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In vitro antibacterial activity of danofloxacin against *Escherichia coli* in Gushi chickens and its residue depletion following multiple oral administration

Jun-Cheng Chen,[*,1] Jun Liang,[†,1] Ze-En Li,[*] Ming-Hui Duan,[*] Yan Dai,[*] Yang-Guang Jin,[*] Yan-Ni Zhang,[*] Yue Liu,[*] Mei Zhang,[*] Guo-Yong Wang,[*] and Fan Yang [*,2]

[*]*College of Animal Science and Technology, Henan University of Science and Technology, Luoyang 471023, China; and* [†]*Zhengzhou Institute of Veterinary Drug and Feed Control, Zhengzhou 450002, China*

ABSTRACT This study aimed to investigate the in vitro antibacterial activity of danofloxacin against *Escherichia coli* isolated from Gushi chickens, as well as the tissue distribution and residue depletion of danofloxacin in Gushi chickens following multiple oral administration. A total of 42 clinical *E. coli* strains were isolated from the cloaca of locally farmed Gushi chickens between August and October 2023. Then the minimum inhibitory concentration (**MIC**) of danofloxacin against these isolates was determined by broth microdilution method. Additionally, 42 healthy Gushi chickens were randomly divided into 6 groups, and danofloxacin was orally administered at a dose of 5 mg/kg body weight (**BW**) for 3 consecutive days. Plasma, intestinal content, and tissue samples, including muscle, skin + fat, liver, kidney, lung, and intestine, were collected at 4, 12, 24, 48, 72, and 120 h after the last administration. Danofloxacin concentrations in all samples were determined using a high-performance liquid chromatography (**HPLC**) method. The average concentration vs. time data were then subjected to non-compartmental analysis using Phoenix software, and withdrawal periods for danofloxacin in Gushi chickens were further determined with WT1.4 software, setting a 95% confidence interval. Results indicated a notable inhibitory effect of danofloxacin on *E. coli*, with an MIC_{50} of 0.5 μg/mL. Additionally, danofloxacin exhibited widespread distribution in Gushi chickens, detectable in all collected samples. Among all tissues, the liver exhibited the highest concentration, followed by the intestine. Even on the fifth day postadministration, danofloxacin persisted in skin + fat, liver, and lung. The elimination half-lives ($t_{1/2\lambda z}$s) of danofloxacin varied across samples: skin + fat (47.87 h), lung (30.61 h), liver (22.07 h), plasma (16.05 h), muscle (12.53 h), intestine (9.83 h), and kidney (6.34 h). Considering residue depletion and the maximum residue limit (**MRL**) of danofloxacin in poultry set by Chinese regulatory authorities, withdrawal periods for the kidney, muscle, liver, and skin + fat were determined as 1.03, 1.38, 3.34, and 5.85 d, respectively, rounded to a final withdrawal time of 6 d.

Key words: danofloxacin, Gushi chickens, in vitro antibacterial activity, residue depletion, withdrawal time

2024 Poultry Science 103:103493
https://doi.org/10.1016/j.psj.2024.103493

INTRODUCTION

Poultry farming holds a pivotal role in China's agricultural production, contributing significantly to economic development. In recent years, spurred by rising living standards and evolving dietary preferences, there has been a steady increase in the demand for poultry meat. The per capita consumption of poultry meat has shown a gradual rise as well (Wen et al., 2019; Chen et al., 2020). Chicken is the primary component of poultry meat, making up over 60%. In China, it stands as a major meat product, witnessing a consistent year-over-year increase in consumption, ultimately becoming the second-largest meat consumer product (Guo et al., 2023; Zhang et al., 2023).

Gushi chicken is renowned for its superior meat and egg quality and is a popular backyard chicken breed in China (Zhai et al., 2023). It possesses exceptional qualities such as remarkable resistance to coarse feeding, robust disease resistance, and the delectable quality of its meat (Zhang et al., 2023). In contrast to commercially farmed chickens confined in cages, Gushi chickens are bred under a free-range model that makes them highly vulnerable to widespread infections, such as *Mycoplasma* and *Escherichia coli* infections (Chen

Received January 4, 2024.
Accepted January 19, 2024.

[1]These authors contributed equally to this manuscript.

[2]Corresponding author: fyang@haust.edu.cn

et al., 2023). Consequently, farmers commonly employ antibiotics to address infections in Gushi chickens.

