Residue depletion of sarafloxacin in black-bone silky fowl tissues after oral administration

Bao-Tao LIU [1], Shi-Kai SUN [2], Li CHEN [2], Jun-Jun YU[2]*

[1]College of Veterinary Medicine, Qingdao Agricultural University, Qingdao, China

[2]Guangdong Wen's Group Academy, Guangdong Wen's Foodstuffs Group Co., Ltd., Xinxing, Guangdong, PR China

*Correspondence: Mr JUN-JUN YU, Guangdong Wen's Group Academy, Guangdong Wen's Foodstuffs Group Co., Ltd., Xinxing, Guangdong, PR China. Email: wens_yu86@163.com.

ORCID iD: BAO-TAO LIU https://orcid.org/0000-0003-1178-2493

SHI-KAI SUN https://orcid.org/0000-0002-9147-1216

LI CHEN https://orcid.org/0000-0002-3636-475X

JUN-JUN YU https://orcid.org/0000-0002-4191-1519

Abstract:

In this study, the residue depletion of sarafloxacin in black-bone silky fowl (BSF) was studied after oral doses of sarafloxacin (10 mg/kg BW for seven consecutive days). Muscle and liver tissues were collected at different withdrawal periods and determined by HPLC-MS/MS method. The limit of detection for sarafloxacin was 1.0 μg/kg, and the recoveries from blank fortified samples were 93.53% ~ 108.47% with coefficients of variation less than 9.28%. At first day after ending sarafloxacin treatment, the mean

concentrations of sarafloxacin in muscle and liver were 366.88 ± 129.51 and 120.35 ± 46.86 μg/kg, respectively, higher than their maximum residue limits (10 μg/kg for muscle, 80 μg/kg for liver). Notably, the sarafloxacin concentrations in muscle depleted very slowly and were still up to 45.46 ± 12.94 μg/kg at 43.25 days after the last administration. Interestingly, the sarafloxacin concentrations in both tissues increased into peak values at 21 days. In addition, the withdrawal time of sarafloxacin in BSFs should be 93 days as calculated in this study, significantly longer than that (0 day) in common broiler chickens. Therefore, our study provides data for a more prudent use of sarafloxacin in BSFs and suggests a withdrawal time of 93 days was necessary to guarantee safety in BSFs for the consumers.

Key words: Sarafloxacin, residue depletion, withdrawal time, black-bone silky fowl

1. Introduction

Sarafloxacin (SAR) was the first fluoroquinolone (FQ) antibacterial agent approved for use in poultry in the United States, and was used for control the early mortality in turkeys and broiler chickens [1]. However, the marketing authorization of this drug in poultry has been withdrawn in the USA owing to concerns about microbial resistance [2]. Because SAR is the primarily metabolite of difloxacin, a synthetic FQ highly effective for a wide variety of Gram-positive and Gram-negative bacteria, it is still important to monitor for the residues depletion of SAR in animals [3].

For monitoring the potentially unsafe residues to ensure the safety of livestock

products, several government authorities have established the maximum residue limits (MRLs) and withdrawal time of SAR. In the Republic of Korea, the MRLs of SAR were established as 10 ~ 80 μg/kg for poultry. In European, no MRLs have been set for SAR in chicken kidney and muscle, because the predicted concentrations in these tissues were below the limit of quantification, and the MRL in liver was 100 μg/kg. In China, the MRLs for chicken muscles and liver were established as 10 and 80 μg/kg, respectively. Importantly, SAR has been widely used in chickens for fattening in China and the withdrawal time of this drug in common broiler chickens was 0 day, because the mean concentrations of SAR in muscle and liver were both below their corresponding MRLs from 12 h after administration of the last dose [4]. Up to date, the depletion studies of SAR were reported in eel and eggs [5-7]. The depletion residues of SAR in certain breeds of chickens with large consumption were rarely studied, although several detection methods for SAR, such as using HPLC fluorometric, broad specific monoclonal antibody and nanocomposite probe, in various types of animal muscle have been reported [1, 8, 9].

