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# Residue Depletion of Cefquinome in Swine Tissues after Intramuscular Administration

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A high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection was developed for the detection of cefquinome (CEQ) residues in swine tissues. The limit of detection (LOD) of the method was 5 ng $g^{-1}$ for muscle and 10 ng $g^{-1}$ for fat, liver, and kidney. Mean recoveries of CEQ in all fortified samples at a concentration range of 20–500 ng $g^{-1}$ were 80.5−86.0% with coefficient of variation (CV) below 10.3%. Residue depletion study of CEQ in swine was conducted after five intramuscular injections at a dose of 2 mg $kg^{-1}$ of body weight with 24 h intervals. CEQ residue concentrations were detected in muscle, fat, liver, and kidney using the HPLC-UV method at 265 nm. The highest CEQ concentration was measured in kidney tissue during the study period, indicating that kidney was the target tissue for CEQ. CEQ concentrations in all examined tissues were below the accepted maximum residue limit (MRL) recommended by the Committee for Veterinary Medical Products of European Medical Evaluation Agency (EMEA) at 3 days post-treatment.

**KEYWORDS: Cefquinome; swine; residue depletion; intramuscular administration; HPLC-UV**

## INTRODUCTION

Cefquinome (CEQ), 1-[[(6R,7R)-7-[[(2Z)-(2-amino-4-thiazolyl)(methoxyimino)acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]-5,6,7,8-tetrahydroquinolinium inner salt, is a member of the fourth generation cephalosporin family (*1*). The molecular structure of CEQ is shown in **Figure 1**. The chemical modifications on the basic cephalosporin structure created the zwitterionic compound with enhanced bioavailability and improved spectrum of antimicrobial activity (*2*). It was developed exclusively for veterinary prescription use. Because CEQ has effective antimicrobial activities against a wide spectrum of Gram-positive as well as Gram-negative bacterial and few adverse effects (*3, 4*), various CEQ injectable and intramammary formulations have been approved for the treatment of respiratory tract diseases and clinical mastitis for livestock in the European Union since 1994 (*5–10*). However, in the United States at present, it is proposed only for the treatment of bovine respiratory disease.

CEQ pharmacokinetics studies had been reported in different animals (*4, 11–17*). The major pharmacokinetic characteristics for CEQ were rapid absorption after intramuscular injection ($t_{max}$ between 0.5 and 2 h), high bioavailability ($F$ > 93%), and short elimination half-time (within 3 h). In addition, the distribution of CEQ was not extensive in animal bodies, and the apparent distribution volume was not large (approximately 0.2 L/kg). A radiolabeled study on pig proved that only a small amount of CEQ in animal bodies was transformed into other metabolites and was mainly excreted by kidney in urine after administration (*5, 6*). The maximum residue limit (MRL) for CEQ in swine and bovine tissues and milk was established by the European Union (EU) with CEQ only as a marker residue (*1, 4–6*). The EU MRLs in swine kidney, liver, muscle, and fat are 200, 100, 50, and 50 $\mu$g $kg^{-1}$, respectively. Some different methods have been described for the determination of CEQ in raw bovine milk and animal tissues, including high-performance liquid chromatography (HPLC) (*18, 19*), liquid chromatography−tandem spectrometry (*20*), screening method (*19, 21, 22*), and other methods (*23–26*). However, few methods have been reported for the analysis of CEQ in swine fat, liver, and kidney.

The aim of the current study is to develop a sensitive and routine HPLC-UV method for the determination of CEQ in swine muscle, fat, liver, and kidney that can be applied to study the residue depletion profiles of CEQ in swine tissues to establish the withdrawal period after intramuscular administration.