Danofloxacin, as a third-generation fluoroquinolone drug, demonstrates superior efficiency and lower toxicity compared to other fluoroquinolones (Fan et al., 2015). Its mechanism of action involves inhibiting bacterial DNA replication and transcription by targeting the DNA gyrase subunit, resulting in bactericidal effects (Wang et al., 2022; Yang et al., 2022). Notably, danofloxacin not only achieves high concentrations in tissues but also can modify bacterial growth rates and morphology even below its inhibitory concentration (Lynch et al., 1994). This unique capability has led to its approval in numerous countries for treating diseases such as *E. coli* infections, *Mycoplasma* infections, and pneumonia in poultry (Chen et al., 2023). Recognized for its efficacy, particularly in addressing respiratory diseases and bacterial infections in poultry, danofloxacin stands as an ideal drug for these applications (Zhang et al., 2020).

The widespread use of antibiotics has brought forth increasingly prominent issues, including escalating bacterial resistance, jeopardizing the safety of animal-derived food, and potentially causing direct harm to the human body (Van Boeckel et al., 2014; Chen et al., 2019). The residual concern related to fluoroquinolone drugs, such as danofloxacin, has garnered widespread attention across various sectors of society (Brown, 1996). Excessive intakes of danofloxacin in the human body may result in symptoms like headaches, nausea, diarrhea, liver damage, joint disease, and allergies (Jin et al., 2023). Currently, numerous studies focus on the residue depletion of danofloxacin in animals (Goudah and Mouneir, 2009; Vardali et al., 2017; Sartini et al., 2021; Song et al., 2023). However, existing research predominantly relies on single-dose or drinking water administration in chickens, with relatively limited exploration into residue depletion, distribution, or extended research timelines (Lynch et al., 1994; Zeng et al., 2011).

To safeguard animal-based food health, various countries, including China and the European Union, have established limit standards (EU, 2010; MARA, 2019). In poultry, these nations have established maximum residue limits (MRL) for danofloxacin. The MRLs are set at 0.1 μg/g and 0.2 μg/g in skin + fat and muscle, respectively. Additionally, the MRL is set at 0.4 μg/g in kidney and liver. It is worth noting that all these MRL values pertain to the same marker residue, danofloxacin. Danofloxacin, available in both powder and solution forms in China, is recommended at a dosage of 2.5 to 5 mg/kg body weight, administered orally once a day for 3 consecutive days. The withdrawal time for both forms in chickens has been established at 5 d (CCVP, 2017). Nevertheless, the established withdrawal period is mainly tailored for captive commercial chickens, like white-feathered broilers. Considering the unique traits of Gushi chickens, it is conceivable that their withdrawal period could differ. Hence, this study aims to investigate the tissue distribution and residue depletion of danofloxacin in Gushi chickens after multiple oral administration. The ultimate goal is to determine a withdrawal time that aligns with the specific characteristics of Gushi chickens.

MATERIALS AND METHODS

Organisms and Chemicals

We isolated 42 clinical *E. coli* strains from the cloaca of locally farmed Gushi chickens between August and October 2023. All strains were identified by Gram staining, 16S rDNA specific primer polymerase chain reaction (**PCR**). The quality control strain was *E. coli* ATCC 25922, which was provided by the Pharmacology Laboratory of Henan University of Science and Technology. MacConkey Agar, Mueller-Hinton Broth (**MHB**), Luria Bertani (**LB**) Broth, and Eosin-Methylene Blue Agar (**EMB**) were purchased from Beijing Aoboxing Bio-Tech Co., Ltd. (Beijing, China).

The danofloxacin mesylate reference substance (Lot No. h0201210; purity 94.2%) was obtained from the China Institute of Veterinary Drugs Control (Beijing, China). The danofloxacin mesylate raw material (Lot No. 201217-1; purity 95.37%) was sourced from Zhejiang Guobang Pharmaceutical Co., Ltd. (Hangzhou, China). Phosphoric acid (H_3PO_4) and tri-ethylamine were acquired from Shanghai Yien Chemical Technology Co., Ltd. (Shanghai, China), and dichloromethane ($\mathbf{CH_2Cl_2}$) was purchased from Shanghai Boer Chemical Reagent Co., Ltd. (Shanghai, China). Chromatographic grade methanol and acetonitrile were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). 0.9% sodium chloride injection was purchased from Sichuan Kelun Pharmaceutical Co., Ltd. (Sichuan, China). All other reagents were obtained from commercial sources.

Experimental Animals

Forty-two healthy 21-wk-old Gushi chickens, evenly distributed between male and female, were procured from a breeding farm. Each chicken was individually housed in a wire cage measuring 55 × 55 × 45 cm. A 1-wk acclimatization period preceded any experimental procedures, during which the chickens had unrestricted access to water and were fed antibiotic-free feed twice daily. All experimental protocols adhered to the guidelines for animal feeding management and use, receiving approval from the Institutional Animal Care and Use Committee (**IACUC**) of Henan University of Science and Technology (Approval Number: 20230303).