Black-bone silky fowl (BSF, *Gallus gallus domesticus* Brisson) with black skin, muscle, and bones is a unique breed of chicken originated from the south of China and is distinguished from common broiler and layer chicken according to the genome analysis [10] . BSF has some health functions and can protect against a range of illnesses such as treating diabetes and anemia, curing women's diseases like menoxenia and postpartum complications [11], thus, consumption of such animals has increased

over the recent years. With the expansion of BSF breeding, the residues of SAR in BSFs are emergent and there is a paucity of data regarding the depletion of SAR in BSFs. Although the residues depletion of SAR in common broiler chickens has been studied using HPLC method [4], however, its limit of detection (LOD) was high up to 50 μg/kg. In this study, we aimed to evaluate the rate of depletion of SAR in edible tissues (muscle and liver) of healthy BSFs using HPLC-MS/MS method after repeat oral administration and a withdrawal time of SAR in BSFs was also determined to guarantee safety for the consumers.

2. Materials and methods

2.1. Animals

One hundred healthy BSFs (7 weeks old, half male and half female) were provided by the Taihe Original Black-Bone Silky Fowl Hennery (Guangdong, China). BSFs were allowed a 7-day acclimation period prior to the study, provided a drug-free pelleted diet and given water ad libitum.

2.2. Chemicals and reagents

SAR hydrochloride (Standard, 91.2%, Lot#G133594) was purchased from Labor Dr. Ehrenstorfer-Schafers (Augsburg, Germany) and SAR hydrochloride (10% Soluble Powder, Lot#18040701) was donated by Henan Royal Federation Biomedical Technology Co., Ltd. (Luoyang, China). Enrofloxacin-d5 (ENR-d5) (hydrochloride salt form, 99.0%) used as internal standard was purchased from Sigma-Aldrich. All

chromatographic solvents used in this study were HPLC grade and the other chemicals were analytical grade.

2.3. Drug application and sampling

All BSFs were placed in cages in the university animal house. Five selected randomly were immediately sacrificed and their muscle tissue was tested for SAR residues. One hundred BSFs without SAR residue were randomly divided into two groups and ten were in the same cage. BSFs of group A (80 BSFs) were used to study tissue depletion of SAR and were given serial daily doses of SAR (10 mg/kg BW every 24 h for seven consecutive days). BSFs of group B (15) did not receive any treatment and were used to determine the validation criteria of the analytical method. All dosages were administered between 8 and 9 AM each day. The solutions for oral administration were daily prepared by dissolving 10% SAR hydrochloride Soluble Powder in sterilized bidistilled water. All BSFs were weighed on the day of drug administration (at a dosage of 10 mg/kg b.w.). BSFs of group A were euthanized using carbon dioxide at 1 d, 2 d, 3 d, 6 d, 9.25 d, 14.25 d, 21 d, 28 d, 36.25 d and 43.25 d after the last dose of SAR. Each time point contained six BSFs and tissue specimens of liver and muscle (breasts and legs) were sampled separately. Each liver sample was minced and frozen at -45 °C until assayed for SAR concentrations. For the muscle tissue, both breasts and legs of each individual were minced and mixed thoroughly before storing at -45 °C.

2.4. Analytical method and validation

Tissue extraction

106 Extraction of SAR in tissue was performed as previously described [12]. Tissue
107 sample (4 g) and 16 mL of 5% acetate-acetonitrile were added to a 50 mL
108 polypropylene centrifuge tube. Anhydrous sodium sulfate (4 g) and sodium chloride (2
109 g) were then added in this tube, followed by homogenizing for 1 min and centrifuging at
110 3155 g for 5 min. The supernatant was injected into a 50 mL tube with 400 mg C18
111 absorbent followed by 5 min horizontal oscillation and centrifugation (3155 g for 5 min).
112 Four milliliter of the organic layer was injected into a 10 mL tube and was evaporated
113 under nitrogen. The residue was redissolved with 1 mL solution consisted of 0.1%
114 formic acid aqueous solution and acetonitrile (9:1, v/v) and then vortexed with 5 mL
115 n-hexane saturated with acetonitrile, followed by centrifugation at 3155 g for 10 min.
116 The n-hexane layer was removed and the aqueous solution was filtered with a 0.22 μm
117 membrane filters for analysis.
118 *HPLC-MS/MS analysis*
119 Liquid chromatography analysis was conducted on UFLC-NEXERA system
120 (LC-30AD, Shimadzu, Kyoto, Japan). The chromatographic separation was performed
121 as previously described with slight modifications [12] and was conducted on a 45 °C
122 waters BECH C18 column (50 mm×2.1 mm, 1.7 μm). The mobile phase was consisted
123 of 0.1% formic acid aqueous solution (A) and methanol solution contains 0.10% (v/v)
124 formic acid (B). The gradient elution was as follows: 95-85% A for 2 min; 85-60% A for
125 3 min; 60-5% A for 2 min; Finally, the gradient was set to 95% A for 2 min to allow
126 equilibration. The flow rate kept at 300 μL/min and an injection volume of 10 μL was

