RESEARCH REPORT
INTERNATIONAL TRADE

Title: **Depletion of penicillin G residues in sows after intramuscular injection**

final

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Industry Summary

A total of 126 heavy sows were treated with a 5x dose of penicillin G procaine via intramuscular (IM) administration for 3 consecutive days using 3 injection patterns. Sets of 18 animals (6 per injection pattern) were slaughtered at 5, 10, 15, 20, 25, 32 and 39 days and penicillin residues were measured in skeletal muscle and kidney samples. Residues in skeletal muscle depleted very rapidly and were not quantifiable by 15 days of withdrawal. The FARAD recommended 15-day withdrawal period is sufficient to ensure that skeletal muscle residues deplete to safe levels. Depletion of residues in kidney was much slower than in skeletal muscle. An estimated withdrawal period of 47 days would be required to ensure that residues in 99% of animals would be depleted below 50 ppb, with 95% confidence; a 51 day withdrawal period would be required for penicillin residues to deplete to the FSIS action level of 25 ppb in a population of heavy sows. Because this withdrawal period is prohibitively long for most commercial sow operations, it is recommended that sows treated IM with extra label doses of penicillin G procaine be slaughtered with a 15-d withdrawal period, with edible offal (kidney, liver) being discarded at the slaughter plant. In a second portion of the study, a commercially available rapid screening assay (Charm-KIS) was used to accurately predict the presence of violative penicillin-G kidney residues in kidneys. When used with urine, there was a very high correlation between positive urine results and penicillin-G positive kidney samples. Thus, the Charm-KIS assay could be employed by commercial sow producers to screen urine of treated animals for the presence of penicillin G residues in kidneys.

Scientific Abstract

Heavy sows (n=126) were treated with penicillin G procaine at a 5x dose (33,000 IU/kg) for 3 consecutive days by intramuscular (IM) injection using 3 separate patterns (treatments) of drug administration (42 animals each). Treatments differed by pattern and volume of penicillin G procaine administration. Sets of 6 animals per treatment were each slaughtered 5, 10, 15, 20, 25, 32, and 39 days after the last treatment; skeletal muscle, kidney, serum, liver, and urine were collected for penicillin G analysis by the Charm-KIS rapid screening assay and by LC-MS/MS. The Charm-KIS rapid screening assay reliably detected penicillin G residues in kidney

juice and in urine, but false positive results were noted when the screening assay was used for skeletal muscle. The limits of detection for the Charm-KIS test were 20, 30, 20, 30, and 100 ppb for kidney, muscle, urine, serum, and liver, respectively. Urine was an excellent surrogate matrix for the detection of penicillin in kidney as assay results between the two matrices were highly consistent. Serum results did not correlate with kidney results at latter withdrawal periods. Penicillin residues in skeletal muscle averaged 23.5 ± 10.5 ppb at withdrawal day 5 for all treatments, but averaged 3,762 ± 1,932 ppb in kidney. By 15 days of withdrawal, skeletal muscle penicillin G residues were quantifiable in only one treated hog (3.4 ppb) but were easily detected in kidneys of 50% of the treated hogs, with kidney residues in all hogs averaging 119 ± 199 ppb (mean includes 8 non-detects counted at ½ the limit of detection). Using a hypothetical tolerance of 50 ppb and a ln-linear depletion model, the withdrawal period required for penicillin depletion to 50 ppb in skeletal muscle was 11 days, whereas a 47 day withdrawal period was required for kidney residues to deplete to 50 ppb. Using the 25 ppb FSIS action level for penicillin G as a tolerance, the withdrawal periods for muscle and kidney would be 13 and 51 days, respectively. The FARAD recommended withdrawal period of 15 days for hogs treated with extra-label doses of penicillin is adequate for skeletal muscle, but is inadequate for kidney. Slaughter of penicillin treated hogs after a 15 day withdrawal period, with edible offal (kidney and liver) discard would ensure the human food safety of skeletal muscle.

Objectives:

- To determine the appropriate pre-slaughter withdrawal period for penicillin G procaine in sows under practical use conditions.

- To determine whether the pattern (sites and volume) of intramuscular penicillin G procaine administration influences penicillin G procaine depletion from edible tissues of sows.

- To determine the incidence of false-positives returned by the Charm-KIS microbial inhibition test under controlled conditions.

Materials and Methods

Animal Housing and Treatment Assignment. Prior to the initiation of the live phase of the study, an animal protocol was submitted to, and approved by, North Dakota State University (NDSU) Animal Care and Use Committee. One-hundred sixty heavy sows, in two lots of eighty each for Trials 1 and 2, respectively, were purchased from the North Dakota Pig Cooperative (Larimore, ND). Sows were delivered to NDSU animal housing facilities on January 3 and March 13, 2012 for Trials 1 and 2, respectively. Upon delivery, sows were group housed in concrete-floored pens covered with ample quantities of straw as bedding. Animals were group fed a corn-soybean ration (Appendix 1) daily at approximately 0800 hours; pens were cleaned daily and water was available on an ad libitum basis.

At delivery, animals were sorted into groups that were lame, had visible abscesses, or other visible anomalies. These animals were separated from sows with no visible problems and were not considered for inclusion in the study. Within the pool of healthy animals available for study, sows were randomly allocated to pen and treatment (injection pattern) so that each pen contained 21 sows. Within treatment, sows were randomly allocated to a post treatment withdrawal slaughter day.

Treatments. Treatments (illustrated in Figure 1) were defined as:

- Treatment 1: "10-mL; Single Site". A maximum injection volume of 10 mL using multiple injection sites within day to deliver the total required drug volume (5 mL/100 lb BW); across day, injections were administered into a single muscle location. (Figure 1, top panel). The total penicillin G dose for treatment 1 was 33,000 U/kg BW or 15,000

IU/lb BW.

- Treatment 2: "10-mL; Multiple Sites". A maximum injection volume of 10 mL using multiple injection sites within day to deliver the total required drug volume (5 mL/100 lb BW); across day, injection sites were separated by approximately 2 inches. (Figure 1, middle panel). The total penicillin G dose for treatment 2 was 33,000 U/kg BW or 15,000 IU/lb BW.
- Treatment 3: "20-mL; Multiple Sites"; A maximum injection volume of 20 mL using multiple injection sites within day to deliver the total required drug volume (5 mL/100 lb BW); across day, injections were separated by approximately 2 inches. (Figure 1, lower panel). The total penicillin G dose for treatment 3 was 33,000 U/kg BW or 15,000 IU/lb BW.

In addition to treatment animals, two positive control hogs received the label dose of penicillin G procaine by IM (1-mL/100 lb BW) injection for 3 consecutive days; during both trials, a single positive control sow was euthanized after a 7-day withdrawal period, consistent with the product label instructions. The other positive control sows were scheduled to be slaughtered after a 15-day withdrawal period; however in Trial 1, the 15 withdrawal day positive control sow died 12 days after penicillin administration due to a ruptured spleen. For both Trials, untreated control sows were administered saline via intramuscular administration at 1 mL per 100 lb BW for 3 consecutive days and slaughtered after 5 days.

Treatment Administration. Approximately 7 days prior to the initiation of penicillin treatment, animals were tattooed according to their treatment assignment. A tattoo apparatus was machined at the NDSU machine shop which allowed multiple patterns of 2 cm "O" s to be tattooed onto necks of sows according to treatment (Figure 1). Tattooing allowed a consistent adherence to treatment protocols and designations (Figure 2).

The day prior to penicillin G procaine administration, sows were weighed. At dosing, penicillin G Procaine (Norocillin; Norbrook Laboratories, Lenexa, KS) was administered via intramuscular injection through 3.8 cm, 16-gauge needles. Dosing was facilitated by holding each sow in a modified metal swine crate (Figure 3) that could be used to lift the animal if it was being difficult. This crate allowed for access to both sides of the animal's neck. Dosing was uneventful.

Slaughter and Tissue Sampling. Sows were killed after 5, 10, 15, 20, 25, 32, or 39 day withdrawal periods relative to the last dosing day. A deviation from the planned 40-day withdrawal period was deemed prudent because of heavy snow and high winds forecasted for Fargo on the calendar day that coincided with withdrawal day 40 of Trial 1. As a consequence, both Trials 1 and 2 were modified to include a 39 day withdrawal period in place a 40 day withdrawal period. Approximately 15 hours prior to slaughter, sows designated for slaughter were withheld from access to feed and animals were transported to the NDSU abattoir at 0630 h the next day. Sows were stunned by electrocution and captive bolt, after which they were quickly exsanguinated. Viscera were removed and kidney, liver, skeletal muscle (mid portion of the longissimus dorsi), and adipose tissue sample aliquots were collected. In addition, urine and blood samples were collected. At collection, samples were placed on dry-ice; they were subsequently transported to the Biosciences Research Laboratory and stored at -80 ˚C until analysis. Aliquots of skeletal muscle, kidney, and liver were also collected for penicillin determination by the Charm-KIS rapid screening assay.

On-Site Analysis of Kidneys. Kidney samples were screened for penicillin residues on the kill floor using the Charm-KIS (Lawrence, MA) microbial inhibition test according to FSIS rapid screening procedures (FSIS, 2011a), but followed the manufacturer recommended incubation time without the automatic shut-off option. Briefly, kidney swabs were saturated with kidney juice after perforating kidneys with the Charm-KIS wand. Swabs were placed into test media and incubated at 64 ± 2 ˚C for approximately 3 hours according to the lot specifications provided by the manufacturer and the presence of penicillin was indicated by a purple color (Figure 4), while samples testing negative for penicillin were indicated by a yellow color (Figure 4). Samples containing penicillin residues below the limit of detection or which contained other classes of antimicrobials/interferences to the Charm-KIS test (Figure 4) could produce ambiguous results, defined as "cautions" by the FSIS (2011a).

