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Residue Depletion and Tissue-Plasma Correlation of Methyl-3-quinoxaline-2-carboxylic Acid after Dietary Administration of Olaquindox in Pigs

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A residue depletion study was performed to investigate the tissue kinetics and tissue-plasma correlation of methyl-3-quinoxaline-2-carboxylic acid (MQCA), the marker residue of olaquindox (OLA), in pigs. Twenty-five pigs were randomly divided into a test and a control group. The former group was treated with 100 mg/kg OLA in its feed for 30 consecutive days, and the latter was given blank feed for the same period. One control and four treated animals were slaughtered at 0.5, 3, 10, 17, and 28 days post-medication. Muscle, liver, kidney, fat, and plasma samples were collected and analyzed using the validated high-performance liquid chromatography method (HPLC). Results showed that the tissue concentration of MQCA in the liver > kidney > fat > muscle, at almost all time points. The half-lives of MQCA in the muscle, liver, kidney, fat, and plasma were 12, 8, 15, 8, and 6 days, respectively. A withdrawal period of 38 days was calculated using the statistical method recommended by the European Medical Evaluation Agency (EMEA). Good correlations between tissue and plasma MQCA levels were found in the present study with correlation coefficients of more than 0.92. These correlations would be helpful in the routine monitoring of OLA in porcine tissues, without sacrificing the animals.

KEYWORDS: Olaquindox; methyl-3-quinoxaline-2-carboxylic acid; pig; residue; correlation

INTRODUCTION

Olaquindox (OLA, methyl-3-(2-quinoalinylmethylene) carbazate-*N*1, *N*4-dioxide) is an antimicrobial drug used as a growth promoter in animal production. Although, OLA has been withdrawn from the market in the European Union, because of possible carcinogenic, mutagenic, and photoallergenic effects (*1*), it is used in Japan and Australia with the maximum residue limit (MRL) of 300 μg/kg in porcine and poultry edible tissues (*2*,*3*). In China, OLA is allowed to be used in pigs weighing less than 35 kg, as a medication premix, at the level of 50−100 mg/kg. The MRL is fixed at 50 μg/kg and 4 μg/kg for the liver and muscle, respectively (*4*).

As a veterinary drug for food-producing animals, a scientific withdrawal time (WDT), based on the MRL and the result of a residue depletion study, is essential to ensure food safety. However, there was inadequate data available for the establishment of a WDT for OLA, until now. Two safety evaluation reports summarized by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) gave some information on the residue depletion of OLA in pigs (*5*, *6*). Methyl-3-quinoxalin-2-carboxylic acid, the major remaining detectable metabolite of the drug, was defined as the marker substance for OLA (*6*). To illustrate the distribution and elimination of OLA residues in pigs, four total residue depletion studies were conducted using ^{14}C-labeled OLA (*6*, *8*). The pigs received an oral dose of 5 mg/kg body weight for seven consecutive days in three studies and 2.5 mg/kg body weight for five consecutive days in the fourth study. The results showed the highest liver residue concentrations for up to 28 days after dosing. According to the official guideline of EMEA (*7*), the liver should be designated as the target tissue for residue analysis and control of OLA. However, the muscle was selected as the target tissue because of the lack of a sensitive assay method for OLA residues in the liver (*6*). Two other studies were performed to illustrate the elimination of MQCA in pigs (*6*, *8*). In one study, 20 pigs received a dose of 25 mg/kg OLA in their feed for 28 consecutive days. In the other study, 25 pigs were dosed with a medicated feed at 100 mg/kg, for 68 consecutive days. Only the data on the muscle was reported. The results showed that a steady state concentration of the OLA residues was reached on the twenty-ninth day after dosing, and the depletion of MQCA was linear over 8−20 days post medication (*8*). On the basis of this information, an MRL of 4 μg/kg was recommended for the muscle tissue, and WDT was not recommended by JECFA (*6*). In China, a WDT of 35 days has been recommended for OLA premix in pigs (*9*). However, whether the current WDT is rational or not still needs further investigation, as there is inadequate data available on tissue residue, and no scientific statistical analysis has been done during the establishment of the