used.

Instrument AB Sciex QTRAP 5500 equipped with ESI source in positive ion mode for mass spectrometric detection was used. The transitions under the multiple reaction monitoring (MRM) mode were of m/z 386.2 → 342.2 for SAR, and 365.0 → 321.3 for ENR-d5 (as internal standard), respectively. The shared mass spectrometry parameters were 5500 V ion spray voltage, 550 °C ion spray temperature, 30 psi curtain gas (CUR), 50 psi nebulizer gas (GS1) and 60 psi heater gas (GS2), as previously described [12]. Table 1 contains the other specific mass parameters for SAR and ENR-d5. The AB Sciex Analyst software (version 1.6.3) was applied for instrument control and original data processing.

Preparation of calibration curves

SAR was weighed accurately and dissolved in methanol at 1 mg/mL, which was stepwise diluted with methanol to obtain series of working solutions (40 ng/mL, 100 ng/mL, 200 ng/mL, 400 ng/mL, 1000 ng/mL, 2000 ng/mL and 4000 ng/mL). The internal reference standard ENR-d5 was dissolved in methanol to obtain working solution of 500 ng/mL. Tissue samples without drugs were processed as described in section 2.4.1, and corresponding working solution of SAR was added into the 1mL solution (0.1% formic acid aqueous solution and acetonitrile (9:1, v/v)) to obtain serials of final calibration standards 2 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL, respectively. The final concentration of ENR-d5 in each standard was 25 ng/mL. The linearity was assessed by plotting the peak area ratios of

the SAR to the ENR-d5 against the concentrations of SAR within blank samples.

Method validation

Method validation parameters including recovery rate, intra-day precision, inter-day precision, limit of detection (LOD), and limit of quantification (LOQ) were determined as previously described [12]. For recovery rate, intra- and inter-day precision, a set of six replicates (tissue samples) added with SAR at three concentration levels (5, 10 and 20 μg/kg) and ENR-d5 at 25 ng/mL, was used. Inter-day precision was evaluated on three consecutive days. LOD and LOQ for each analyte were calculated using the standard deviation of six matrix blanks (s_{blank}) and the slope of the matrix-calibration (s) : LOD = $3.3 \times s_{blank}/s$ LOQ = $10 \times s_{blank}/s$ [13].

3. Results

3.1. HPLC separation analysis

The typical MRM chromatograms were displayed in Figure 1. No endogenous interference was detected at the elution times of SAR and ENR-d5, which were 4.66, and 4.28 min, respectively. Carry-over was not observed.

3.2. Method validation results

As shown in Table 2, both the calibration curves of SAR in muscle and liver samples showed good linearity over the studied concentration ranges with correlation coefficients (r^2) > 0.9994. The representative calibration curves were $f = 0.01270 \times C + 0.00342$ for muscle, and $f = 0.01018 \times C + 0.04177$ for liver, where f means the peak

area ratio of SAR to ENR-d5, and C is drug concentration. The linear concentration range of SAR in the two tissues was both from 2 μg/kg to 200 μg/kg.

Method validation results using the sample preparation described in section 2.4 are summarized in Table 3. The recoveries of SAR from muscle and liver samples were 93.53%~99.18% and 102.46% ~ 108.47%, respectively. Intra-day precision for all tissue samples and spiked concentration levels ranged from 1.52% to 5.09% and the inter-day precision was less than 9.28% (Table 3). LOD for SAR in spiked tissue samples was 1.0 μg/kg (S/N=3) and the corresponding LOQ was 5.0 μg/kg (S/N=10). The recoveries of SAR from both tissue samples at 5.0 μg/kg spiked concentration level were more than 90%, with RSD < 5.09% (Table 3), showing the LOQ was accurate.