Minimum Inhibitory Concentration (MIC) Assays

The minimum inhibitory concentration (MIC) of danofloxacin against *E. coli* was determined using the microbroth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute

(CLSI Clinical and Laboratory Standards Institute, 2023). Initially, each strain of *E. coli* underwent overnight incubation in LB broth at 37°C. Subsequently, 3 μL of the bacterial solution was transferred to a new MHB and incubated at 37°C and 170 rpm for 4 h. The bacterial liquid concentration was then adjusted using MHB to achieve a turbidity corresponding to the 0.5 McFarland Standard. The bacterial solution, diluted to a content of 10^6 CFU/mL, was distributed into a 96-well plate. The initial danofloxacin concentration for all strains was set at 64 μg/mL. A double dilution of danofloxacin was prepared in MHB on a 96-well plate. Each strain was allocated to a negative control group (200 μL MHB) and a positive control group (200 μL bacterial solution), respectively. Results were observed after 20 h of incubation in a constant temperature incubator at 37°C. The effective MIC value was determined as the minimum concentration of antibacterial drugs observed without visible bacterial growth to the naked eye. This process was repeated 3 times for each *E. coli* strain.

Administration and Sampling

All chickens were individually weighed and randomly assigned to 6 groups, each consisting of 7 chickens, comprising 3 males, 3 females, and 1 chicken exempt from dosing. The weight range for males and females was 1.62 to 2.53 kg and 1.24 to 1.86 kg, respectively. The raw material of danofloxacin mesylate was diluted into a 5 mg/mL oral solution using purified water. Except for the chicken in each group exempt from dosing, every other chicken received a 5 mg/kg dose of danofloxacin via gavage, administered every 24 h for 3 consecutive times. The oral solution was freshly prepared on the same day.

At intervals of 4, 12, 24, 48, 72, and 120 h after the last administration, a group was randomly selected, and blood samples (approximately 2 mL) were collected from the inferior vein and placed in heparin sodium tubes. Following blood collection, chickens were euthanized via cervical dislocation, and samples, including muscle, skin plus fat, liver, kidney, lung, intestine, and intestinal content, were collected at each time point. To prevent contamination, all tissue samples were left unwashed and stored independently in zip-lock bags at −20°C. Blood samples were centrifuged at 4,000 × g for 10 min to obtain plasma, which were subsequently stored at −20°C until further analysis. The samples collected from the untreated chicken were utilized as blank samples for the quantification of danofloxacin.

Sample Preparation

The sample processing method, optimized based on previously published papers in our laboratory (Song et al., 2023), involves the following steps. Briefly, the plasma sample was thawed at room temperature, and then 0.5 mL of the sample was combined with 1 mL of acetonitrile. The mixture was thoroughly vortexed for 3 min and subsequently centrifuged at 12,000 × g for 10 min. The resulting supernatant was collected in a clean glass tube, and the extraction process was repeated. The glass tube was then subjected to evaporation to dryness in a nitrogen stream at 50°C. The residue was dissolved in 2 mL of the mobile phase, vortexed for 1 min, and the liquid was filtered through a 0.22-μm filter into a glass vial for the automatic sampler. Finally, 20 μL of the supernatant was injected into the HPLC system.

The tissue sample processing involved several key steps. Initially, all samples were thawed to room temperature and homogenized. A gram of tissue homogenate was taken, and 2 mL of acidified acetonitrile (1.8 mL acetonitrile + 0.2 mL phosphate buffer) was added. After thorough vortexing for 5 min, the mixture underwent centrifugation at 10,000 × g for 10 min. The resulting supernatant was carefully collected in a clean glass tube, and the extraction process was repeated. The collected supernatants were combined and subsequently evaporated using a stream of nitrogen at 50°C. The residue was then dissolved in 2 mL of the mobile phase. Following this, 2 mL of dichloromethane was added, and the mixture underwent complete vortexing for 3 min. After centrifugation at 4,000 × g for 5 min, the supernatant underwent filtration using a 0.22-μm filter. Finally, 20 μL of the resulting filtrate was injected into the HPLC system.

Danofloxacin in plasma and tissues was detected using the Waters e2695 HPLC system coupled with a 2475 fluorescence detector. The separation process employed a Hypersil BDS C18 column (4.6 × 250 mm, 5 μm, Elite Analytical Instruments Co., Ltd.; Dalian, China). A mobile phase consisting of 0.05 mol/L phosphate buffer (adjusted to pH 2.6 with triethylamine) and acetonitrile (83:17) facilitated the separation. The column temperature was consistently maintained at 30°C. Excitation and emission wavelengths were set at 280 nm and 450 nm, respectively. Elution occurred over 12 min at a flow rate of 1 mL/min, ensuring accurate and efficient detection of danofloxacin in the samples.