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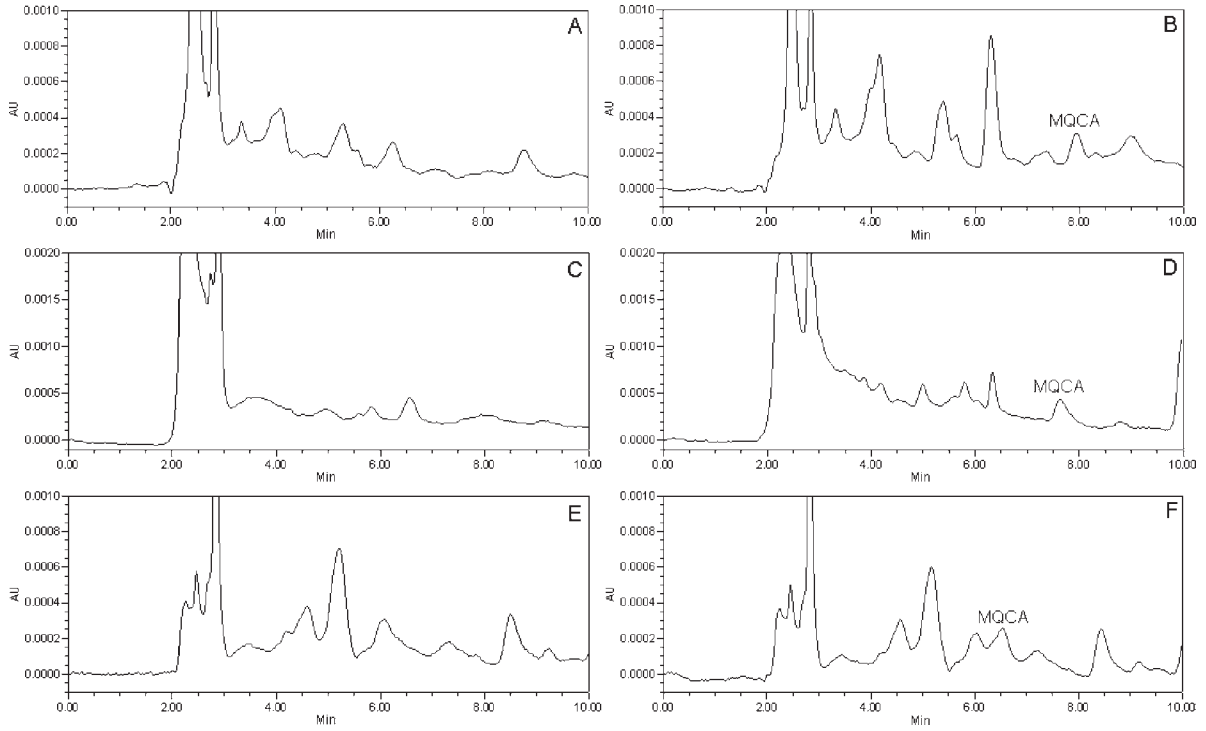

Figure 1. Chromatograms of blank plasma (**A**), blank fat (**C**), blank kidney (**E**), and plasma sample (**B**), fat sample (**D**), and kidney sample (**F**) spiked with MQCA at 4 μg/kg (plasma and fat sample) and 20 μg/kg (kidney sample). The mobile phase was acetonitrile/water containing 1% acetic acid (18:82 v/v, plasma and fat sample; 20:80 v/v, kidney sample) at a flow rate of 1.0 mL/min.

WDT. To establish a rational WDT, a further study on residue depletion of OLA in pigs should be undertaken.

In vivo prediction of residues is an efficient, low-cost method to decrease the risk of human exposure to food contaminated by veterinary drugs. Using the in vivo prediction procedure, unnecessary waste of animals and tedious sample processing can be avoided. The literature survey shows that considerable research has been performed on the correlation of tissue-body fluids (*10*−*13*). These studies show the feasibility and rationality of estimating tissue MQCA concentration using body fluids.

In the current study, residue depletion of OLA in pigs was investigated to characterize the kinetics process of MQCA in the muscle, liver, kidneys, fat, and plasma. Subsequently, the WDT of OLA in pigs was calculated on the basis of the residue depletion data. Meanwhile, the correlations of MQCA between edible tissues and plasma were investigated for the first time. These results would provide a scientific food safety standard for residue monitoring of OLA and a suggestive method for estimating the concentration of MQCA in edible porcine tissues during breeding, without sacrificing the animals.