## MATERIALS AND METHODS

**Reagents and Materials.** The CEQ injectable formulation (2.5%, w/v) used in the present study was provided by Animal Medicine Corp. Ltd. (Zhejiang Province, People's Republic of China). Acetonitrile (ACN) and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Hexane, triethanolamine, phosphoric acid ($H_3PO_4$, 85%), and isopropanol (analytical grade) were obtained from Beijing Chemical Reagent Corp. (Beijing, People's Republic of China). Solid-phase extraction (SPE) cartridges (Oasis HLB, 3 $cm^3$/60 mg) were

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from Waters Co. Water used for HPLC analysis was purified through a Milli-Q system (Millipore, Bedford, MA). Phosphate buffer (0.085 M) was prepared by adding 7 mL of phosphoric acid (85%) into 850 mL of water. The pH was adjusted to 2.8 using triethanolamine, and the volume was made up to 1000 mL with water. It was passed through a 0.2 $\mu$m membrane and degasified before use. Buffer was stored at 4 °C and used within a week after preparation.

**Standard.** The CEQ reference standard (80.9% purity) was supplied by the China Institute of Veterinary Drug Control (Beijing, People's Republic of China). A stock solution of 1000 $\mu$g mL$^{-1}$ was prepared by dissolving 5 mg of CEQ standard in 5 mL of water. It was stable for at least 2 weeks at 4 °C, even after several freeze–thaw cycles. The stock solution was freshly diluted with water to prepare the working standard solutions.

**Sample Preparation.** Swine tissues (muscle, fat, liver, and kidney) were minced and homogenized in a homogenizer for 2 min. Five grams of homogenate was accurately weighed into a 50 mL polypropylene centrifuge tube. Ten milliliters of ACN/$H_2O$ (95:5, v/v) was added, and the mixture was mixed for 30 s using a vortex mixer before centrifugation at 3800 rpm (2400*g*) for 10 min. The supernatant was collected into a pear-shape flask, and the extraction was repeated. The supernatants were combined, and 3 mL of isopropanol was added. The combined extracts were evaporated to <1 mL in a 37 °C water bath with a rotary evaporator. The residue was dissolved in 10 mL of water and defatted by adding 5 mL of hexane. The mixture was transferred to a centrifuge tube, gently mixed, and then centrifuged as before. After removal of the hexane layer, the extract was cleaned up by HLB SPE cartridge, which was preconditioned with 2 mL of methanol and 2 mL of water. The combined extraction was drained through the cartridge with gravity. The centrifuge tube was rinsed with 5 mL of water, and the rinsewater was also added to the column. Then the cartridge was washed with 3 mL of ACN/$H_2O$ (5:95, v/v) and dried by vacuum for at least 1 min. The analytes were eluted with 2 mL of ACN/$H_2O$ 10:90 (v/v) for liver and kidney, whereas 2 mL of ACN/$H_2O$ 20:80 (v/v) was used for muscle and fat. The collected eluate was evaporated to near dryness in a water bath at 40 °C under a stream of nitrogen gas. The residue was reconstituted in 1 mL of water and filtered through a 0.2 $\mu$m syringe filter before injection into the HPLC system.

**Fortification.** To test the stability of the HPLC system and the feasibility of the detection method for CEQ in swine tissues, a fortifying test was performed on muscle, fat, liver, and kidney at 20, 50, 100, and 500 ng g$^{-1}$ fortification levels. The precision (interday and intraday) of the method was assessed using five replicates of control and fortified samples at four fortification levels on three different days.

**Method Calibration.** The calibration curves were prepared with the peak areas and the working standard solution concentration. The standard curve for CEQ was constructed with standard working solution concentrations of 50, 100, 200, 500, 1000, 2500, 5000, and 10000 ng mL$^{-1}$.

**Animal Treatment.** The trial was conducted in 40 healthy Chester white−Yorkshire cross-breed swine with an average body weight of 45 kg. During the acclimation for 20 days and subsequent treatment periods, the animals were fed an antibiotic-free balanced diet ad libitum with free access to fresh water. They were kept in individual metabolic cages in an environmentally controlled room. Their health condition was checked twice daily by a professional veterinarian. Five animals were kept as control. Thirty-five animals were weighed on the same day and injected five times on the right side of the neck at a dose of 2 mg kg$^{-1}$ of body weight with 24 h intervals. Five animals of the treated group were killed at 12 h and 1, 2, 3, 5, 7, and 9 days of withdrawal after the last injection. The untreated animals were sacrificed on day 9 to obtain blank tissues. Samples of muscle, fat, liver, and kidney collected from each animal were thoroughly minced, homogenized, and stored at −70 °C until they were assayed.