Rapid Screening Assay Analysis of Frozen Skeletal Muscle and Kidney Samples. While on the kill floor, skeletal muscle, additional kidney, and liver samples were removed from carcasses using a coring device. Cored samples were placed into Driploss containers (Danish Meat Research Institute; Taastrup, Denmark) and were frozen for a minimum of 16 h at -80 ˚C. Samples were subsequently thawed and the collected tissue juices were used to saturate Charm-KIS swabs. Microbial inhibition tests were then conducted as described by the FSIS (2011a) for determination of the presence of penicillin residues.

Rapid Screening Assay Analysis of Liver Samples. Liver samples were cored, placed into Driploss containers, frozen, thawed and juice collected as described for kidney and muscle samples. After the addition of an equal volume of water, boiling for 1 min, and centrifugation (14,000 x g) for 10 min, the Charm-KIS cotton applicator swab was immersed in the liver juice supernatant for 10 second prior to performing the Charm-KIS microbial inhibition assay as described previously.

Analysis of Serum and Urine Samples. Serum or urine aliquots (500 µL) were combined with a neutralizing tablet (Charm Sciences, Inc., Lawrence, MA) and vortexed; particulates were allowed to settle for 1 min. Serum or urine supernatant was adsorbed for 10 seconds with a cotton swab, after which, the CHARM-KIS microbial inhibition assay was performed.

Liquid Chromatography-Mass Spectrometry. Frozen tissues were processed by homogenization on dry ice to prevent thaw and degradation of penicillin residues. After tissue processing, kidney and muscle samples were extracted using the FSIS method CLG-BLAC.02 with some modifications (FSIS, 2011b). Blank (negative control) and fortified samples (spiked with 25 ng/g penicillin G procaine) were extracted with each sample set in duplicate. Trial samples were extracted in duplicate by withdrawal day. Before analysis, a deuterated internal standard (Penicillin G-d7; Sigma Aldrich, St Louis, MO) was added to each sample extract at an end concentration of 100 ng/mL for quantification purposes. Blank sample extracts were utilized to prepare matrix matched standard curves ranging from 2 ng/mL to 500 ng/mL. A new standard curve was made fresh for each sample set to avoid any Penicillin degradation. Samples and curves were analyzed within 24 hours of extraction and preparation. A modified version of a UHPLC-MS/MS method from Apley et al. (2009) was used for extract analysis. Extracts were analyzed using a Waters (Milford, MA) Ultra High Performance Liquid Chromatograph coupled to a tandem quadrupole mass spectrometer. The chromatography was modified slightly to be a slower gradient for longer retention of Penicillin G. The detection method was also modified to monitor additional fragment ions for Penicillin G to improve confirmation and quantification of residues. If samples contained residues greater than the highest penicillin concentration of the standard curve then samples were re-extracted as above but with an additional dilution step to bring concentrations of residues within the range of the standard curve. If sample duplicate concentrations differed by more than 25% they were re-extracted and re-analyzed in duplicate to achieve better agreement between replicates. Reported data are not corrected for recovery (FDA CVM, 2006).

Estimation of Withdrawal Period. The European Union and the United States estimate withdrawal periods using essentially identical techniques. That is, withdrawal periods are calculated by determining time required for tissue residues in 99 (US) or 95 (Europe) percent of a population of animals to deplete to an established tolerance with 95% confidence. Tolerance limits are calculated using linear regression of the natural logs (ln) of tissue residues in the linear depletion phase of the residue depletion curve. In calculating the 95^{th} percentile confidence interval, the US assumes a non-central t-distribution (FDA CVM, Guide 3, 2006), whereas the European Union allows the 95^{th} percentile confidence interval to be calculated using the standardized normal distribution (CVMP, 1995). The two methods generally return similar results (CVMP, 1995; Damte et al, 2012), depending upon a number of variables, the most important being the handling of data which fall below the method limit of quantification (Damte et al., 2012). Both the US and the CVMP methods of establishing withdrawal periods have been criticized (Concordet and Toutain, 1997; Sanquer et al., 2006) because of the

difficulty of meeting assumptions of normality and equal variance of data at each withdrawal time.

In the estimation of withdrawal periods for kidney, the traditional target tissue for penicillin residues in swine, the following criteria were used. These criteria are a combination of European Union and US FDA approaches to handling data from residue depletion trials.

- For a given time point to be included in the analysis, at least three animals had to have returned residues above the method limit of quantification (FDA CVM, 1996)
- Values below the limit of quantification, but above the limit of detection, were used as returned by the assay if there were at least three points at the withdrawal period above the limit of quantification
- Values falling below the limit of detection were included at ½ the method limit of detection and were included in withdrawal period calculations (CVMP, 1995)

For kidney calculations, data obtained from withdrawal days 10, 15, 20, and 25 were used in the analysis. Data from day 32 were not employed even though there were 3 animals with penicillin residues above the LOQ because the 32 day data did not continue the linear trend with respect to days 20 and 25. One could argue that data from withdrawal days 20, 25, and 32 days should have been used for estimating a withdrawal period because these data appear to represent a terminal depletion phase for penicillin depletion. However, the data were not linear ($P = 0.45$) with respect to each other and could not be used. Thus, the kidney data that best fit the criteria for estimating withdrawal periods using a log-linear approach were those of days 10, 15, 20 and 25.

For skeletal muscle, only one animal out of 18 had residues above the LOQ at the 15-d withdrawal period; 3 animals had residues above the LOD, but below the LOQ. Because a minimum of 3 days of data are required to determine the terminal, linear elimination period, the 15-d withdrawal period data were used for estimation of a withdrawal period, even though these data did not strictly comply with FDA guidelines.

Because the US has no tolerance established for penicillin residues in any edible tissue of swine, in practice the default tolerance becomes the sensitivity of the Charm-KIS test used to screen residues at federally inspected abattoirs. In our hands this was approximately 20 ppb for kidney juice. Therefore, for US calculations a tolerance of 20 ppb could be used to calculate withdrawal periods. However, the US FDA CVM does allow 50 ppb penicillin residues in edible beef tissues (FAS online, 2013), so 50 ppb was also used for swine tissues. A tolerance of 50 ppb in edible tissues is used, and would be applicable, for most other countries of the world (FAS online, 2013). For practical purposes, the FSIS has established an 'action level' for penicillin of 25 ppb in swine tissues (FSIS, 2013) and this action level serves as a *de facto* tolerance. Additional withdrawal calculations were therefore performed using the FSIS 25 ppb action level.

Estimations of withdrawal period were completed for kidney and skeletal muscle tissues using FDA (FDA, 1996) guidelines. Excel 7.0 was used in conjunction with the tables of Owen (1962) to calculate the critical values or the non-central t-distribution used in FDA calculations.

Modifications of project from original proposal

Other than reducing the longest withdrawal time from 40 to 39 days (due to inclement weather during Trial 1), no material modifications to the original proposal were required. A positive control sow, treated with a 1x dose of penicillin G procaine and scheduled to be slaughtered with a 15-d withdrawal period, died prior to slaughter. This was the only experimental animal that died unexpectedly during the study. As a consequence, Trial 1, did not contain a sow dosed according to label directions, with a 15-d withdrawal period. During Trial 2, a positive control sow farrowed and was removed from the study. A replacement sow was added during the trial. At slaughter, 6 study sows were found to be pregnant. These animals were not dropped from the study.

Results

Animal Receipt and Dosing. Table 1 summarizes sow weights by treatment and withdrawal period. A simple one-way ANOVA of hog weights between Trials 1 and 2 indicated that sows in Trial 1 (221.1 ± 2.9 kg, n= 63; mean ± SEM) were lighter ($P < 0.01$) than sows in Trial 2 (235.4 ± 4.4 kg, n= 63; mean ± SEM). Because the objective of the study was to capture variation in typical commercial settings, and because sow doses were normalized on a kg body weight basis, weights of animals in Trials 1 and 2 were combined for further statistical analyses (2-way ANOVA with treatment and withdrawal period as main factors). As shown in Table 1, within the study as a whole, no differences ($P > 0.05$) in body weights of sows occurred between treatments or withdrawal period. Variations of sow starting weights shown in Table 1 are representative of the range of heavy sows which might be candidates for penicillin G procaine treatment in commercial sow operations.

Slaughter and Tissue Collection. Slaughter weights, by treatment and withdrawal period, are shown in Table 2. Differences in sow body weights across treatment and withdrawal period were not significant ($P > 0.05$).

Effect of Withdrawal Period on the Detection of Penicillin G by the Charm-KIS Rapid Screening Assay. Table 3 summarizes the effects of treatment and withdrawal period on Charm-KIS positive results from regulated tissues including kidney and skeletal muscle. Data for kidney on-site and for skeletal muscle are shown graphically in Figure 5. Table 4 shows Charm-KIS results from non-target matrices including serum, urine, and liver. The limit of detection for the Charm-KIS test was 20, 30, 20, 30, and 100 ppb for kidney, muscle, urine, serum, and liver, respectively.