MATERIALS AND METHODS

Chemicals and Drugs. All solvents were of analytical reagent grade. Deionized water (Milli-Q; Millipore, Bedford, MA, USA) was used throughout the study. The standard of MQCA was obtained from the Institute of Veterinary Pharmaceuticals (Huazhong Agricultural University, Wuhan, China), with a purity of over 97%. Stock standard solution (1.0 mg/mL) was prepared by dissolving MQCA in methanol. Working standard solution (100 μg/mL) was prepared by dilution of the stock standard in methanol. These solutions were found to be stable for at least three months when stored in amber vials below 4 °C. The extraction solution consisted of 5% (w/v) metaphosphoric acid in 10% methanol (v/v) and 0.01 M dipotassium phosphate buffer (prepared from K_2HPO_4, with the necessary pH adjustment being made with phosphoric acid, pH 7.0).

OLA was purchased from Hebei Smart Chemicals Co., Ltd. (>98.0% purity, Shijiazhuang, China).

Feed. The feed used in this study was purchased from Wuhan Luhong Bioscience and Technology Co. Ltd. (Wuhan, China). The blank feed and the medicated feed were analyzed using the HPLC method by Wu (*14*) to determine the drug content. The results indicated that no quinoxaline compounds were detected in the blank feed, and only OLA (93 ± 6 mg/kg feed, n = 9) was found in the medicated feed.

Animals and Sampling. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals provided by the Institutional Animal Care and Use Committee. Twenty-five castrated male pigs (healthy large Landrace-Large white cross-bred) weighing 18.08 ± 1.64 kg were purchased from Breeding Swine Testing Center, Wuhan, China. They were housed in four 10 × 12 m pig beds with ad libitum access to fresh water. Temperature and humidity in the house were maintained at a constant level of 22 ± 5 °C and 80−90%, respectively. All of the pigs were fed a standard ration twice a day. The average daily intake for each pig was 1.08 kg/day. All of the animals were provided seven days for acclimation prior to the experiment.

All of the pigs were randomly divided into a control group (n = 5) and a test group (n = 20). Pigs in the control group were supplied with blank feed. Pigs in the test group were provided with medicated feed for 30 consecutive days. At 0.5, 3, 10, 17, and 28 days post-medication, one control and four medicated pigs were sacrificed by captive bolt stunning and exsanguinations according to the guidance provided by American Veterinary Medical Association (AVMA) for euthanasia (*15*). Blood samples (approximately 10 mL) were collected with a disposable vacutainer-heparinized tube. These blood containing tubes were placed in an ice bath for at least 30 min and centrifuged at 5 °C for 10 min at 3000 rpm, to prepare plasma. Edible tissues such as muscle, liver, kidney, and fat were collected and homogenized, and subsequently placed in labeled plastic bags. All samples were assayed immediately or frozen at −20 °C until the analysis could be completed.

Assay of MQCA in Edible Tissues and Plasma. *Sample Preparation.* Muscle, liver, and kidney samples were handled using a modified procedure by Wu (*16*). Briefly, tissue samples (2.0 ± 0.01 g) were extracted with 5% (w/v) metaphosphoric acid in 10% methanol (v/v), ethyl acetate, and dipotassium phosphate buffer, in turn. Then, the extracts were purified by an Oasis MAX cartridge (60 mg, 3 mL, Milford, MA, USA). After the residues were evaporated to dryness, they were dissolved with acetonitrile/water (30/70 v/v). An aliquot of 50 μL was analyzed by HPLC.