**HPLC Conditions.** The analyses of standards, fortified samples, and incurred samples were performed at room temperature by means of a HPLC-UV system that included a Waters 600 controller pump and a Waters 2487 UV detector with a Waters 717 autosampler (Waters Co., Milford, MA). The reverse phase analytical column was a Symmetry $C_{18}$ (250 mm × 4.5 mm i.d., 5 $\mu$m) from Waters Co. The HPLC mobile phase was ACN/phosphate buffer (0.085 M, pH 2.8), and an isocratic HPLC gradient (15:85, v/v) was carried out. The flow rate was set at 0.8 mL min$^{-1}$, and the injection volume was 100 $\mu$L. The UV detector wavelength was set at 265 nm to monitor for CEQ.

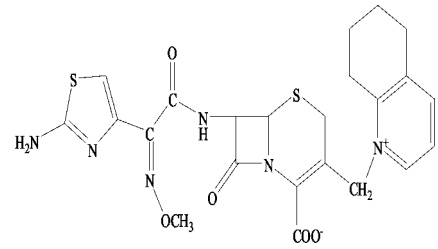

**Figure 1.** Molecular structure of CEQ.

**Data Analysis.** Student's *t* test was performed to test the CEQ concentrations for significant differences in different swine tissues. The withdrawal time was established by linear regression analysis of the log-transformed tissue concentrations and determined at the time when the one-sided 95% upper tolerance limit was below the EU MRL (*27*).

## RESULTS AND DISCUSSION

**Method Validation.** A sensitive and efficient analytical method plays a key role in drug residue analysis and depletion study. The crucial parameters for a method are linearity, accuracy, and precision. The standard calibration curve was linear at the concentration range of 50–10000 ng mL$^{-1}$, with correlation coefficients (*R*) of 0.9999. (The equation was $Y = 3.54 \times 10^2X - 8.10 \times 10^3$.) The limit of detection (LOD) and the limit of quantification (LOQ) were defined on the basis of signal-to-noise ratios (S/N) of 3:1 and 6:1, respectively (*28*). The LOD and LOQ were 5 and 10 ng g$^{-1}$ for muscle and 10 and 20 ng g$^{-1}$ for fat, liver, and kidney, respectively. The LOD and LOQ in our study were lower than those found in previous studies (*5*, *6*). The typical chromatograms of CEQ standard, blank, and fortified samples are presented in **Figure 2**. CEQ was well detected by this HPLC method with UV detection at 265 nm. No obvious interfering peaks were found in the control samples, which indicated high selectivity of the extraction, cleanup, and chromatographic method. The accuracy and precision of the method were assessed by using blank swine tissues spiked with CEQ at 20, 50, 100, and 500 ng g$^{-1}$ on three different days. Recoveries of all fortification levels are presented in **Table 1**. The interday mean recovery of CEQ was between 80.5 and 86% with CVs of 4.3–10.3%. The intraday mean recoveries for all samples ranged from 80.1 to 83.9% with CVs of 5.1–9.2%. EMEA described an analytical method (HPLC−diode array assay, ISO 78/2) for which the recoveries at LOQ in swine muscle, fat, liver, and kidney were not more than 50% (*5*).

**Sample Extraction.** Phosphate buffer (pH 9) (*29*), 0.4% dithioerythritol (DTE) in pH 9 borate buffer (*30*), and ACN/$H_2O$/$Et_4NCl$ (*31*) were used as extraction solution to extract ceftiofur and/or cephalosporin from animal tissues in previously reported methods. During the extraction, $\beta$-lactamase was added to samples in some studies (*20*, *31*). Compared with the previous methods, the extraction process in this study used less buffer/organic reagents and fewer steps, but good recoveries, LOD, and LOQ were obtained. The sample extraction and purification for CEQ from fortified and incurred swine samples in the current study was developed following a previously reported method with some modifications (*20*). In contrast to this method, the modifications were as follows: ACN/$H_2O$ (95:5, v/v) was used as extraction solution instead of ACN/$H_2O$ (90:10, v/v); 3 mL of isopropanol was added to the supernatant to avoid foaming during

