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ARTICLE

Residue Depletion of Gentamicin in Swine Tissues after Intramuscular Administration

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A sensitive and robust high-performance liquid chromatographic method with fluorescence detection (HPLC-FLD) was developed for the determination of gentamicin (GEN) residues in swine tissues. The limit of quantification (LOQ) of the method was 50 ng/g for muscle and 100 ng/g for liver and kidney. Mean recoveries at all fortification levels ranged from 82.34 to 93.20% with coefficient of variation (CV) below 5.39%. Residue depletion study of GEN in swine was performed after intramuscular injections twice daily at a dose of 4 mg/kg of bw with 12 h intervals for 5 consecutive days. The concentrations of GEN were determined in injection site, muscle, liver, and kidney by the HPLC-FLD method. The highest GEN concentration was measured in kidney, indicating that kidney was the primary target tissue for GEN residue. GEN concentrations in all examined tissues were below the maximum residue limit (MRL) recommended by the European Union (EU) at 50 days post-treatment.

KEYWORDS: Gentamicin; swine tissues; residue depletion; intramuscular administration; HPLC-FLD

INTRODUCTION

Gentamicin (GEN) is an aminoglycoside antibiotic produced by fermentation of *Micromonospora purpurea*. It consists of four major components, C_1, C_{1a}, C_2, and C_{2a} (**Figure 1**), and has a broad spectrum of antibacterial activity that inhibits both Gram-positive and Gram-negative bacteria. Generally, it is used for the treatment of serious infections in pigs and cattle (*1*) and also used to promote animal growth for prophylactic reasons (*2*).

Because of its polar nature and high aqueous solubility, GEN is poorly absorbed after oral administration. However, the absorption in most species is good, with peak concentrations in blood occurring within 30−90 min after intramuscular injection. It is recognized that GEN was not catabolized in the animal body and was eliminated unchanged in the urine by glomerular filtration (*3*−*5*). Owing to its pharmacokinetic properties and high tissue affinity, the use of GEN often results in high and persistent residues and prolonged withdrawal times in animal tissues. Unfortunately, it has potential ototoxicity and nephrotoxicity (*6*), and, in order to avoid exposure of humans to GEN in edible tissues of animal origin, the European Agency for the Evaluation of Medical Products (*7*) and the Ministry of Agricultural of China (*8*) have established maximum residue limits (MRLs) for edible tissues and milk (**Table 1**). Therefore, numerous analytical methods have been developed to monitor GEN residues in biological matrices, such as microbiological assay (*9*), ELISA (*10*−*13*), gas chromatographic (GC) (*14*), and high-performance liquid chromatography (HPLC) (*15*−*20*). The analysis of GEN by HPLC is complicated. First, its polar nature aggravates its extraction and chromatographic separation. Second, it consists of four structurally similar components. Third, it has no ultraviolet (UV) or visible absorption. To solve the detection problems, a sensitive and robust high-performance liquid chromatographic method with fluorescence detection (HPLC-FLD) was developed in this study.

GEN injection has been approved in many countries for the treatment of a variety of bacterial infections in pig and cattle, the recommended dose regimen is about 4 mg/kg of body weight (bw) for cattle and pig once or twice daily for 3−5 days either by parenteral administration or by oral administration. However, there are few studies for the residue depletion profile of GEN in swine. The aim of the current study is to develop a sensitive and reliable HPLC-FLD method for the determination of GEN in swine tissues and apply this method to study the residue depletion profile of GEN in swine tissues after intramuscular administration.

MATERIALS AND METHODS

Reagents and Materials. GEN standard (54.6% purity) and GEN sulfate injection were obtained from the National Institute for the Control of Pharmaceutical and Biological products (Beijing, China). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ). 9-Fluorenylmethyl chloroformate (FMOC-Cl) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Trichloroacetic acid (TCA), glycine, boric acid, and sodium hydroxide (NaOH) were supplied by Beijing Regent Corp. (Beijing, PRC). The water used was prepared with a Milli-Q system (Millipore, Bedford, MA). SPE cartridges (Oasis MCX, 3 cm^3/60 mg, 6 cm^3/150 mg) were from Waters Co., and borate buffer (0.4 M) was prepared by adding 24.73 g of boric acid into 1000 mL of water, with the pH being adjusted to 8.8 using 7.5 M NaOH.

