RESEARCH REPORT

INTERNATIONAL TRADE

pork checkoff

Title: Chlortetracycline, Oxytetracycline, Tetracycline and Bacitracin Tissue Residue Studies in Swine Conducted in Reference to Foreign Export Markets – **NPB #09-257**

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II. Industry Summary

The objective of this study was to describe tissue residues of chlortetracycline, oxytetracycline, and tetracycline in slaughter hogs and also bacitracin in cull sows. For the tetracyclines, the residues were monitored out to 28 days after ending the treatments. All analysis was carried out on a mass spectrometer representative of advanced equipment used for residue detection around the world. The methods used were developed in the analytical lab conducting this study, and are not necessarily equivalent to regulatory methods in any country. However, tissue concentration levels of detection and quantitation are similar to the most sensitive methods in use. In addition to mass spectrometry analysis, Charm II analysis was performed on muscle for all 5 treatment groups in the tetracycline portion of the study.

In the tetracycline category study, 5 groups of pigs received the following treatments.

1. Chlortetracycline in the water calculated to deliver 10 mg/lb body weight daily for 5 days
2. Oxytetracycline in the water calculated to deliver 10 mg/lb body weight daily for 5 days
3. Tetracycline in the water calculated to deliver 10 mg/lb body weight daily for 5 days
4. Chlortetracycline in the feed calculated to deliver 10 mg/lb body weight daily for 14 days
5. Oxytetracycline in the feed calculated to deliver 10 mg/lb body weight daily for 14 days

Within each treatment, 5 pigs were euthanized and samples collected at the time that drug administration was stopped (day 0), and at 7, 14, 21, and 28 days following the end of drug administration. The timing of drug administration resulted in the first samples being collected 28 days prior to the projected finish date of the pigs. Weights on the first sample date were approximately 200 lbs, and were approximately 270 lbs on day 28.

Samples collected included muscle, liver, kidney, fat, colon contents (at 21 and 28 days only), bone (21 and 28 days only), plasma, and urine. Detailed results are reported for each tissue in the final report. For the purposes of this summary, emphasis will be placed on muscle. For all of the tetracyclines in the study, all tissues displayed a dramatic drop in concentrations from day 0 to day 7. The discussion here focuses on days 7 through 28. Results are discussed in parts per billion (ppb) which is equivalent to nanograms per gram (ng/g). A nanogram is 1 billionth of a gram.

Results are discussed in light of the level of detection (the concentration at which we are sure the compound is there, but cannot reliably quantify it) and level of quantitation (the concentration at which we can reliably say how much is present). These two levels are abbreviated as LOD and LOQ, respectively. Half-lives will also be discussed, which is the time required for the concentration of a drug in a tissue to decrease by one half.

Chlortetracycline results: (LOD 1 ppb, LOQ 4 ppb in muscle) At day 7, muscle residues ranged from 11.8 to 29.0 ppb in the feed group and from 5.8 to 13.0 ppb in the water group. At 28 days withdrawal time, the feed group residues ranged from 4.7 to 12.0 ppb. At 28 days, the pigs in the water group displayed detectable residues only in 2 pigs, and concentrations ranging from 4.9 to 6.0 ppb in the other 3 pigs. The elimination half-life calculated in muscle after feed administration was 18.0 days, explaining the similar concentrations in the muscle at 7 and 28 days. Due to the

number of detected but non-quantifiable concentrations in the water group, a tissue withdrawal for muscle could not be calculated, but calculated withdrawal times for liver and kidney were very similar to the feed group, suggesting muscle would also be similar.

Very low plasma concentrations were detected at 28 days in 3 of 5 pigs in the feed group, but none of the pigs in the water group. Significant concentrations could still be detected in the urine at 28 days in all 4 pigs from which a urine sample could be collected in the feed group, and 3 pigs in the water group (28 to 397 ppb).

Concentrations in bone suggest a significant reservoir of chlortetracycline contributing to these persistent low concentrations in other tissues. At 28 days, chlortetracycline concentrations in bone ranged from 375 to 1120 ppb in the feed group (100 to 766 ppb in the water group), which were very similar to the concentrations found at 21 days for both groups. No chlortetracycline could be detected in colon contents at 28 days in either the feed or water groups. Pen floor manure samples taken on the day that drug administration ceased for both groups were found to contain chlortetracycline ranging from 163,103 to 274,903 ppb on a dry matter basis.

Charm II analysis of muscle samples for chlortetracycline (LOD reported as 100 ppb) identified all 0 withdrawal time samples in both the feed and water groups as suspect (161.0 to 545.0 ppb). No other residues at withdrawal times 7 to 28 days were identified in muscle (maximum residue of 29.0 ppb).

Oxytetracycline results: (LOD 1 ppb, LOQ 4 ppb in muscle) At day 7, muscle residues ranged from 10.0 to 15.7 ppb in the feed group and from 4.5 to 18.8 ppb in the water group. At 28 days withdrawal time, the feed group residues ranged from 4.7 to 9.9 ppb. At 28 days, the pigs in the water group displayed detectable residues only in 4 pigs, and a concentration of 5.1 ppb in the fifth pig. The elimination half-life calculated in muscle after feed administration was 31.6 days. Due to the number of detected but non-quantifiable concentrations in the water group, a tissue withdrawal for muscle could not be calculated.

Low plasma concentrations were detected at 28 days in 4 of 4 pigs in the feed group (one pig died during the study), and all 5 pigs in the water group. Significant concentrations could still be detected in the urine at 28 days in 2 pigs from which a urine sample could be collected in the feed group and 4 pigs from which urine could be collected in the water group (8.9 to 526 ppb).

Bone samples retained significant residues at 28 days for all pigs in both the feed and water groups (12.7 to 117 ppb). In the feed group, colon contents at 28 days were not detected in one pig and ranged from 404 to 713 ppb in 3 other pigs. Colon contents at 28 days in the water group displayed nondetectable (2 pigs), detectable (2 pigs) and quantifiable (276 ppb) residues.

Pen floor manure samples taken on the day that drug administration ceased ranged from 593,857 to 1,400,000 ppb on a dry matter basis.

Charm II analysis of muscle samples for oxytetracycline (LOD reported at 100 ppb) identified no residues in the feed and water groups as suspect (maximum residue of 223.0 ppb).

Tetracycline Results: (LOD 0.5 ppb, LOQ 4 ppb in muscle) There was only a water administration group for tetracycline. At day 7, muscle concentrations ranged from 16.6 to 24.1 ppb. At day 28, muscle concentrations ranged from 12.5 to 26.9 ppb. The muscle elimination half-life for tetracycline was calculated at 118.5 days, with kidney being similar at 92.7 days. Plasma concentrations were detectable in all 5 pigs at day 28, as were concentrations in the urine. Concentrations in colon contents were only detected in 1 of the 5 pigs at day 28. Bone concentrations ranged from 44.6 to 75.0 ppb at 28 days, which were very similar concentrations to the 5 pigs on day 21.

Charm II analysis of muscle samples for tetracycline (LOD reported at 25 ppb) identified all 0 withdrawal time samples as suspect (161.0 to 899.0 ppb). Four other samples were identified as suspect on days 7-21 (18.0 to 36.2 ppb), with all other samples on these days determined as negative (9.8 to 26.9 ppb).

Results summary for the tetracyclines: Muscle tissue analyzed from chlortetracycline, oxytetracycline, and tetracycline treatment groups maintained concentrations well below the U.S. tolerance (1000 ppb) and some foreign export maximum residue limits (MRLs, 50 ppb) out to 28 days. The concentrations were high enough that they could be detected and quantified by advanced analytical techniques, which could pose trade issues in markets where detected, or very low, quantifiable residues are used as a regulatory standard. Bone samples displayed prolonged residues, which may be a major component of the persistent, low-level residues demonstrated in this study. Colon contents and urine displayed quantifiable residues for all treatment groups in at least some pigs at 21 and 28 days withdrawal depending on the group. The high initial manure concentrations at 0 withdrawal time, and the persistent manure and urine contributions to the pen floor environment, could contribute to recycling of drug in environments where manure consumption is possible.

In the hands of this laboratory, and in incurred samples (residues in the muscle from drug given to the animal rather than added in the lab), the chlortetracycline and tetracycline CHARM II assays detected residues at or near the stated level of detection. The oxytetracycline CHARM II assay failed to detect oxytetracycline residues up to 223 ppb with a stated level of detection of 100 ppb.

Bacitracin results: Bacitracin was fed to sows at a rate of 750 g/ton in 5 lbs of feed daily for 2 weeks. Residues were detected (LOD 38 ppb) in one muscle sample at 0 days withdrawal and two samples at 7 days withdrawal. Multiple samples had detected or quantifiable concentrations in the liver at 0 and 7 days withdrawal (LOD 97 ppb, LOQ 292 ppb).

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III. Keywords

Chlortetracycline, Oxytetracycline, Tetracycline, Bacitracin, Swine, Residues, Meat, Kidney, Liver, Fat, Manure, Bone, Urine

IV. Scientific Abstract

The objective of this study was to describe tissue residues of chlortetracycline, oxytetracycline, and tetracycline in slaughter hogs and also bacitracin in sows.

In the tetracycline category study, 5 groups of pigs received the following treatments. (1) Chlortetracycline in the water calculated to deliver 10 mg/lb body weight daily for 5 days, (2) Oxytetracycline in the water calculated to deliver 10 mg/lb body weight daily for 5 days, (3) Tetracycline in the water calculated to deliver 10 mg/lb body weight daily for 5 days, (4) Chlortetracycline in the feed calculated to deliver 10 mg/lb body weight daily for 14 days, and (5) Oxytetracycline in the feed calculated to deliver 10 mg/lb body weight daily for 14 days. Bacitracin was fed at 750 g/ton in 5 lbs of feed daily to the cull sows.

For the tetracyclines, the residues were monitored at 0, 7, 14, 21, and 28 days after ending the treatments. The pigs weighed approximately 91 kg on day 0, and 123 kg on day 28. Bacitracin was monitored at 0 and 7days withdrawal. Samples collected included muscle, liver, kidney, and fat. Additional samples in the tetracycline groups included plasma, urine, colon contents (at 21 and 28 days only), and bone (21 and 28 days only), Residue analysis was by HPLC/MS/MS using an API 4000 detector. Assays were developed in the study laboratory and are not approved regulatory methods.

Results for the tetracyclines: All edible tissues rapidly decreased in residue concentrations from withdrawal day 0 to withdrawal day 7. Concentrations in the muscle at 28 days withdrawal time for chlortetracycline, oxytetracycline, and tetracycline ranged from detectable to 12.0 ppb, detectable to 9.9 ppb, and 12.5 to 26.9 ppb respectively. Calculated muscle residue elimination half-lives from withdrawal day 7 to withdrawal day 28 were 18.0, 31.6, and 118.5 days for chlortetracycline, oxytetracycline, and tetracycline respectively.

Bone samples displayed prolonged residues, which may be a major component of the persistent, low-level residues demonstrated in this study. Bone concentrations were detected in all samples for all treatment groups at 28 days, with the highest being chlortetracycline, ranging from 375 to 1120 ppb. Colon contents and urine displayed quantifiable residues for all treatment groups in at least some pigs at 21 and 28 days withdrawal depending on the group. The high initial manure concentrations at 0 withdrawal time, and the persistent manure and urine contributions to the pen floor environment, could contribute to recycling of drug in environments where manure consumption is possible.

Bacitracin results: Bacitracin was fed to sows at a rate of 750 g/ton in 5 lbs of feed daily for 2 weeks. Residues were detected (LOD 38 ppb) in one muscle sample at 0 days withdrawal and two

samples at 7 days withdrawal. Multiple liver samples had detected or quantifiable concentrations at 0 and 7 days withdrawal (LOD 97 ppb, LOQ 292 ppb).

V. Introduction

The ability to export pork is based on meeting the requirements of the importing jurisdiction, with the presence of very low residue concentrations being the basis for enforcement actions in some areas. Modern analytical techniques make it possible to detect very low residue concentrations, well below the tissue tolerances or maximum residue limits (MRLs) defined in some countries. This study was designed to describe the magnitude and duration of very low residue concentrations for the tetracyclines in feeder pigs and bacitracin in sows. By comparing multiple tissue residues resulting from differing drugs and routes of administration, the results enable an understanding of residue characteristics of these regimens.

VI. Objectives

To determine the tissue residue characteristics, and therefore the appropriate slaughter withdrawal times, for drugs used in swine production. These studies will target withdrawal times appropriate for export markets.