Table 1. Regression Equations and Coefficient Correlations of the Calibration Curve of MQCA in Tissues and Plasma

matrix	regression equation	coefficient correlation
liver	$y = 125.06x + 366.93$	0.9990
kidney	$y = 130.42x + 2747.80$	0.9959
fat	$y = 404.61x + 1925.50$	0.9968
muscle	$y = 321.66x + 732.79$	0.9960
plasma	$y = 354.38x + 132.67$	0.9662

Table 2. Individual Pig Tissue Residue Concentrations (μg/kg) of MQCA at Various Times after Treatment with 100 mg/kg OLA in Feed for 30 Consecutive Days[a]

pig no.	time (d)	liver	kidney	fat	muscle	plasma
1	0.5	1382	1332	308	205	469
2	0.5	1853	1451	273	213	673
3	0.5	1491	1184	260	192	650
4	0.5	1721	1111	217	182	793
5	3	369	306	75	62	98
6	3	442	328	82	67	118
7	3	423	357	71	63	100
8	3	467	390	93	85	133
9	10	53	61	35	11	56
10	10	65	50	30	14	38
11	10	108	50	29	13	44
12	10	79	47	45	12	53
13	17	51	42	15	7	26
14	17	46	38	17	6	36
15	17	58	49	30	12	41
16	17	55	47	28	9	38
17	28	15	ND	7	ND	ND
18	28	11	ND	5	ND	ND
19	28	15	ND	8	ND	5
20	28	18	23	8	4	7

[a] ND: samples were below the LOQ of analysis method.

Adipose samples (2.00 ± 0.01 g) were extracted twice with 8 mL of 0.05 M dipotassium phosphate buffer. Two supernatants (16 mL) were combined and filtrated through a filter paper. Then, the filtrate was cleaned up using a Waters Oasis MAX cartridge (60 mg, 3 mL, Milford, MA, USA). After the residues were evaporated to dryness under a stream of nitrogen at 45 °C, they were dissolved with 400 μL of acetonitrile/water (30/70 v/v). An aliquot of 50 μL was analyzed by HPLC.

Plasma samples (1.0 ± 0.01 mL) were acidified by 150 μL of 5 M hydrochloric acid and extracted twice with 4 mL of ethyl acetate. The two supernatants (8 mL) were collected and evaporated to dryness under a stream of nitrogen at 45 °C. The residues were dissolved with 200 μL of acetonitrile/water (30/70 v/v). Next, the sample was filtered through a 0.22 μm microbore cellulose membrane. An aliquot of 50 μL was analyzed by HPLC.

HPLC Anaylsis. All HPLC analyses were performed using a WATERS HPLC system, comprising a 2695 ternary pump and 2487 UV detection. An Eclipse XDB-C_{18} (250 mm × 4.6 mm I.D) (Agilent Technology, USA) HPLC column was used for sample separation. The temperature of the HPLC column was set at 30 °C. The mobile phase was acetonitrile/water containing 1% acetic acid (18:82 v/v for the plasma, muscle, liver, and fat samples; 20:80 v/v for the kidney samples). It was pumped at a flow rate of 1.0 mL/min. The spectra of all of the samples were obtained from detecting at the wavelength of 320 nm.

Method Validation. The method for plasma and tissue samples was validated for sensitivity, accuracy, precision, and stability of MQCA in the samples. The limit of quantification (LOQ) was 4 μg/kg for the plasma, muscle, and fat samples, 10 μg/kg for the liver samples, and 20 μg/kg for the kidney samples. The specificity of the method was checked by comparing the blank tissue with the fortified samples. A representative chromatogram is shown in **Figure 1**. The recovery of MQCA from plasma and tissues ranged from 74 to 118%, with the intraday relative standard deviation less than 15%. The concentration of MQCA in incurred samples was quantified using calibration curves, as shown in **Table 1**. Good linear correlation ($r > 0.96$) was achieved within the concentration range of 4−640 μg/kg. Stability experiments were also carried out to investigate the stability of MQCA in incurred samples stored at −20 °C. The results indicated that MQCA was stable for at least three weeks in incurred samples during storage at −20 °C.