Penicillin residues were detected by the Charm-KIS test in kidney swab samples for the duration of the study, but with decreasing frequency as the study progressed. There appeared to be no clear effects of treatment (penicillin administration pattern) on the depletion of penicillin from the kidney, as assessed by Charm-KIS detection. When Charm-KIS detection frequency (% positive) data were plotted against time for each treatment and fit with a mono-exponential decay curve, no difference ($P > 0.05$) in the decay rate constant between the three treatments was observed; data between treatments were therefore pooled and all the frequency of detection data were fit to a one-phase mono-exponential decay curve. Figure 5 shows the curve fit by all of the data; the half-life for the Charm-KIS detection in kidney was estimated to be 9.4 days with a 95% confidence interval of 8.1 to 11.1 days. It is clear from Figure 5 that penicillin residues had not depleted from kidneys of all animals even after 39 days of withdrawal.

The Charm-KIS test had a limit of detection of approximately 20 ppb in kidney juice. Mass spectral analysis indicated that each kidney sample that tested positive by the Charm-KIS test, had penicillin G resides above 15 ppb. The screening test returned no false positives for kidney swab samples and there were no instances of false negatives for kidney. In practice, the detection limit of the Charm-KIS test serves as a *de facto* tolerance in the United States, even though there is no set Maximum Residue Limit (MRL) for penicillin residues in swine tissues. In slaughter facilities, kidney swabs testing positive for residue will be sent for confirmatory penicillin residue testing. Because the LC-MS/MS cut-off value used by the FSIS for penicillin residues is 25 ppb (FSIS, 2013) the Charm-KIS test in kidney will return positive LC-MS/MS values when concentrations exceed 25 ppb. Data from this study suggest that approximately 11% of sows treated IM with a 5x dose of penicillin for 3 consecutive days will have Charm-KIS detectable residues in kidneys even after a prolonged withdrawal period of 39 days. While these animals would test positive by the screening assay, they would likely have penicillin residues below 25 ppb and would not meet the FSIS confirmation level (FSIS, 2013). For animals slaughtered at the FARAD recommended 15-day withdrawal period, it would be reasonable to expect that approximately 50% of penicillin-treated sows will have kidneys that test positive for penicillin residues.

There were very few differences between Charm-KIS positive kidney and frozen-kidney samples (Table 3), suggesting that the assay could be valuable for research purposes and for screening in instances where testing

fresh tissue is not possible.

In contrast to kidney, the frequency of Charm-KIS positives in skeletal muscle diminished rapidly with increased duration of the withdrawal period. There were no discernible effects of treatment on skeletal muscle detection frequency so further analysis was conducted with results from the 3 treatments pooled (Table 3). For skeletal muscle, 16 of 18 hogs returned positive Charm-KIS results after a 5-day withdrawal period, but by 10 days, only 1 of 18 hogs tested returned a positive kidney Charm-KIS test result. Two instances occurred in which the Charm-KIS returned positive assay results (Treatment 2, days 20 and 39; Table 3) which could not be verified with LC-MS/MS. The Charm-KIS assay had a limit of detection of approximately 30 ppb penicillin G. In general terms, skeletal muscle was a difficult, and somewhat unreliable, matrix for use with the Charm-KIS test.

Applicability of using the Charm-KIS for pre-slaughter screening of treated animals was tested with assays of serum and urine (Table 4). No false-positive Charm-KIS assay results were returned for either tissue (LC-MS/MS data not shown). With regards to sensitivity, the urine was a much better surrogate matrix for detecting potential violative penicillin G residues in kidney because Charm-KIS results in urine replicated results in kidney fairly well whereas serum results did not (Figure 6). The liver proved to be a relatively poor tissue for the determination of penicillin G residues by the Charm-KIS assay (Table 4).

Effect of Withdrawal Period on the Depletion of Penicillin G Residues as Determined by LC-MS/MS. Table 5 shows the depletion of penicillin G residues from kidneys of heavy sows as measured by LC-MS/MS. The same data are presented graphically, after natural log (ln) transformation, in Figure 7. Residues were measured in kidneys of all animals 5-days after the final treatment, but by 10 days 6 of 18 animals had residues below the assay LOD. By 20 days of withdrawal, 5 of 18 animals had residues above the LOQ with 3 of these animals having kidney residues above 100 ppb and one animal with penicillin residues greater than 300 ppb. Table 5 clearly shows that penicillin residues were present in the kidneys of two hogs after a 39-d withdrawal period. Data presented in Table 3 indicate that the Charm-KIS test accurately predicted the presence of these residues in these kidney samples. The presence of penicillin residues in 11% of animals at withdrawal day 39 suggests that an extensive withdrawal period would be required for the complete depletion of penicillin from kidneys of a population of treated animals.

A withdrawal period was estimated for kidney tissues using the log-linear approach promulgated by the US-FDA CVM (2006) with modifications suggested by the CVMP (1995). In making a withdrawal period estimation, the essential assumptions of equal variance and normal distributions (Shapiro-Wilk) of data were violated ($P < 0.001$), thus the estimated withdrawal period presented here was admittedly calculated with data that did not conform to statistical ideals. As discussed by Concordet and Toutain (1997a) and documented by Sanquer et al. (2006) these assumptions are, at best, difficult, and are sometimes impossible to meet. Non parametric approaches to withdrawal period calculations proffered by Sanquer et al (2006) and Concordet and Toutain (1997b) were not attempted on this data set.

Figure 8 shows that a 47-day withdrawal period would be required for kidney residues to deplete to 50 ppb in 99% of the animals (with 95% certainty) in a population of heavy sows treated with a 5x dose of penicillin G procaine for 3 consecutive days. In order for residues to fall below the FSIS action level of 25 ppb in a population of treated sows, a 51-day withdrawal period would be required. Again, these estimates are not statistically valid because the data from which they were calculated were not normally distributed and did not have an equal variance. Nevertheless, the estimates show that a withdrawal period based on kidney residues is prohibitively long with regards to commercial sow production systems.

In contrast to kidney tissues, Penicillin G residues depleted quickly from skeletal muscle (Table 6 and Figures 9 and 10). Residues in skeletal muscle at 5 days of withdrawal averaged only 23.5 ± 10.5 ng/g (ppb) and depleted

rapidly thereafter. By the 15th day of withdrawal, only 1 sow had skeletal muscle residues greater than the method LOQ (2.4 ppb) with 4 other sows having residues greater than the method LOD (0.7 ppb). Thus, the estimated withdrawal period for skeletal muscle was calculated to be 11 days (Figure 7) for the worldwide tolerance of 50 ppb, and 13 days for the FSIS action level of 25 ppb. Regardless of dosing pattern, the FARAD estimated withdrawal time of 15 days was sufficient for penicillin residues to deplete from skeletal muscle. It should be noted that an 11-day withdrawal period does not represent a regulatory withdrawal period because the normality assumption inherent in regression analyses was not met by the skeletal muscle data set ($P < 0.001$; Shapiro-Wilk). Critics of the FDA method have maintained that many of the underlying assumptions required by the use of a statistical approach for withdrawal period determination are difficult, if not impossible, to meet (Concordet and Toutain, 1997).

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Table 1. Starting weights of sows by treatment and withdrawal period.

Withdrawal	Treatment					Pooled	
Period	1	2	3	Mean	n	SEM	P
d	*kg*	*kg*	*kg*	*kg*		*kg*	
5	245.8	233.0	222.2	233.7	18		
10	249.7	200.7	219.3	223.2	18		
15	219.0	211.5	215.3	215.4	18		
20	240.3	246.8	207.6	231.5	18	6.8	0.34
25	224.4	215.4	236.2	225.4	18		
32	242.2	225.7	240.7	236.2	18		
39	218.1	233.6	246.1	232.6	18		
Mean:	234.2	223.8	226.8				
n:	42	42	42		126		
Pooled SEM:		4.5					
P:		0.24					

Table 2. Slaughter weights of sows by treatment and withdrawal period.

Withdrawal	Treatment						
Period	1	2	3	Mean	n	SEM	P
d	*kg*	*kg*	*kg*	*kg*		*kg*	
5	245.3	229.5	225.2	233.3	18		
10	251.5	201.4	217.0	223.3	18		
15	220.3	217.7	216.5	218.2	18	6.7	0.19
20	241.2	251.0	209.4	233.9	18		
25	226.2	215.8	244.1	228.7	18		
32	253.0	230.8	242.9	242.3	18		
39	224.4	239.5	247.3	237.0	18		
Mean:	237.4	226.5	228.9				
n:	42	42	42		126		
SEM:		4.4					
P:		0.19					

Table 3. Results of the Charm-KIS rapid screening assay in tissues of heavy sows treated with a 5x penicillin G procaine dose for 3 consecutive days and slaughtered with varying withdrawal periods. Plus signs (+) indicate positive test results, minus signs (-) represent negative test results and (C) represent "caution" results, which FSIS deems negative.