VII. Materials and Methods

VII. a: Materials and Methods: Chlortetracycline, Oxytetracycline, and Tetracycline Residue Study

Concurrent study: A non-challenge vaccine performance study for a masters project was carried out concurrently in this group of pigs. Each treatment group for the residue study reported here was allocated such that each pen and treatment group contained an equal distribution of the vaccine study treatment groups. Slaughter dates were allocated among treatment groups such that vaccine treatment groups were equally represented on each slaughter day.

Test article administration methods – Chlortetracycline and Oxytetracycline Feed medication study:

The feed test articles were administered to the pigs starting on Thursday, April 1, 2010 and were terminated on Thursday, April 15th, 2010, the same day as the first sample collection.

The oxytetracycline and chlortetracycline feed medications were prepared to provide a target of 10 mg/lb bodyweight per day and administered for 14 days. Chlortetracycline and oxytetracycline were included in the respective experimental rations at 507.7 g/ton (10.15# of product per ton). The products were Alpharma CTC 50 g/lb (CGA 90328) and Phibro Terramycin 50 g/lb (N63083745XA). This inclusion rate was based off of starting with 165# pigs (weighed the week prior to starting the medicated diets) eating 6.5# of feed per day, gaining 2.5# per day, and ending at 7# of feed per head per day.

Samples of feed were taken every-other-day to allow for confirmation of feed concentrations and also to confirm correct placement of feed. Samples of the test diets were provided to Alpharma for analysis.

Appendix A contains a history of oxytetracycline use in the feedmill in relation to diets delivered to the bin for this room prior to and during the study. The preparation of the test diets was the only use of chlortetracycline in the feed mill in the calendar year prior to the study and in the period from diet preparation to study conclusion.

No antimicrobial-medicated feeds had been used in the bins or feeding system of the room housing the study pigs prior to this study, nor was medicated feed placed in the feeding system during the study; the facility was approximately 1.5 years old at the time of the study. At the start of the feed portion of the study, the feeding system was shut off to all study animal feeders and the feeders were scooped out followed by final cleaning with a vacuum. Medicated feed was hand fed by the use of bagged feed. The bagged feed was transported to a pen, the tag was checked against the study group designation above the feeder, and the feed was then placed in the feeder. The feed tags were retained in the records with the number of the pen in which they were dispensed, the date of dispensing, and the initials and date of the principal investigator. Medicated feed was present in the feeders for the entire 14 days of the study.

At the end of the feed study, all medicated feed had been dispensed into feeders, and the remainder was removed from the feeders by scoop, followed by a final cleaning with a vacuum. All treatment groups had feed left at the end of the feed administration period. The feed system was then turned back on to each study feeder.

Test article administration methods – Chlortetracycline, oxytetracycline, and tetracycline water medication study:

Water test articles:

Chlortetracycline: Aureomycin Chlortetracycline Soluble Powder Concentrate (Fort Dodge, Lot 080575, Exp 5-11)

Oxytetracycline: Oxytet Soluble Oxytetracycline Hydrochloride (Alpharma, TTH00015, Exp 02/15)

Tetracycline: Tet-Sol 324 Tetracycline Hydrochloride Soluble Powder (Alpharma, TBH00001, Exp 01/15)

The gravity flow water system to allow dosing of the water medications was constructed by the primary investigator and installed to provide water through the cup waterers already installed in the finishing barn (Figure 1). The system was constructed specifically for this study, consisting of new 65 gallon plastic tanks and new, commercially available flexible plastic pipe, clamps, and fittings. The lines from the main water line to the waterers were disconnected and connected to

the test system at the start of the water study and reconnected to the house water system at the completion of drug administration.

The water medications were calculated to provide 10 mg/lb bodyweight per day and were administered for 5 days. Water consumption was estimated for the day preceding the start of the study and this consumption was used to calculate the water inclusion rates for the first day. The initial consumption estimate was in excess of the actual consumption during test article administration, resulting in a low first day dose, but adjustments then brought consumptions within +/- 15% of the 10 mg/lb per day target except for one day for each of two treatments, which were estimated at 8.2 and 8.5 mg/lb (Table 1).

Table 1: Estimated mg/lb dosing by treatment and study day.

Date	Pig weight (lbs estimated)	g active per gallon	Estimated consumption (total gal)	Gallons of water/pig	g active/pig	Estimated mg/lb
Chlortetracycline						
4/10/2010	183	0.763	42	1.7	1.282	7.0
4/11/2010	185	0.953	44	1.8	1.676	9.1
4/12/2010	187	1.039	45	1.8	1.870	10.0
4/13/2010	189	1.016	39	1.6	1.585	8.4
4/14/2010	191	1.120	42	1.7	1.882	9.9
Oxytetracycline						
4/10/2010	183	0.763	50	2.0	1.526	8.3
4/11/2010	185	0.954	40	1.6	1.526	8.2
4/12/2010	187	1.145	39	1.6	1.786	9.5
4/13/2010	189	1.174	44	1.8	2.066	10.9
4/14/2010	191	1.166	42	1.7	1.958	10.3
Tetracycline						
4/10/2010	183	0.763	45	1.8	1.373	7.5
4/11/2010	185	0.952	42	1.7	1.600	8.6
4/12/2010	187	1.089	45	1.8	1.959	10.5
4/13/2010	189	1.016	40	1.6	1.626	8.6
4/14/2010	191	1.128	45	1.8	2.031	10.6

On study days 2, 3, and 4, the previous day water consumption was used to calculate the inclusion rate. On study day 5 an average of 3 previous days was used for each group.

New medication batches in water were mixed each day in plastic tanks with adjustment in concentration based on revised water intake estimates. Samples of water were taken immediately after mixing to allow for confirmation of water concentrations.

Figure 1: Water medication system

After the first day of water medication administration, the tanks were drained each morning after recording the approximate remaining volume. Approximately 20 gallons was then added to each tank and then allowed to drain again. The chlortetracycline, oxytetracycline, or tetracycline product amount to be added was then pre-mixed in a 1 gallon ziplock bag prior to introduction into the tank. This was added to the tank after approximately 20 gallons of the 65 gallon total fill was reached. The additional 45 gallons were added to top off the tank and mix the product.

Sample collection:

The harvest dates for the study pigs were April 15th, April 22nd, April 29th, May 6th, and May 13th, resulting in withdrawal times of 0, 7, 14, 21, and 28 days respectively. Samples of muscle, fat, liver and kidney were collected in whirlpack bags and frozen at -80° C until analysis. Plasma samples were collected post-mortem from all pigs. Samples of urine from the bladder were collected whenever present, resulting in multiple missing samples due to animals with no urine present. Feces from the colon and bone from the femur were collected at the last two sample times.

Composite manure samples from the pen floor were collected from each pen on withdrawal day zero. These samples consisted of 3 – 5 different fecal locations in the pen, with emphasis placed on collecting fresh feces. In some cases, drier, compacted feces was scraped from the pen floor for collection.

Mass Spectrometry Analytical Methods for muscle, liver, kidney, and fat:

Tissue preparation and extraction: Samples were prepared by placing 5 g of minced pork tissue in a 50-ml centrifuge tube and homogenizing with a Polytron homogenizer. A 0.5-g sample of homogenate was weighed into a 15-ml centrifuge tube. The internal standard, demeclocycline, was then added (50µl of a 1,000 ng/ml solution; 100 ng/g final concentration). One ml of 0.4 M oxalate buffer, pH 4.0, was added, followed by 1 ml of 5% trichloracetic acid (v/v). After vortexing, tubes were centrifuged for 15 min. at 3,400 x g. The supernate was loaded on a methanol & water-conditioned Varian Nexus C18 3cc, 60 mg column, washed with 5% methanol, and eluted with 100% methanol. The extract was dried and reconstituted in 200 µl 0.01 M oxalic acid:acetonitrile 88:12 and placed in vials for LC/MS analysis.

Standards and Controls: Blank pork tissue was prepared in the same manner as samples and spiked with 50 µl of 10X spiking solutions of the tetracycline that was to be analyzed. A fresh (same day) 1 mg/ml solution of the compound was prepared and diluted in methanol to spiking concentrations that yielded concentrations of 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 ng/g in tissue. The internal standard was also prepared as a 1 mg/ml solution in methanol and diluted serially to obtain the 1,000 ng/ml spiking solution. Quality Control (QC) samples were prepared in the same way as standards and spiked at final concentrations of 8 and 64 ng/g in tissue. Extraction of standards, QC samples, blanks, and unknowns was carried out in an identical manner as described above. LOD and LOQ were determined by calculating the standard deviation of the analyte concentration found in a set of tissue blanks and multiplying by 3. Then LOQ = 3*LOD. The standard curves for each of the treatments are contained in Appendix B.

HPLC Conditions: A Waters XBridge C18 HPLC column, 5 µ, 2.1 x 50 mm, columns was eluted with a 12 to 30% mobile phase B gradient (A: 0.01 M oxalic acid; B: 100% acetonitrile) at a flow rate of 0.35 ml/min over 6 minutes, achieving baseline separation of the three tetracyclines and the internal standard demeclocycline.

Mass spec. conditions: The API 4000 mass spectrometer was operated in electrospray positive mode, optimized for each of the four compounds, scanning the following precursor/product transitions: chlortetracycline: 478.5>443.8, oxytetracycline: 461.7>427.1, tetracycline: 444.4>410, and demeclocycline: 465.1>289.1. Quantitation was performed using the Analyst 1.5 software.

CHARM II Analytical Methods for muscle

CHARM II testing was performed the same day as for the mass spectrometry analysis. The only difference in methodology from the CHARM II manual was that the series of samples were incubated in a water bath set at the standard temperature as opposed to the supplied heat block. This was due to the need to put 25 samples through the system in the same time frame as extraction of the samples for mass spectrometry analysis. This alteration in procedure was validated to demonstrate that the results were consistent with using the supplied heat block.

The standard CHARM II method for tetracyclines in pork muscle as described in the manufacturer's directions was used. In order to provide a direct comparison with HPLC-Mass Spectrometry results, however, the following modifications were incorporated:

1. Muscle tissue homogenate from the same sample as used for Mass Spec analysis was used.
2. The extraction amounts were scaled down 4X from 30 ml/10 g to 7.5 ml/2.5 g, but the same amount of tissue was analyzed as in the food processor extraction.
3. The 80^{o}C incubation of the diluted homogenate was carried out with a covered water bath set at 80^{o}C rather than the heater block to facilitate timely processing of multiple samples. A 30-minute incubation was found to give the same result as a 45-minute incubation.

Tissue preparation and extraction: Samples were prepared by placing 5 g of minced pork tissue in a 50-ml centrifuge tube and homogenizing with a Polytron homogenizer. For extraction for mass spectrometric analysis, a 0.5-g sample of homogenate was weighed into a 15-ml centrifuge tube. For extraction for the CHARM II assay, 2.5 g of the homogenate was placed in a separate 15-ml tube. The following procedure was then used for the CHARM II assay on these samples:

Sample Prep

1. Homogenize a 5-g sample and weigh out 2.5-g into a 15 ml tube.
2. Add 7.5 ml of MSU Extraction Buffer, replace cap, and vortex.
3. Incubate at 80°C in water bath for 30 minutes.
4. Place tube in ice water bath for 10 minutes.
5. Centrifuge for 10 minutes at 3400 rpm.
6. Pipette off the top layer (approximately 5 ml) into a 15 ml tube, avoiding fat particles.
7. Check pH with pH indicator strips. Extract should be equivalent to 7.5.
8. If pH is low, add 1-2 drops (using a transfer pipet) of M2 Buffer; vortex and retest.
9. Pipet 3 ml of extract into a new 15 ml tube and add 3 ml of prepared negative control. Vortex 30 seconds.

Assay*

1. Add white tablet to empty glass test tube.
2. Add 300 µl of water. Vortex 10 seconds to break up tablet.
3. Add 4.0 ml of extract.
4. Add orange tablet and immediately vortex by swirling sample up and down 10 times for 10 seconds.
5. Incubate at 35 °C for 5 minutes.
6. Centrifuge for 5 minutes at 3300 rpm.
7. Immediately pour off extract to avoid the pellet from sliding out with it. Blot edge of test tube on towel.
8. Add 300µl water. Vortex 10 seconds to break up pellet.
9. Add 3.0 ml scintillation fluid. Cap test tube. Vortex for 5 seconds or until mixture has a uniform cloudy appearance.
10. Count in Analyzer for 60 seconds.