Method Validation

A standard stock solution of danofloxacin (500 μg/mL) was prepared using methanol and stored in the dark at 4°C. Calibration standards were prepared to cover a broad range of concentrations in plasma (0.005−5 μg/mL) and tissues (0.01 or 0.05−20 μg/g), which were achieved by spiking serial working solutions into blank samples. The accuracy and precision of the methods were evaluated by spiking 5 replicate blank samples with 3 distinct concentrations of danofloxacin. For each spiked tissue sample, 0.9 g of drug-free sample was combined with 0.1 mL of the danofloxacin standard; for spiked plasma samples, 0.45 mL of drug-free sample was mixed with 0.05 mL of danofloxacin standard. And the spiked concentrations in plasma, muscle, lung, and skin + fat, were 0.01, 0.5, and 2 μg/mL or μg/g, while in the liver, kidney, intestine, and intestinal content, the spiked concentrations were 0.01, 1, and 5 μg/g. Each replicate sample underwent continuous measurement for 3 d to calculate the recovery rate, intra-day, and

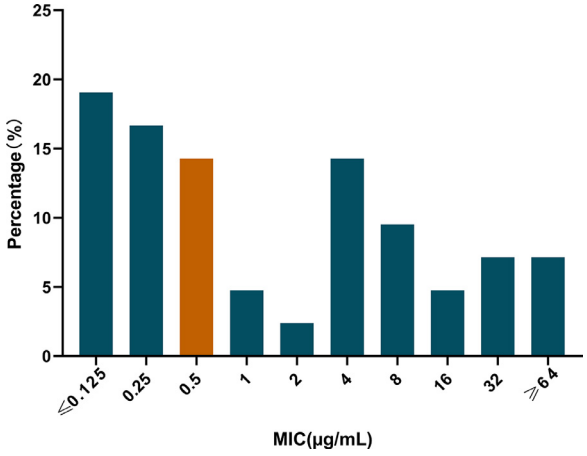

Figure 1. MIC distribution of danofloxacin against 42 *Escherichia coli* strains.

inter-day coefficients of variation. The limits of detection (**LOD**) and quantification (**LOQ**) were determined based on signal-to-noise (**S/N**) ratios, with thresholds set at ≥ 3 for LOD and ≥ 10 for LOQ.

Data Analysis

In plasma and all tissues, concentrations have been expressed as Mean ± SD. Noncompartment model analysis (**NCA**) in Phoenix software was used to analyze the elimination equation, terminal half-life ($\mathbf{t_{1/2\lambda z}}$), area under the concentration-time curve (**AUC**), and mean residence time (**MRT**) of danofloxacin in plasma and tissues of Gushi chickens. To determine the withdrawal period, we utilized WT1.4 software in conjunction with tissue concentration to establish the elimination curve. This approach ensured a 95% confidence interval, guaranteeing that 99% of the drug residue concentrations in the tissue remained lower than the MRL.

RESULTS

In vitro Antibacterial Activity

We conducted an evaluation of the in vitro antibacterial activity of danofloxacin against clinical isolates of *E. coli*. Figure 1 illustrates the MIC results of danofloxacin against 42 strains of *E. coli* isolated from Gushi chickens. The MIC ranges varied from ≤ 0.125 to ≥ 64 μg/mL. Notably, 19% of the strains exhibited MIC values lower than 0.125 μg/mL. The MIC_{50} of danofloxacin against *E. coli* was determined to be 0.5 μg/mL, the MIC against ATCC 25922 is 0.125 μg/mL, reflecting a significant antimicrobial effect within the tested strains.

Validation of Analytical Methods

The extraction and detection method employed in this study effectively circumvents endogenous interference, demonstrating high selectivity. Table 1 illustrates the robust linear range of danofloxacin across different samples at varying concentration levels, with correlation coefficients ($\mathbf{R^2}$) consistently exceeding 0.99. The LOD across all samples ranged from 0.001 to 0.005 μg/g (μg/mL), while the LOQ were within the range of 0.005 to 0.01 μg/g (μg/mL). In cases where danofloxacin concentrations exceed the upper limit of quantification, a mobile phase-based dilution approach was employed to ensure the accuracy of sample quantification. The recovery rates for different concentrations of danofloxacin in various samples ranged from 74.98 to 97.93% (Table 2). Both the intraday and interday coefficients of variation were found to be lower than 7.38% and 5.94%, respectively, as detailed in Table 2.

Residue Depletion

Throughout the experiment, all Gushi chickens maintained good health, with no observable adverse reactions, female chickens have not laid any eggs. Normal water and food intake were recorded, and during dissection, all tissues and organs exhibited good condition without any lesions. None of the samples from the untreated chickens tested positive for danofloxacin.

Following multiple oral administration of danofloxacin, Table 3 displays the average concentrations across all collected samples, and Figure 2 illustrates the drug concentrations over time in each tissue. The findings indicate a gradual elimination of danofloxacin in various tissues of Gushi chickens, with low concentrations still detectable in the lung, liver, and skin + fat on the fifth day postadministration cessation. Danofloxacin

Table 1. The results of the current analysis method include the limit of detection (LOD) and limit of quantification (LOQ), linear range, regression equation, and correlation coefficient (R^2) of all samples.