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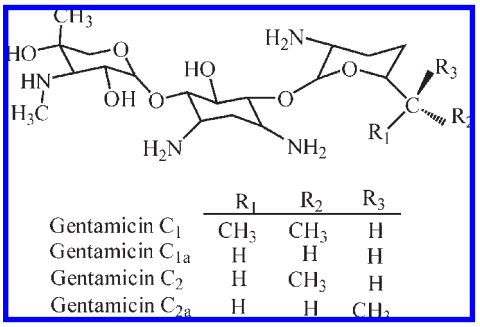

	R_1	R_2	R_3
Gentamicin C_1	CH_3	CH_3	H
Gentamicin C_{1a}	H	H	H
Gentamicin C_2	H	CH_3	H
Gentamicin C_{2a}	H	H	CH_2

Figure 1. Structure of the gentamicin components.

Table 1. Maximum Residue Limits of Gentamicin in Edible Tissues and Milk

		MRL[a] (μg/kg)	
animal species	target tissue	EU	China
pig/cattle	muscle	50	100
	fat	50	100
	liver	200	2000
	kidney	750	5000
cattle	milk	100	200
chicken	edible tissues	NE	100

[a] NE, not established.

Standards. A stock solution of 1 mg/mL was prepared by dissolving 18.32 mg of GEN standard in 10 mL of water. The working standard solutions of 0.025, 0.05, 0.1, 0.2, 0.5, 1, and 5 μg/mL were prepared with borate buffer.

Sample Extraction. Swine tissues (muscle, liver, kidney) were minced and homogenized in a homogenizer for 2 min. Two grams of homogenate was accurately weighed into a 50 mL polypropylene centrifuge tube. Three milliliters of 0.01 M phosphate buffer saline (PBS, pH 7.4) was added and mixed for 30 s using a vortex mixer, then 7 mL of TCA (10%, w/v) was added to the mixture and mixed for 1 min before centrifugation at 3800 rpm (2400*g*) for 10 min. The supernatant was transferred to another 50 mL polypropylene centrifuge tube. The tissue pellet was extracted again following the above-mentioned procedure. The supernatants were combined and subjected to SPE cleanup.

Purification. In this paper, the MCX (3 cm^3/60 mg) cartridge was used for muscle purification and the 6 cm^3/150 mg MCX cartridge was used for liver and kidney purification, because muscle has much less matrix interference than liver and kidney. The purification procedures for the two kinds of MCX cartridges were identical. The MCX cartridge was preconditioned with 5 mL of methanol followed by 5 mL of water prior to the addition of the extracted supernatant. After the extract was drained through the cartridge, the cartridge was washed with 5 mL of water, 5 mL of methanol/water (1:1, v/v), and 5 mL of 0.25% ammonia/methanol solution. The SPE cartridge was dried for at least 1 min. GEN was eluted from the cartridge with 5 mL of 2% ammonia/methanol solution. The collected eluate was evaporated to dryness under a nitrogen stream at 50 °C in a water bath and then reconstituted in 2 mL of borate buffer.

Derivatization. This procedure mainly followed the method described by Stead et al. (*19*) with some modification. Briefly, 500 μL of reconstituted borate buffer was added to a 2 mL polypropylene centrifuge tube and derivatized with 500 μL of FMOC-Cl (2 mM in acetonitrile) at ambient temperature for 10 min; the reaction was then stopped by adding 50 μL of glycine (0.1 M in water), and an aliquot (100 μL) of the derivative was subjected to HPLC analysis.

Fortification. To test the stability of the HPLC system and the feasibility of the detection method for GEN in swine tissues, a fortifying test was performed on muscle, liver, and kidney. For recovery study, GEN standard solution (1000 ng/mL, prepared in 0.01 M PBS) was added into homogenized tissue samples to produce spiked concentrations of 0.05, 0.1, and 0.2 mg/kg in muscle, 0.1, 0.2, and 0.5 mg/kg in liver, and 0.1, 0.5, and 0.75 mg/kg in kidney. The precision (interday and intraday) of the method was assessed using five replicates of control and fortified samples at three fortification levels on three different days.