*All instrument settings (control points, etc.) were set according to CHARM Sciences protocol for the CHARM II Assay.

Extraction method for colon contents (feces) and manure from pen floors:

Because of the variability in moisture content among samples of feces and manure, a dry weight to wet weight ratio was obtained. One g of each sample was weighed for extraction into a 15-ml conical tube and a second gram (or lesser exact amount) was weighed into an aluminum weigh boat. The samples in the weigh boats were dried in an oven at 100-105^{o}C for 48 hours in a fume hood, cooled, and weighed again to obtain the dry weight ratio. The final result of analysis was divided by this ratio to normalize all values to a dry weight basis.

The extraction protocol was adapted from separate methods described by Arikan, Blackwell, and O'Connor. An organically raised, untreated pig was the source for the feces as the blank matrix for standards, QC samples, and blanks. Samples were spiked with 50 µl of demeclocycline internal standard, 1,000 ng/ml in methanol, for all standards, samples, and blanks, except the double blank. For spiking standards, 50 µl of a mix of chlortetracycline, oxytetracycline, and tetracycline pure standards was added. Four ml of McIlvaine-EDTA buffer, pH 4.0, was added and mixed, followed by sonication for 10 minutes. After centrifugation for 7 min at 3,000xg, the supernatant fraction was loaded on an Abs-Elut Nexus C18 cartridge (3 cc, 60 mg, Agilent #121031101) conditioned with 3 ml of methanol, 3 ml of water, and 2 ml of citric acid buffer, 0.04 M, pH 4.0. The column was washed with 3 ml water, then 3 ml of 5% methanol, dried 15-30 min with vacuum, and eluted with 3 ml of 100% methanol. A 0.5-ml volume of the citric acid buffer was added, mixed briefly, and the mixture was loaded on a Sep-Pak Plus Alumina N cartridge (1.2 ml, 1710 mg, Waters #WAT020510) fitted with a 3-ml syringe barrel and conditioned with 3 ml of methanol and 3 ml of the citric acid buffer. The column was washed with 1 ml of methanol and the entire volume was collected. The extracts were dried under nitrogen at 40^{o}C and reconstituted in 200 µl 10% acetonitrile in water and filtered through a nylon syringe filter, 13 mm, 0.2 µ (Fisher #09-720-5) into vials for LC/MS/MS analysis.

Extraction method for pig bone:

Methods used by Junior and Korner were utilized in the extraction procedure. An organically raised, untreated pig was the source for bone as the blank matrix for standards, QC samples, and blanks. Bone samples were cleaned thoroughly and mid-femur cross-sections ½ to 1" in width were cut with a power saw. Membranes and marrow were removed and the sections were placed in a stainless steel bowl and covered with liquid nitrogen, allowing 3-4 minutes for cooling. The sections were then crushed by repeated mechanical impact until nearly complete fragmentation was achieved. The crushed sample was passed through a 5-mm mesh and 2 g of this preparation was weighed and stored at -20^{o}C until extraction. All equipment was washed with water and methanol between samples to prevent any potential carryover.

Samples were spiked with 50µl of demeclocycline internal standard, 1,000 ng/ml in methanol, for all standards, samples, and blanks, except the double blank. For spiking standards, 50 µl of a mix of chlortetracycline, oxytetracycline, and tetracycline pure standards was added. One ml of McIlvaine-EDTA buffer, pH 4.0, and 3 ml of methanol were added, followed by 5 min of vortexing and 15 min on a rocker. After centrifugation for 6 min at 3,000xg, the supernatant fraction was dried under nitrogen at 40^{o}C to a volume of 1-1.5 ml. Three ml of water were added and mixed and the solution was loaded on an Abs-Elut Nexus C18 cartridge (3 cc, 60 mg, Agilent #121031101) conditioned with 3 ml of methanol and 3 ml of water. The column was washed with 3 ml water, 3 ml of 20% methanol, dried 10 min under vacuum, and eluted with 3 ml of 100% methanol. The extracts were dried under nitrogen at 40^{o}C and reconstituted in 200 µl 10% acetonitrile in water and filtered through a nylon syringe filter, 13 mm, 0.2 µ (Fisher #09-720-5) into vials for LC/MS/MS analysis.

Extraction method for pig plasma:

The method of Cheng was adapted for the extraction. An organically raised, untreated pig was the source for plasma as the blank matrix for standards, QC samples, and blanks. A 0.5-ml volume of plasma was spiked with 25 µl of demeclocycline internal standard, 1,000 ng/ml in methanol, for all standards, samples, and blanks, except the double blank. For spiking standards, 25 µl of increasing concentrations of a mix of chlortetracycline, oxytetracycline, and tetracycline pure standards was added. Then 20 µl of 50% phosphoric acid was added, followed by 3 min of vortexing . The solution was loaded on an Oasis HLB C18 cartridge (1 cc, 30 mg, Waters #WAT094225) conditioned with 1 ml of methanol and 1 ml of water. The column was washed with 1 ml of 5% methanol, dried 5 min with vacuum, and eluted with 1 ml of 100% methanol. The extracts were dried under nitrogen at 40^{o}C and reconstituted in 200 µl 10% acetonitrile in water and transferred into vials for LC/MS/MS analysis.

Extraction method for pig urine:

The method of Jin was adapted for the extraction. An organically raised, untreated pig was the source for urine as the blank matrix for standards, QC samples, and blanks. For all standards, samples, and blanks, two ml of urine were centrifuged 3 min at 3,000Xg and the supernatant fraction was spiked with 100µl of demeclocycline internal standard, 400 ng/ml in methanol. For spiking standards, 100 µl of increasing concentrations of a mix of chlortetracycline, oxytetracycline, and tetracycline pure standards was added. Then 200 µl of 5% EDTA and 100 µl of 1M HCl (final pH 4.0) were added, followed by 5 min of vortexing. The solution was loaded on an Abs-Elut Nexus C18 cartridge (3 cc, 60 mg, Agilent #121031101) conditioned with 3 ml of methanol, 2 ml of 0.5 M HCl, and 3 ml of water. The column was washed with 3 ml of water and 3 ml of 20% methanol , dried 10 min with vacuum, and eluted with 3 ml of 100% methanol. The extracts were dried under nitrogen at 40^{o}C and reconstituted in 200 µl 10% acetonitrile in water and filtered through a nylon syringe filter, 13 mm, 0.2 µ (Fisher #09-720-5) into vials for LC/MS/MS analysis.

Standards and controls for feces, manure, bone, and urine

Separate 1 mg/ml solutions in methanol of chlortetracycline, oxytetracycline, and tetracycline were prepared and combined to make a composite 100,000 ng/ml stock solution. This stock was diluted to 20X spiking solutions that yielded final concentrations in the matrix of 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 ng/ml (or higher as required). When analyte levels exceeded the range of this set of standards, extracts were diluted and higher range standards were prepared. The internal standard demeclocycline was prepared by dilution of a 1 mg/ml solution in methanol to a 20X 400 ng/ml solution, yielding 20 ng/ml in urine and dilution to a 20X 1,000 or 2,000 ng/ml solution yielding 50 ng/g in feces/manure and bone matrices and 50 ng/ml in plasma. QC samples were prepared in the same way as standards and spiked at final concentrations appropriate to the analyte level. Extractions of standards, QC samples, blanks, and unknowns were carried out in an identical manner as described for each matrix. The LOD was determined by calculating the standard deviation of a low-level analyte concentration found in a set of spiked blanks, multiplying by 1.645, and adding the average blank value. Then the LOQ was the LOD plus 3 times that standard deviation.

Analytical conditions for feces, manure, bone, and urine

HPLC conditions: A Waters XBridge C18 HPLC column, 5 µ, 2.1 x 50 mm was eluted with a 12 to 30% mobile phase B gradient (A:0.1% formic acid in water; B: 0.1% formic acid in acetonitrile) at a flow rate of 0..35 ml/min over 4.5 minutes.

Mass spectrometry conditions: The API 4000 mass spectrometer was operated in electrospray positive mode, optimized with pure standards for each of the four compounds, scanning the following precursor/product transitions: chlortetracycline: 478.5>443.8, oxytetracycline: 461.7>427.1, tetracycline: 444.4>410, and demeclocycline: 465.1>289.1. Quantitation was performed using the Analyst 1.5 software.

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Detection of Epimers in Chlortetracycline Standards and Extracts

Pure standards of chlortetracycline (CTC) and epichlortetracycline (epi-CTC) were prepared individually in methanol and diluted to 100 ng/ml. Organic blank pork muscle was homogenized and weighed as 0.5-g samples and spiked at 500 ng/g final concentration. Selected pig muscle samples with incurred chlortetracycline in the 200-500 ng/g range (as analyzed previously) were also weighed as 0.5-g homogenates for extraction.

Chromatography was performed with a Shimadzu Prominence system using a Waters XBridge 2.1x50mm C18 column equipped with a Waters 4.6x20mm C18 Guard Cartridge and mobile phases of 0.01M oxalic acid and 100% acetonitrile in a 12 to 20% gradient of acetonitrile and a flow rate of 0.3 ml/min. Chlortetracycline elutes as a single peak in the 2.1-minute range. Detection of both the epi-CTC and the parent CTC was done by an API 4000 mass spectrometer using a transition of 478.5>443.8.

Separate injection of the pure standard CTC and the pure standard epi-CTC showed identical detection and identical elution times, within a few seconds. Injection of a mixture of CTC and epi-CTC pure standards at identical concentration also showed no significant difference in elution times. When extracts of blank muscle spiked at the 500 ng/g level were injected, there was only a single major peak in the elution profile. Finally, extracts of muscle incurred with CTC were analyzed and showed again only a single peak in the elution profile. No adjustment of the gradient was found that resulted in separation or identification of two separate peaks for CTC and epi-CTC.

Since workers who have reported on the measurement of epi-CTC in incurred samples rely upon differences in chromatographic properties and not mass (Blanchflower, 1997; Cherlet, 2003), it may be concluded that under our extraction and/or chromatographic conditions and equipment, the epimers of chlortetracycline are not distinguishable or detectable separate from the parent CTC in these extracted pig muscle samples. Thus, quantification of CTC in our system reports the total of all isomers of CTC as a single value.

Detection of Epimers in tetracycline Standards and Extracts

Additional work done with a tetracycline epimer in relation to the parent compound indicated that, as for chlortetracycline, there was neither chromatographic or transition separation between the parent compound and the epimer. It was concluded that the tetracycline concentrations reported in this study may be considered as representing both tetracycline and the primary epimer.

Oxytetracycline epimers were not evaluated in relation to these procedures.

VII.b: Materials and methods - Bacitracin Residue Study

Test article administration:

Sows were individually administered 5 lbs of feed daily for 14 days. The feed contained 750 mg /ton of feed added in the form of BMD30. Mixing was on a daily basis using a small mixer. Sows were fed starting with a random sow each day and then moving through the feeding progression.

Samples were submitted from 5 sows which were euthanized on the 14th day of feeding. An additional 5 sows were euthanized and samples were collected 7 days after cessation of bacitracin feeding.

Bacitracin extraction from tissue:

1. Weigh out 0.5g of homogenate in 15ml tube
2. Add 50µl of Polymyxin B I.S. Solution (5,000ng/ml)
3. Add 0.5ml of 4% TCA in Acetonitrile
4. Vortex 5 minutes
5. Add 2.5ml of 0.1% Formic Acid
6. Vortex Briefly
7. Centrifuge 5 minutes at 3900 xG
8. Condition Nexus 3cc column with 3ml of Methanol and 3ml of Water
9. Decant supernatant into column
10. Wash columns with 3ml of 2% Acetone
11. Dry columns with vacuum
12. Elute with 3ml of 0.2% Formic Acid:Methanol (3:7) into 15ml tube
13. Evaporate to dryness
14. Reconstitute with 200µl of 0.1% Formic Acid: Acetonitrile (95:5)

HPLC conditions: A Waters XBridge C18 HPLC column, 5µ, 2.1 x 50 mm, was eluted with a 50 to 95% mobile phase B gradient (A: 0.1% formic acid; B: 100% acetonitrile) at a flow rate of 0.3 ml/min for 7 minutes.

Bacitracin Mass Spectrometry Methods: The API 4000 mass spectrometer was operated in electrospray positive mode, optimized with pure standards for each of the compounds, scanning the following precursor/product transitions: bacitracin A: 712.2>199.1 and polymyxin B: 602.7>233.0. Quantitation was performed using the Analyst 1.5 software.