Samples	LOD (μg/mL or μg/g)	LOQ (μg/mL or μg/g)	Linear range (μg/mL or μg/g)	Regression equation	Correlation coefficient (R^2)
Plasma	0.001	0.005	0.005∼2	y=7e-09x+0.0021	0.9999
Muscle	0.005	0.01	0.01∼5	y=5e-09x+0.0144	0.9996
Skin and Fat	0.001	0.005	0.005∼5	y=4e-09x−0.0116	0.9999
Liver	0.001	0.005	0.005∼5	y=4e-09x−0.0027	0.9999
Kidney	0.001	0.005	0.005∼5	y=4e-09x−0.0297	0.9996
Lung	0.001	0.005	0.005∼5	y=4e-09x−0.0338	1
Intestine	0.001	0.005	0.005∼5	y=4e-09x-0.0054	0.9999
Intestinal content	0.001	0.005	0.005∼5	y=4e-09x−0.0459	0.9982

In the regression equation of each sample, "x" represents the peak area of danofloxacin in the chromatogram, and "y" represents the concentration of danofloxacin.

Table 2. Recovery, intraday and interday coefficient of variation of different concentrations of danofloxacin in each tissue of Gushi chickens.

Samples	Concentrations (μg/mL or μg/g)	Recovery (%)	Intraday CV (%)	Interday CV (%)
Plasma	0.01	87.14±4.38	1.50−4.79	5.03
	0.5	85.23±3.21	2.57−4.70	3.76
	2	95.05±2.15	1.93−2.85	2.26
Muscle	0.01	83.05±4.24	3.94−5.38	5.1
	0.5	75.24±3.88	0.10−8.47	5.16
	2	74.98±3.05	3.55−5.10	4.93
Skin and Fat	0.01	92.89±4.18	2.50−6.81	4.5
	0.5	84.59±2.07	1.01−3.12	2.45
	2	83.94±3.86	2.70−5.86	4.6
Liver	0.01	87.14±4.38	1.50−4.79	5.03
	1	85.23±3.21	2.57−4.70	3.76
	5	95.05±2.15	1.93−2.85	2.26
Kidney	0.01	91.17±4.97	1.85−4.98	5.45
	1	86.04±2.75	1.02−3.89	3.19
	5	84.75±2.60	1.99−4.40	3.06
Lung	0.01	88.13±2.14	0.92−3.16	2.42
	0.5	85.51±1.80	0.97−3.50	2.1
	2	86.78±3.11	2.08−4.59	3.59
Intestine	0.01	97.93±5.82	4.68−6.27	5.94
	1	90.66±1.36	1.37−1.70	1.49
	5	95.92±2.31	1.54−3.06	2.41
Intestinal content	0.01	81.02±2.50	2.22−3.39	3.08
	1	94.71±4.46	3.15−5.11	4.7
	5	96.92±3.30	1.82−4.23	3.41

exhibited rapid absorption in the bodies of Gushi chickens after oral administration, with the liver demonstrating the highest concentration among all tissues, except for intestinal content. Notably, the C_{max} values at the initial sampling time point of 4 h were 0.394, 0.415, 1.216, 3.05, 3.157, 4.826, and 44.28 μg/g in skin + fat, muscle, lung, kidney, intestine, liver, and intestinal content, respectively—surpassing the C_{max} in plasma at 0.17 μg/mL. This distribution pattern suggests widespread distribution of danofloxacin in various tissues.

Table 4 outlines key pharmacokinetic parameters derived through a noncompartmental model. It is important to note that due to the rapid elimination of danofloxacin from the intestinal contents, acquiring sufficient data for parameter estimation proved challenging. The AUC values of danofloxacin in plasma, muscle, skin + fat, lung, intestine, kidney, and liver were estimated as 1.71, 5.51, 8.64, 15.50, 21.52, 22.28, and 39.32 h·μg/g (or mL), respectively. The tissue-specific $t_{1/2\lambda z}$ in each tissue was as follows: skin + fat (47.87 h), lung (30.61 h), liver (22.07 h), plasma (16.05 h), muscle (12.53 h), intestine (9.38 h), kidney (6.34 h).