3.3. Tissue residue depletion

Residues of SAR in tissue specimens after oral administration of SAR (10 mg/kg BW, daily for 7 consecutive days) were determined. The tissue concentration-time profiles for muscle and liver tissues were presented in Table 4. Mean concentrations of SAR in muscle and liver were 366.88 ± 129.51 and 120.35 ± 46.86, respectively at first day after ending SAR treatment (Table 4). The SAR concentrations depleted much slower from the muscle tissue than liver tissue. SAR concentrations in liver were below the MRL (80 μg/kg) since three days after the end of treatment and depleted slowly. The SAR concentrations in muscle samples decreased rapidly after the last treatment, however, the concentration has been increased since 9.25 days and decreased after 21 days. The concentration of SAR in muscle at 43.25 days after the administration was

still 45.46 ± 12.94 μg/kg, which was higher than the MRL (10 μg/kg) for muscle (Table 4 and Figure 2).

3.4. Withdrawal time estimation

To calculate the withdrawal periods of SAR in the tissues studied, linear regression analysis was performed using the logarithmic transformed data and the withdrawal time was determined as the time when the one-sided, 99% upper tolerance limit of the regression line with 95% confidence level was below the MRL as previously described [14]. Based on the MRLs of SAR in liver (80 μg/kg) and muscle (10 μg/kg), the withdrawal time for liver and muscle was calculated as 20 days and 93 days, respectively, after oral administration (10 mg/kg BW, daily for 7 consecutive days) (Figure 3). Thus, the final withdrawal time in BSFs should be 93 days.

4. Discussion

The pharmacokinetics of SAR has been described in Atlantic salmon [15], Carassius auratus gibelio [16], pigs and chickens [17], however, the depletion of SAR was rarely reported [4-7]. SAR has been widely used in chickens for fattening and fishes in China, and therefore there is a great need to study the residues depletion of SAR in BSFs, a unique breed of chicken, with an increasing consumption in China. Our results from the oral administrations of SAR in BSFs showed that SAR had quite different residues depletion characteristics compared to that in common chickens.

In BSFs, the concentrations of SAR in muscle depleted very slowly and its

concentrations at 43.25 days after the last administration were still 45.46 ± 12.94 μg/kg, which was significantly higher than the MRL (10 μg/kg) for muscle. However, the SAR concentrations in muscle of broiler chickens were below the MRL (10 μg/kg) since 12h after administration of the last dose [4]. Interestingly, the residues of ofloxacin in BSFs also depleted very slowly and its concentrations in muscles were up to 193.5 μg/kg at 40 days (longer than the withdrawn time of ofloxacin in common chickens) after the last administration [18], significantly higher than the MRL for common chicken edible tissues (30 μg/kg) in the USA. This finding was consistent with that of our study and both studies indicate the FQs in BSFs have different depletion characteristics when compared to common chickens. A unique feature of BSF when compared with other common chickens is the presence of melanosomes in various organs including muscles, periosteum, trachea, mesentery, digestive canals, ovary and testis [19, 20], and the high level of melanin might be the reason for the high residual concentrations of FQs in muscles in our study because FQs have been proved to bind with melanin through the basic nitrogen atom at position 7 of the quinolone ring [21]. The affinity of FQs for organs containing abundant melanin have been reported in various fishes [22]. Actually besides FQs, several drugs also had affinity to melanin [23], and melanin had been proved to act as an adsorbent for drug residues [24], resulting in the long term therapeutic/toxicological activities [25]. For example, 35S-sulfadiazine and 14C-trimethoprim could combine with melanin, resulting in high residual concentrations of these two drugs in the organs containing abundant melanin in rainbow

trout [26].