		Treatment[a]									
		1			2			3			Pooled
Tissue	Day	Trial 1	Trial 2	Total	Trial 1	Trial 2	Total	Trial 1	Trial 2	Total	Treatments
Kidney On-Site[b]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	– – +	+ + +	4/6	– – +	– – +	2/6	+ + +	+ + +	6/6	12/18
	15	– – +	– + +	3/6	– – –	– + +	2/6	– + +	c[c] + +	4/6	9/18
	20	– – +	– + +	3/6	– – –	– – +	1/6	– – –	– – +	1/6	5/18
	25	– – +	– – –	1/6	~[d] – –	– + +	2/6	– – –	– – –	0/6	3/18
	32	– – –	– + +	2/6	– – –	– – +	1/6	– – –	– – –	0/6	3/18
	39	– – –	– – –	0/6	– – +	– – –	1/6	– – –	– – +	1/6	2/18
Frozen Kidney[e]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	– – +	+ + +	4/6	– – +	~[d] – +	2/6	+ + +	+ + +	6/6	12/18
	15	– – +	– + +	3/6	~[d] – –	– + +	2/6	– + +	– + +	4/6	9/18
	20	– – c[c]	– + +	2/6	– – –	– – +	1/6	– – –	– – +	1/6	4/18
	25	– – +	– – –	1/6	– – –	– + +	2/6	~[d] – –	– – –	0/6	3/18
	32	– – –	– + +	2/6	– – –	– – +	1/6	– – –	– – –	0/6	3/18
	39	– – –	– – –	0/6	– – +	– – –	1/6	– – –	– – +	1/6	2/18
Frozen Muscle[e]	5	+ + +	+ + +	6/6	+ + ~[d]	+ + +	5/6	+ + +	c[c] + +	5/6	16/18
	10	– – ~[d]	– – ~[d]	0/6	– – –	– ~[d] ~[d]	0/6	– – –	– – +	1/6	1/18
	15	– – –	– – –	0/6	~[d] – –	– – –	0/6	– – –	– – ~[d]	0/6	0/18
	20	– – –	– – –	0/6	FP[f] – –	– – –	0/6	– – –	– – –	0/6	0/18
	25	– – –	– – –	0/6	– – –	– – –	0/6	– – –	– – –	0/6	0/18
	32	– – –	– – –	0/6	– – –	– – ~[d]	0/6	– – –	– – –	0/6	0/18
	39	– – –	– – –	0/6	FP[f] – –	– – –	0/6	– – –	– – –	0/6	0/18

[a]Treatments: (1) Maximum of 10 mL penicillin G procaine per injection; injections on consecutive days were in the same location as the previous day; (2) Maximum of 10 mL penicillin G procaine per injection site; injections on consecutive days were separated by approximately 2-3 inches; (3) Injection volumes of 20 mL penicillin G procaine; injections on consecutive days were separated by approximately 2-3 inches.

[b]Charm-KIS tests of kidney swabs were performed on the kill floor; results were obtained 3-4 hours after animal harvest.

[c]"c" Duplicate, simultaneously run Charm-KIS tests each returned "caution" results. FSIS regards cautions as a negative result.

[d]A tilde (~) represents ambiguous results in which duplicate Charm-KIS assays of the tissue scored either +/-, -/+, -/c, c/-, +/c, or c/+ or in which independent analyst interpretation of the Charm-KIS assay were in disagreement Ambiguous results were never clearly positive, so they were regarded as a negative result.

[e]Aliquots of kidney and skeletal muscle were placed in Driploss tubes and were frozen at sampling; tubes were thawed after approximately 16 hours and Charm-KIS tests were performed on kidney and muscle juice.

[f]False Positive. Presence of penicillin in positive Charm-KIS test results could not be verified with mass spectral analysis.

Table 4. Results of the Charm-KIS rapid screening assay in serum, urine, and liver of heavy sows treated with a 5x penicillin G procaine dose for 3 consecutive days and slaughtered with varying withdrawal periods.

		Treatment[a]									
		1			2			3			Pooled
Tissue	Day	Trial 1	Trial 2	Total	Trial 1	Trial 2	Total	Trial 1	Trial 2	Total	Treatments
Serum[b]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	– – +	+ + +	4/6	~[c] – –	– – +	1/6	– + +	+ + +	6/6	10/18
	15	~[c] – –	– – +	1/6	– – –	– c[d] c[d]	0/6	~[c] – –	c[d] c[d] –	0/6	1/18
	20	~[c] – –	c[d] – –	0/6	– – –	~[c] – –	0/6	– – –	– ~[c] +	1/6	1/18
	25	c[d] – –	~[c] – –	0/6	– – –	– – –	0/6	– – –	– – –	0/6	0/18
	32	– – –	– – –	0/6	– – –	~[c] ~[c] –	0/6	– – –	~[c] – –	0/6	0/18
	39	– – –	– – –	0/6	– – –	– – –	0/6	– – –	– – –	0/6	0/18
Urine[b]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	– + +	+ + +	5/6	c + +	– – +	3/6	+ + +	+ + +	6/6	14/18
	15	– – +	– + +	3/6	– – –	– + +	2/6	– + +	– + +	4/6	9/18
	20	– – –	– + +	2/6	~[c] – –	– – +	1/6	– – –	– – +	1/6	4/18
	25	x[e] – +	– – –	1/5	x[e] c[d] –	– + +	2/5	~[c] – –	– – –	0/6	3/16
	32	x[e] – –	– + +	2/5	– – –	– – +	1/6	– – –	– – –	0/6	3/17
	39	x[e] – –	– – –	0/5	~[c] – x[e]	– – –	0/5	– – –	– – +	1/6	1/16
Liver[f]	5	– – +	– + +	3/6	– + +	– – –	2/6	~[c] – –	– – +	1/6	6/18
	10	– – –	– – –	0/6	– – –	– – –	0/6	– – –	– – +	1/6	1/18
	15	– – –	– – +	1/6	– – –	– – –	0/6	– – –	– – –	0/6	1/18
	20	– – –	– – –	0/6	– – –	– – –	0/6	– – –	– – –	0/6	0/18
	25	– – –	– – –	0/6	– – –	– – –	0/6	– – –	– – –	0/6	0/18
	32	– – –	– – –	0/6	– – –	– – –	0/6	– – –	– – –	0/6	0/18
	39	– – –	– – –	0/6	– – –	– – –	0/6	– – –	– – –	0/6	0/18

[a]Treatments:

1. Maximum of 10 mL penicillin G procaine per injection; injections on consecutive days were in the same location as the previous day
2. Maximum of 10 mL penicillin G procaine per injection site; injections on consecutive days were separated by approximately 2-3 inches
3. Injection volumes of 20 mL penicillin G procaine; injections on consecutive days were separated by approximately 2-3 inches

[b]Serum and urine aliquots were combined with Charm-KIS serum/urine neutralizing tablets, mixed, and supernatant were used for Charm-KIS tests.

[c]A tilde (~) represents ambiguous results in which duplicate Charm-KIS assays of the tissue scored either +/-, -/+, -/c, c/-, +/c, or c/+ or in which independent analyst interpretation of the Charm-KIS assay were in disagreement Ambiguous results were never clearly positive, so they were regarded as a negative result.

[d]"c" Duplicate, simultaneously run Charm-KIS tests each returned "caution" results. FSIS regards cautions as a negative result.

[e]x, no urine sample was obtained.

[f]Aliquots of liver were placed in Driploss tubes and were frozen at sampling; Charm-KIS tests were performed on liver and injection site juice after thawing.

Table 5. Depletion of penicillin G residues from kidneys of heavy sows treated with a 5x penicillin G procaine dose for 3 consecutive days and slaughtered with varying withdrawal periods. Data are expressed as parts per billion (ppb) penicillin G equivalents; values shown in italic font were below the method limit of quantification (LOQ; 6.1 ppb) but above the method limit of detection (LOD; 1.8 ppb); values shown in bold font were below the method LOD and are expressed at one-half the LOD, Within treatment, means and standard deviations are calculated from 6 observations per withdrawal period. Across treatment means were calculated from 18 observations within withdrawal period.

		Treatment									
	Withdrawal	1			2			3			Overall
Tissue	Period	Residue		Mean ± SD	Residue		Mean±SD	Residue		Mean±SD	Mean±SD
	d	*ppb*	*ppb*	*ppb*	*ppb*	*ppb*	*ppb*	*ppb*	*ppb*	*ppb*	*ppb*
Kidney	5	3,182	4,259		3,494	2,597		5,850	9,900		
		2,948	3,819		4,264	1,498		1,194	3,435		
		3,540	4,756	3,751 ± 676	3,077	4,494	3,238 ± 1,110	1,618	3,791	4298 ± 3219	3762 ± 1932
	10	**0.9**	492		**0.9**	**0.9**		42.1	1,916		
		1,516	2,540		74.1	**0.9**		332	502		
		0.9	143	782 ±1,034	**0.9**	524	100 ± 210	2,036	1,887	1119 ± 919	667 ± 875
	15	**0.9**	804		**0.9**	95.3		**0.9**	355		
		214	76.8		*3.1*	152		62.3	185		
		0.9	**0.9**	183 ± 315	**0.9**	**0.9**	42.1 ± 65.5	194	**0.9**	133 ± 138	119 ± 199
	20	**0.9**	**0.9**		**0.9**	**0.9**		**0.9**	273		
		4.9	35.3		**0.9**	128		**0.9**	**0.9**		
		0.9	350	65.4 ± 140	**0.9**	**0.9**	22.0 ± 51.7	**0.9**	**0.9**	46.2 ± 111	44.5 ± 103
	25	149	**0.9**		**0.9**	**0.9**		**0.9**	**0.9**		
		0.9	**0.9**		**0.9**	49.5		**0.9**	**0.9**		
		0.9	**0.9**	25.6 ± 60.4	**0.9**	41.0	15.7 ± 23.1	**0.9**	**0.9**	**0.9**	14.1 ± 36.6
	32	**0.9**	28.9		**0.9**	**0.9**		**0.9**	**0.9**		
		0.9	64.0		**0.9**	167		**0.9**	**0.9**		
		0.9	**0.9**	16.1 ± 26.0	**0.9**	**0.9**	28.5 ± 67.6	**0.9**	**0.9**	**0.9**	15.2 ± 41.0
	39	**0.9**	**0.9**		**0.9**	**0.9**		**0.9**	**0.9**		
		0.9	**0.9**		22.7	**0.9**		**0.9**	17.0		
		0.9	**0.9**	**0.9**	**0.9**	**0.9**	4.5 ± 8.9	**0.9**	**0.9**	3.6 ± 6.6	3.0 ± 6.2

[a]Data are not corrected for recovery which averaged 72.7 ± 10.7% across all kidney assays (n = 26).
[b]Method LOQ, 6.1 ng/g (ppb); LOD, 1.8 ng/g; one-half LOD, 0.9 ng/g.