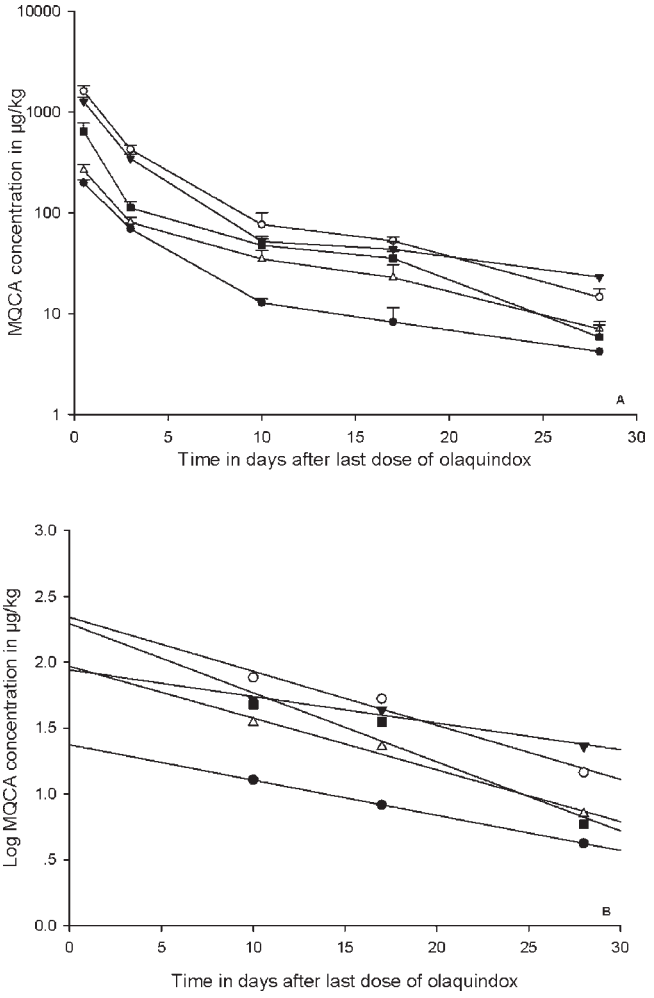

Figure 2. Concentration−time curve of MQCA in porcine muscle (●), liver (○), kidney (▼), fat (△), and plasma (■) after treatment with 100 mg/kg OLA in feed for 30 consecutive days. Regression curves were generated using the average value of the last three time points without standard deviation.

Data Analysis. The concentrations of MQCA in the edible tissues and plasma were calculated using SigmaPlot 11.0 (SPSS, Inc.). The linear regression algorithm in this program was used to determine the slope, intercept, and half-lives ($t_{1/2}$) of plasma and tissue residue data. The WDT was estimated by linear regression analysis of the $\log_e$-transformed tissue concentration using the WT1.4 program and determined at the time when the upper one-sided tolerance limit, with a confidence of 99%, was below the accepted MRL of 4 μg/kg (in muscle) and 50 μg/kg (in liver), set by the Agriculture Ministry of People's Republic of China (*4*). The correlation analysis was also performed using the linear regression algorithm in SigmaPlot 11.0.

RESULTS AND DISCUSSION

Elimination of MQCA. The concentrations of MQCA in the plasma, muscle, liver, kidney, and fat are presented in **Table 2**. Liver and muscle contained the highest and the lowest concentrations of MQCA, respectively. The MQCA level in the kidney was higher than that in the fat. The results were in agreement with the depletion profile of OLA-related residues in pigs (*6*,*8*). According to the principle of pharmacokinetics, the distribution of MQCA

