83.0	7.7	80.2	9.7
	500	84.5	5.2	83.6	8.2
	2000	88.8	7.9	83.8	9.9

v/v). After it was filtered with a 0.2 µm syringe filter, the sample solution was applied to HPLC analysis.

**LC Analysis.** The analysis of standards, fortified samples, and incurred samples was performed using an HPLC system at room temperature. The HPLC system was composed of a Waters 2695 Separations Module and a Waters 2996 Photodiode Array Detector with an autosampler (Waters Co., Milford, MA). The chromatographic column was a reversed-phase column (Inertsil ODS-3 columns, 4.6 mm i.d. × 250 mm, 5 µm, GL Sciences Inc. Tokyo, Japan). The injection volume was 100 µL, and the detection wavelength was 225 nm.

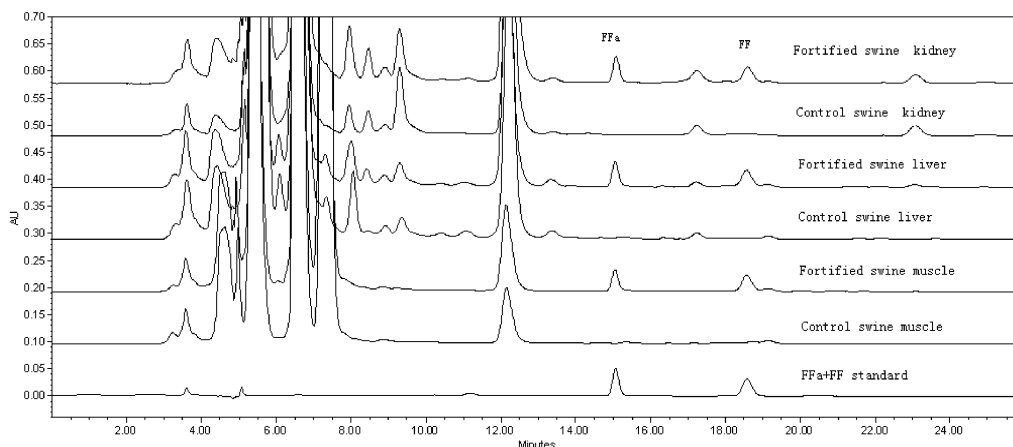
**Calibration.** The calibration curves were prepared on the basis of the peak areas and the working standard solution concentrations. A series of working standard solutions of 100, 500, 1000, 2000, 5000, 10000, and 20000 ng mL<sup>-1</sup> were injected into HPLC and analyzed as described above.

**Data Analysis.** The Student's *t*-test was performed to test for significant differences between the drug and its metabolite concentrations in the same or different tissues. The withdrawal period was estimated by linear regression analysis of log-transformed tissue sum of FF and FFa concentrations and determined at the time when the one-side 95% upper tolerance limit was below the MRL (23).