Animal Treatment. The trial was conducted in 45 healthy Chester white−Yorkshire cross-bred swine with an average body weight of 35 kg. During the acclimation for 3 weeks and the subsequent treatment periods, they were fed drug-free assorted feed ad libitum with free access to water and were kept in individual metabolic cages in a closed room. Their health condition was checked twice daily by a professional veterinarian. All of the pigs were randomly divided into nine groups of five pigs each. Five animals were kept as control. Forty animals were weighed and injected twice daily at 12 h intervals on the right side of the neck at a dose of 4 mg/kg of bw for 5 consecutive days. Five animals of the treated groups were killed at 1, 4, 7, 14, 21, 28, 35, and 42 days of withdrawal after the last injection. The untreated animals were sacrificed on day 42 to obtain blank tissues. Samples of muscle at the injection site and of muscle (hind thigh), liver, and kidney collected from each animal were thoroughly minced, homogenized, and stored at −20 °C until they were assayed.

HPLC Analysis. The analyses of standards, fortified samples, and incurred samples were preformed at room temperature. The HPLC system included a Waters 2695 separation module, a reverse phase C18 column (Kromasil 100 C18 column, 4.6 mm i.d. × 250 mm, 5 μm, MZ-Analysentechnik GmbH), and a Waters 2475 fluorescence detector. The excitation wavelength was 260 nm, and the emission wavelength was 315 nm. The mobile phase was acetonitrile/water (95:5, v/v) and was pumped at a flow rate of 1.0 mL/min. The injection volume was 100 μL, and the column temperature was set at 30 °C. The run time was 20 min for each sample.

Method Calibration. The calibration curves were prepared with the summed peak areas of C_1, C_{1a}, C_2, and C_{2a} and the working standard solution concentration. The standard curve for GEN was constructed with standard working solution concentrations of 0.025, 0.05, 0.1, 0.2, 0.5, 1, and 5 μg/mL.

Data Analysis. Student's *t* test was used to estimate the differences of the GEN concentration observed in the different swine tissues. The withdrawal time was estimated 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 MRLs established by the European Medical Evaluation Agency (EMEA) (*7*).

RESULTS AND DISCUSSION

Method Validation. The method was validated for use with respect to three critical aspects: linearity, accuracy, and precision. The standard calibration curve for GEN showed a high correlation coefficient over the concentration range of 0.025−5 μg/mL (r = 0.9999), and its equation was $Y = 207531000X + 3703210$. The limit of detection (LOD) and the limit of quantification (LOQ), defined on the basis of signal-to-noise ratios (S/N) of 3:1 and 10:1 (*21*), respectively, were 15 and 50 ng/g for muscle and 30 and 100 ng/g for liver and kidney, respectively. The LOD and LOQ in the current HPLC-FLD study for animal tissues were lower than those reported in the previous studies (*20*), indicating high sensitivity of the method developed in this study. The accuracy and precision were assessed by fortifying blank tissue samples with GEN standard at three fortification levels for muscle, liver, and kidney and analyzing five replicates per level each day on three separate days. Typical chromatograms of GEN standard, blank, and fortified samples are presented in **Figure 2**. No interference in the separation suggests a high specificity of the chromatographic method and a good selectivity of the extraction procedure. Recoveries of the GEN at all fortification levels in swine tissues are summarized in **Table 2**. Briefly, the intra-assay average recovery of GEN from all tissues was from 82.34 to 91.09% with CVs between 0.20 and 2.86% and the interassay average recovery was between 82.63 and 93.20% with CVs from 1.76 to 5.39%. The results obtained in this validation procedure indicated that a sensitive, simple, rapid, and reliable analytical method for the detection of GEN residues in swine tissues is available.