The SAR concentrations in muscle and liver samples decreased after the last treatment, however, the concentrations in both tissues increased into peak values at 21 days (Figure 2), presenting elimination curves with two peaks in our study, which might because that FQs were combined with melanin and glucuronic acid in these tissues [24]. As the withdrawal time calculation software (WT1.4) is designed to analyze data with first-order kinetics [27], the four concentrations after 21 days were selected to calculate the withdrawal time of SAR and its withdrawal time in BSFs was 93 days, which was significantly longer than that (0 day) in common broiler chickens [4]. This finding suggest that species-to-species extrapolation of the residue depletion of SAR in chickens should be more prudent because the main parameters of residue depletion of this drug varied in different chicken species.

5. Conclusion

SAR has been widely used in chickens for fattening in China, including BSFs (a unique breed of chicken in China) which is consumed largely because of its high nutritional and medicinal values. However, there is a paucity of data regarding the depletion of SAR in BSFs. This paper first reported the residue depletion of SAR in BSFs. Results showed that SAR was eliminated slowly in vivo and its concentration at 43.25 days after the last administration in BSF muscle was still significantly higher than the MRL (10 μg/kg) for muscle. In addition, the withdrawal time of SAR in BSFs

should be 93 days as calculated in the current study, significantly longer than that (0 day) in common broiler chickens. Therefore, our study provides data for a more prudent use of SAR in BSFs and suggests a withdrawal time after treatment in order to guarantee safety in BSFs for the consumers.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgments

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Authors' Contribution

Conceived and designed the experiments: BTL JJY. Performed the experiments: BTL SKS LC. Analyzed the data: BTL SKS JJY. Wrote the manuscript: BTL JJY. All authors have read and approved the final manuscript.

Ethical statements

In this study, animal experimental protocol was approved by the Institutional Animal Care and Use Committee at Qingdao Agricultural University (Approval No. 2019-288). All animal experimental tests were carried out in accordance with the 2016 standards of laboratory animal in China and other related regulations in Animal Welfare Act.

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379

380 **Table 1.** Optimized mass parameters for sarafloxacin

Analytes	Precursor and product ion (m/z)	Dwell time (ms)	Declustering potential (V)	Entrance potential (ev)	Retention time (min)
Sarafloxacin	386.2/342.2*	80	80	25	4.74
	386.2/299.2	80	80	38	
enrofloxacin-d5	365.0/321.3	80	80	26	4.35

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382 **Table 2.** Linear regression parameters of the calibration curves.

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Samples	Calibration range (μg/kg)	Standard calibration curve		
		Intercept	Slope	R^2
muscle	2~200	0.00342	0.01270	0.9994
liver	2~200	0.04177	0.01018	0.9994

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391 **Table 3.** Accuracy and precision for the analysis of sarafloxacin in BSF muscle and

392 liver tissues (n=6).

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Samples	Spiked level (μg/kg)	Recovery (%)	Intra-day RSD (%)	inter-day RSD (%)
muscle	5	99.18	4.28	7.79
	10	93.53	3.52	4.01
	20	94.49	1.69	2.30
liver	5	108.47	5.09	9.28
	10	104.06	4.51	7.89
	20	102.46	1.52	3.95

394

395 **Table 4.** Sarafloxacin residues in BSF tissues after a 7 day medication period（n=6,

396 $\bar{x} \pm s$）

Time after last dose (days)	sarafloxacin residues in tissues (μg/kg)	
	muscle	liver
1.00	366.88 ± 129.51	120.35 ± 46.86
2.00	450.85 ± 336.30	71.87 ± 85.45
3.00	277.10 ± 104.21	35.06 ± 9.10
6.00	200.27 ± 96.18	35.55 ± 25.47
9.25	91.86 ± 42.29	16.95 ± 8.80
14.25	117.30 ± 62.56	8.63 ± 4.31

21.00	216.81 ± 59.87	13.10 ± 8.37
28.00	103.44 ± 36.06	7.61 ± 4.41
36.25	82.68 ± 32.13	5.01 ± 3.05
43.25	45.46 ± 12.94	2.97 ± 1.58

