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RESEARCH ARTICLE

Depletion of Residual Amoxicillin and Its Major Metabolites in Muscle, Liver and Kidney of Chicken

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A B S T R A C T

The depletion of residual amoxicillin (AMO) and its metabolites, amoxicillin acid (AMA) and 2,5-diketopiperazine (DIKETO), in Jinghai chickens was studied. Chicken tissue samples (muscle, liver and kidney) were deproteinized with acetonitrile and water and extracted with saturated dichloromethane, and the supernatants were analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). After the drug was withdrawn, the depletion times of AMA and DIKETO were longer in the liver and kidneys than in muscle. In the chicken tissue, the AMA residue levels were higher than the AMO and DIKETO residue levels, and the concentrations of AMA were highest in the kidney and liver. Because AMA is an allergen, we recommend monitoring AMA levels even though maximum residue limits (MRLs) for the metabolites of AMO have not been specified. In addition, the calculated withdrawal times for AMO at doses of 30 and 60 mg/kg chicken body weight were 4.01 and 4.33 days in muscle, 5.17 and 5.78 days in the liver, and 3.92 and 5.19 days in the kidney, respectively. To guarantee food safety, AMO withdrawal times of 6 days are required for doses of 30 or 60 mg/kg chicken body weight.

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INTRODUCTION

Amoxicillin (AMO), which contains a penicillin-type β-lactam moiety with a side chain, is one of the most commonly used broad-spectrum antibiotics (Lara *et al*., 2012). The bactericidal and antibacterial activity of AMO is related to the β-lactam structure (Anfossi *et al*., 2002). The activity of AMO against gram-negative and gram-positive bacteria is broad, and AMO can permeate tissue. Given its wide-ranging antibacterial/bactericidal activity and relatively low cost, AMO is commonly used in veterinary products and feeds.

However, drugs such as AMO are overused and abused, which can lead to trace amounts of the drug in materials such as milk and animal tissue. Because these drugs are transmitted via the food chain, they pose a risk to human health. Trace amounts of penicillin have been reported to be harmful to people who are allergic to penicillin (Ang *et al*., 1996; Fagerquis *et al*., 2005). The major AMO-associated metabolites are amoxicillin diketopiperazine-2',5'-dione (DIKETO), which is generated by the degradation of AMA and formation a new stable six-membered ring, and amoxicillin acid (AMA), which is generated by β-lactam cleavage of AMO (Nägele and Moritz, 2005). Studies have shown that the anaphylaxis caused by penicillin is related to these metabolites (Blaha *et al*., 1976; Marimuthu *et al*., 2015). Currently, many countries and organizations around the world have strict limits on the maximum residue levels (MRLs) of veterinary drugs. According to the regulations set by the European Commission **(**Commission Regulation No. 37, 2010), the MRLs for AMO in edible tissue (kidneys, liver, muscle and fat) and milk are 50 μg/kg and 4 μg/kg, respectively, and inspections for AMO are required for imported animal foods. To protect human health and guarantee proper conduct in the export trade,

residual AMO detection methods must be developed, and its elimination kinetics in animal tissue must be determined. The MRLs used in this study for AMO in chicken tissue were in accordance with the EU standards (50 μg/kg).

At present, a number of methods, including ultraviolet detection (UV) (Sørensen *et al*., 1999), fluorescence detection (FLD) (Ang *et al*., 2000) and mass spectrometry (Bogialli *et al*., 2004; Morenogonzález *et al*., 2017; Bessaire *et al*., 2018), have been developed for detecting residual β-lactam antibiotics in animal-derived foods. Several single- and multi-residue methods have been developed for the extraction and detection of β-lactam compounds in animal tissue or milk (Liu *et al*., 2011). Additionally, many studies have used liquid chromatography-tandem mass spectrometry (LC-MS/MS) approaches to detect AMO in milk (Liu *et al*., 2011), beef tissue (Fagerquist *et al*., 2005), chicken tissue (Hermo *et al*., 2013, 2014; Wang *et al*., 2017), and pig tissue (Reyns *et al*., 2008a). However, studies on the depletion of trace amounts of AMO and its primary metabolites in tissue derived from chicken are not available. Therefore, in our study, the residues of AMO and its major metabolites were detected using LC-MS/MS, and the depletions of residual AMO and its primary metabolites in chicken tissue were compared at two different doses (30 and 60 mg/kg) to provide a scientific basis for the use of AMO in the clinic.