Table 6. Depletion of penicillin G residues from skeletal muscle of heavy sows treated with a 5x penicillin G procaine dose for 3 consecutive days and slaughtered with varying withdrawal periods. Data are expressed as parts per billion (ppb) penicillin G equivalents; values shown in italic font were below the method limit of quantification (LOQ) but above the method limit of detection (LOD); values shown in bold font were below the method LOD (0.7 ppb) and are expressed at one-half the LOD, Within treatment, means and standard deviations are calculated from 6 observations per withdrawal period. Accoss treatment means were calculated from 18 observations within withdrawal period.

		Treatment								
	Withdrawal	1			2			3			Overall
Tissue	Period	Residue[a]		Mean±SD	Residue[a]		Mean±SD	Residue[a]		Mean ± SD	Mean ± SD
	d	*ppb*		*ppb*	*ppb*		*ppb*	*ppb*		*ppb*	*ppb*
Skeletal Muscle[b]	5	21.6	22.7		16.0	34.6		26.8	51.9		
		15.7	22.3		11.9	12.2		23.8	33.4		
		10.0	28.0	20.0 ± 6.3	13.4	32.4	20.1 ± 10.5	17.0	29.7	30.4 ± 11.9	23.5 ± 10.5
	10	**0.4**	2.8		**0.4**	**0.4**		7.9	9.2		
		8.6	15.1		**0.4**	**0.4**		2.8	7.8		
		0.4	*1.4*	4.8 ± 5.9	**0.4**	5.6	*1.3 ± 2.1*	9.5	21.9	9.8 ± 6.4	5.3 ± 6.1
	15	**0.4**	*2.0*		**0.4**	3.4		**0.4**	*2.0*		
		1.5	**0.4**		**0.4**	*1.2*		**0.4**	**0.4**		
		0.4	**0.4**	*0.8 ± 0.7*	**0.4**	**0.4**	*1.0 ± 1.2*	**0.4**	**0.4**	*0.7 ± 0.6*	*0.8 ± 0.8*
	20	**0.4**	2.7		**0.4**	**0.4**		**0.4**	*1.5*		
		0.4	**0.4**		**0.4**	3.8		**0.4**	**0.4**		
		0.4	*2.0*	*1.1 ± 1.0*	**0.4**	**0.4**	*1.0 ± 1.4*	**0.4**	**0.4**	*0.6 ± 0.4*	*0.9 ± 1.0*
	25	**0.4**	**0.4**		**0.4**	**0.4**		**0.4**	**0.4**		
		0.4	**0.4**		**0.4**	**0.4**		**0.4**	**0.4**		
		0.4	**0.4**	**0.4**	**0.4**	**0.4**	**0.40**	**0.4**	**0.4**	**0.4**	**0.4**
	32	**0.4**	**0.4**		**0.4**	**0.4**		**0.4**	**0.4**		
		0.4	**0.4**		**0.4**	*1.2*		**0.4**	**0.4**		
		0.4	**0.4**	**0.4**	**0.4**	*0.7*	*0.6 ± 0.3*	**0.4**	**0.4**	**0.4**	*0.5 ± 0.2*
	39	**0.4**	**0.4**		**0.4**	**0.4**		**0.4**	**0.4**		
		0.4	**0.4**		**0.4**	**0.4**		**0.4**	**0.4**		
		0.4	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**	**0.4**

[a]Data are not corrected for recovery which averaged 55.8 ± 9.4% across all skeletal muscle assays (n = 14).
[b]Method LOQ, 2.4 ng/g (ppb); LOD, 0.7 ng/g; one-half LOD, 0.4 ng/g.

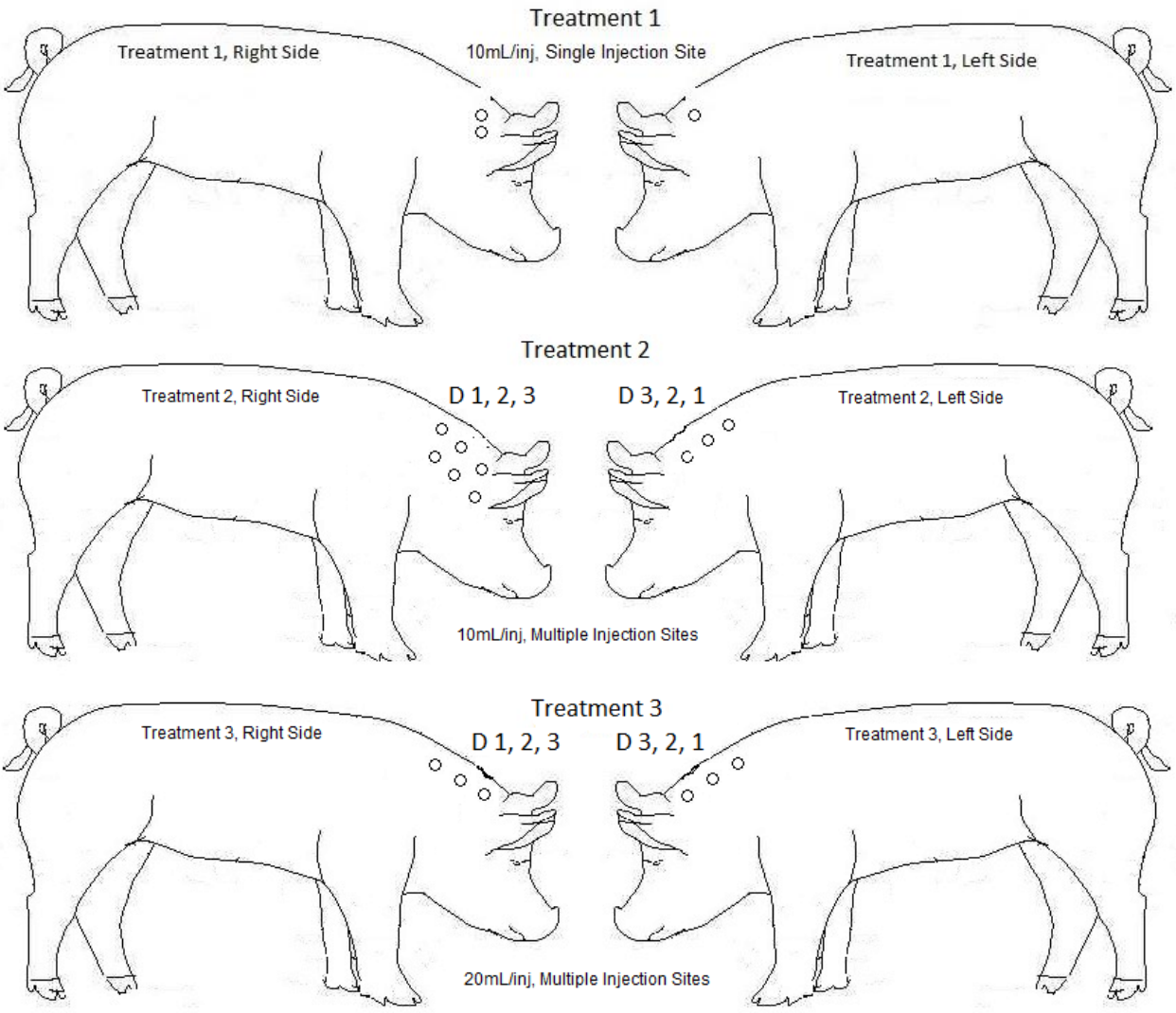

Figure 1. Penicillin G procaine treatments. Sows were administered 5x doses (15,000 IU/lb BW) of penicillin G procaine on each of three consecutive days as either 10 mL injections (Treatments 1 and 2) or as 20 mL injections (Treatment 3). Across day, treatment 1 sows received injections in a single location; sows in treatments 2 and 3 received consecutive injections at locations separated by approximately 2 inch intervals. Because sows were very large, total injection volumes were typically greater than 20 mL; thus, for Treatments 1 and 2, the pattern of injections was 10 mL right, 10 mL left and the remaining volume (for total doses greater than 20 mL) would be injected into the right side approximately 2 inches below the initial 10 mL injection. The injection pattern for Treatment 3 was 20 mL in the right side with overflow injections occurring in the left neck muscles. Injection sites were marked by tattoos 7 days prior to the initial treatment.