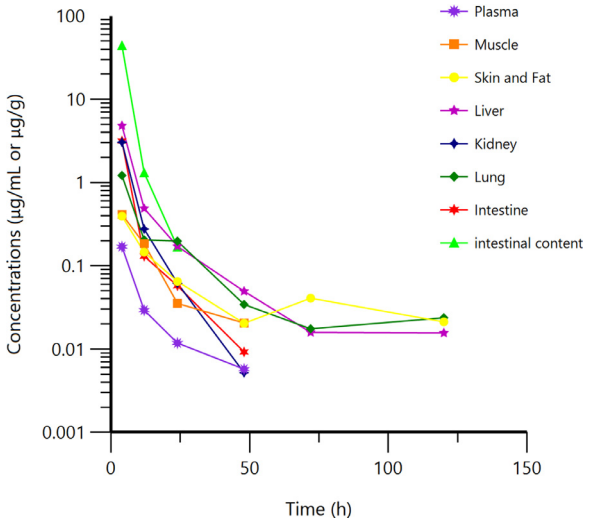

Figure 2. Mean ± SD plasma concentrations (μg/g or μg/mL) of danofloxacin in different samples after oral administration at 5 mg/kg BW per day for 3 consecutive days in Gushi chickens.

Considering residue depletion and adherence to the MRL values of danofloxacin in poultry set by Chinese regulatory authorities, withdrawal periods in kidney, muscle, liver, and skin + fat were determined as 1.03, 1.38, 3.34, and 5.85 d (Figure 3), respectively, rounded to 6 d.

DISCUSSION

In this study, we determine the tissue distribution and depletion of danofloxacin in Gushi chickens for the first time. After multiple administration of danofloxacin at 5 mg/kg BW for 3 d, the C_{max} in plasma was only 0.17 ± 0.119 μg/mL. This result starkly contrasts with a single administration of the same dose in Gushi chickens (0.53 ± 0.19 μg/mL), where the time to peak was 4 h (Chen et al., 2023). The difference in C_{max} values between multiple and single administration may be attributed to the current sampling design. In our study, the initial sampling time point was set at 4 h. We suspect that the peak time might occur earlier after multiple administration. Unfortunately, due to the absence of sampling at an earlier time point, we may have overlooked the true peak concentration. Adjusting the

Table 3. Tissue concentration (μg/g or μg/mL) in Gushi chickens after multiple oral administration of danofloxacin (5 mg/kg BW for 3 consecutive days) (Mean ± SD).

Tissue/Time	4 h	12 h	24 h	48 h	72 h	120 h
Plasma	0.170±0.119	0.029±0.015	0.012±0.004	0.006±0.001	ND	ND
Muscle	0.415±0.258	0.183±0.111	0.035±0.016	0.021±0.005	ND	ND
Skin and Fat	0.394±0.238	0.146±0.105	0.065±0.051	0.020±0.015	0.041±0.033	0.021±0.013
Liver	4.826±3.710	0.489±0.272	0.171±0.151	0.049±0.037	0.016±0.013	0.016±0.003
Kidney	3.050±1.690	0.276±0.184	0.062±0.039	0.005	ND	ND
Lung	1.216±0.825	0.205±0.101	0.197±0.241	0.034±0.028	0.018±0.008	0.024±0.019
Intestine	3.157±3.031	0.130±0.065	0.057±0.068	0.009±0.001	ND	ND
Intestinal content	44.28±20.52	1.309±1.299	0.168±0.184	ND	ND	ND

Time represents the time after the last administration, "ND" indicates "not detected."

Table 4. Elimination equation, elimination half-life ($t_{1/2\lambda z}$), the area under the concentration-time curve (AUC), and mean residence time (MRT) of tissues in Gushi chickens after multiple oral administration of danofloxacin (5 mg/kg BW for 3 consecutive days).

Samples	Elimination equation	R^2	$t_{1/2\lambda z}$ (h)	AUC (h·μg/mL or h·μg/g)	MRT (h)
Plasma	$C = 0.4074e^{-0.043t}$	0.936	16.05	1.71	14.42
Muscle	$C = 0.6238e^{-0.055t}$	0.863	12.53	5.51	14.38
Skin and Fat	$C = 0.6487e^{-0.014t}$	0.625	47.87	8.64	54.41
Liver	$C = 15.1672e^{-0.031t}$	0.799	22.07	39.32	10.97
Kidney	$C = 10.1312e^{-0.109t}$	0.998	6.34	22.28	6.22
Lung	$C = 2.9623e^{-0.023t}$	0.691	30.61	15.50	24.96
Intestine	$C = 15.5373e^{-0.074t}$	0.999	9.38	21.52	6.01

In each elimination equation, C represents danofloxacin concentration, and t represents the time after the last dosing.

sampling schedule to capture earlier time points could provide a more comprehensive understanding of the peak concentration dynamics after repeated administration. Similar trends were observed in other studies, such as the research on Yellow River carp (Song et al., 2022; Song et al., 2023), where the C_{max} after a single dose (0.166 ± 0.321 μg/mL) exceeded that after 5 consecutive days of oral administration (0.121 ± 0.066 μg/mL). In broilers, the C_{max} after a single dose (0.73 μg/mL) was higher than that after 5 consecutive days of drinking water administration (0.044 μg/mL) (Lynch et al., 1994), suggesting a potential establishment of a stable state for danofloxacin in the body. Notably, danofloxacin's high lipid solubility and cell permeability contribute to increased tissue concentrations despite a decrease in blood concentration (Nix et al., 1991). Continuous administration further enhances the accumulation of the drug in the bloodstream within tissues, consequently accelerating its transformation rate (Brocks and Mehvar, 2010).