MATERIALS AND METHODS

Standard reagents: Analytical standards of AMO (purity 98.0%), AMA (purity 92.6%), DIKETO (purity 95.4%) and penicillin V (PV) (purity 98.8%) were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Soluble AMO powder (purity 97.7%) was obtained from Jiangsu Beikang Pharmaceutical Co. (Taizhou, China).

Experimental design and sample collection: In total, 162 Jinghai yellow chickens (Nantong, Jiangsu Province, China) at 16 weeks of age were randomly divided into Groups A, B and C. The chickens were fed a formula feed without any antimicrobial drugs. After a 7-day adaptation period, soluble AMO powder was mixed with sterile ultrapure water to a final concentration of 50 μg/mL. According to the veterinary pharmacopoeia of China, Groups A and B were administered AMO at 30 (normal dose, Group A) or 60 mg/kg (twice the normal dose, Group B) via crop injection twice daily for 7 consecutive days. Group C did not receive any further treatment. The drug was withdrawn after 4 hours. Then, at 1, 3, 5, 7, 9, 11, 13, and 15 days, the breast muscle, liver, and kidneys of 6 chickens were sampled and stored at –70°C.

Sample preparation: Aliquots of 2±0.02 g was placed into 50-mL centrifuge tubes with 100 μL of the internal standard solution of PV and vortexed for 1 min. Then, acetonitrile (4 mL) and water (4 mL) were added, and samples were placed in an ultrasonic bath for 20 min at 25°C for extraction followed by centrifugation for 10 min at 7000 rpm. Next, the supernatants were collected, and the precipitates were removed. The supernatants were combined, 5 mL of water-saturated n-hexane was added, and after standing for 1 min, the mixture was vortexed. The n-hexane layers were discarded to afford the final extracts, which were concentrated and freeze-dried at -55°C. The dried samples were completely dissolved in 10 mL of 3% acetonitrile and centrifuged for 10 min at 13000 rpm. The supernatants were filtered through 0.22 μm filters, and the filtrates were analyzed using HPLC-MS/MS.

Instrumentation and conditions: LC analysis was conducted on a Waters Alliance e2695 device. The samples were analyzed on a Waters SunFire^{TM} C_{18} column (5 μm, 150 mm×4.6 mm). The flow rates were 1.0 mL/min, the injection volumes were 20 μL, and the temperature of the column was set to 35°C. A solution of AMO, AMA, DIKETO and PV was separated by gradient elution using 0.1% formic acid in water (A) combined with an acetonitrile solution (B). The chromatographic gradient elution was performed as follows: 0-2 min, 97% A; 5 min, 80% A; 12-14 min, 30% A; 15-20 min, 97% A.

An AB SCIEX Triple Quad 5500 mass spectrometer was used for analysis. The sample analysis was carried out in a multiple reaction monitoring setting using positive electrospray ionization. The ion spray voltage setting was 5500 V, the ion source gas temperature was 550°C, the nebulizer and heater gas were set to 50 psi, and the curtain gas and collision gas were at 40 and 8 psi, respectively. Table 1 presents the specific retention times, molecular weights, and MS parameters for each analyte.

Table 1: The specific retention times and MS parameters for each analyte

Compounds	Retention Time (min)	Mass Transitions (m/z)	Declustering Potential (V)	Collision Energy (eV)
AMO	8.06	366.2>114.0*	50	29
		366.2>208.0		19
		366.2>160.0		29
AMA	7.95	384.2>323.1*	45	19
		384.2>189.0		29
		384.2>160.0		34
DIKETO	9.25	366.2>160.1*	52	22
		366.2>114.1		52
		366.2>207.1		18
PV	15.12	351.2>160.1*	50	19
		351.2>114.1		46
		351.2>192.2		15

Note: *Quantification ion pair.

Method validation: This method for the quantitative determination of AMO and its major metabolites was validated based on parameters such as linearity, recovery, precision, limit of detection (LOD), and limit of quantitation (LOQ) (Feng *et al*., 2012; Sharmili *et al*., 2016; Wang *et al*., 2017).

Statistical analysis: Experimental data are presented as the mean±standard deviation. Withdrawal times were estimated using WT1.4 software.

RESULTS

Method validation: Linearity: In muscle samples, concentrations of AMO, AMA, and DIKETO between 0.45-2000 μg/kg exhibited good linearity with correlation coefficients r>0.9994. In the liver samples, AMO, AMA and DIKETO concentrations in the range of 0.90-2000 μg/kg exhibited good linearity with r>0.9999. In the

kidney samples, concentrations of AMO, AMA and DIKETO between 1.38-2000 µg/kg exhibited good linearity with r>0.9997.