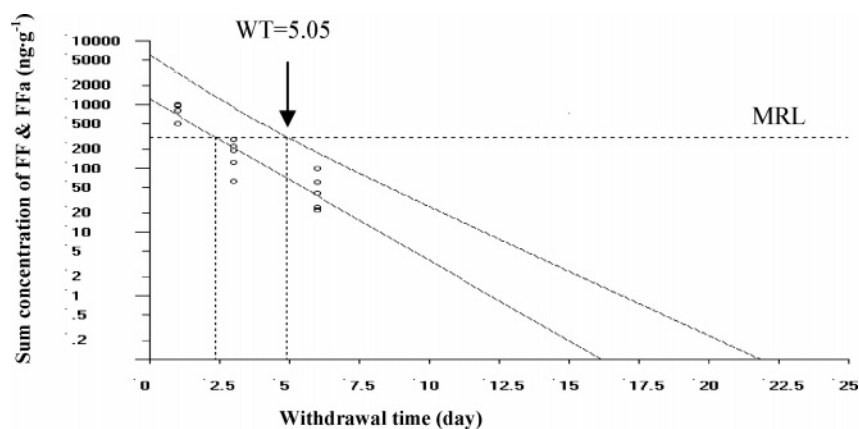
## RESULTS AND DISCUSSION

**Analytical Method.** The sample extraction and cleanup procedure is an important factor for drug residue analysis, during which the analytes of interest are separated from biological matrix components. There are a few reported methods to extract FF and/or FFa from aquatic tissues (15–21), but no published reports described the simultaneous determination of FF and FFa residues in swine tissues. Ethyl acetate, methanol, acetonitrile, or acetone was individually tested to extract the drug from swine tissues in our study, but the results were not satisfactory. In the present study, FF and FFa in swine tissues were first extracted with acetone and water followed extraction by acetonitrile, and higher recoveries of both FF and FFa were obtained with no matrix interferences.

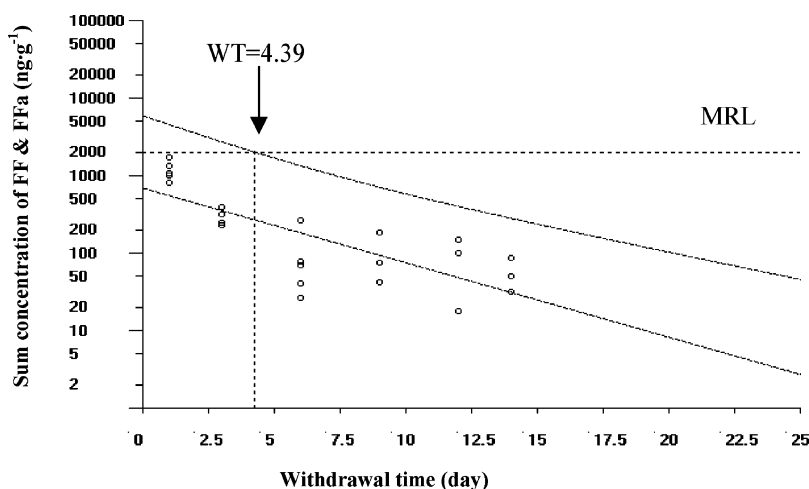
Nagata and Saeiki (18) used Sep-Pak Florisil to clean up tissue samples, Christopher et al. (19) used Chem Elut CE1020 Sorbant



**Figure 1.** Chromatograms of FF and FFa standard (2500 ng mL<sup>-1</sup>); control of swine muscle, liver, and kidney; fortified swine muscle, liver, and kidney (500 ng g<sup>-1</sup>). Peaks: FF, florfenicol; FFa, florfenicol amine.



**Figure 2.** Plot of withdrawal time (WT) calculation for swine muscle at the time when the one-sided 95% upper tolerance limit was below the MRL of 300 ng g<sup>-1</sup>.

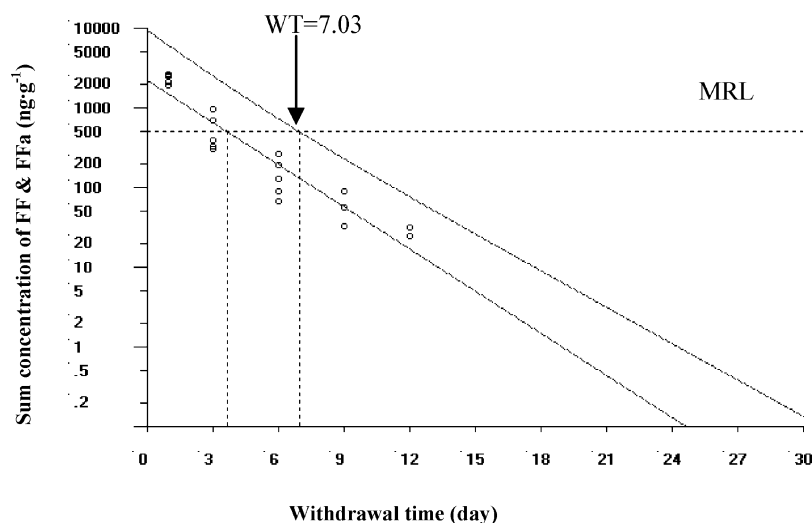


**Figure 3.** Plot of withdrawal time (WT) calculation for swine liver at the time when the one-sided 95% upper tolerance limit was below the MRL of 2000 ng g<sup>-1</sup>.