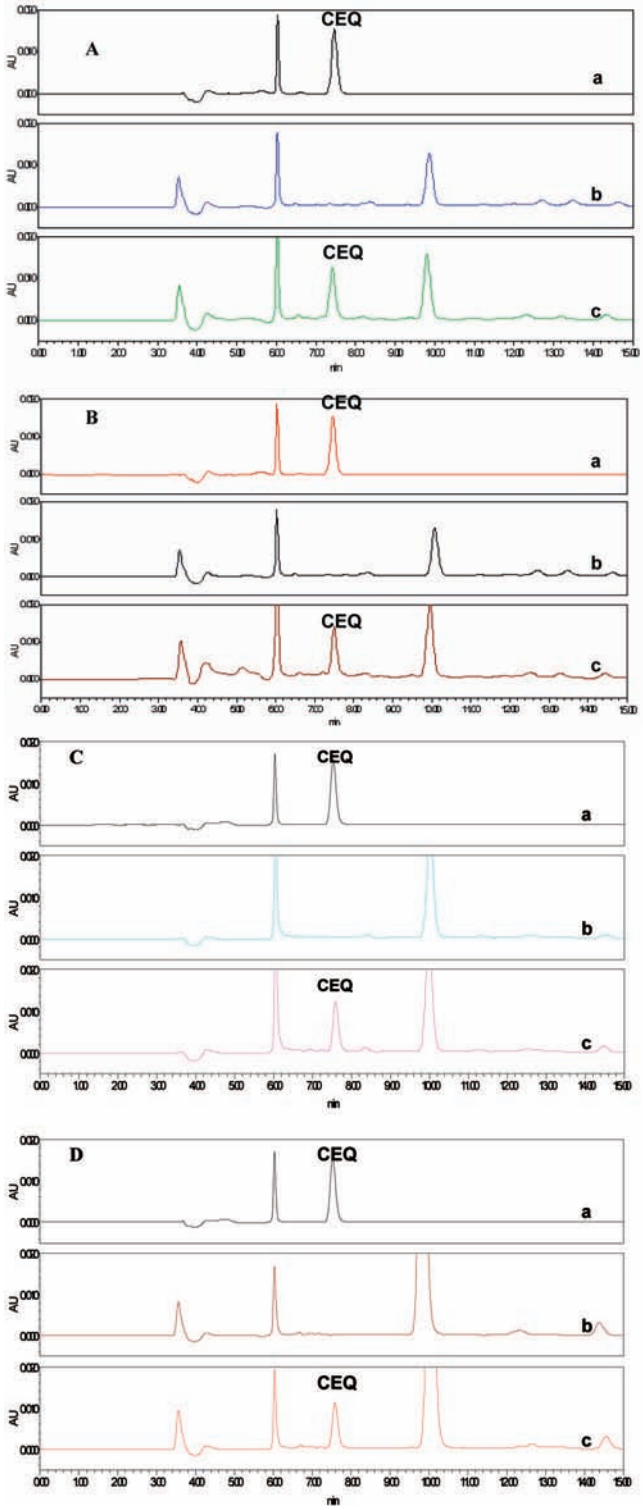


**Figure 2.** Chromatograms of (**A**) liver, (**B**) kidney, (**C**) muscle, and (**D**) fat: (a) standard of 250 ng $g^{-1}$; (b) blank of sample; (c) spiked sample at 50 ng $g^{-1}$.

the ACN evaporation, replacing 4 mL of saturated sodium chloride solution; water was used to dissolve the residue instead of the pH 8.5 phosphate buffer; moreover, hexane was indispensable to defatting during the extraction. It was found that ACN was a key for the extraction and cleanup procedure. Different ratios of ACN/$H_2O$ (v/v) were tested to obtain the optimal extraction solution. For example, the CEQ extraction efficiency of different ratios of ACN/$H_2O$ (v/v) at the fortification level of 500 ng $g^{-1}$ for liver is shown in **Figure 3**. The conclusion of CEQ extraction efficiency obtained from muscle, fat, and kidney complied with liver.