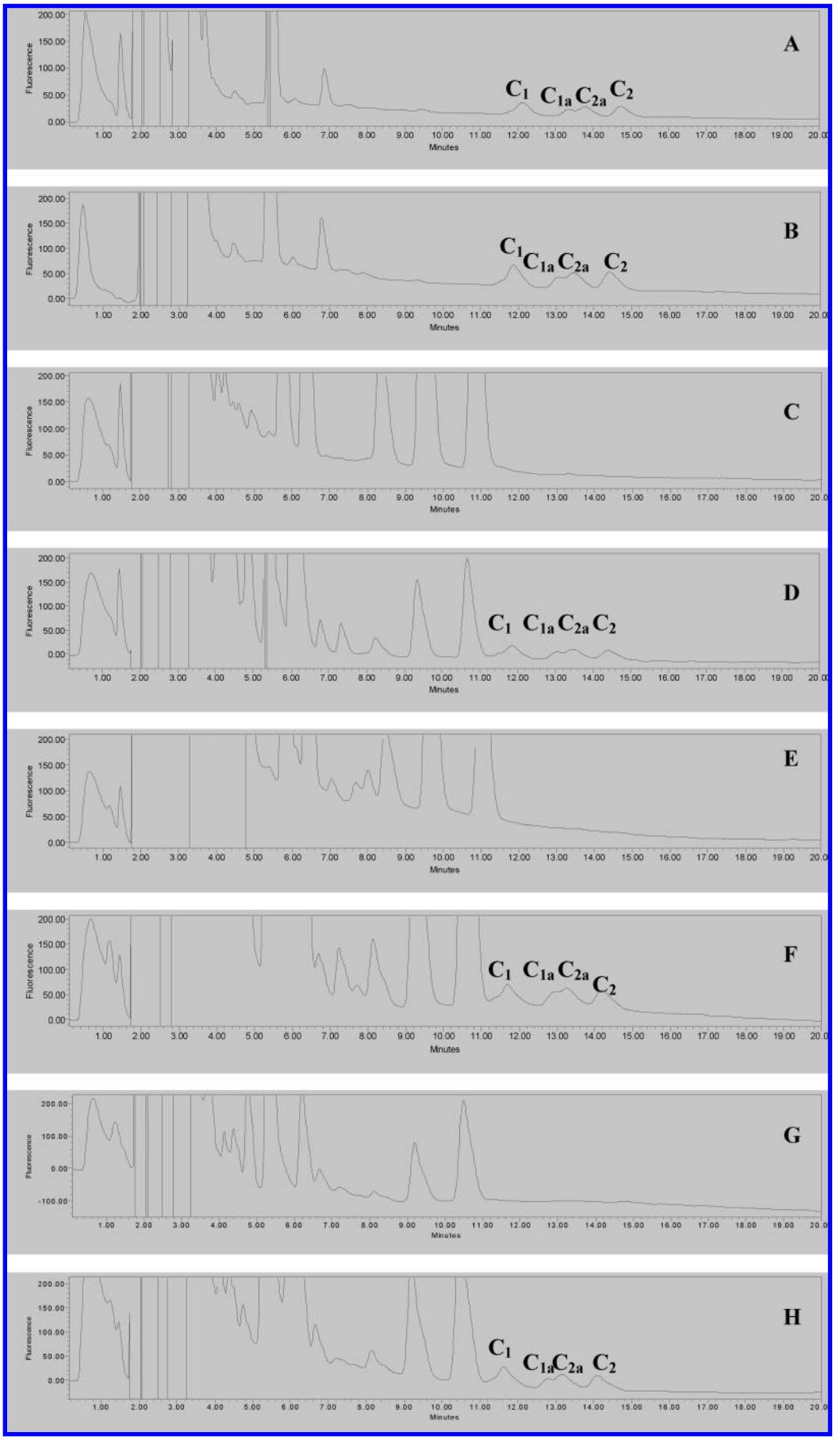

Figure 2. Representative chromatograms of swine samples fortified with GEN: (**A**) 50 ng/mL GEN standard; (**B**) 100 ng/mL GEN standard; (**C**) control muscle; (**D**) control muscle fortified at 50 ng/g; (**E**) control liver; (**F**) control liver fortified at 100 ng/g; (**G**) control kidney; (**H**) control kidney fortified at 100 ng/g.