Column, and Allen et al. (21) employed a propylsulfonic acid and C<sub>18</sub> SPE system. In this study, we compared several SPE columns. The results showed that the MCX column has a better extraction effect.

The analytical method in this study was a modification from Hormazabal et al. (16) and was demonstrated to be reliable and stable. The standard curves were linear from 100 to 20000 ng mL<sup>-1</sup> ( $R = 0.9997$  for FF and  $R = 0.9999$  for FFa, respectively). The limits of detection (LODs) in muscle were 10 and 15 ng

g<sup>-1</sup> in liver and kidney for both FF and FFa, respectively, and the limits of quantification (LOQs) for both the drug and the metabolite were 20 ng g<sup>-1</sup> in muscle and 30 ng g<sup>-1</sup> in liver and kidney, respectively. The accuracy and precision of the method were determined using swine muscle, liver, and kidney samples fortified at levels of 100, 500, and 2000 ng g<sup>-1</sup>. The interday mean recovery of FF was between 76.1 and 87.9% with coefficients of variation (CVs) of 2.1–7.2%. The intra-assay mean recovery of FF was between 75.2 and 85.5% with



**Figure 4.** Plot of withdrawal time (WT) calculation for swine kidney at the time when the one-sided 95% upper tolerance limit was below the MRL of 500 ng g<sup>-1</sup>.

CVs of 6.1–9.5% (**Table 2**). The interday mean recovery of FFa was between 74.1 and 88.8% with CVs of 4.0–7.9%. The intra-assay mean recovery of FFa was between 72.4 and 83.8% with CVs of 6.3–9.9% (**Table 3**). The recoveries of FF and FFa in the present method were slightly lower than the previous method. The method can meet the international general criteria of residue study that an average recovery was between 70 and 110% and a CV was 15% or less for each group of sample. The chromatograms of FF and FFa standards, control tissues, and fortified tissues were shown in **Figure 1**. The lack of interferences in the chromatographic separation demonstrates a high specificity of the analytical method and a good selectivity obtained in the extraction procedure.

**Residue Depletion.** The potency and broad spectrum of activity of FF make it a good antibiotic to replace chloramphenicol, which has been banned from use in food-producing animals because of possible induction of aplastic anemia in humans. There are several studies on FF pharmacokinetics (24–33) but few reports on FF residues in swine. In this depletion study, the concentrations of FF and FFa measured in muscle, liver, and kidney from swine administered intramuscularly twice at the dose of 20 mg kg<sup>-1</sup> of body weight at 24 h intervals and slaughtered at 1, 3, 6, 9, 12, 14, 17, and 21 days posttreatment are summarized in **Table 4**. At 1 day postadministration, the concentrations of both FF and FFa in all tissues peaked (muscle: FF, 469–929 ng g<sup>-1</sup>; FFa, 40–163 ng g<sup>-1</sup>; liver: FF, 189–669 ng g<sup>-1</sup>; FFa, 606–1062 ng g<sup>-1</sup>; and kidney: FF, 1439–1939 ng g<sup>-1</sup>; FFa, 434–862 ng g<sup>-1</sup>, respectively). The results showed that kidney should be considered to be the target organ for FF in swine. At 9 days posttreatment, the concentrations of FF in kidney tissue ranged from 30 to 34 μg g<sup>-1</sup>, while it was not detected in muscle or liver tissues. The concentrations of FFa in liver tissue ranged from 42 (near LOQ) to 186 ng g<sup>-1</sup> at 9 days posttreatment, but it was only detected in kidney tissue of two animals and in no samples of muscle tissues. Moreover, as shown in **Table 4**, at 1 day posttreatment, there were significant differences between the mean concentrations of FF and FFa in all tissue samples ( $P > 0.05$ ). The concentrations of FF were higher than FFa in muscle and kidney tissues at 1 and 3 days postadministration. However, in liver tissue, the concentrations of FFa were higher than FF, and FFa residues were depleted faster than FF at the 1 and 3 days posttreatment.