**Table 1.** Mean Recoveries and Precisions of CEQ in Fortified Tissue Samples ($n = 5$)

| | | interday | | intraday | |
|---|---|---|---|---|---|
| sample | fortification (ng $g^{-1}$) | mean recovery (%) | CV[a] (%) | mean recovery (%) | CV (%) |
| muscle | 20 | 84.9 | 7.7 | 80.9 | 9.2 |
| | 50 | 81.1 | 4.3 | 79.2 | 5.8 |
| | 100 | 80.9 | 9.3 | 81.4 | 6.9 |
| | 500 | 82.2 | 9.3 | 79.7 | 8.2 |
| fat | 20 | 86.0 | 9.8 | 83.9 | 8.1 |
| | 50 | 82.8 | 8.9 | 81.0 | 7.0 |
| | 100 | 83.7 | 7.7 | 82.2 | 7.5 |
| | 500 | 83.9 | 8.9 | 81.8 | 6.9 |
| liver | 20 | 83.0 | 7.1 | 82.4 | 7.4 |
| | 50 | 82.8 | 6.2 | 81.0 | 6.5 |
| | 100 | 80.5 | 7.5 | 80.4 | 5.6 |
| | 500 | 81.7 | 9.0 | 80.3 | 8.3 |
| kidney | 20 | 81.5 | 6.1 | 81.2 | 5.1 |
| | 50 | 84.6 | 7.5 | 81.2 | 7.0 |
| | 100 | 84.5 | 7.4 | 80.4 | 6.9 |
| | 500 | 81.7 | 10.3 | 80.1 | 7.9 |

[a] CV, coefficient of variation.

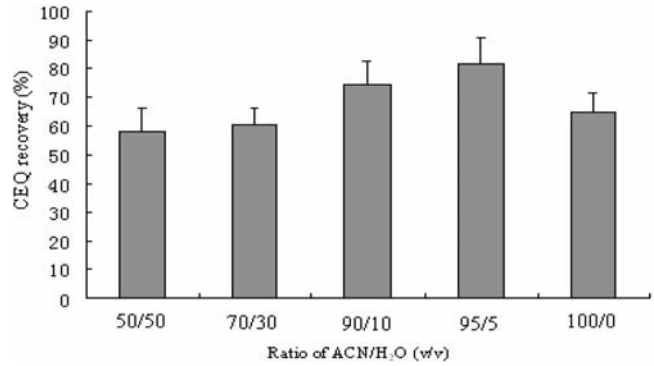


**Figure 3.** CEQ extraction efficiency of different ratios of ACN/$H_2O$ (v/v) at the fortification level of 500 ng $g^{-1}$ for liver ($n = 3$).

**Residue Depletion Study.** In this depletion study, the incurred swine tissue samples were collected from 35 pigs, which were divided into seven groups randomly. The concentrations of CEQ measured in muscle, fat, liver, and kidney tissue from swine administered intramuscularly five times at a dose of 2 mg $kg^{-1}$ of body weight on five consecutive days and slaughtered at 12 h and 1, 2, 3, 5, 7, and 9 days post-treatment are summarized in **Table 2**. The concentration of CEQ in all collected muscle and fat incurred samples was not detected using the present analytic method. At 12 h postadministration, the peak CEQ concentrations in liver and kidney (liver, 218.9–392.3 ng $g^{-1}$; kidney, 602.9–952.1 ng $g^{-1}$) were measured. The highest CEQ residue concentration was detected in kidney, which indicated that the kidney should be considered to be the target organ for CEQ in swine. At 3 days post-treatment, the concentrations of CEQ in kidney tissue ranged from 20.2 to 28.4 ng $g^{-1}$, which were detected in liver tissue of three animals (22.5–25.9 ng $g^{-1}$). Moreover, as shown in **Table 2** and **Figure 4**, at 12 h post-treatment there were significant differences between the CEQ concentrations obtained from liver and kidney ($P < 0.01$); there were no significant differences of the CEQ concentrations between liver and kidney at 1 and 2 days post-