Table 2. Recoveries of GEN Fortified in Swine Tissues ($n = 5$)

		intraday		interday	
sample	spike level (μg/kg)	recovery (%)	CV (%)	recovery (%)	CV (%)
muscle	50	84.10	2.60	84.00	1.96
	100	88.78	1.52	89.69	1.76
	200	91.09	2.07	90.67	2.97
liver	100	82.66	0.38	82.63	3.08
	200	86.04	1.61	85.00	4.87
	500	88.99	0.20	88.29	2.93
kidney	100	85.30	2.86	85.09	3.21
	500	82.34	1.00	84.01	5.39
	750	90.16	2.67	93.20	4.79

Extraction. Preliminary experiments showed that high recovery could be obtained by using PBS and TCA solution as the extraction solvent. The tissue samples were adequately dispersed by adding PBS solution, in which the proteins in the tissue samples were precipitated with TCA solution. The whole procedure is less laborious and time-consuming compared to previous studies (*18*, *20*, *22*).

SPE Cleanup. In most previous SPE procedures of GEN in tissues, a carboxypropyl (CBA) column (*18*, *19*) or an octadecyl (C_{18}) column (*15*) was applied. However, low recoveries were obtained in our preliminary experiments when using CBA or C_{18} cartridges. MCX is a strong cation-exchanged cartridge; it is suitable for basic analytes in highly complex matrices and for basic analytes that are too hydrophilic for Oasis HLB extraction. The SO_3^- groups of the MCX stationary phase are especially suitable for combination with hydrophilic amines. Therefore, the MCX cartridge was used in this paper and the recovery of the method was improved successfully. Washing with 5 mL of water and 5 mL of methanol/water (1:1, v/v) removed most of the interfering matrix components, but it was necessary to introduce a third wash of 5 mL of 0.25% ammonia/methanol solution to completely remove all residual interferences, especially some free amino acids including arginine, threonine, or tryptophan and so on that remain in tissue samples. All of these kinds of amino acids could react with the FMOC-Cl and could be possibly eluted. Therefore, it is very important to make sure that free amino acids have no interferences with the peak of GEN. Increasing the concentration of the ammonia solution can elute the GEN from the cartridge. The results showed that there were no interferences with the peaks of GEN and high recovery for GEN.

Derivatization. FMOC-Cl was used as the derivative reagent in this paper. GEN (1 mg/mL) was reacted with FMOC-Cl, as described above, and the pH, reaction time, and concentrations of acetonitrile, borate buffer, and FMOC-Cl were independently varied in turn; all of these parameters affected the peak heights of GEN. The results indicated that the most optimal reaction of GEN with FMOC-Cl was at pH 8.0−8.8, and the reaction was complete after 10 min. When the reaction was in 50% (v/v) acetonitrile, at a concentration of 0.4 M borate buffer, and the concentration of FMOC-Cl between 1 and 4 mM, the maximal yields of the derivatives were detected. The obtained results were identical to those found by Stead (*19*).

Residue Depletion Study. The incurred swine tissue samples were collected from 40 pigs, which were administered intramuscularly twice daily at a dose of 4 mg/kg of bw with 12 h intervals for 5 consecutive days and then 5 pigs randomly selected and slaughtered at 1, 4, 7, 14, 21, 28, 35, and 42 days after the last dose. The concentrations of GEN measured in the injection site, muscle, liver, and kidney tissue are summarized in **Table 3**. At 1 day of withdrawal, the concentrations of GEN in all tissues peaked (injection site, 7.186−10.296 μg/g; muscle, 0.325−0.498 μg/g; liver, 6.011−7.762 μg/g; and kidney, 241.856−487.107 μg/g, respectively). The results showed that kidney should be considered to be the target organ for GEN in swine because the residue in kidney is highest. At 28 days of withdrawal, the concentrations of GEN in kidney tissue ranged from 1.137 to 1.695 μg/g, in liver and injection site; the concentrations of GEN detected ranged from 0.682 to 0.962 μg/g and from 0.028 to 0.042 μg/g, respectively, whereas it was not detected in muscle tissues. At 42 days of withdrawal, the concentrations of GEN in liver tissue ranged from 0.189 to 0.290 μg/g and in kidney tissue from 0.330 to 0.613 μg/g. The depletion curve (**Figure 3**) for liver and kidney tissues was prepared with the average residue concentrations in swine tissues and the withdrawal time.