VIII.a: Results - Chlortetracycline, Oxytetracycline, and Tetracycline

While this study was not designed as a bioequivalence study, the zero withdrawal concentrations in muscle, kidney, and liver may be compared between routes of administration to give an idea of how the routes compare. Figures 2, 3, 4, and 5 report time zero muscle, kidney, liver, and plasma concentrations.

Figure 2: Zero withdrawal concentrations in muscle by treatment. (CTC = chlortetracycline, OTC = oxytetracycline, TC = tetracycline)

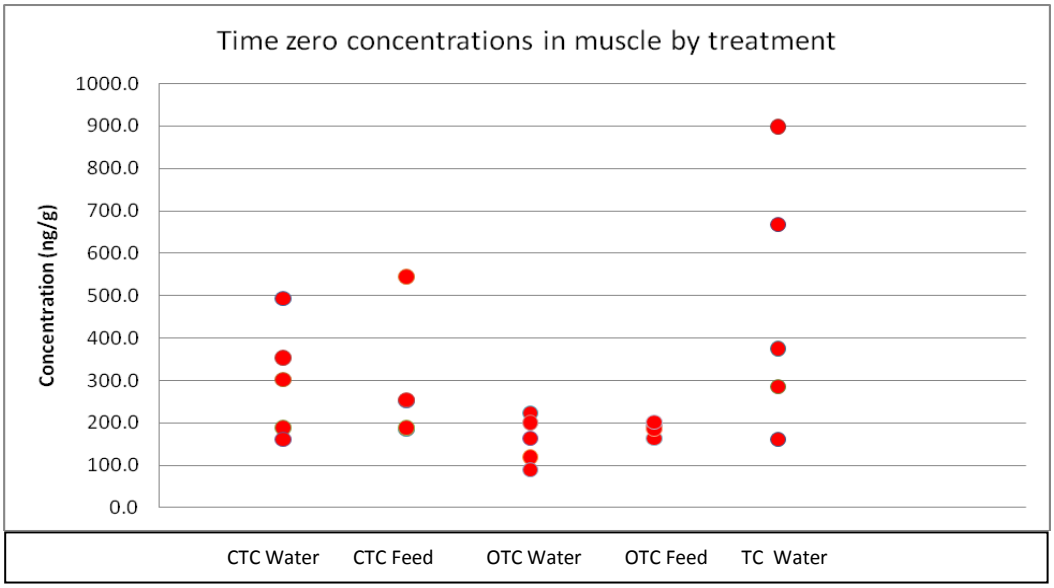


Figure 3: Zero withdrawal concentrations in kidney by treatment. (CTC = chlortetracycline, OTC = oxytetracycline, TC = tetracycline)

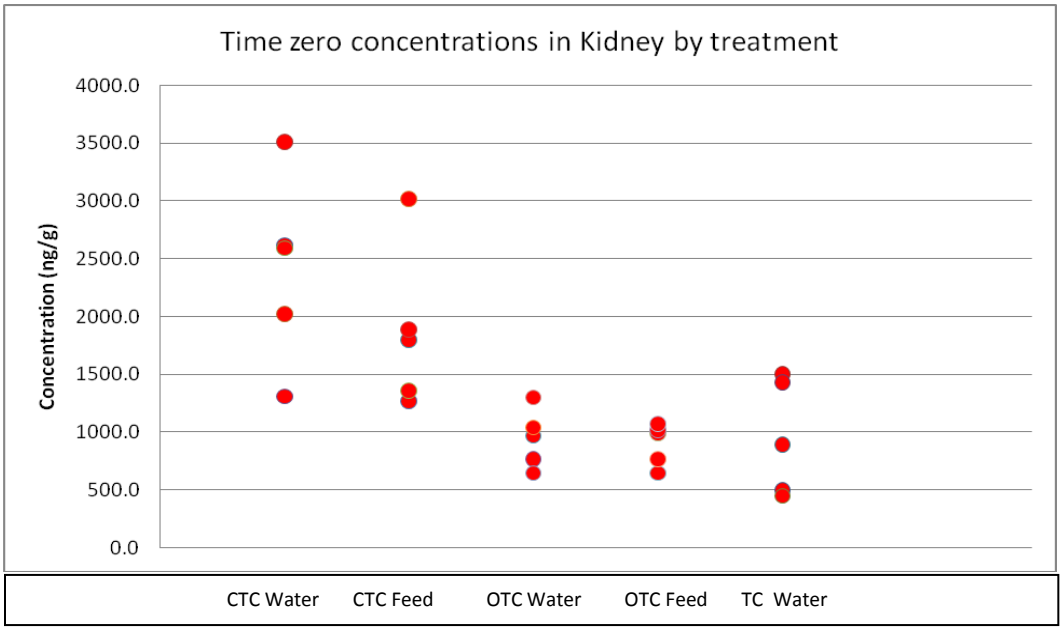


Figure 4: Zero withdrawal concentrations in liver by treatment. (CTC = chlortetracycline, OTC = oxytetracycline, TC = tetracycline)

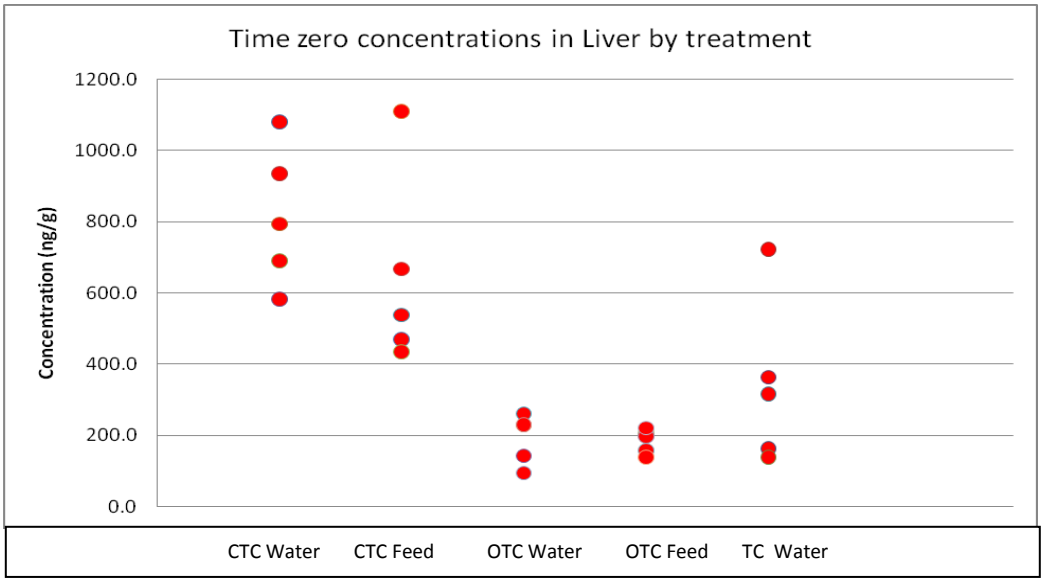
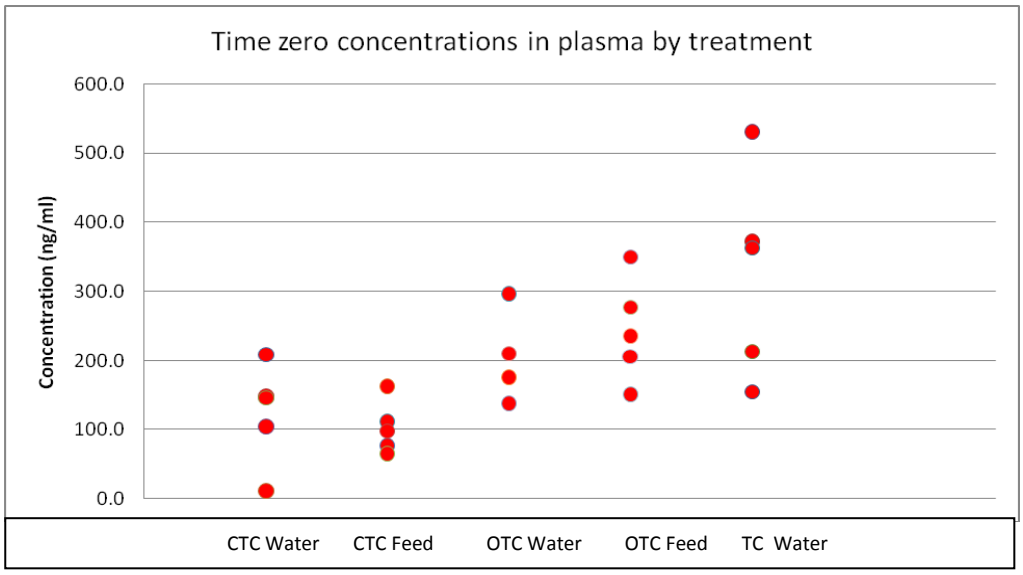


Figure 5: Zero withdrawal concentrations in plasma by treatment. (CTC = chlortetracycline, OTC = oxytetracycline, TC = tetracycline)



Calculated Tissue Elimination Half-Lives in edible tissues

Tissue concentrations on withdrawal days 7, 14, 21, and 28 were used to calculate elimination half-lives in muscle, liver, and kidney. It is possible in some cases that further evaluation beyond 28 days might indicate the regression analysis should be started at a later withdrawal day (e.g., day 14 or 21). While these estimated half-lives reflect the data out to 28 days as determined in this study, they may or may not reflect the behavior of drug residues in these tissues after 28 days. The liver half-lives for both oxytetracycline regimens were not calculated due to insufficient values above level of quantification. The muscle half-lives for chlortetracycline and oxytetracycline administered in the water were also not calculated due to the effect of detected but not quantifiable concentrations.

		Calculated Tissue Half-Life for Days 7-28 (Days)		
Drug	Regimen	Muscle	Kidney	Liver
Chlortetracycline	10 mg/lb per day in **feed f**or 14 days	18.0	19.6	24.7
Chlortetracycline	10 mg/lb per day in **water** for 5 days	Not Calculated	18.3	29.5
Oxytetracycline	10 mg/lb per day in **feed** for 14 days	31.6	47.8	Not Calculated
Oxytetracycline	10 mg/lb per day in **water** for 5 days	Not Calculated	23.9	Not Calculated
Tetracycline	10 mg/lb per day in **water** for 5 days	118.5	92.7	28.5

Important notes on presentation of results:

Individual tissue sample results are reported by drug and administration route with comparison to Charm II testing on muscle samples conducted on the same day as conduct of mass spectrometry analysis.

Results Section	Antimicrobial	Target Dose (mg/lb per day	Route of Administration	Duration of Administration
1	Chlortetracycline	10	Feed	14 days
2	Oxytetracycline	10	Feed	14 days
3	Chlortetracycline	10	Water	5 days
4	Oxytetracycline	10	Water	5 days
5	Tetracycline	10	Water	5 days

The Level of Quantitation (LOQ) and the Level of Detection (LOD) are reported for each tissue in each results table.

The results tables contain all individual tissue results. They are either reported as a quantified amount, detected (“D”, below the LOQ but above the LOD), or not detected (“ND”, below the LOD).

The mean value table following the main results table in each results section only reports a mean if there were at least two quantified results for that period.

The mean value chart in each results section only displays a value for a withdrawal day for a tissue if there were at least two quantified results. Zero withdrawal time values are not displayed in any of the charts in the report due to this initially very large concentration creating a very high Y axis value which results in trends at the later, lower concentrations being obscured. It is important to interpret the mean value chart in light of how many of the tissues for that withdrawal day had quantified concentrations. When not all tissues had quantified concentrations, but there were at least two, the value in the table (and therefore presented on the chart) represents only the mean of those quantified concentrations.

The scatter plots for muscle, kidney, and liver in each results section present a value for each quantified concentration. In some cases, the values fell between the LOD and the LOQ. In this case, a box spanning the "detected" value range is presented with the number of samples detected but not above the level of quantitation displayed in the box (highlighted in yellow). All samples for muscle, kidney, and liver resulted in either quantifiable or "detected" concentrations.

The concentrations detected in fat have not been presented in the mean tables, charts, or any of the individual tissue scatter plots due to the majority of findings being either "detected' or "not detected".