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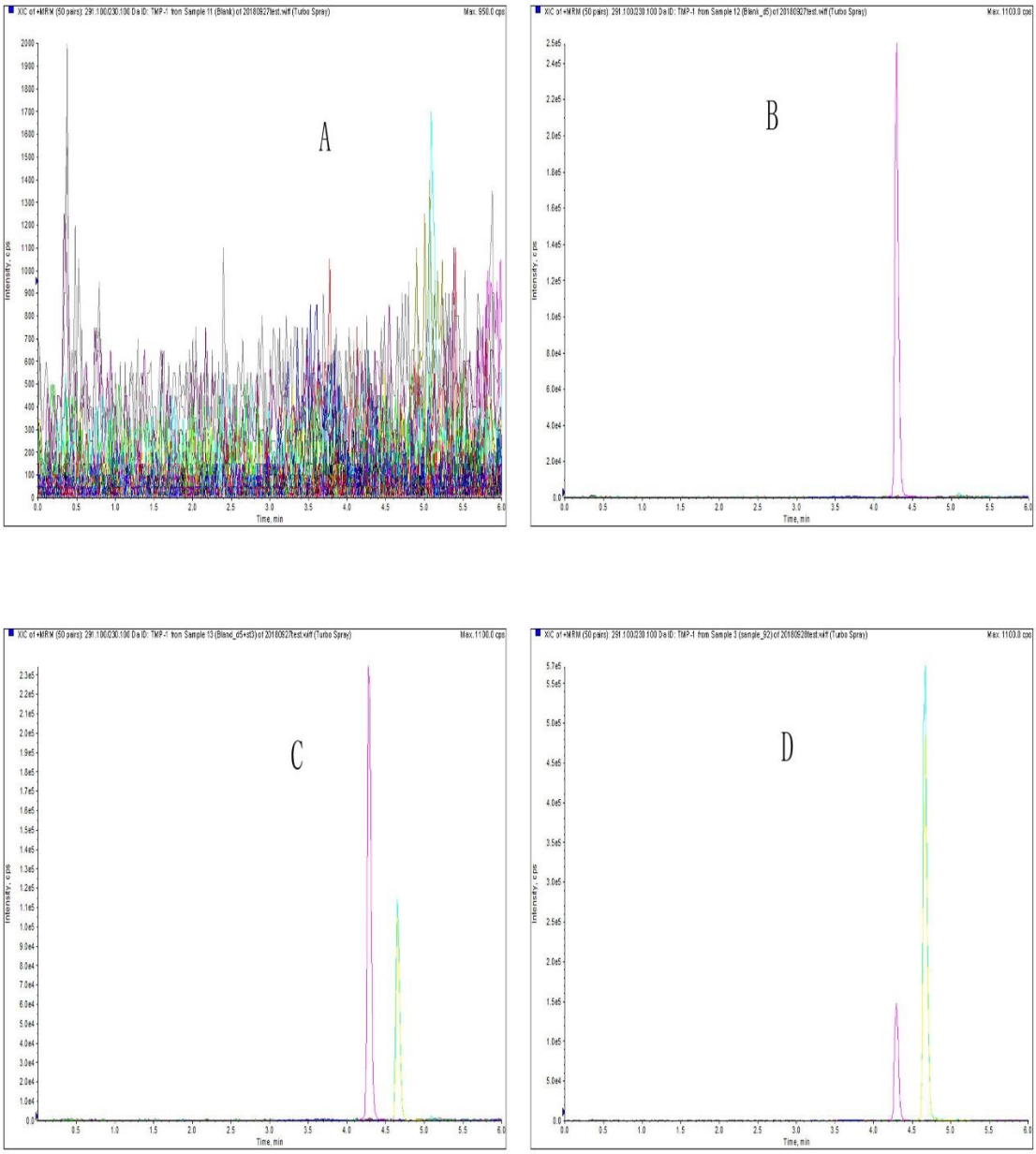
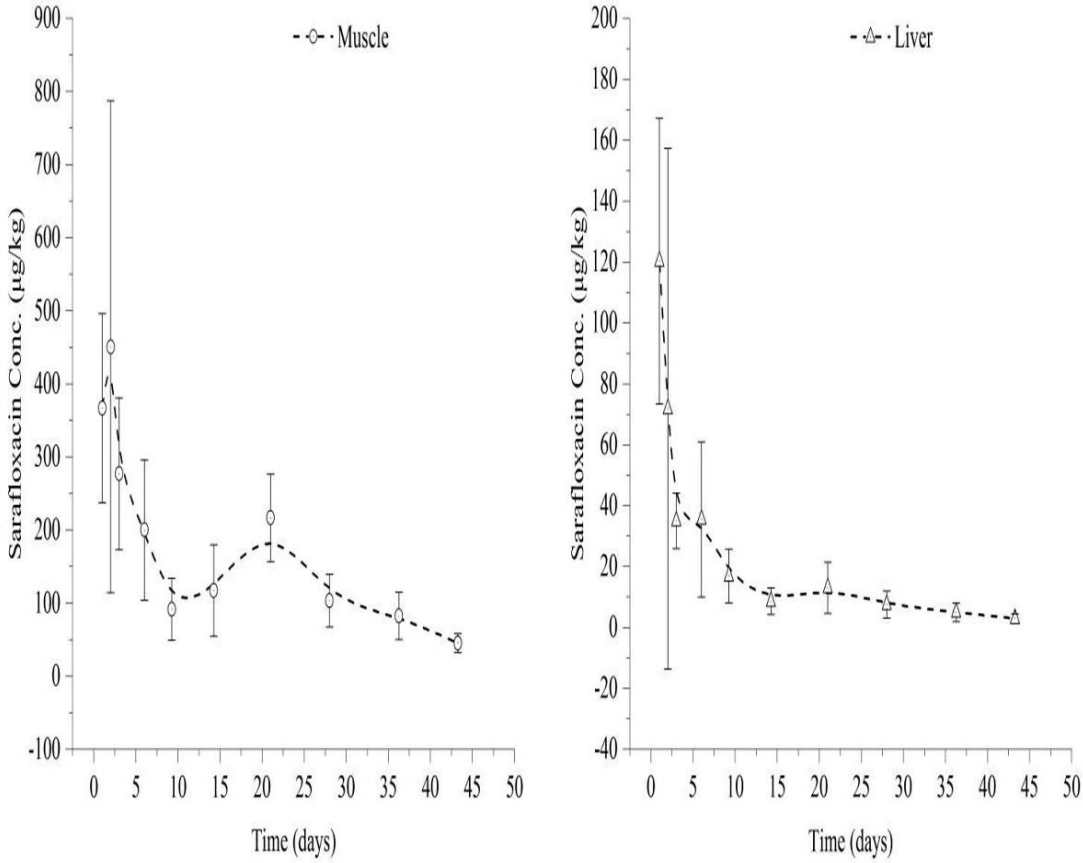