After multiple doses, Gushi chickens exhibited liver and kidney concentrations of 4.826 ± 3.71 and 3.05 ± 1.69 μg/g at 4 h, which had declined to 0.489 and 0.276 μg/g at 12 h. Previous studies on animals like Muscovy ducks (Goudah and Mouneir, 2009), broilers (Knoll et al., 1999), and Yellow River carp (Song et al., 2023) have consistently shown that danofloxacin concentrations in the liver and kidney surpass those in other viscera and exhibit rapid elimination. This implies that the liver and kidney play pivotal roles as crucial eliminating organs for danofloxacin. However, an interesting finding in our study was that the concentration in the intestine was not markedly different from that in the kidney and liver but significantly higher than in other tissues. This observation can be attributed to the predominant absorption of fluoroquinolones from the intestine, subsequently entering the portal vein system—a crucial process emphasized in existing literature (Chen et al., 2019; Sartini et al., 2021).

The persistence of danofloxacin detection in the lung even on the fifth day postadministration suggests a slow elimination process in this tissue. Remarkably, danofloxacin remained detectable in the intestine 48 h after administration. Following multiple doses, the peak

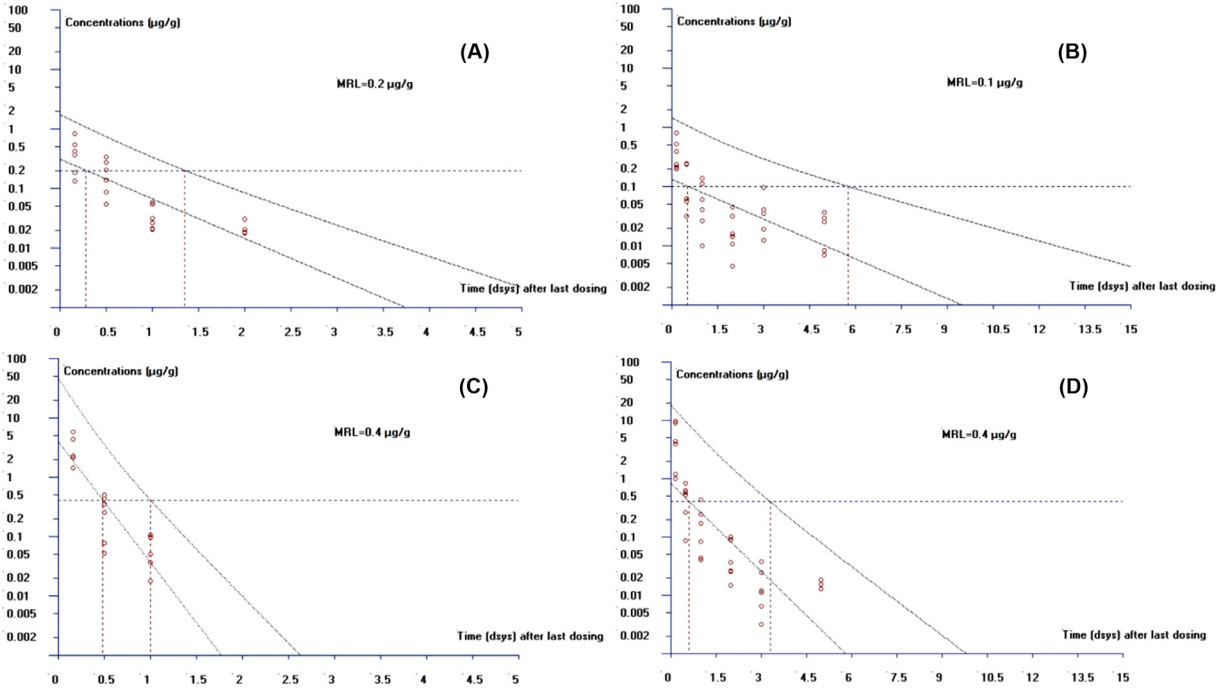

Figure 3. Semi-log plot of the depletion of danofloxacin from the tissues [(A) for muscle, (B) for skin + fat, (C) for kidney, and (D) for liver] of Gushi chickens following the last oral administration. Both regression lines represent the linear regression line and 95% confidence interval of the upper bound. Dots represent measured concentrations at each sampling point, and the horizontal dotted line represents the MRL values.