Table 2 - Chlortetracycline 10 mg/lb per Day in Feed for 14 Days

Chlortetracycline in feed at 10 mg/lb Per Day for 14 Days													
Tag	**Sex**	**Pen**	**Slaughter Date**	**Days**	**Muscle CHARM Result**	**Muscle MS (ppb)**	**Kidney (ppb)**	**Liver (ppb)**	**Fat (ppb)**	**Bone (ppb)**	**Colon (ppb)**	**Plasma (ppb)**	**Urine (ppb)**
Level of Detection (LOD)					100 ppb	1.0	1.0	0.3	0.1	N/A	19.1	1.5	5.6
Level of Quantitation (LOQ)					NA	4.0	8.0	8.0	8.0	32.0	32.0	2.0	8.0
61	G	7	4/15/2010		Suspect	185.0	1800.0	538.0	54.3			112.0	
62	B	6	4/15/2010		Suspect	545.0	3020.0	1110.0	543.0			163.0	
209	G	7	4/15/2010	0	Suspect	252.0	1270.0	469.0	14.2			77.5	
242	G	6	4/15/2010		Suspect	254.0	1890.0	667.0	36.0			98.4	
249	G	6	4/15/2010		Suspect	188.0	1360.0	434.0	26.4			65.6	1031
86	B	8	4/22/2010		Not Found	11.8	58.4	31.5	D			2.7	
135	G	6	4/22/2010		Not Found	15.5	92.4	43.6	D			5.8	
142	B	7	4/22/2010	7	Not Found	20.3	110.0	43.1	ND			7.5	
219	G	6	4/22/2010		Not Found	21.6	130.0	47.9	D			5.5	419
330	B	8	4/22/2010		Not Found	29.0	111.0	54.1	D			7.7	321
17	G	8	4/29/2010		Not Found	10.6	98.4	47.9	ND			4.9	
97	B	7	4/29/2010		Not Found	11.0	59.6	38.5	D			4.4	
217	G	8	4/29/2010	14	Not Found	11.0	86.3	22.2	D			3.8	252
256	B	7	4/29/2010		Not Found	10.7	59.9	32.0	D			5.5	340
303	G	8	4/29/2010		Not Found	16.1	70.8	24.4	D			5.3	253
16	G	6	5/6/2010		Not Found	8.5	41.8	31.6	D	710	ND	2.0	
146	G	7	5/6/2010		Not Found	15.7	94.9	58.8	D	1578	ND	5.1	
161	G	7	5/6/2010	21	Not Found	8.8	69.7	21.0	D	798	220	3.8	
234	G	7	5/6/2010		Not Found	12.3	90.7	36.4	D	812	ND	4.5	561
333	G	6	5/6/2010		Not Found	15.9	67.9	23.0	D	773		3.6	
156	G	8	5/13/2010		Not Found	12.0	67.9	32.8	D	1010	ND	3.9	
198	B	6	5/13/2010		Not Found	4.7	22.3	11.6	D	375	ND	ND	128
205	B	8	5/13/2010	28	Not Found	10.1	60.9	24.6	ND	671	ND	2.7	397
225	B	8	5/13/2010		Not Found	4.7	27.2	26.4	ND	442	ND	ND	37
283	B	6	5/13/2010		Not Found	9.5	59.8	23.2	D	1120	ND	2.3	371

Table 3: Mean edible tissue concentrations by days withdrawal after chlortetracycline in the feed at 10 mg/lb bodyweight for 14 days.

Chlortetracycline 10 mg/lb Per Day in Feed for 14 Days				
Withdrawal Days	Muscle mean (ppb)	Kidney mean (ppb)	Liver mean (ppb)	Fat mean (ppb)
0	284.8	1868.0	643.6	134.8
7	19.6	100.4	44.0	D
14	11.9	75.0	33.0	D
21	12.2	73.0	34.2	D
28	8.2	47.6	23.7	D

Figure 6: Mean edible tissue concentrations by days withdrawal after chlortetracycline in the feed at 10 mg/lb bodyweight for 14 days.

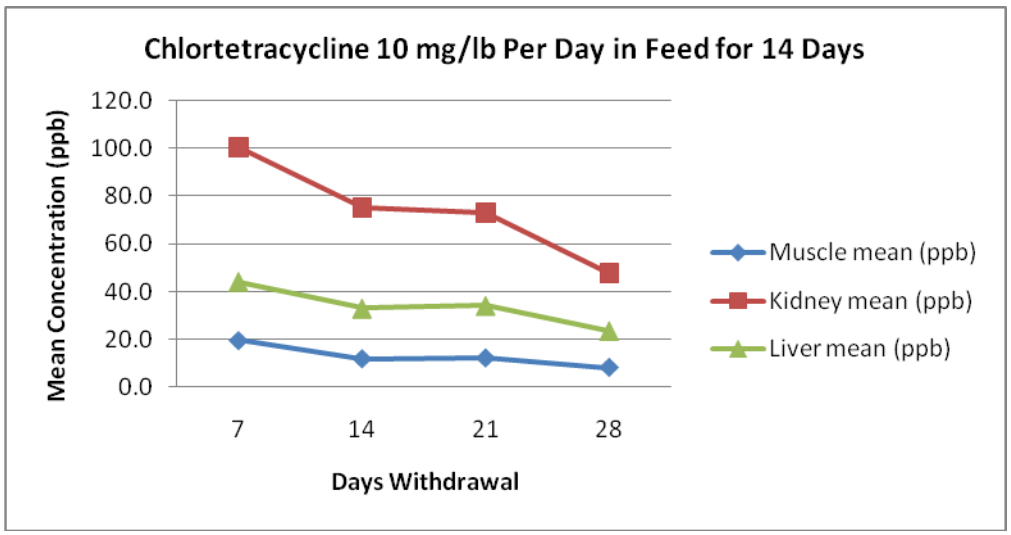


Figure 7:

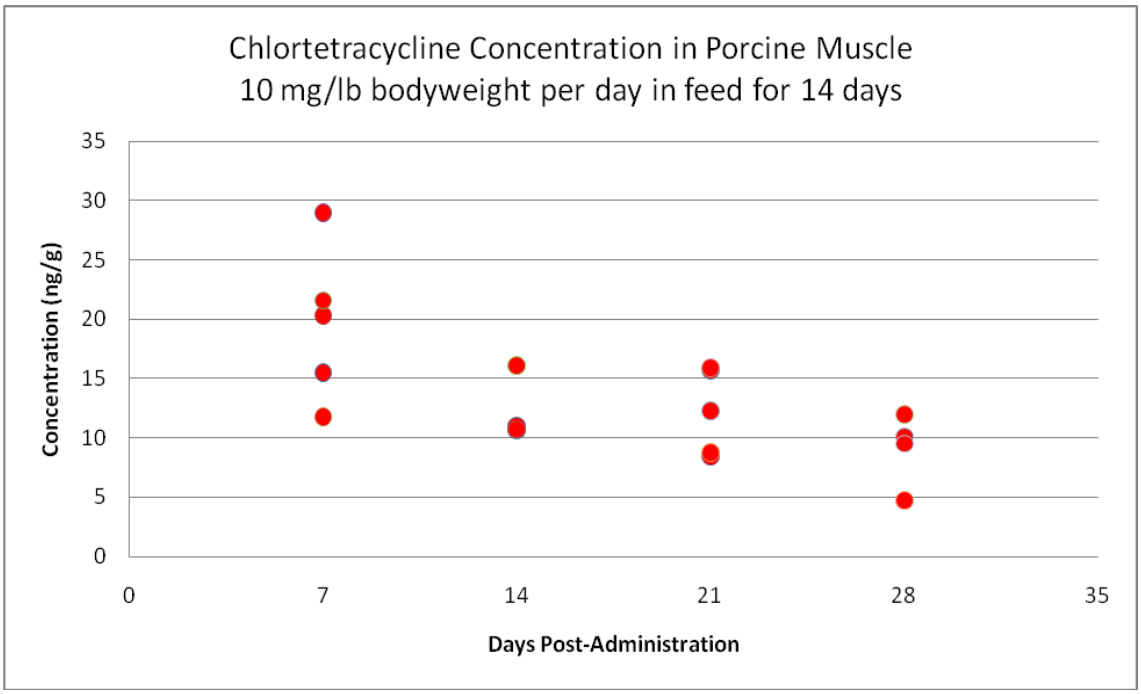


Figure 8:

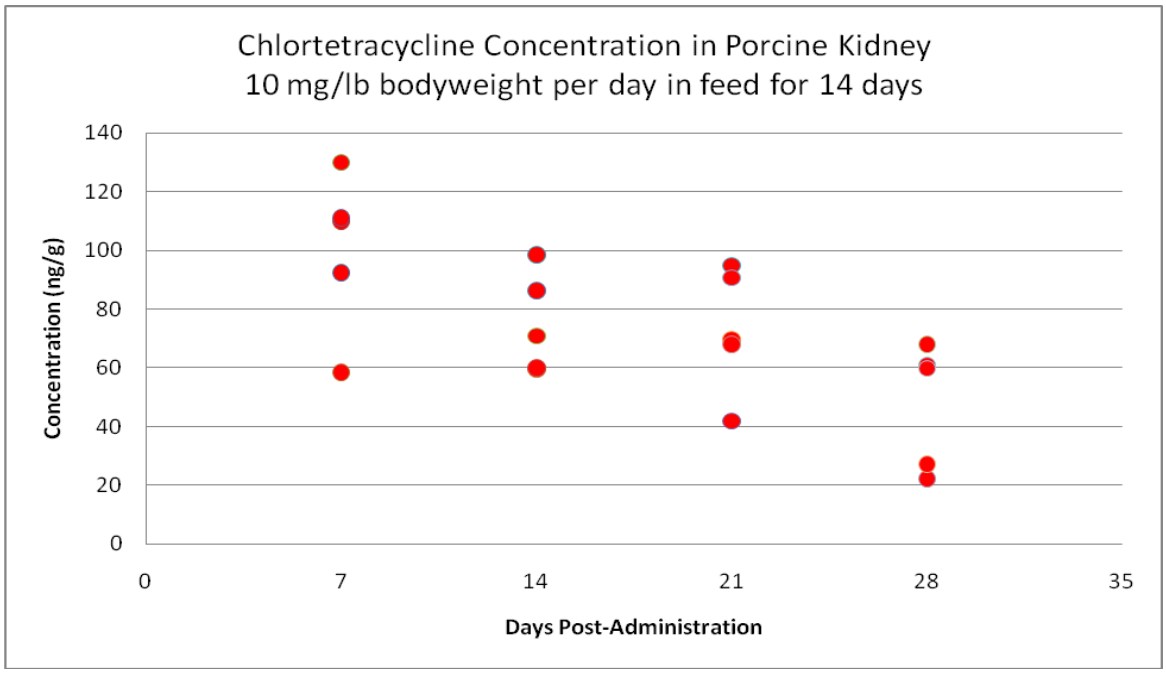


Figure 9:

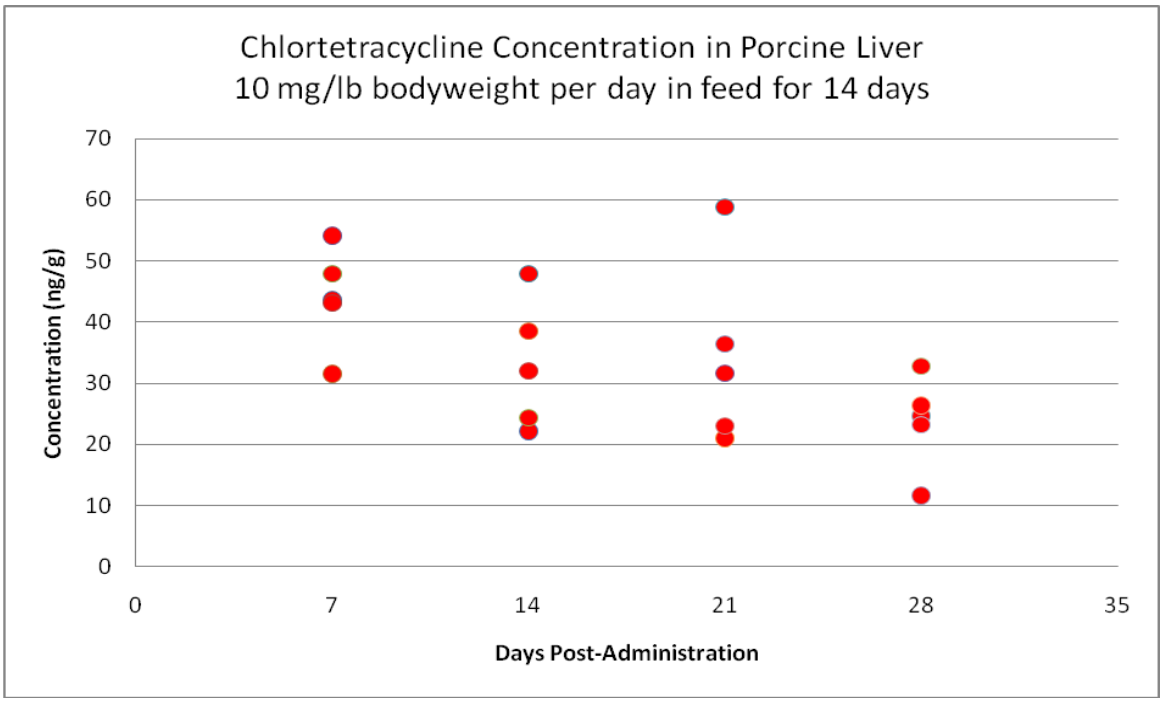


Figure 10:

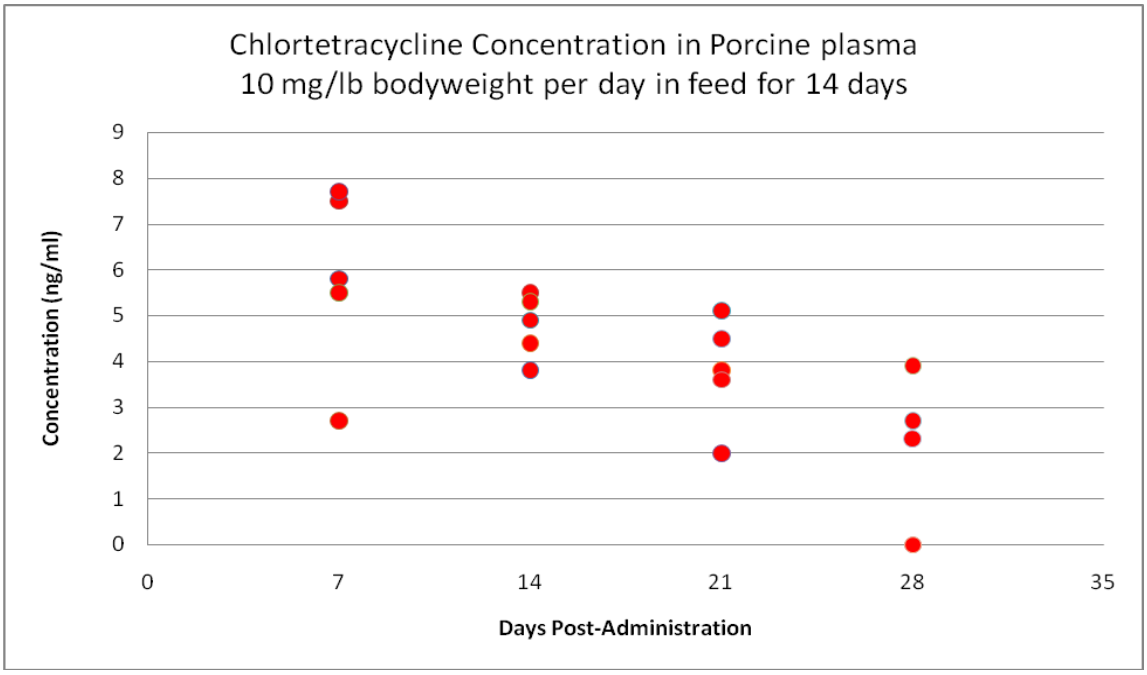


Figure 11:

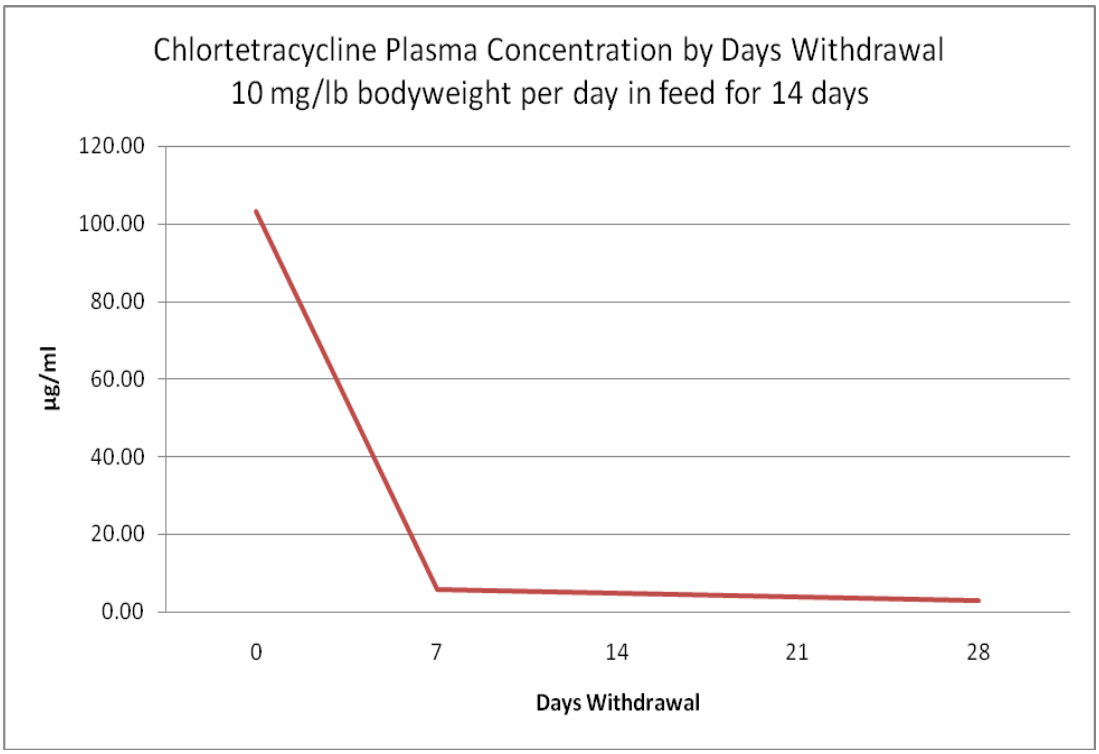


Figure 12:

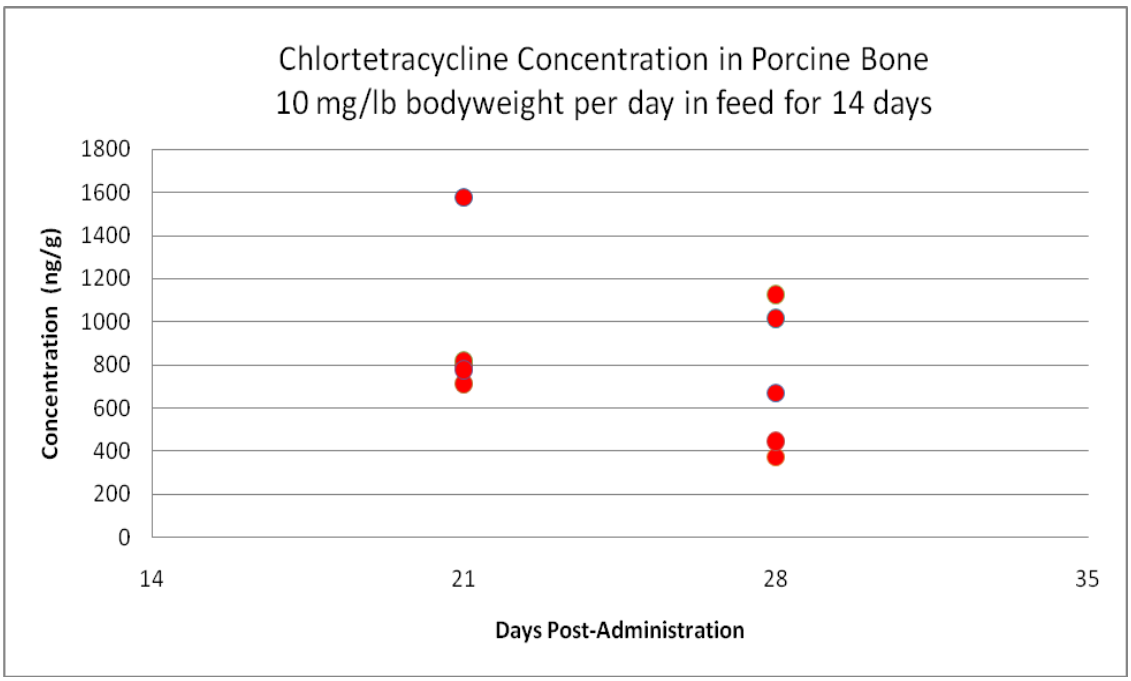


Table 4 - Oxytetracycline 10 mg/lb Per Day in Feed for 14 Days

Oxytetracycline in Feed at 10 mg/lb Per Day for 14 Days													
Tag	**Sex**	**Pen**	**Slaughter Date**	**Days**	**Muscle CHARM Result**	**Muscle MS (ppb)**	**Kidney (ppb)**	**Liver (ppb)**	**Fat (ppb)**	**Bone (ppb)**	**Colon (ppb)**	**Plasma (ppb)**	**Urine (ppb)**
Level of Detection (LOD)					100 ppb	1.0	1.2	1.0	0.7	2.0	53.7	0.6	2.2
Level of Quantitation (LOQ)					NA	4.0	8.0	8.0	4.0	4.0	74.2	1.0	4.0
67	G	10	4/15/2010	0	Not Found	190.0	988.0	206.0	43.0			277.0	
204	B	9	4/15/2010		Not Found	190.0	1020.0	197.0	11.2			350.0	2138
210	G	9	4/15/2010		Not Found	164.0	646.0	158.0	4.0			151.0	
243	G	9	4/15/2010		Not Found	186.0	768.0	139.0	5.8			236.0	
285	G	10	4/15/2010		Not Found	202.0	1070.0	220.0	8.5			206.0	
73	B	11	4/22/2010	7	Not Found	13.0	100.0	8.2	D			16.5	953
77	G	9	4/22/2010		Not Found	10.0	53.3	D	D			16.4	
87	B	11	4/22/2010		Not Found	10.3	49.9	D	D			14.2	326
92	G	9	4/22/2010		Not Found	10.0	70.6	D	D				
271	B	10	4/22/2010		Not Found	15.7	141.0	8.7	D			13.1	735
14	B	10	4/29/2010	14	Not Found	21.0	96.6	15.2	D			18.9	714
197	G	11	4/29/2010		Not Found	8.8	95.0	11.2	D			10.2	496
228	B	10	4/29/2010		Not Found	12.6	124.0	17.9	D			12.9	576
269	B	9	4/29/2010		Not Found	7.4	141.0	10.2	ND			11.1	614
322	B	9	4/29/2010		Not Found	8.7	91.8	D	D			9.3	416
180	G	9	5/6/2010	21	Not Found	4.9	44.7	D	D	24.9	833	11.3	
208	G	10	5/6/2010		Not Found	19.1	41.5	D	D	47.5	ND	9.2	
279	G	10	5/6/2010		Not Found	13.3	81.2	8.7	ND	94.0	D	15.3	
317	G	10	5/6/2010		Not Found	7.6	72.0	D	D	38.7	827	8.7	
324	G	9	5/6/2010		Not Found	9.9	89.0	D	D	55.0	1347	10.7	548
155	G	11	5/13/2010	28	Not Found	9.2	69.0	D	ND	117.0	ND	9.4	
165	G	11	5/13/2010		Not Found	8.8	70.6	D	D	59.5	404	13.2	69
306	B	11	5/13/2010		Not Found	6.5	75.4	D	D	54.0	713	8.9	282
313	B	11	5/13/2010		Not Found	4.7	50.0	D	D	57.5	508	68.4	

5th pig for the 28 day group died during study

Table 5: Mean edible tissue concentrations by days withdrawal after oxytetracycline in the feed at 10 mg/lb bodyweight for 14 days.

Oxytetracycline 10 mg/lb Per Day in Feed for 14 Days				
Withdrawal Days	Muscle mean (ppb)	Kidney mean (ppb)	Liver mean (ppb)	Fat mean (ppb)
0	186.4	898.4	184.0	14.5
7	11.8	83.0	8.4	D
14	11.7	109.7	13.6	D
21	11.0	65.7	D	D
28	7.3	66.3	D	D

Figure 13: Mean edible tissue concentrations by days withdrawal after oxytetracycline in the feed at 10 mg/lb bodyweight for 14 days.