Table 3. GEN Residue Concentrations in Swine Tissues after Twice Daily Intramuscular Injections at a Dose of 4 mg/kg of Body Weight with 12 h Intervals for 5 Consecutive Days

		GEN residue concn[a] (μg/g)			
withdrawal time (days)	animal	injection site	muscle	liver	kidney
1	1	7.186	0.325	6.272	283.931
	2	10.296	0.498	7.726	487.107
	3	9.564	0.439	7.762	416.285
	4	8.252	0.427	6.011	241.856
	5	9.312	0.377	6.614	361.793
4	6	3.119	0.113	5.365	134.783
	7	3.338	0.112	5.573	151.543
	8	4.245	0.097	5.761	161.598
	9	2.866	0.096	5.250	129.340
	10	2.069	0.074	5.132	114.952
7	11	1.106	0.068	4.488	24.508
	12	0.986	0.064	4.146	16.685
	13	1.038	0.086	4.735	29.322
	14	1.091	0.055	4.322	20.850
	15	0.891	0.050	4.099	14.134
14	16	0.279	0.059	3.542	9.808
	17	0.139	0.055	2.940	9.140
	18	0.136	0.046	2.925	8.789
	19	0.120	0.042	2.736	8.344
	20	0.159	0.050	3.012	9.465
21	21	0.067	0.035	1.279	4.323
	22	0.087	0.039	1.925	5.055
	23	0.050	0.023	1.040	3.673
	24	0.073	0.037	1.304	4.931
	25	0.055	0.033	1.218	4.196
28	26	0.032	nd	0.734	1.257
	27	0.030	nd	0.704	1.253
	28	0.028	nd	0.682	1.137
	29	0.039	nd	0.879	1.407
	30	0.042	nd	0.962	1.695
35	31	nd	nd	0.487	0.676
	32	nd	nd	0.581	1.075
	33	nd	nd	0.387	0.733
	34	0.020	nd	0.484	0.976
	35	nd	nd	0.339	0.626
42	36	nd	nd	0.189	0.330
	37	nd	nd	0.290	0.613
	38	nd	nd	0.276	0.526
	39	nd	nd	0.198	0.405
	40	nd	nd	0.229	0.344

[a] nd, not detected.

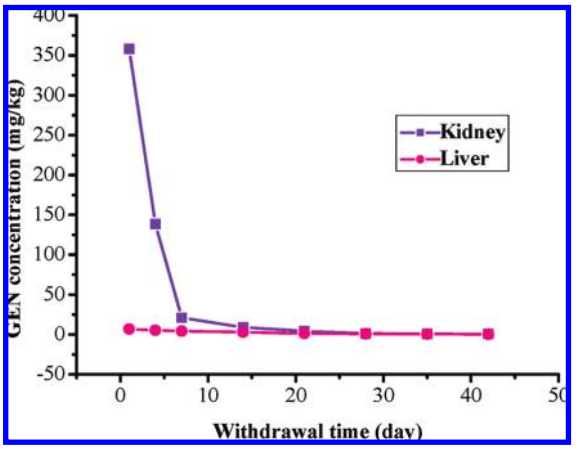

Figure 3. Residue depletion curves of GEN from swine liver and kidney after twice daily intramuscular injections at a dose of 4 mg/kg of bw with 12 h intervals for 5 consecutive days.

Comparison of the two curves clearly showed that the rate of GEN residue elimination from kidney was much more rapid than that of liver between 1, 4, 7, 14, and 21 days postadministration, but the elimination rates of GEN from liver and kidney were similar from 28 to 42 days postadministration.

As shown in **Table 3** and **Figures 4**−**6**, the concentrations of GEN residues in muscle and tissues were below the accepted EU MRL at 42 days of withdrawal time except for liver. 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 (*23*), which were 27.55 days for muscle, 49.51 days for liver, and 45.79 days for kidney. Moreover, when withdrawal periods are established for parenterally administered drugs, it is important to take into account the residue of the injection site. In this paper, the estimated withdrawal time for GEN residue in muscle samples at the injection site was 31.18 days (**Figure 7**), which is much longer than the period for muscle.