LOD and LOQ: In this study, the LOD and LOQ values of AMO were 0.52 and 4.10 µg/kg in the muscles, 0.85 and 3.60 µg/kg in the liver and 1.20 and 4.50 µg/kg in the kidneys, respectively. Comparatively, the LOD and LOQ values of AMA were 1.04 and 4.10 µg/kg in the muscles, 1.65 and 6.40 µg/kg in the liver, and 2.20 and 8.50 µg/kg in the kidneys, respectively. Finally, the LOD and LOQ values of DIKETO were 0.15 and 0.45 µg/kg in muscles, 0.30 and 0.90 µg/kg in the liver, and 0.46 and 1.38 µg/kg in the kidneys, respectively.

Precision: The intra-day relative standard deviation (RSD) values when AMO (25 µg/kg), AMA (50 µg/kg) and DIKETO (100 µg/kg) were added were 4.20~13.73% in the blank muscles, 3.09~11.44% in the blank livers, and 3.52~10.58% in the blank kidneys, and the inter-day RSD values were 6.32~15.39% in the blank muscles, 6.41~11.84% in the blank livers, and 6.38~13.66% in the blank kidneys, respectively.

Recovery: The recovery rates were determined by adding standard solutions to blank samples. The recovery rates for AMO, AMA and DIKETO were 90.8~106.3%, 90.5~94.8% and 95.2~104.5% in the blank muscles, 92.9~97.2%, 83.1~97.7% and 93.5~101.0% in the blank livers, and 92.4~102.9%, 95.1~103.6% and 99.9~101.4% in the blank kidneys, respectively. These tests were conducted by adding AMO, AMA and DIKETO at concentrations of 25, 50 and 100 µg/kg, respectively.

Therefore, the linearity, LOD, LOQ, precision and recovery values of the samples are consistent with the regulations of the Commission Decision 2002/657/EC, which indicates that the testing method is reliable.

Residue depletion: For AMO doses of 30 and 60 mg/kg per body weight, after the drug was withdrawn, the residual concentrations were determined in chicken tissues (Tables 2-4).

The experimental groups were administered AMO at doses of 30 or 60 mg/kg of body weight. The concentrations of AMA and DIKETO in the three chicken tissues peaked 4 hours after the drug was withdrawn. The concentrations of AMO and its key metabolites in the kidney were higher than those in the muscle and liver, and elimination from the kidney was rapid. The first day after the drug was withdrawn, the elimination rates of AMO, AMA and DIKETO rapidly decreased and the elimination rate was slow at the final measured time point. Higher concentrations of AMA and DIKETO were present in the liver and kidneys than in the muscle at the same time points after the drug was withdrawn. Elimination was slow, and the elimination times were longer in the liver and kidney. The level of AMO residue in the muscle was below the MRL on the first day after the drug was withdrawn, and the levels of AMO residue in the liver and kidneys were below the MRL on the third day after the drug was withdrawn. In addition, we also found that AMO, AMA, and DIKETO were present in muscle, liver, and kidney, and the levels of AMA residue were the highest among the analytes.

Table 2: Residual AMO in chicken tissue (n=6)

Group	Withdrawal Time	Residue (µg/kg) Mean ± SD		
		Muscle	Liver	Kidney
30 mg/kg b.w.d	4 hours	124.97±23.50	178.67±10.69	221.00±22.26
	1 day	34.76±12.20	45.46±7.65	51.64±15.26
	3 days	6.55±0.33	22.84±9.78	12.48±3.07
	5 days	5.20±1.93	17.45±6.68	7.33±1.10
	7 days	4.09±0.53	12.63±4.83	4.86±1.11
	9 days	3.53±0.23	6.45±2.37	<LOQ
	11 days	<LOQ	<LOQ	<LOQ
	13 days	<LOD	<LOQ	<LOD
	15 days	<LOD	<LOD	<LOD
60 mg/kg b.w.d	4 hours	166.94±40.31	252.95±16.55	334.52±48.61
	1 day	32.33±8.35	65.65±8.21	84.80±15.77
	3 days	20.70±5.44	31.36±5.01	19.08±2.90
	5 days	10.44±2.06	25.75±4.10	9.37±1.85
	7 days	7.76±1.50	18.64±3.65	5.97±1.59
	9 days	4.58±0.98	9.06±2.10	4.60±1.05
	11 days	3.25±0.65	4.12±1.23	<LOQ
	13 days	<LOD	<LOQ	<LOD
	15 days	<LOD	<LOD	<LOD