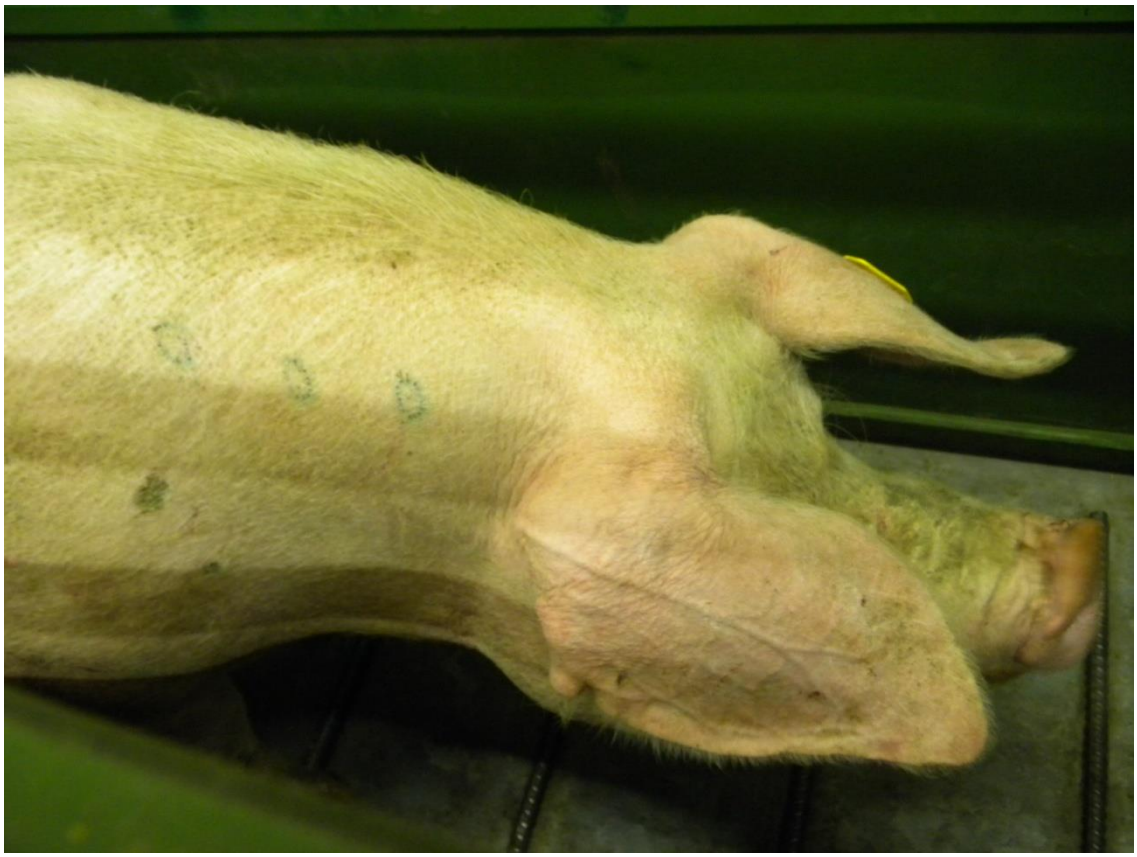

Figure 2. The right aspect of a Treatment 3 sow clearly showing tattooing demarcating injection sites for penicillin G procaine on treatment days 1 (cranial tattoo), 2 (middle tattoo), and 3 (distal tattoo). On each of treatment days 1, 2, and 3, 20 mL of penicillin G procaine was injected into each tattooed area; volumes of penicillin G procaine in excess of 20 mL were injected into similar tattooed areas on the left side of the animal. Photo was taken on withdrawal day 5 (15 days after tattoo application).

Figure 3. A modified crate used for dosing that allowed access to right and left neck sides of heavy sows. The crate was not anchored to the floor and could be lifted if an animal became unruly; the heavily padded bar longitudinally bisecting the bottom of the crate would lift the unruly sow so that the sow could no longer contact the floor.

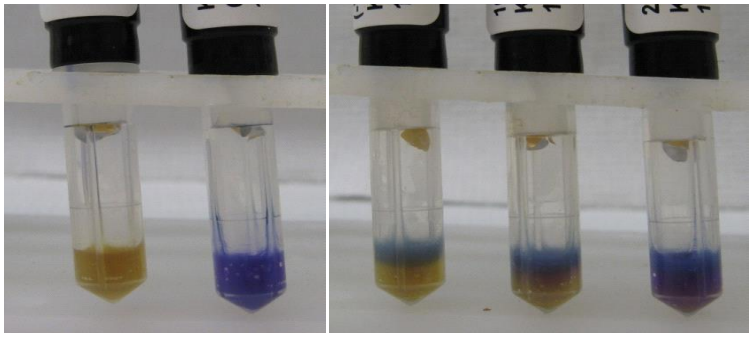

Figure 4. Negative (-) and positive (+) controls (left panel) and negative (-), caution (c) and (+) test results returned from kidney samples by the Charm-KIS rapid screening assay.

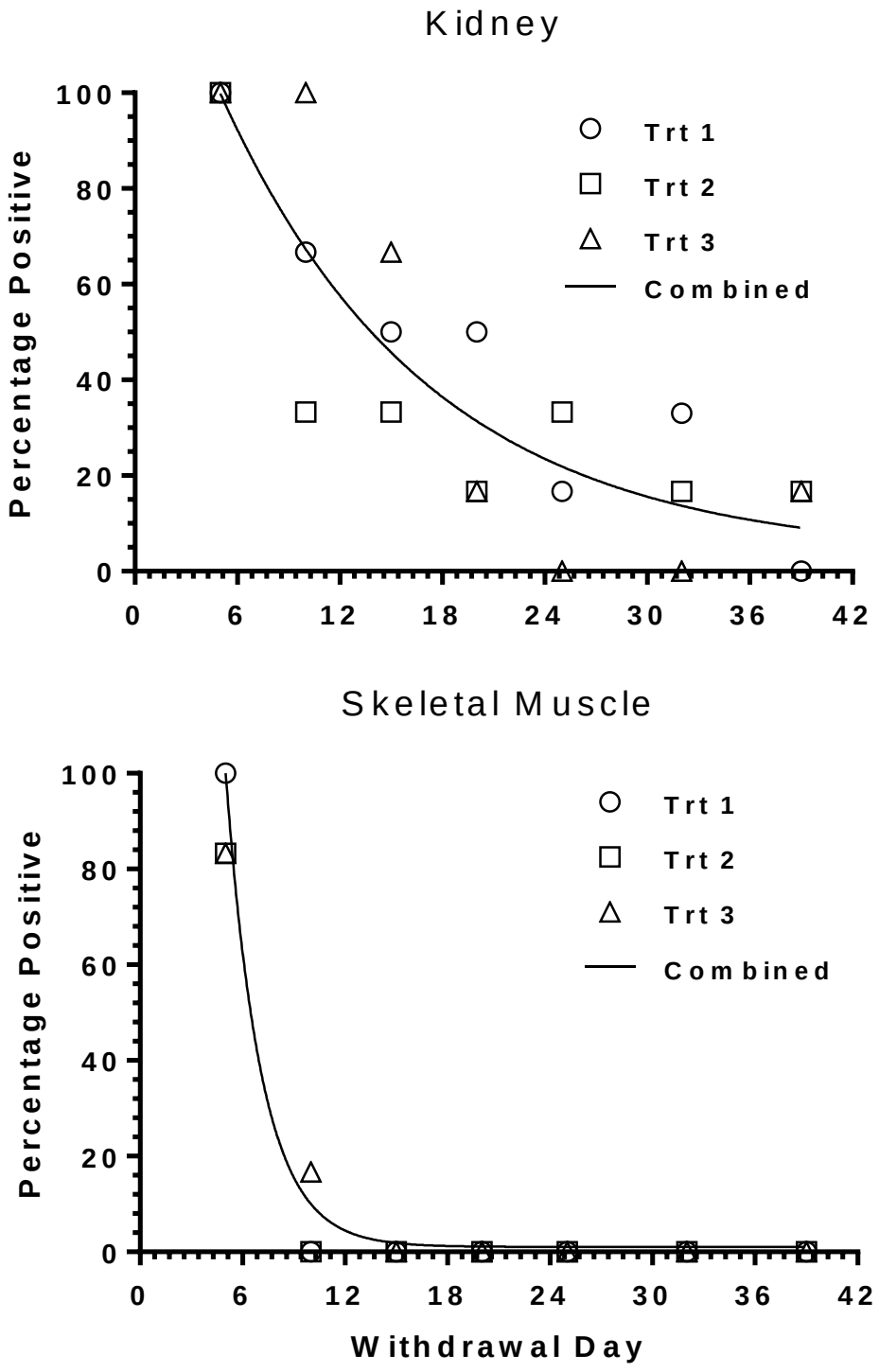

Figure 5. Depletion of Penicillin G residues in kidneys and muscle of heavy sows as detected by the Charm-KIS rapid screening assay. Line is a first order depletion fit of combined data from Treatments; false positive results were removed from the skeletal muscle data prior to plotting.

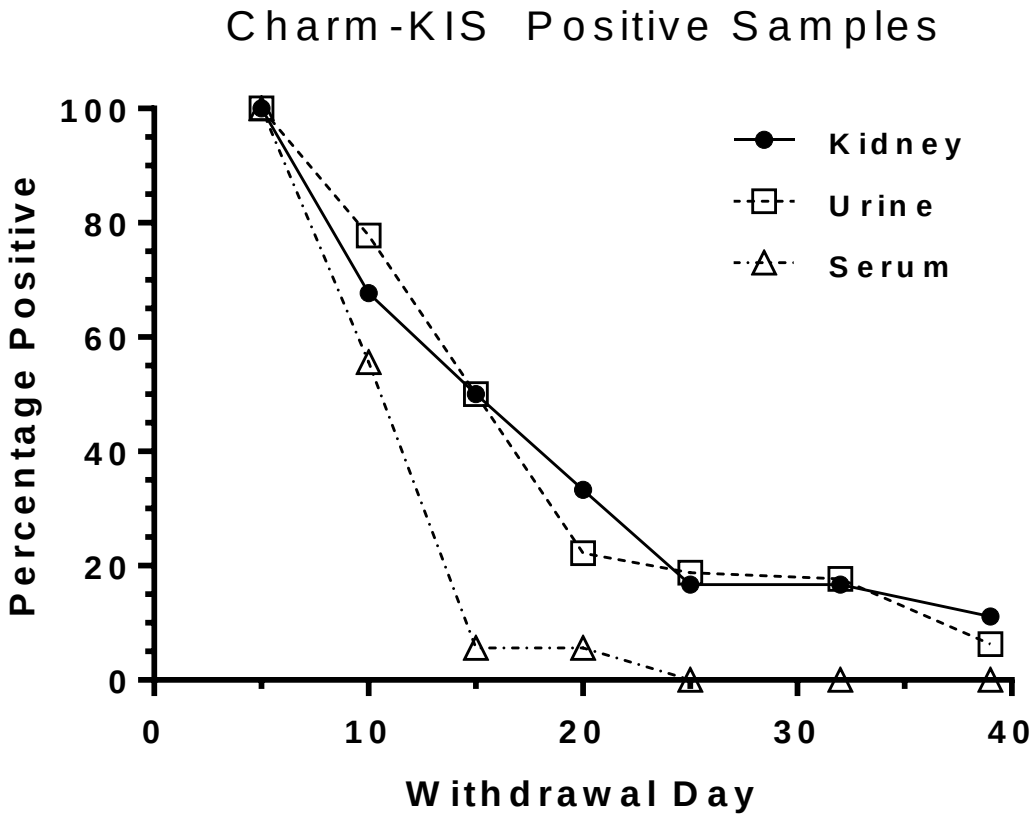

Figure 6. Comparison of Charm-KIS test results in urine (square, hatched line), serum (triangles, hatched line) and kidneys (circle, solid line) of treated sows as a function of withdrawal day.