398

399 **Figure 1.** Typical MRM chromatograms of sarafloxacin and reference standard
400 enrofloxacin-d5 : (A) Blank chicken muscle; (B) Blank chicken muscle added with
401 enorfloxacin-d5; (C) Blank chicken muscle added with enorfloxacin d-5 and
402 sarafloxacin (10 μg/kg); (D) Chicken muscle sample after oral administration of
403 sarafloxacin (10 mg/kg BW, daily for 7 days).



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405 **Figure 2.** Depletion curves of sarafloxacin in different samples after cessation of

406 sarafloxacin at 1, 2, 3, 6, 9.25, 14.25, 21, 28, 36.25 and 43.25 days.

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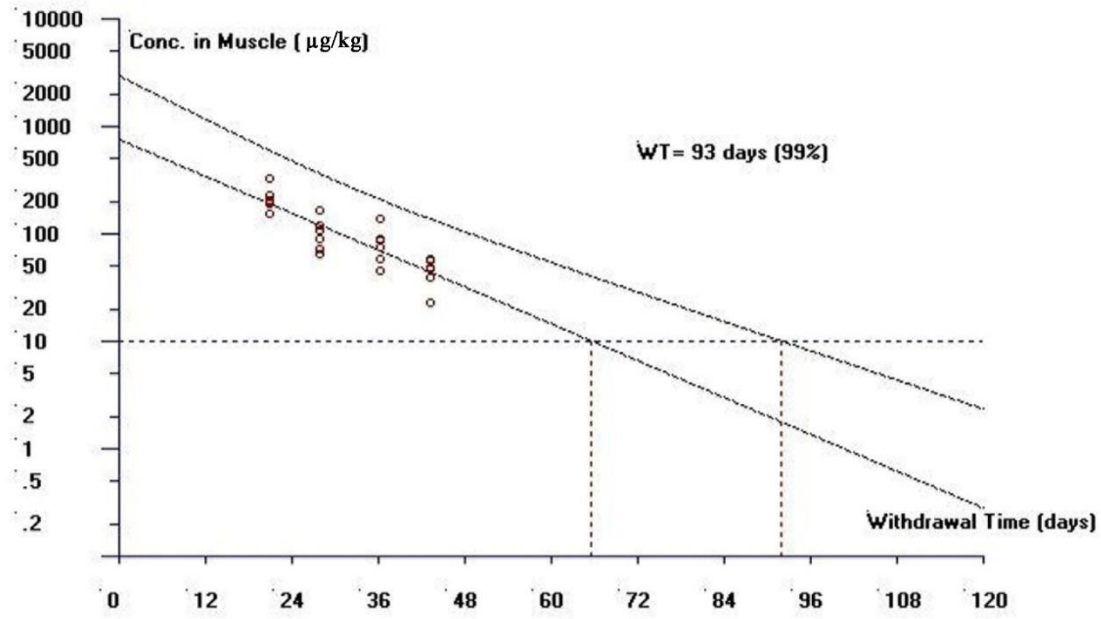

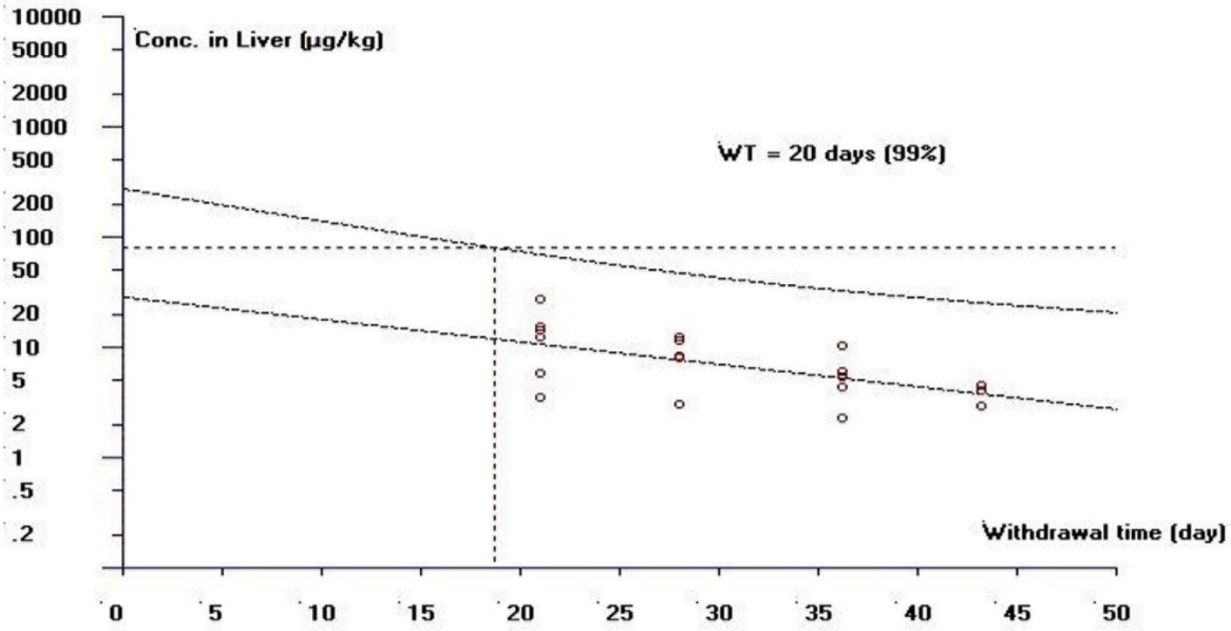

408

409 **Figure 3.** Plot of the withdrawal time calculation for sarafloxacin in muscle and liver at
410 the time when the one-sided 99% upper tolerance limit is below the MRL for
411 sarafloxacin (10 µg/kg for muscle, and 80 µg/kg for liver) after oral administration of
412 sarafloxacin (10 mg/kg BW, daily for 7 days).