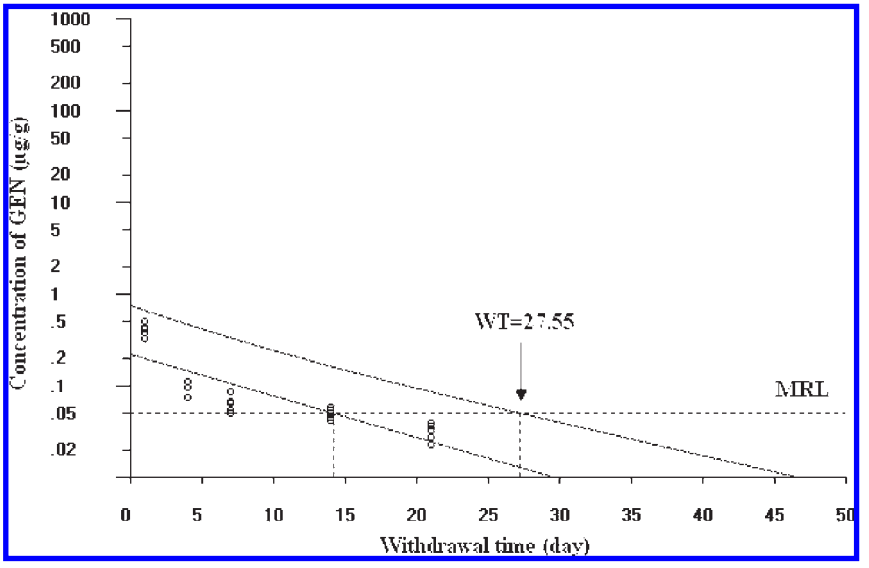

Figure 4. Plot of withdrawal time (WT) calculation for swine muscle at a time when the one-sided 95% upper tolerance limit was below the EU MRL of 50 ng/g.

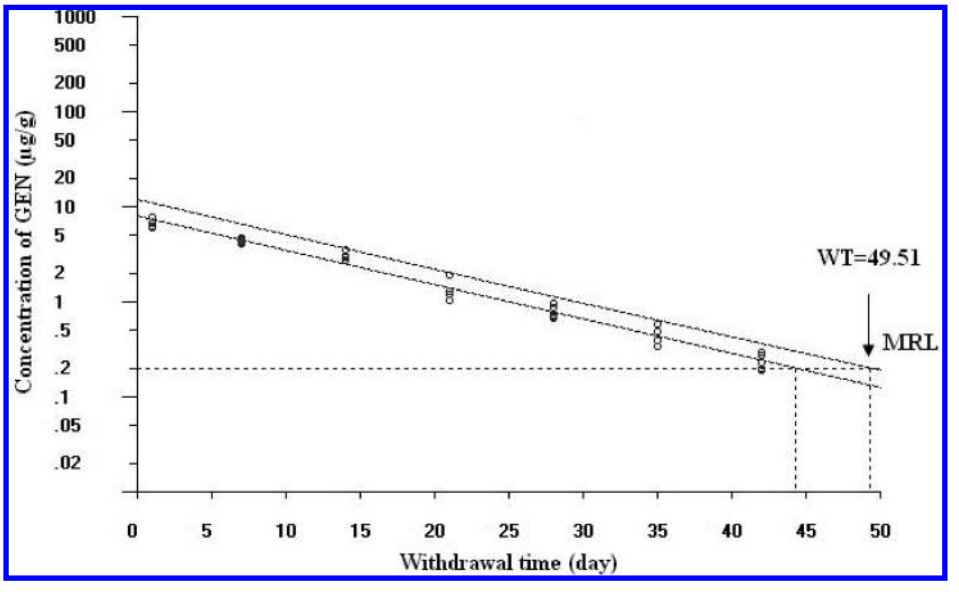

Figure 5. Plot of withdrawal time (WT) calculation for swine liver at a time when the one-sided 95% upper tolerance limit was below the EU MRL of 200 ng/g.