Table 3: Residual AMA in chicken tissue (n=6)

Group	Withdrawal Time	Residue (µg/kg) Mean ± SD		
		Muscle	Liver	Kidney
30 mg/kg b.w.d	4 hours	404.67±64.37	1123.35±131.77	4282.20±144.24
	1 day	45.43±23.36	182.23±70.72	241.30±40.60
	3 days	21.19±9.34	40.40±5.52	28.76±7.80
	5 days	10.18±5.84	16.56±7.23	15.24±6.26
	7 days	<LOQ	11.20±4.09	9.43±2.72
	9 days	<LOQ	6.50±3.47	<LOQ
	11 days	<LOQ	<LOQ	<LOQ
	13 days	<LOD	<LOQ	<LOQ
	15 days	<LOD	<LOD	<LOD
60 mg/kg b.w.d	4 hours	3159.30±42.55	6234.41±80.23	8735.75±73.16
	1 day	124.86±39.93	328.79±43.25	584.53±35.42
	3 days	36.66±8.99	69.15±10.45	57.22±8.83
	5 days	26.88±5.43	30.80±4.45	30.74±5.98
	7 days	19.36±4.87	22.68±4.20	18.73±4.36
	9 days	6.53±2.34	15.49±3.17	10.98±3.21
	11 days	<LOQ	8.35±2.09	<LOQ
	13 days	<LOD	<LOQ	<LOQ
	15 days	<LOD	<LOD	<LOD

Table 4: Residual DIKETO in chicken tissue (n=6)

Group	Withdrawal Time	Residue (µg/kg) Mean ± SD		
		Muscle	Liver	Kidney
30 mg/kg b.w.d	4 hours	50.97±17.68	38.03±8.63	237.30±32.83
	1 day	8.16±3.28	16.23±4.82	26.44±4.22
	3 days	3.58±1.27	4.69±0.65	3.95±0.77
	5 days	1.55±1.03	4.25±0.74	2.77±1.01
	7 days	1.21±0.65	3.98±0.92	2.16±0.73
	9 days	1.06±0.72	2.79±0.42	1.95±0.71
	11 days	0.83±0.26	2.03±1.02	1.39±0.56
	13 days	<LOD	1.58±0.56	<LOQ
	15 days	<LOD	<LOD	<LOD
60 mg/kg b.w.d	4 hours	144.60±24.53	279.24±15.40	326.27±45.23
	1 day	20.28±5.32	11.65±3.04	36.41±8.67
	3 days	10.40±4.25	6.88±4.92	14.62±3.80
	5 days	3.71±1.21	4.69±2.35	7.44±2.27
	7 days	2.90±1.37	4.27±1.57	5.13±1.38
	9 days	2.05±0.54	3.45±0.52	3.89±1.25
	11 days	1.52±0.87	2.50±1.05	2.43±1.58
	13 days	<LOD	1.78±0.96	1.43±0.23
	15 days	<LOD	<LOD	<LOD

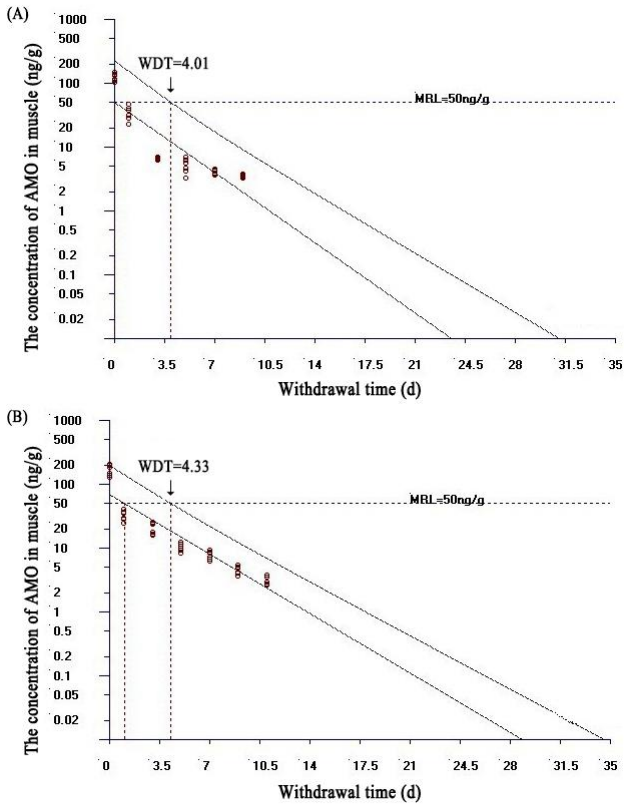

Fig. 1: Withdrawal times, i.e., the time point at which the one-sided 95% upper tolerance limit fell below the MRL of AMO, for AMO residues in chicken muscle after the administration of 30 mg AMO/kg body weight (A) and 60 mg AMO/kg body weight (B).