**Table 2.** CEQ Residue Concentrations (Nanograms per Gram) in Swine Tissues after Five Intramuscular Injections at a Dose of 2 mg $kg^{-1}$ of Body Weight with 24 h Intervals

| withdrawal time (days) | animal | muscle | fat | kidney | liver |
|---|---|---|---|---|---|
| 0.5 | 1 | ND | ND | 952.1 | 337.5 |
| | 2 | ND | ND | 602.9 | 218.9 |
| | 3 | ND | ND | 825.0 | 303.2 |
| | 4 | ND | ND | 785.8 | 241.2 |
| | 5 | ND | ND | 855.9 | 392.3 |
| 1 | 6 | ND | ND | 115.1 | 114.9 |
| | 7 | ND | ND | 107.6 | 78.05 |
| | 8 | ND | ND | 123.9 | 50.71 |
| | 9 | ND | ND | 114.5 | 96.77 |
| | 10 | ND | ND | 151.2 | 100.1 |
| 2 | 11 | ND | ND | 40.80 | 29.72 |
| | 12 | ND | ND | 70.73 | 36.99 |
| | 13 | ND | ND | 42.30 | 35.44 |
| | 14 | ND | ND | 56.83 | 36.47 |
| | 15 | ND | ND | 46.42 | 29.12 |
| 3 | 16 | [b] | | 28.38 | 23.12 |
| | 17 | | | 20.23 | 25.87 |
| | 18 | | | 26.92 | <LOQ |
| | 19 | | | 28.26 | 22.55 |
| | 20 | | | 26.41 | <LOQ |
| 5 | 21 | | | ND | ND |
| | 22 | | | ND | ND |
| | 23 | | | ND | ND |
| | 24 | | | ND | ND |
| | 25 | | | ND | ND |
| 7 | 26 | | | | |
| | 27 | | | | |
| | 28 | | | | |
| | 29 | | | | |
| | 30 | | | | |

[a] ND, not detected. [b] Blank entries were not measured.

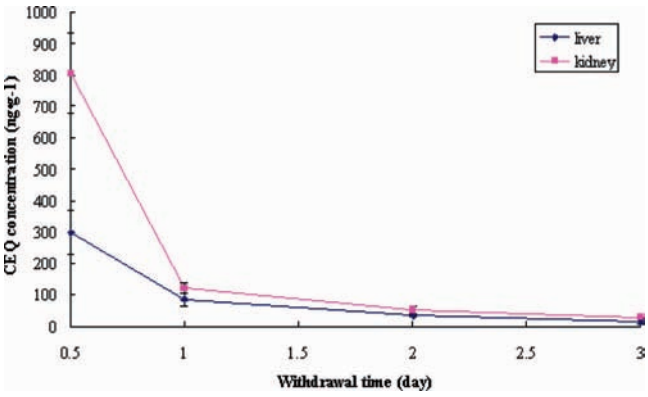


**Figure 4.** Residue depletion curves of CEQ from swine liver and kidney after five intramuscular injections at a dose of 2 mg $kg^{-1}$ of body weight with 24 h intervals.

treatment ($P > 0.05$). The results of Student's $t$ test further indicated that kidney was the target tissue for CEQ in swine and that CEQ residue in kidney was eliminated more rapidly than that in liver. The depletion curve (**Figure 4**) was prepared with the average residue concentrations in swine tissues and the withdrawal time. Comparison of the two curves clearly showed that the rate of CEQ residue elimination from kidney was much more rapid than that of liver between 12 h and 1 day postadministration, but the elimination rates of CEQ from liver and kidney were similar from 1 to 3 days postadministration.

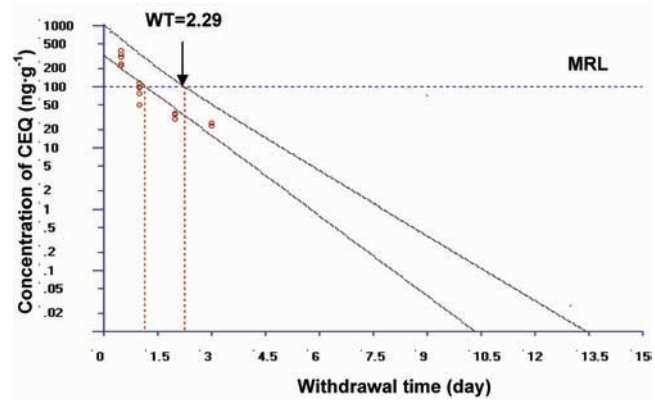


**Figure 5.** Plot of withdrawal time calculation for swine liver at the time when the one-sided 95% upper tolerance limit was below the EU MRL of 100 ng $g^{-1}$.