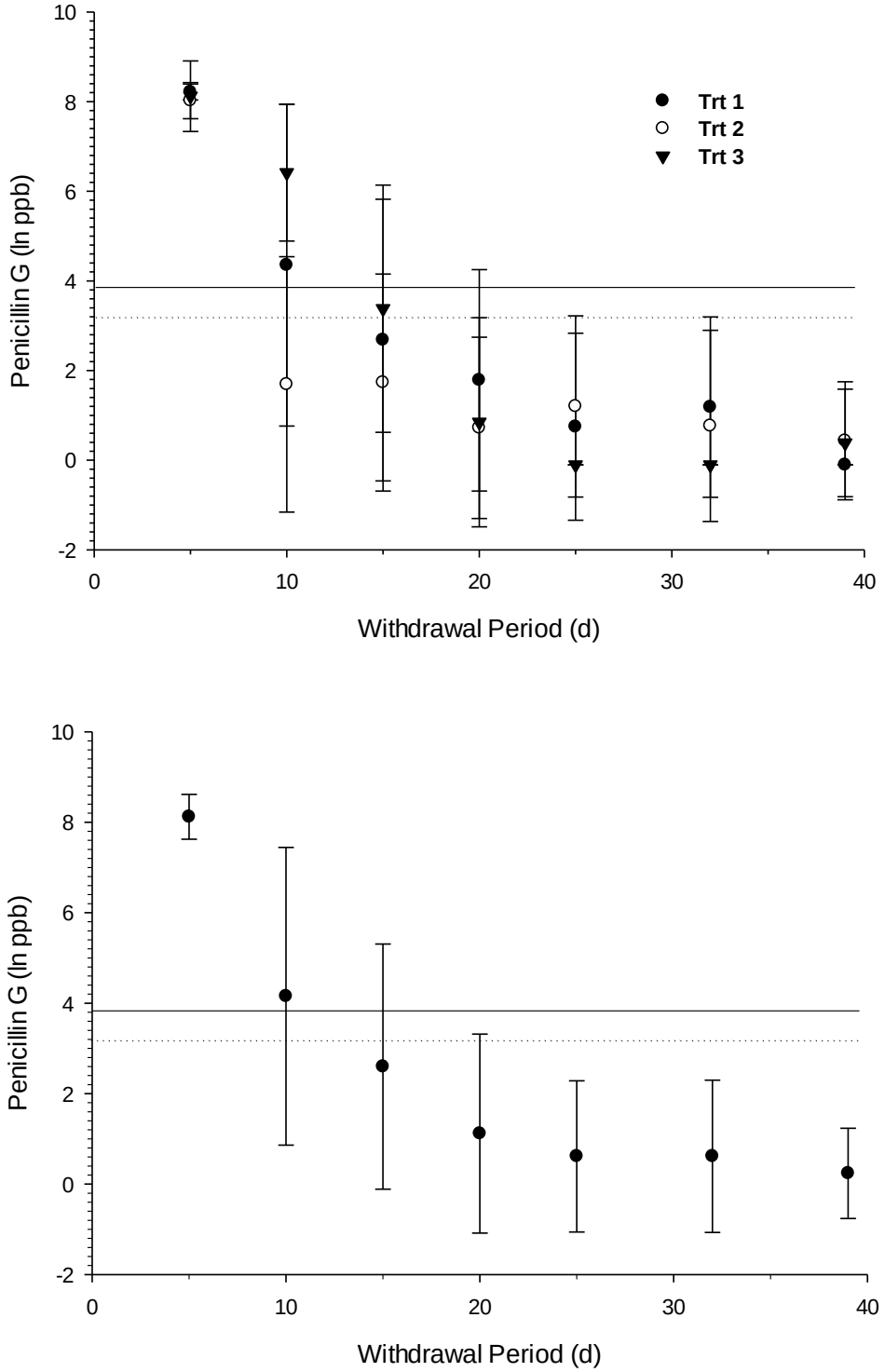

Figure 7. Depletion of penicillin G procaine from kidney tissues of heavy sows as detected by LC-MS/MS. Data are expressed as the geometric means of the natural log (ln) of kidney penicillin G concentrations (ng/g or ppb) by treatment (top graph) or by combined (bottom graph) means. The horizontal solid line is equivalent to the ln of the penicillin G tolerance for most of the world (50 ppb) and the horizontal dotted line is equivalent to the FSIS action limit of 25 ppb.

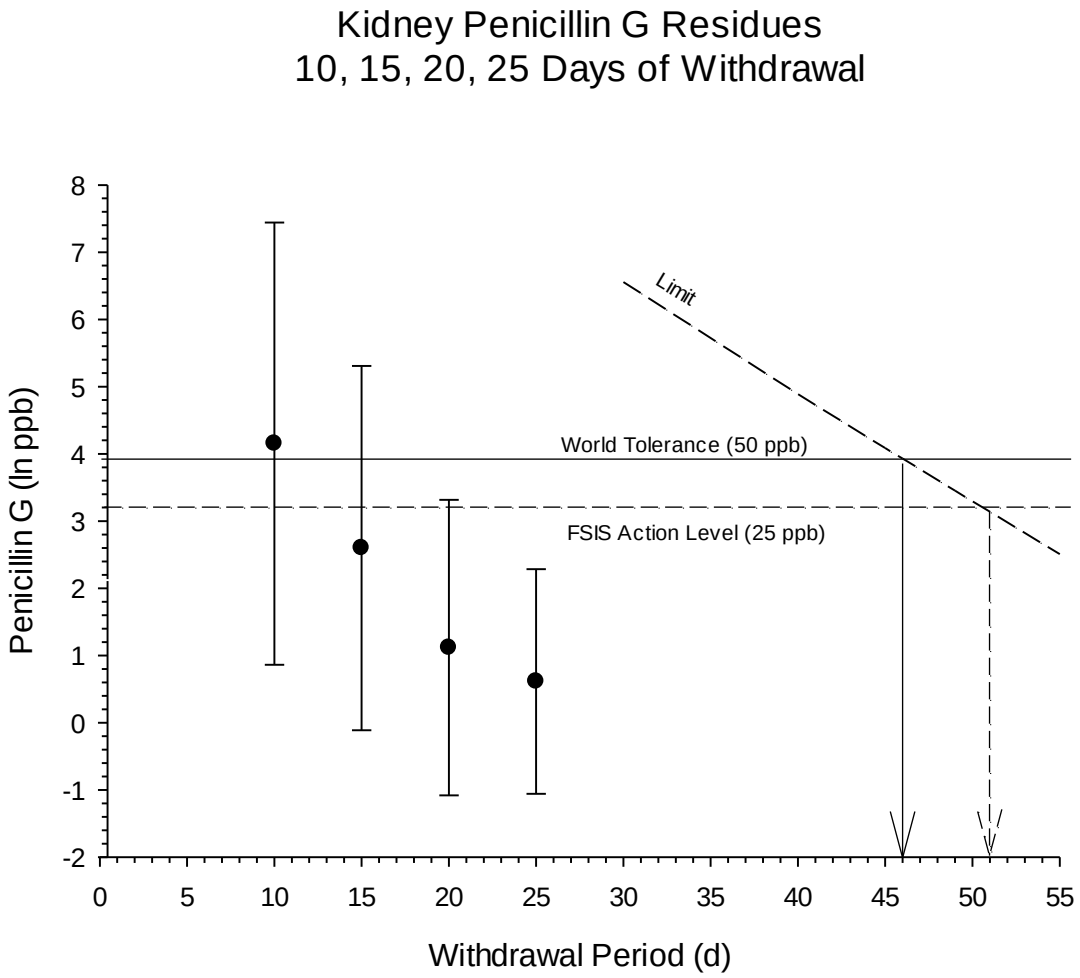

Figure 8. Estimation of pre-slaughter withdrawal period in heavy sows treated with a 5x dose of penicillin-G procaine, based on depletion of penicillin-G residues from kidney tissues. Data taken from the linear portion of the depletion curve were analyzed as described by FDA Guidance Document no. 3 (US FDA CVM, 2006). In contrast to US FDA guidance, 15-d withdrawal data below the method LOD were assigned values of ½ the LOD (CMVP, 1995). Kidney penicillin-G concentrations that were between the LOQ and LOD were included in the analysis as their nominal concentration. The residue tolerance used by most of the world (50 ppb or ln ppb 3.91) crossed the 99^{th} percentile with a 95^{th} percent confidence interval tolerance limit at approximately 46.5 days (solid vertical arrow). The kidney withdrawal period for a tolerance of 50 ppb was established at 47 days after rounding up to the nearest whole-day. In the United States, there is no tolerance established for penicillin G in swine tissues, but FSIS has established an action limit of 25 ppb for penicillin residues detected in swine tissues (FSIS, 2013). In order for penicillin residues to deplete below the action limit of 25 ppb in a population of swine, just over 50 days would be required; rounding up to the nearest whole-day, the withdrawal period would be 51 days.