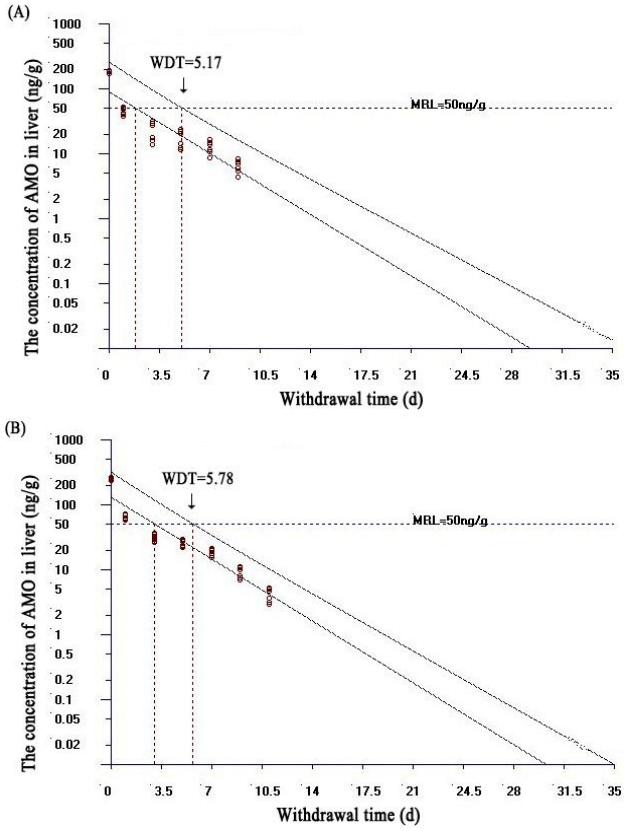

Fig. 2: Withdrawal times, i.e., the time point at which the one-sided 95% upper tolerance limit fell below the MRL of AMO, for the residues of AMO in chicken liver after the administration of 30 mg AMO/kg body weight (A) and 60 mg AMO/kg body weight (B).

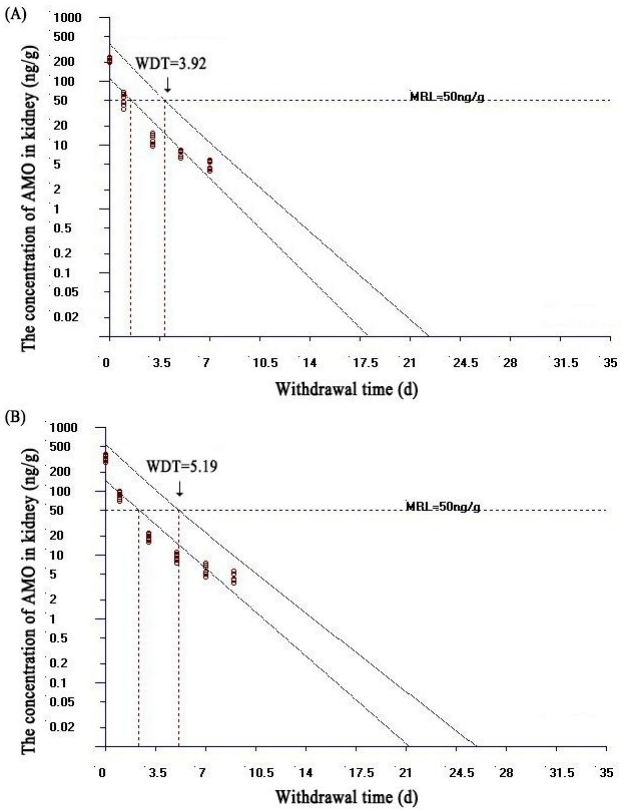

Fig. 3: Withdrawal times, i.e., the time point at which the one-sided 95% upper tolerance limit fell below the MRL of AMO, for the residues of AMO in chicken kidney after the administration of 30 mg AMO/kg body weight (A) and 60 mg AMO/kg body weight (B).