**Table 4.** FF and FFa Residue Concentrations (ng g<sup>-1</sup>) in Swine Tissues after Two Intramuscular Injections at 24 h Intervals at a Dose Rate of 20 mg kg<sup>-1</sup> of Body Weight<sup>a</sup>

withdrawal time (day)	animal no.	muscle		liver		kidney	
		FF	FFa	FF	FFa	FF	FFa
1	1	789	163	669	1062	1817	862
	2	887	92	304	759	1484	434
	3	929	88	424	933	1939	555
	4	469	40	189	606	1439	678
	5	746	56	219	772	1762	755
3	6	256	25	235	156	703	264
	7	192	31	333	56	346	360
	8	153	41	44	187	338	59
	9	99	27	191	54	147	175
	10	62	ND	195	123	154	153
6	11	61	ND	ND	78	103	165
	12	ND	40	ND	27	24	65
	13	58	41	ND	262	117	76
	14	ND	25	ND	41	30	37
	15	22	ND	ND	69	39	90
9	16	ND	ND	ND	186	ND	ND
	17	ND	ND	ND	75	34	56
	18	ND	ND	ND	42	30	60
	19	ND	ND	ND	ND	32	ND
	20	ND	ND	ND	ND	30	<LOQ
12	21	ND	ND	ND	148	ND	<LOQ
	22	ND	ND	ND	ND	ND	ND
	23	ND	ND	ND	99	ND	32
	24	ND	ND	ND	ND	ND	ND
	25	ND	ND	ND	<LOQ	ND	ND
14	26	ND	ND	ND	ND	ND	ND
	27	ND	ND	ND	50	ND	ND
	28	ND	ND	ND	87	ND	ND
	29	ND	ND	ND	ND	ND	ND
	30	ND	ND	ND	31	ND	ND

<sup>a</sup> ND, not detected.

Then, the FFa residues were eliminated slowly. Even at 14 days postadministration, FFa was detected in three out five pigs.

The residue study showed that FF determined was highest in kidney and lowest in liver at 24 h after intramuscular administration, which was similar to Afifi and El-Sooud (29) about FF concentration in chicken tissue and Adams et al. (33) concerning FF residue concentrations in male veal calves given repeated doses.



The European Agency for the Evaluation of Medicinal Products reported a residue study on pigs, and the results showed that about 45–60% of FF was excreted through urine in unchanged form, 11.2–17% was excreted as FFa, <10% was excreted as FF oxamic acid, and 1.1% was excreted as FF alcohol (12). These results were similar to ours. FFa was also detected based on the LOD after 9 days of withdrawal, which showed that FFa was excreted slowly from the kidneys.

The veterinary drug residue regulations of the China Ministry of Agriculture and the Committee for Veterinary Medicinal Products of the European Union had established the MRLs for FF in swine (12, 13). The MRLs for FF are 300, 2000, and 500 ng g<sup>-1</sup> in muscle, liver, and kidney, respectively, and the residue marker is the sum of FF and its metabolite FFa. As shown in **Table 4** and **Figures 2–4**, at 6 days of withdrawal, the sum of FF and FFa concentrations in all tissues from swine intramuscularly administration twice at a dose rate of 20 mg kg<sup>-1</sup> of body weight at 24 h intervals was below the accepted MRLs. However, because of the interindividual variability, the limited test number of animals, and to avoid potential hazards to human health, the withdrawal periods were estimated as 5.05 days for muscle, 4.39 days for liver, and 7.03 days for kidney by the statistical method stated in the guidance (21). The longest withdrawal time of 8 days has to be selected as the conclusive withdrawal time.

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