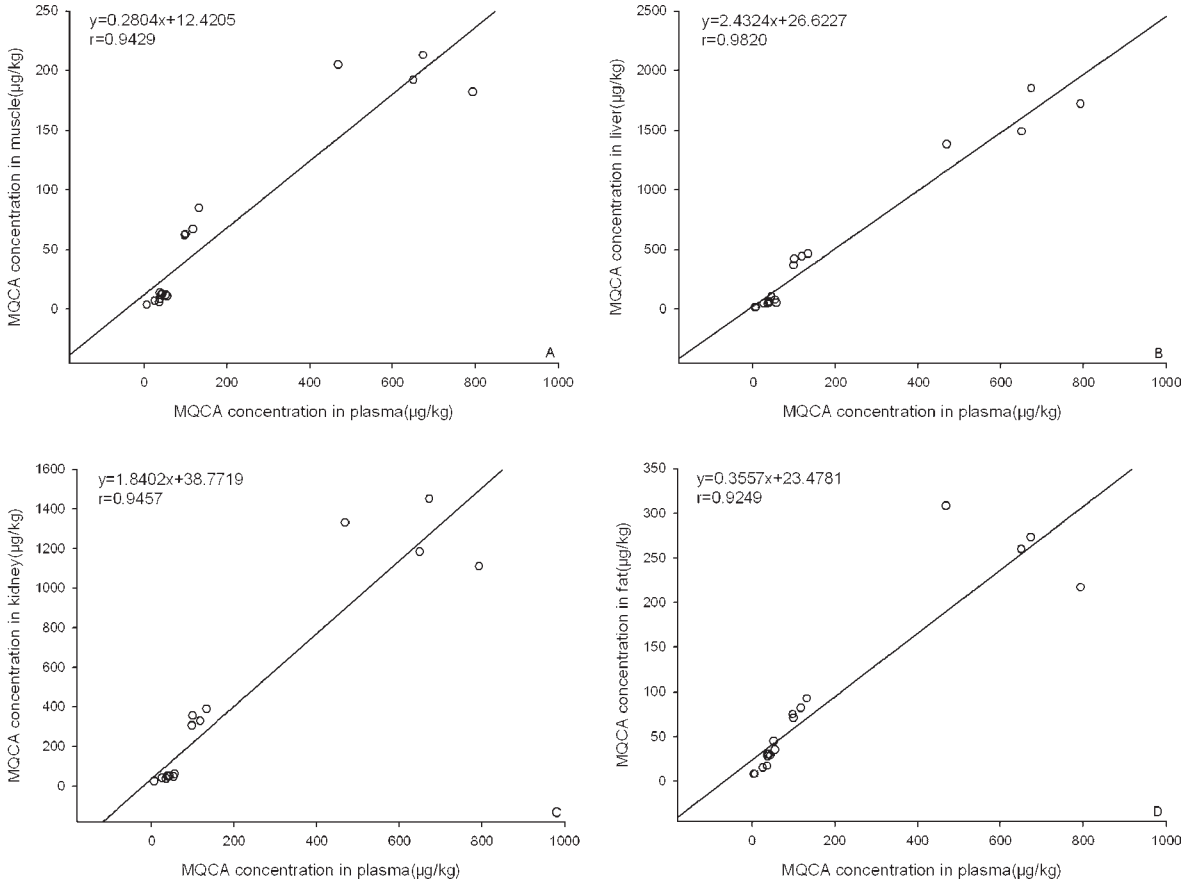

Figure 3. Correlation of MQCA concentration in plasma and muscle (**A**), liver (**B**), kidney (**C**), and fat (**D**).

in the body depended on the organ blood flow and tissue affinity. The blood supply to the liver and kidneys was more than that to the muscle and fat. Therefore, a comparatively higher level of MQCA was found in the liver and kidneys. The quinoxaline ring in MQCA increased the lipophilicity of the compound, which led to a higher MQCA level in fat than in muscle.

At zero withdrawal intervals, on 0.5 day post-medication, the concentration of MQCA was 1382−1853 μg/kg in the liver, 1111−1451 μg/kg in the kidney, 182−213 μg/kg in the muscle, and 217−308 μg/kg in the fat. On day 17 post-medication, the MQCA concentration in the liver was around the acceptable MRL (50 μg/kg). On day 28 post-medication, the MQCA concentrations in all liver and fat samples were above the LOQ of the method, while three out of four kidney samples, two out of four plasma samples, and three out of four muscle samples did not have enough MQCA to be quantified using the HPLC method. The concentration−time curves of MQCA are shown in **Figure 2A**. Similar two-phase decay in tissue and plasma were observed. The MQCA level in tissues and plasma decreased significantly at 0−3 days post-medication. Subsequently, the depletion of MQCA from the pig became very slow. The elimination half-lives of MQCA in the plasma and tissues were calculated using the regression curves derived from **Figure 2B**, which were 6 days for plasma, 12 days for muscle, 8 days for liver, 15 days for kidney, and 8 days for fat.