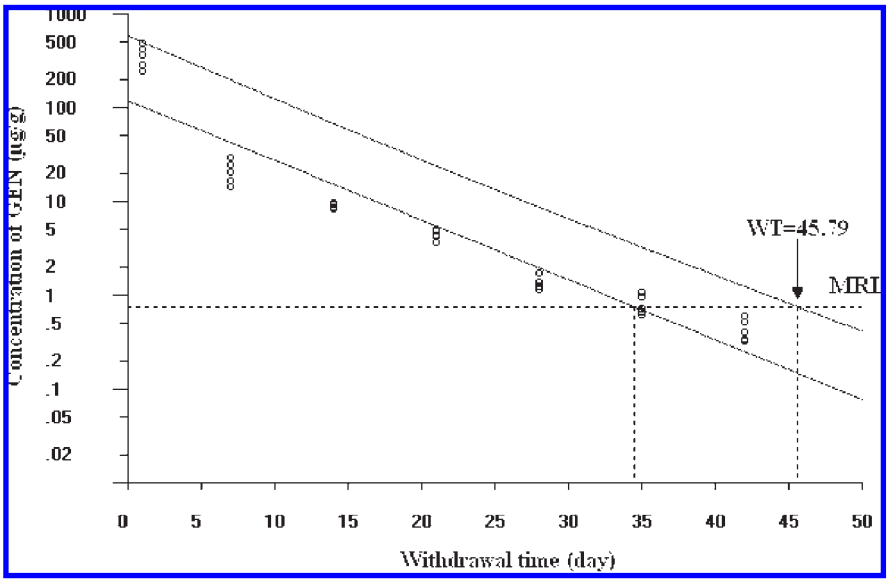

Figure 6. Plot of withdrawal time (WT) calculation for swine kidney at a time when the one-sided 95% upper tolerance limit was below the EU MRL of 750 ng/g.

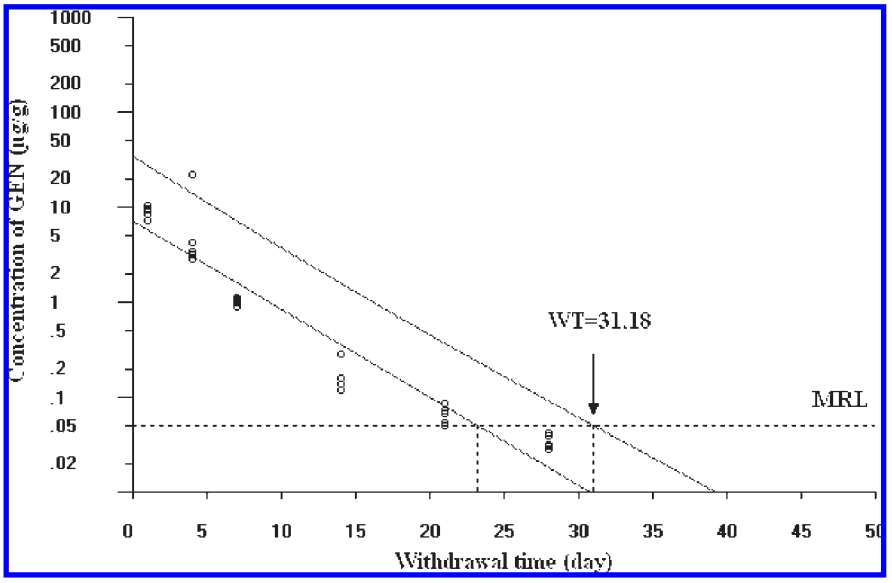

Figure 7. Plot of withdrawal time (WT) calculation for swine muscle at the injection site at a time when the one-sided 95% upper tolerance limit was below the EU MRL of 50 ng/g.

In this study, an improved HPLC-FLD method was developed for the determination of GEN in swine muscle, liver, and kidney. All results of the depletion study clearly indicate the elimination and distribution characteristics of GEN in swine tissues, which can provide a scientific basis for administering GEN in clinical practice and recommending a rational withdrawal period. A withdrawal period for the concentrations of GEN residues below the EU MRL in all examined swine tissues was 50 days according to the statistical method suggested by EMEA.

ABBREVIATIONS USED

HPLC, high-performance chromatography; FLD, fluorescence detection; GEN, gentamicin; LOD, limit of detection; LOQ, limit of quantification; CV, coefficient of variation; MRL, maximum residue limit; EMEA, European Medical Evaluation Agency; EU, European Union; S/N, signal-to-noise ratio; FMOC-Cl, 9-fluorenylmethyl chloroformate; SPE, solid-phase extraction.

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