For doses of 30 or 60 mg/kg of body weight, the residual concentrations of AMO in the muscle, kidney, and liver were below the LOD after 13 days, 15 days, and 13 days after the drug was withdrawn, respectively. Moreover, the levels of AMA and DIKETO in muscle, kidney, and liver were below the LOD after 13 days, 15 days and 15 days after the drug was withdrawn, respectively (Fig 1-3). The amounts of residual AMO, AMA and DIKETO in each tissue sample were positively correlated with the dose.

DISCUSSION

AMA and DIKETO were found to be degradation products of AMO. In the present study, we found that AMO, AMA, and DIKETO are present in chicken tissues. In the chicken tissues, the levels of AMA residues are highest among the analytes, and the levels may be affected by the pH of the tissue. Freitas *et al.* (2012) found that AMO has different degradation products at different pH levels; acidic pH levels can lead to AMA, DIKETO can be generated under weak alkaline conditions. Reyns *et al.* (2008b) only found high concentrations of AMA in the liver and kidneys of pigs. At the same time, Freitas *et al.* (2012) noted that temperature changes could influence the degradation of AMO. Furthermore, the concentration of AMA was highest in the kidney and liver. The metabolite levels were high in the kidney, which may be related to the excretion of the drug from the kidneys. However, the liver is a detoxifying and metabolic organ; therefore, it also contained high concentrations of the metabolites.

Previous studies have primarily focused on AMO consumption and subsequent concentrations, and few studies have analyzed AMA. Baere *et al.* (2002) developed a quantitative assay for AMO, AMA and DIKETO in animal tissues and described the long-term presence of AMA as a metabolite of AMO in kidney and liver samples of swine. In practice, AMO metabolites have lost the antimicrobial activity of the parent compound (Liu *et al*., 2017); however, the metabolites, AMA and DIKETO, have been reported to be allergens (Fagerquist *et al*., 2005; Reyns *et al*., 2008a). Therefore, AMA residues should be determined to ensure the safety of animal-derived foods despite MRLs for the metabolites of AMO not being specified. Therefore, we recommend that the MRL of AMO should depend not only on the amount of parent compound present but also on the sum of the levels of AMO, AMA and DIKETO.

Because AMO and its metabolites may pose various unknown risks, estimating the withdrawal time of AMO and its metabolites in chicken tissue is important. The EU has established MRLs for AMO in edible chicken tissues (muscle, liver, and kidney), but MRLs for its metabolites have not yet been established. Consequently, the withdrawal time for AMO metabolites cannot be calculated. Therefore, residual AMO was used as the basis for determining the drug withdrawal time in this study, and the MRL of AMO was used to determine the most reasonable withdrawal time. The withdrawal time was estimated by linear regression analysis of the log-transformed tissue concentrations and was calculated as the time point at which the 95% upper one-sided tolerance limit was below the MRL with 95% confidence (Zhao *et al*., 2015). The AMO concentrations versus the withdrawal time are shown in Figs. 1-3. For AMO doses of 30 and 60 mg/kg body weight, the withdrawal time of AMO was 4.01 and 4.33 days in the muscle, 5.17 and 5.78 days in the liver, and 3.92 and 5.19 days in the kidney, respectively. To ensure food safety, a withdrawal period of 6 days is warranted for AMO doses of 30 and 60 mg/kg body weight. Reports indicate that a residual amount of penicillin as low as 0.6 μg can cause an allergic reaction (Dayan 1993; Beyene, 2015). Therefore, MRL standards for AMO should be developed, which requires the establishment of the withdrawal period of AMO. Moreover, monitoring the residual levels of AMA and DIKETO is also recommended.

Conclusions: This study indicated that AMO and its major metabolites were detectable in chicken tissue. AMA was observed at higher concentrations in chicken tissue than AMO or DIKETO. However, the EU defined an MRL for only AMO and not its metabolites. Because AMA has associated health risks, we recommend monitoring AMA levels. For AMO administered to broilers at 30 or 60 mg/kg body weight, we recommend a withdrawal time of 6 days to ensure food safety.

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Authors contribution: Xie KZ, Wang R, Wu HQ, Zhang GX, Dai GJ and Wang JY designed, guided and supervised this research. Zhao M and Zhang YY conducted the experiments and drafted the manuscript. Wang B, Zhao X and Wang YJ assisted in sample collection. Pang MD and Xing X helped in the statistical analysis.

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