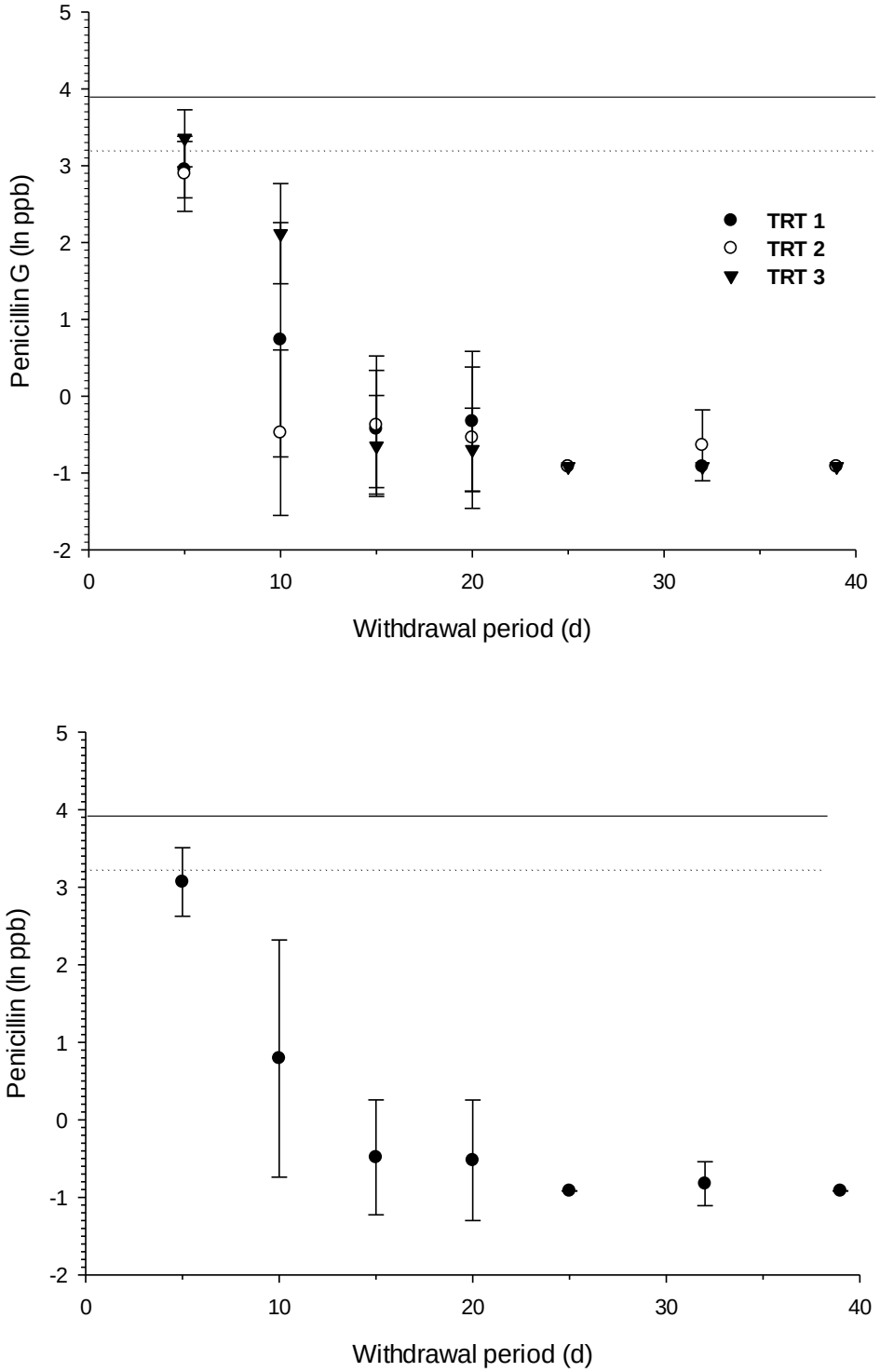

Figure 9. Depletion of penicillin G procaine from skeletal muscle of heavy sows as detected by LC-MS/MS. Data are expressed as geometric means of the natural log (ln) of muscle penicillin G concentrations (ng/g or ppb). by treatment (top graph) or by combined (bottom graph) means. The horizontal solid line is equivalent to the ln of the penicillin G tolerance for most of the world (50 ppb) and the horizontal dotted line is equivalent to the FSIS action limit of 25 ppb.

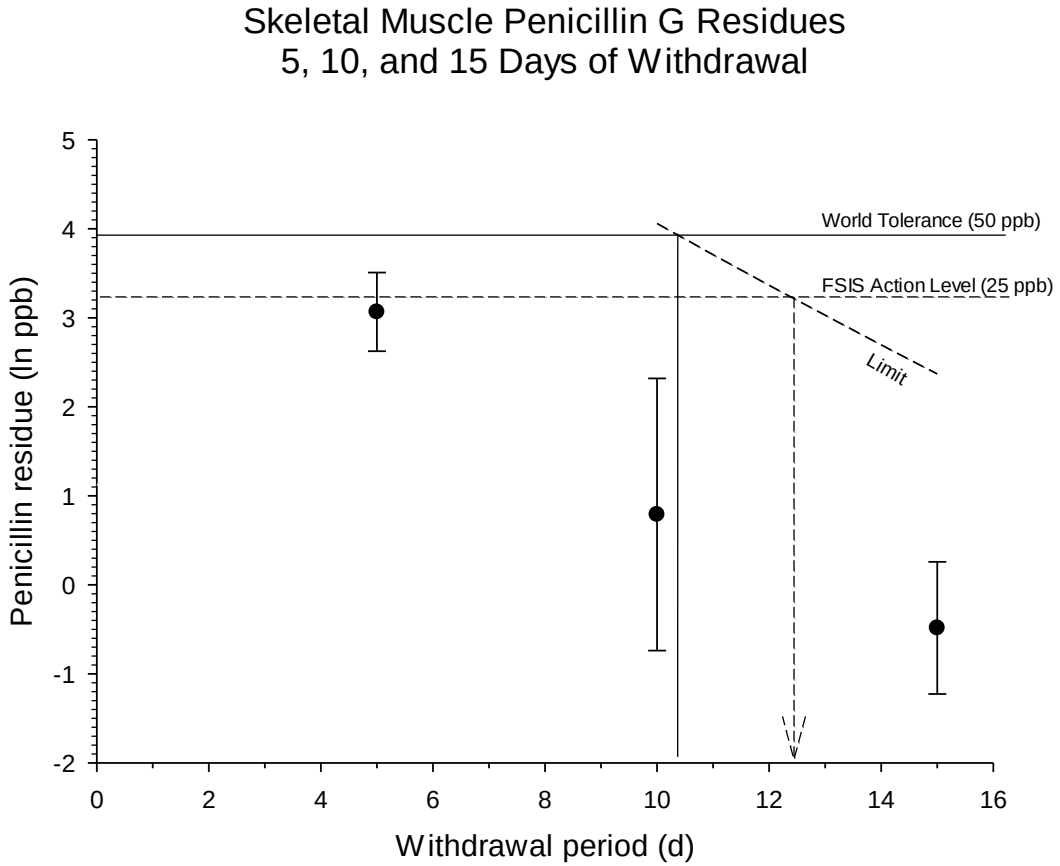

Figure 10. Estimation of pre-slaughter withdrawal period in heavy sows treated with a 5x dose of penicillin-G procaine, based on skeletal muscle penicillin-G residue depletion. Data taken from the linear portion of the depletion curve were analyzed as described by FDA Guidance Document no. 3 (US FDA CVM, 2006). In contrast to US FDA guidance, 15-d withdrawal data below the method LOD were assigned values of ½ the LOD (CMVP, 1995). Muscle penicillin-G concentrations that were between the LOQ and LOD were included in the analysis as their nominal concentration. The residue tolerance used by most of the world (50 ppb or ln ppb 3.91) crossed the 99th percentile with a 95th percent confidence interval tolerance limit at approximately 10.5 days (solid vertical arrow). The skeletal muscle withdrawal period for a 50 ppb tolerance was established at 11 days after rounding up to the nearest whole-day. In the United States, there is no tolerance established for penicillin G in swine tissues, but FSIS has established an action limit of 25 ppb for penicillin residues detected in swine tissues (FSIS, 2013). In order for penicillin residues to deplete below the action limit of 25 ppb in a population of swine, just over 12 days would be required; rounding up to the nearest whole-day, the withdrawal period would be 13 days.

Appendix 1. Sow diets (NDSU SW-50) fed on a restricted basis (2 kg/animal per day).

Ingredient	lb/used	Percentage
Corn, grd	1592.00	79.6
Soybean meal (46.0%)	271.00	13.5
Malt sprouts	100.00	5.0
Mono-cal, 21%	1.90	0.1
Limestone	17.20	0.9
Salt	8.00	0.4
RalCo Sow	10.00	0.5
	2000.10	100.0