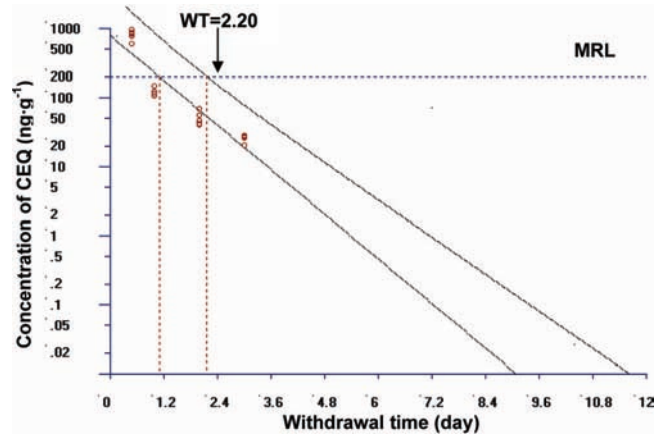


**Figure 6.** Plot of withdrawal time calculation for swine kidney at the time when the one-sided 95% upper tolerance limit was below the EU MRL of 200 ng $g^{-1}$.

These results further revealed that CEQ was absorbed and excreted rapidly in the animal's body.

**The European Agency for Evaluation of Medicinal Products** reported a non-radiolabeled residue depletion study on pigs (*5*, *6*). Comparison of the two studies showed no significant differences in the tissue distribution of CEQ in swine after intramuscular injection. At 1 day postadministration, the highest CEQ concentration (below the EU MRL) was measured in kidney, and no CEQ was detected in muscle and fat. The results were the same as in the present study. At 3 days post-treatment, no CEQ residue was detected in kidney, liver, fat, and muscle samples except for one 72 h sample of fat tissue containing 27 $\mu$g $kg^{-1}$. However, in the current study, the CEQ concentrations of kidney were all above the LOQ (20.2–28.4 ng $g^{-1}$), which were detected in three liver samples (22.5–25.9 ng $g^{-1}$) at 3 days postadministration. The reason may be due to the experimental animals, CEQ formulation, and analytical method.

As shown in **Table 2** and **Figures 5** and **6**, the concentrations of CEQ residues in muscle, fat, liver, and kidney from swine intramuscularly administered five times at a dose of 2 mg $kg^{-1}$ of body weight with 24 h intervals were below the accepted EU MRL at 2 days of withdrawal time. Because of the limited number of test animals, high animal individual variability, and potential hazards to human health, the withdrawal periods were established on the basis of EU MRL using the statistical method (95% tolerance limit and 95% confidence) stated in the guidance (*27*), which were 2.29 days for liver and 2.20 days for kidney.

Because the time points do not make up a full day, the withdrawal periods have to be rounded up to the next day. Therefore, the longest withdrawal time of 3 days can be selected as the conclusive withdrawal time to guarantee consumer safety.

**Conclusions.** In this paper, an improved HPLC-UV method was developed for the determination of CEQ in swine muscle, fat, liver, and kidney. All results of the depletion study clearly indicate the elimination and distribution characteristics of CEQ in swine tissues, which can provide a scientific basis for administering CEQ in clinical practice and recommending a rational withdrawal period and safety assurance for food consumption. A withdrawal period for the concentrations of CEQ residues below the EU MRL in all examined swine tissues was 3 days according to the statistical method suggested by EMEA.

## ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; UV, ultraviolet detector; CEQ, cefquinome; LOD, limit of detection; CV, coefficient of variation; MRL, maximum residue limit; EMEA, European Medical Evaluation Agency; EU, European Union; ACN, acetonitrile; SPE, solid-phase extraction; *R*, correlation coefficient; LOQ, limit of quantification; S/N, signal-to-noise ratio; DTE, dithioerythritol; $Et_4NCl$, tetraethylammonium chloride.

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