These long half-lives of MQCA in tissues may be ascribed to the following reasons. The main metabolic pathways from OLA to MQCA include reduction and hydrolysis (*6*). The distribution and elimination of the intermediate metabolites in tissues and blood also affect the dynamics of MQCA in pigs. Desoxyolaquindox is one of the reduced metabolites of OLA. Its polarity is much lower than OLA and MQCA. As a result, desoxyolaquindox may be easily retained by the organs and tissues. As MQCA is derived from the hydrolysis of desoxyolaquindox (*8*), it may exist in the body for a long time. Meanwhile, the binding of OLA metabolites to tissues, enterohepatic circulation of MQCA, and its plasma protein binding can also cause the long half-lives of MQCA in tissues. However, no bound residues of OLA have been found in pigs, according to the report by JECFA (*6*, *8*). Accumulation of OLA metabolites in tissues is therefore excluded. Compounds with large molecular weight and some conjugated metabolites (glucuronide conjugates) are found to be transported into bile more easily than the small ones (*17*). OLA-related residues are small molecular compounds, and no phase II metabolites are found in pigs (*6*). Therefore, there is a small possibility of enterohepatic circulation of MQCA in pigs. Nevertheless, further study is needed to provide information on the impaction of enterohepatic circulation and plasma protein binding on the elimination of MQCA.

Estimation of the Withdrawal Time. The prolonged presence of OLA-related residues in edible tissues was a potential risk for consumers. To ensure the safety of porcine edible tissues, the WDT for OLA was established on the basis of tissue residue data and the acceptable MRL recommended by the Ministry of Agriculture of the People's Republic of China. In the current study, the linear regression analysis recommended by EMEA was used for calculation. According to the official guidelines of EMEA, the data used for calculation of the WDT had to satisfy the linear regression analysis assumptions, and the WDT could not be calculated by extrapolation (*18*). However, the obvious deviation from linearity at early time points was found ($p < 0.05$, F-test), excluding day 0.5, and day 3 from the calculation was, therefore, taken into account. To avoid extrapolation far removed from the range of the observed data, day 28 (muscle) was retained, and three less than

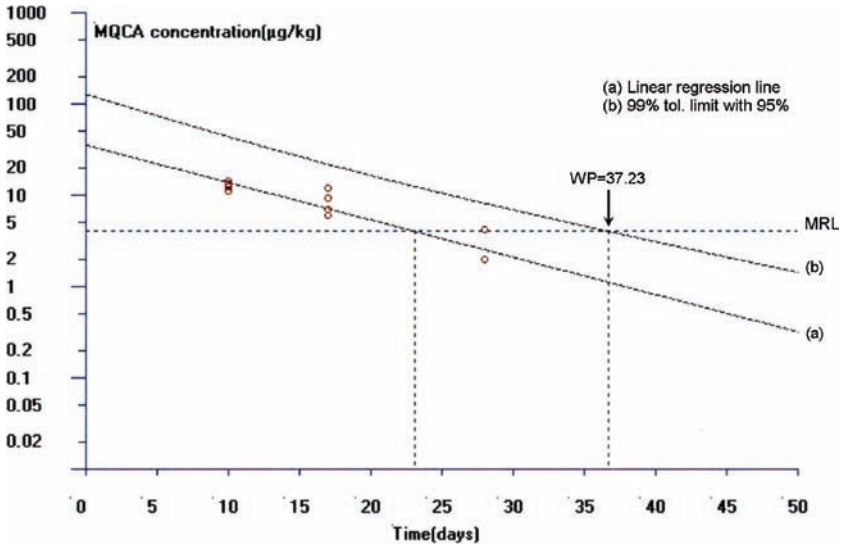

Figure 4. Plot of WDT calculation for porcine muscle.