concentration of danofloxacin in the lung reached 1.216 μg/g, with an AUC of 15.5 h μg/g, while in the intestines, the peak concentration was 3.157 μg/g, with an AUC of 21.52 h μg/g. Despite the relatively low plasma concentration, it is crucial to recognize that, in disease treatment, the focus should not solely be on blood drug concentration. The concentration of antibiotics at the local infection site plays a critical role in determining the therapeutic efficacy of infectious diseases (Zhang et al., 2023). *Escherichia coli*, a susceptible bacterium in the intestine, and *Mycoplasma*, which can cause lung lesions, underscore the significance of site-specific considerations (Collingwood et al., 2014; Wang et al., 2022). After multiple administrations, the AUC values in the intestine and lung are 12.6 and 9.1 times higher than that in plasma, respectively. Elevated drug content in tissues enhances the bactericidal effect on pathogens. Therefore, when formulating treatment plans, the drug concentration at the target site must not be overlooked.

The MIC values for danofloxacin were determined against 42 *E. coli* isolates, with the MIC_{50} established at 0.5 μg/mL (Figure 1). Although this result is higher than previously reported values ranging from 0.015 to 0.25 μg/mL (Ozawa et al., 2010; Rusch et al., 2015; Wang et al., 2022), the findings suggest that the concentration of danofloxacin in the intestine is adequate for effective eradication of *E. coli* (Chen et al., 2023). However, it is undeniable that *E. coli* has developed increased resistance to danofloxacin. This trend is not limited to *E. coli*, as enhanced resistance to fluoroquinolones has been observed in various bacterial strains (Watts et al., 1997; Kong et al., 2023; Wu et al., 2023). The misuse of individual drugs poses a significant risk of amplifying resistance within entire drug classes, potentially leading to substantial challenges in drug selection and disease treatment over time.

To the best of our knowledge, this study marks the first instance of collecting intestinal content from poultry following danofloxacin administration. Post multiple doses, the concentration in the intestinal content peaked at 44.28 μg/g. While there is a possibility of reabsorption in the intestine, this concentration is notably high. Ultimately, both danofloxacin and its metabolite, demethyldanofloxacin, will be excreted from the body through feces. This finding is particularly significant for free-range chickens like Gushi chickens and backyard chickens. As these high concentrations of danofloxacin enter the environment, circulating through soil and water resources (Picó and Andreu, 2007; Van Boeckel et al., 2014), they can directly contribute to an increase in the resistance of other livestock and poultry to fluoroquinolones (Sasaki et al., 2022). This poses a potential threat to human life and health. Therefore, the risks associated with animal excrement, especially in free-range environments, cannot be overlooked.

Muscle and skin + fat represent the most significant edible tissues in poultry. In this study, the elimination half-lives ($t_{1/2\lambda z}$) of danofloxacin in Gushi chicken muscle and skin + fat reached 12.53 h and 47.87 h, respectively. Notably, the $t_{1/2\lambda z}$ in muscle surpassed that observed in broiler chickens (9.71 h) and geese (8.19 h) after a single dose of 5 mg/kg (Zeng et al., 2011; Sartini et al., 2021). Danofloxacin demonstrated prolonged accumulation in the skin + fat of Gushi chickens, with a slow elimination process. Even at 72 h postadministration cessation, there was a continued upward trend. This extended elimination period may be attributed to the high fat solubility of danofloxacin and the elevated abdominal fat content in Gushi chickens (Zhai et al., 2021). The elimination of danofloxacin in skin + fat appears to necessitate more time, contributing to the observed extended elimination profile.

In conclusion, this study determined the MIC values of danofloxacin against *E. coli*. Additionally, we investigated the distribution and residue depletion of danofloxacin in Gushi chickens following 3 consecutive days of oral administration at 5 mg/kg BW. Among tissues, the liver exhibited the highest concentration, trailed by the intestine and kidney. Prolonged accumulation was observed in the liver, lung, and skin + fat. Postadministration, proper management of feces is essential to prevent environmental pollution that could escalate drug resistance, posing risks to human health. In China, the official withdrawal time for danofloxacin in poultry has been set at 5 d. However, our findings suggest that this timeframe may not be accurate for Gushi chickens. Considering residue depletion and the MRL levels in various tissues, we recommend extending the withdrawal period to 6 d.

ACKNOWLEDGMENTS

This work was supported by funding from the Foundation for the University Young Key Teacher Program of Henan Province (no. 2021GGJS044).

Ethical Approval: All applicable international, national, and institutional guidelines for the care and use of animals were followed. And the animal experimentation protocol adhered to the guidelines and approvals set forth by the Experimental Animal Management and Use Committee of Henan University of Science and Technology (approved # 20230303).

DISCLOSURES

All authors declare that they have no conflict of interest.

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