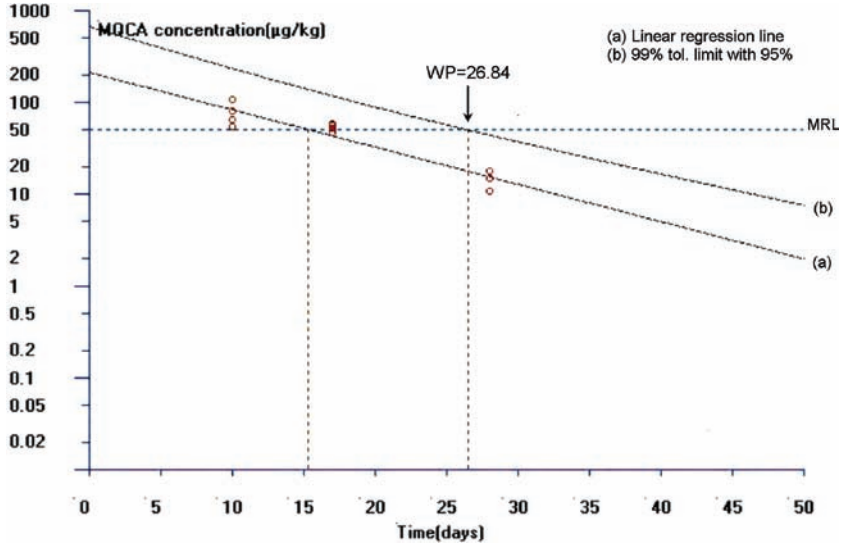

Figure 5. Plot of WDT calculation for porcine liver.

data at this time point were set at 2 μg/kg (1/2 LOQ) (*18*). In addition, 99% tolerance limits were applied to get a safer and better protection for the consumer. Using the WT1.4 software program, a period of 37.23 days and 26.84 days (shown in Figures **4** and **5**) was calculated as the WDT of OLA in pig muscle and liver, respectively. As these time points did not make up full days, we had to round off the WDT to the next day. To guarantee consumer safety, the longest WDT of 38 days was selected as the conclusive withdrawal period, at the dose used in this study. As the established WDT was based on tissue residue data and the consideration of the ninety-ninth percentile with 95% confidence, we could use it for residue monitoring of OLA in pigs.

Correlation of MQCA in Plasma and Tissue. The correlation between plasma and edible tissue concentration was developed using the regression equations shown in **Figure 3**. The correlation coefficients were 0.98 between the liver and plasma, 0.95 between the kidney and plasma, 0.92 between the fat and plasma, and 0.92 between the muscle and plasma. Judging by these correlation coefficients, an excellent correlation existed between the plasma and tissues. A t test was also carried out to evaluate the correlation between plasma and edible tissues. A high, significant correlation ($P < 0.01$) was found between the tissues and plasma.

Good tissue−plasma correlations were ascribed to the similar dynamics of MQCA in tissues and blood. Equations shown in **Figure 3** described the functional relationship between the MQCA concentrations of plasma and tissues. These equations would be useful for the estimation of the MQCA concentration in tissues, without unreasonable extrapolation. As shown in **Table 2**, the MQCA level in the plasma was always higher than that in the muscle. When the concentration of MQCA in the muscle decreased to around that of the MRL (4 μg/kg), the corresponding concentration in the plasma was still quantifiable. Although plasma had a lower MQCA level than the liver, the MQCA concentration in the liver could be estimated using the plasma data. When the liver MQCA concentration decreased around the acceptable MRL (50 μg/kg), the corresponding concentration in the plasma was still above the LOQ of the assay method. Plasma is the most appropriate sample for in vivo drug monitoring compared to urine and bile because data obtained from plasma is more homogeneous than that obtained from urine and bile. Meanwhile, the drug concentration in the plasma represents instantaneous concentration (not an average concentration over a period of time), which makes the estimated tissue concentration more meaningful for the routine residue monitoring of OLA.

In practice, this routine monitoring would be very useful for the judgment of suitable time for pig slaughter. If the estimated MQCA concentrations in the tissues are above the MRL, additional time for OLA elimination needs to be provided, and further plasma testing at a later time should be performed until the estimated MQCA levels in the tissues are below the MRL.

Conclusions. The results of the present study provided the residue profile of MQCA in edible tissues and plasma. With the residue data of MQCA in edible tissues, a withdrawal period of 38 days was estimated for OLA in pigs, at 100 mg/kg in the feed, using the linear regression method recommended by EMEA. In addition, excellent correlations were observed between the plasma and edible tissues. On the basis of these correlations, a low-cost procedure could be used for the routine residue monitoring of OLA in pigs.

ABBREVIATIONS USED

MQCA, methyl-3-quinoxaline-2-carboxylic acid; OLA, olaquindox; WDT, withdrawal time; MRL, maximum residue limit; LOQ, limit of quantification; HPLC, high-performance liquid chromatography method; EMEA, European Medical Evaluation Agency; JECFA, Joint FAO/WHO Expert Committee on Food Additives.

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