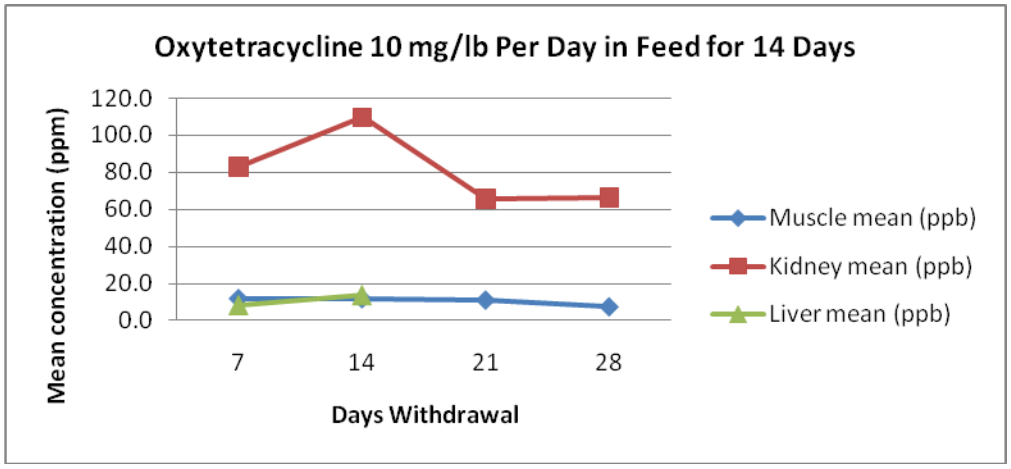


Figure 14:

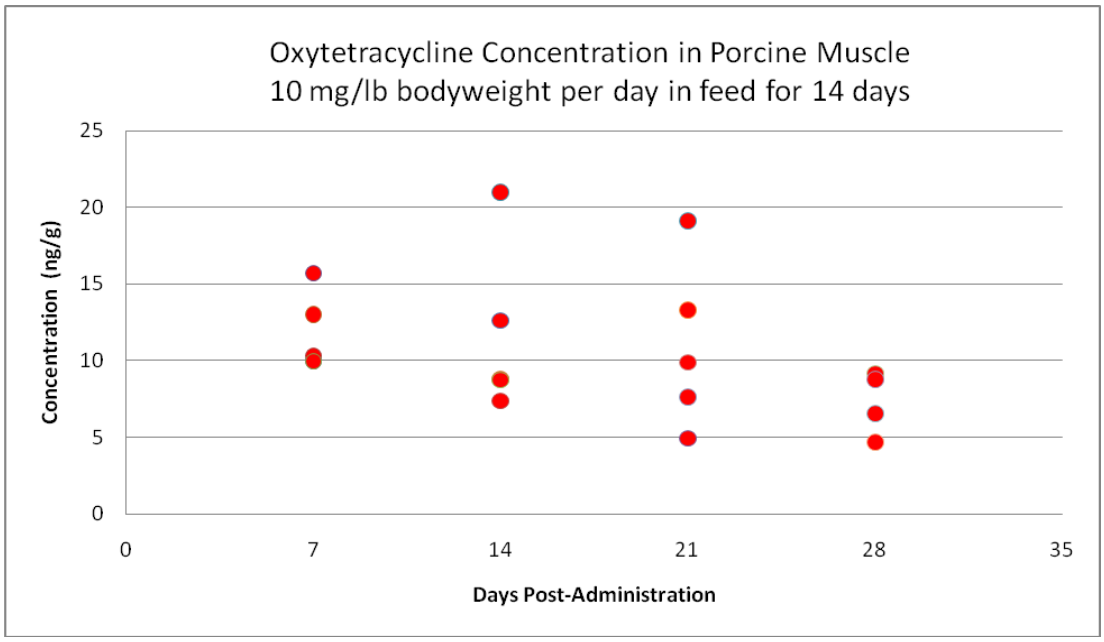


Figure 15:

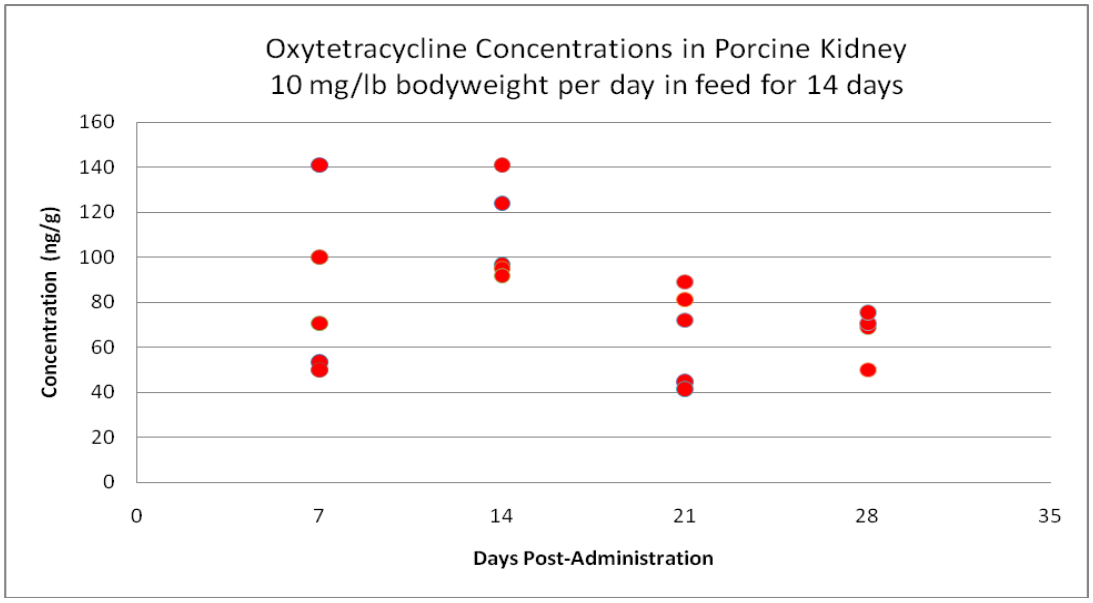
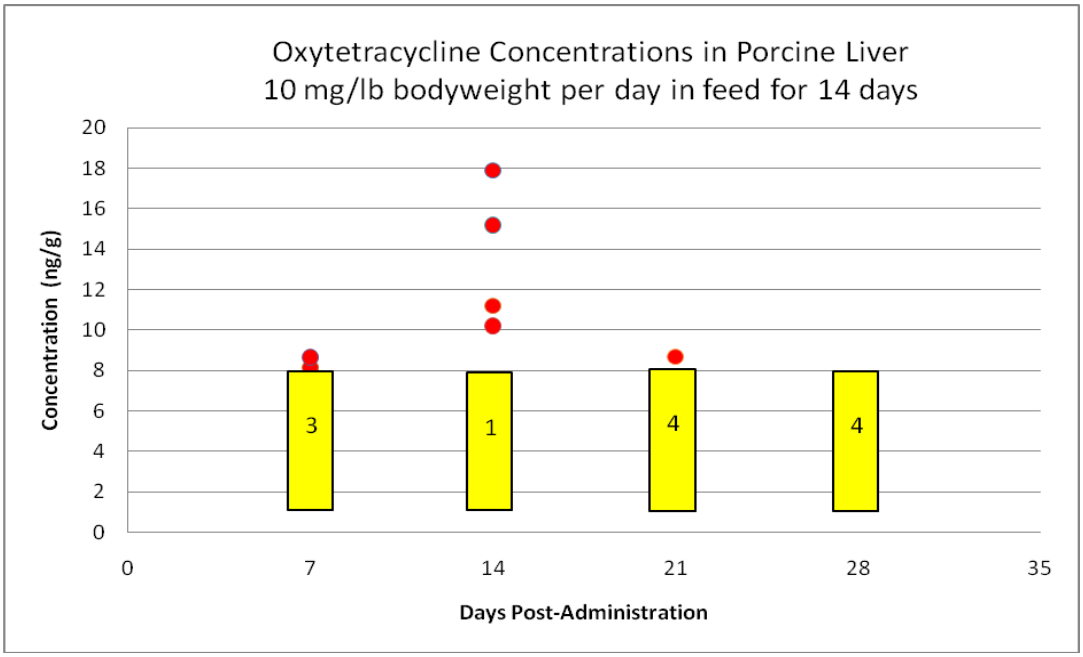


Figure 16:



Yellow boxes indicate number of samples below level of quantitation (LOQ) but above level of detection (LOD)

Figure 17:

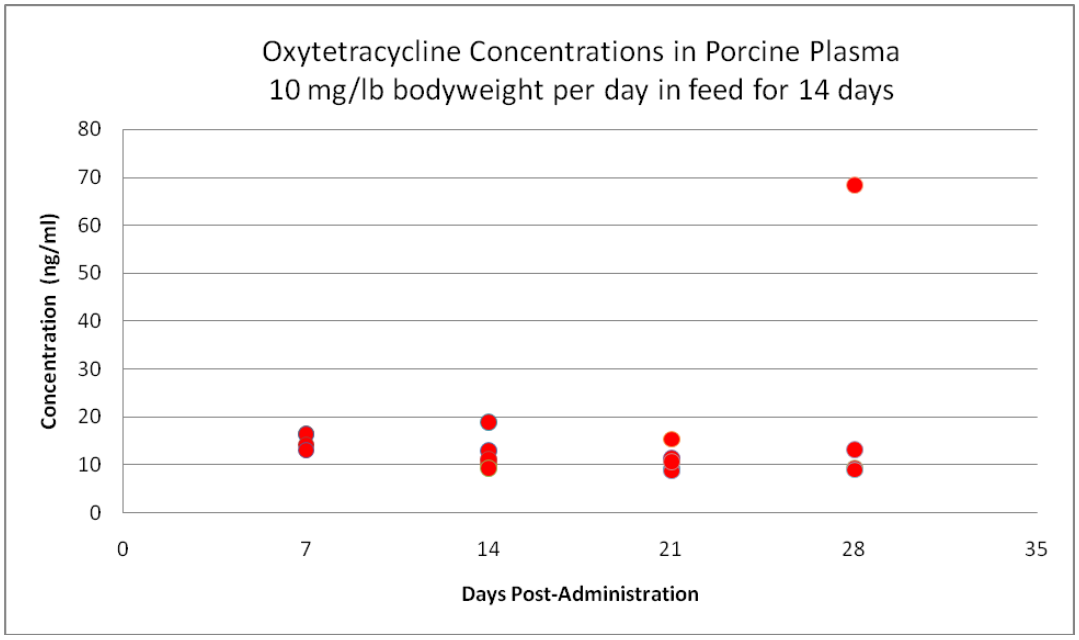


Figure 18:

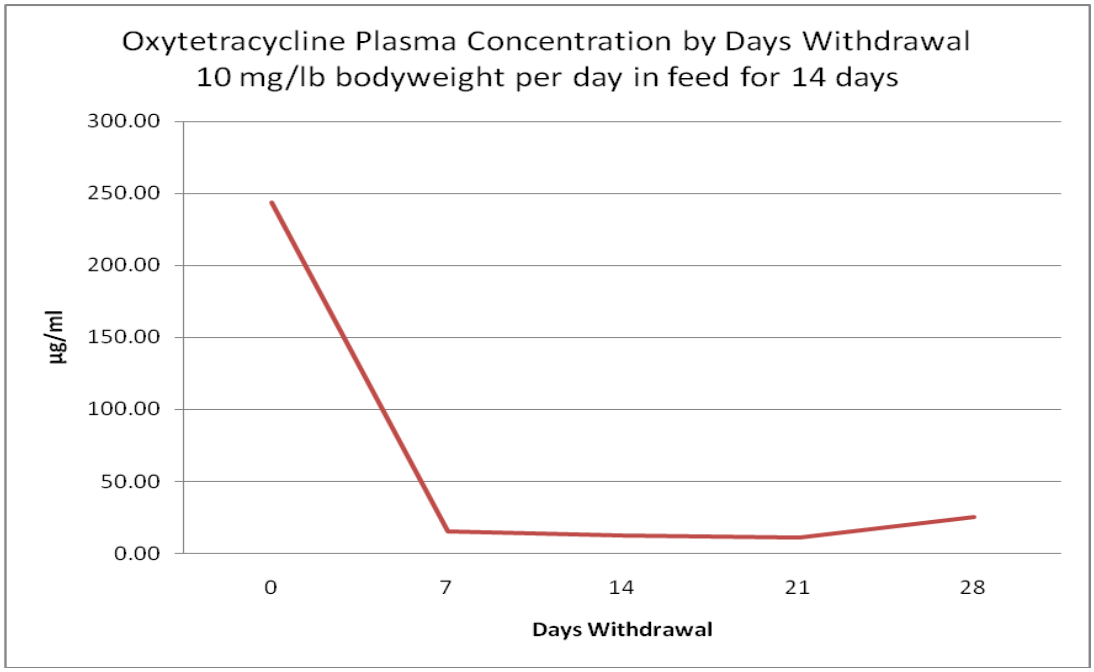


Figure 19:

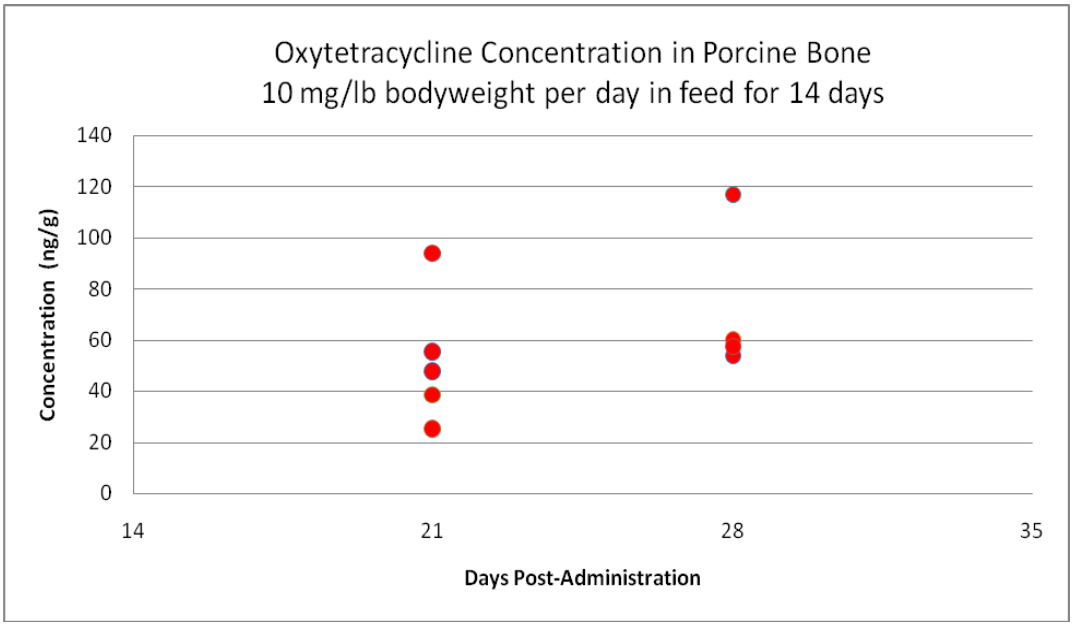


Table 6 - Chlortetracycline 10 mg/lb Per Day in Water for 5 Days

Chlortetracycline in Water at 10 mg/lb Per Day for 5 Days													
Tag	Sex	Pen	Slaughter Date	Days	Muscle CHARM Result	Muscle MS (ppb)	Kidney (ppb)	Liver (ppb)	Fat (ppb)	Bone (ppb)	Colon (ppb)	Plasma (ppb)	Urine (ppb)
Level of Detection (LOD)					100 ppb	1.0	1.0	0.3	0.1	N/A	19.1	1.5	5.6
Level of Quantitation (LOQ)					NA	4.0	8.0	8.0	8.0	32.0	32.0	2.0	8.0
90	B	3	4/15/2010	0	Suspect	302.0	2020.0	794.0	26.2			11.8	1940
195	B	3	4/15/2010		Suspect	493.0	2620.0	1080.0	127.0			209.0	
213	G	4	4/15/2010		Suspect	353.0	3510.0	935.0	13.1			148.0	
246	G	4	4/15/2010		Suspect	189.0	2590.0	690.0	38.3			146.0	
290	B	3	4/15/2010		Suspect	161.0	1310.0	582.0	14.9			105.0	1400
98	G	3	4/22/2010	7	Not Found	8.1	44.6	27.5	D			10.3	267
121	G	3	4/22/2010		Not Found	6.9	47.9	30.9	D			5.2	
212	B	5	4/22/2010		Not Found	7.2	60.1	50.6	8.7			7.3	81
238	B	4	4/22/2010		Not Found	13.0	81.4	40.9	D			5.9	204
241	B	5	4/22/2010		Not Found	5.8	38.1	19.8	D			ND	162
190	B	4	4/29/2010	14	Not Found	6.9	30.9	28.0	8.1			3.7	169
194	B	4	4/29/2010		Not Found	D	42.0	31.1	D			5.6	140
247	G	5	4/29/2010		Not Found	6.7	48.0	32.4	D			3.8	
258	B	3	4/29/2010		Not Found	4.0	27.8	25.4	D			3.0	99
305	B	3	4/29/2010		Not Found	6.1	35.4	23.5	D			2.5	301
60	G	4	5/6/2010	21	Not Found	6.3	38.3	40.8	D	746	ND	4.0	90
94	G	3	5/6/2010		Not Found	4.0	15.8	13.3	D	444	489	ND	
99	G	4	5/6/2010		Not Found	4.2	25.1	15.7	D	472	ND	ND	
154	G	4	5/6/2010		Not Found	D	18.9	16.6	D	441	387	D	
166	G	3	5/6/2010		Not Found	4.4	47.9	58.4	D	538	908	2.8	
96	B	5	5/13/2010	28	Not Found	D	25.7	22.2	D	676	ND	ND	369
181	G	5	5/13/2010		Not Found	6.0	43.8	30.2	D	766	ND	2.4	
220	G	5	5/13/2010		Not Found	4.9	26.7	20.8	D	471	ND	ND	28
251	G	5	5/13/2010		Not Found	4.9	19.5	14.1	D	669	ND	ND	
263	B	5	5/13/2010		Not Found	D	13.4	14.4	D	100	ND	ND	91

Table 7: Mean edible tissue concentrations by days withdrawal after chlortetracycline in the water at 10 mg/lb bodyweight for 5 days.

Chlortetracycline 10 mg/lb Per Day in Water for 5 Days				
Withdrawal Days	Muscle mean (ppb)	Kidney mean (ppb)	Liver mean (ppb)	Fat mean (ppb)
0	299.6	2410.0	816.2	43.9
7	8.2	54.4	33.9	8.7
14	5.9	36.8	28.1	8.1
21	4.7	29.2	29.0	D
28	5.2	25.8	20.3	D

Figure 20: Mean edible tissue concentrations by days withdrawal after chlortetracycline in the water at 10 mg/lb bodyweight for 5 days.

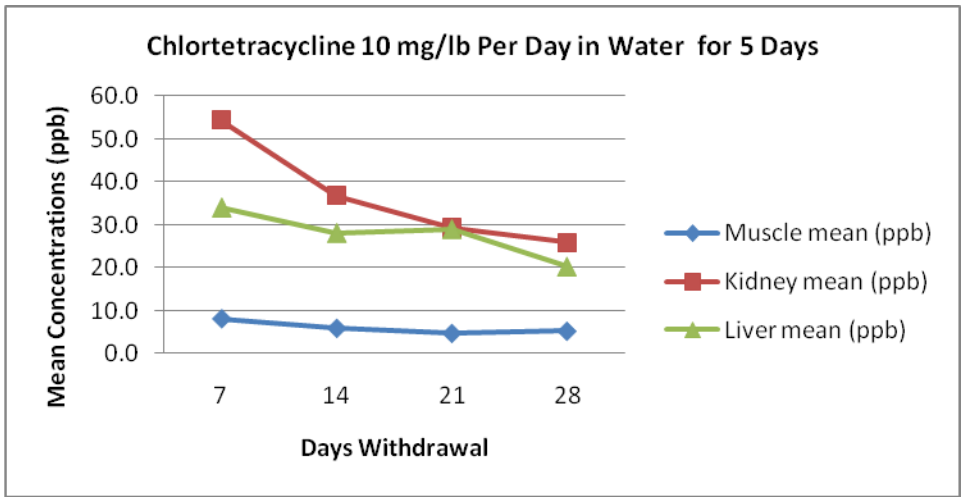
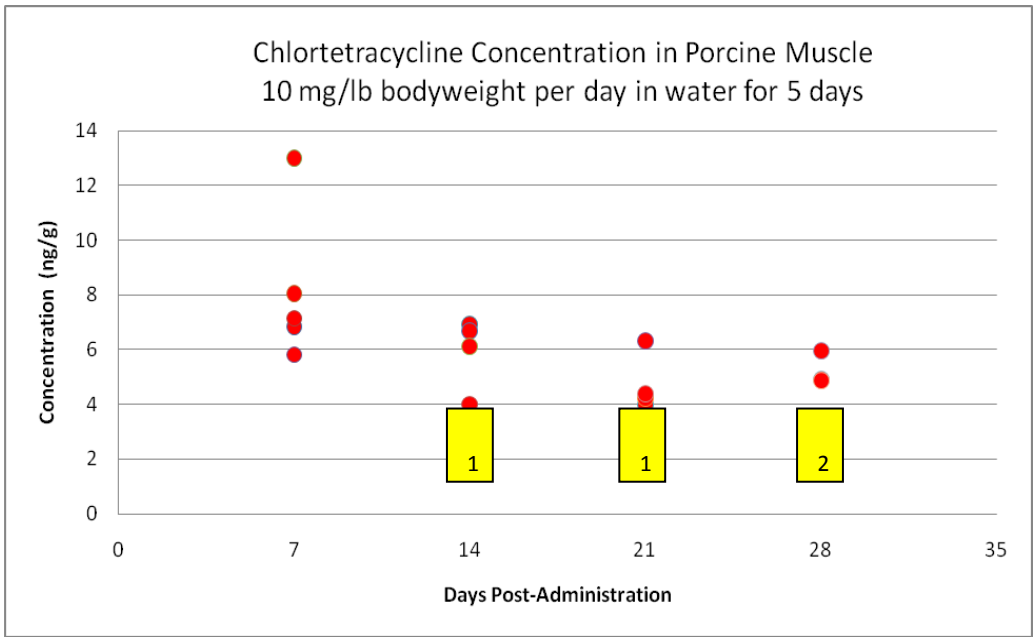


Figure 21:



Yellow boxes indicate number of samples below level of quantitation (LOQ) but above level of detection (LOD)

Figure 22:

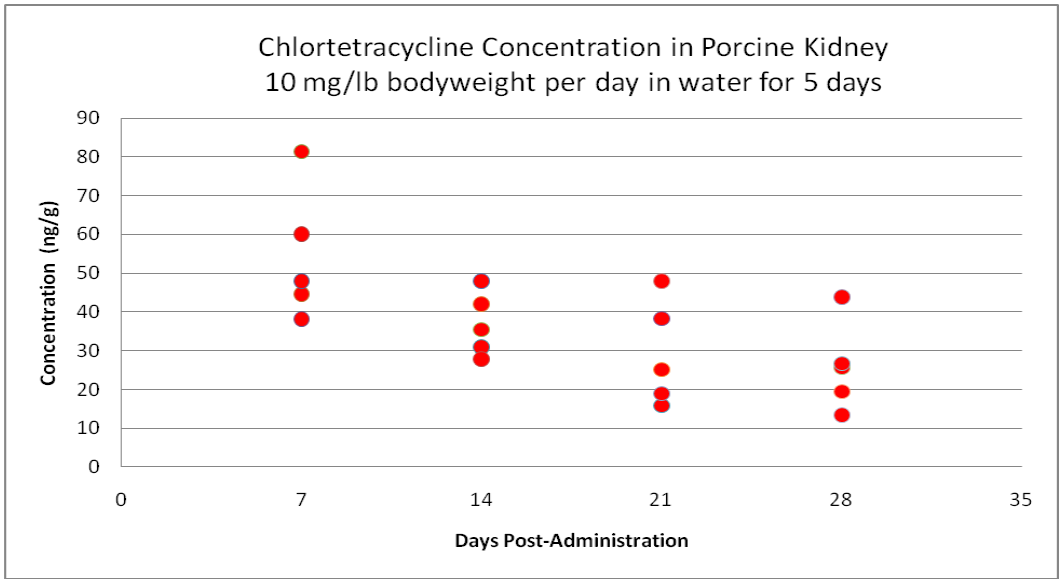


Figure 23:

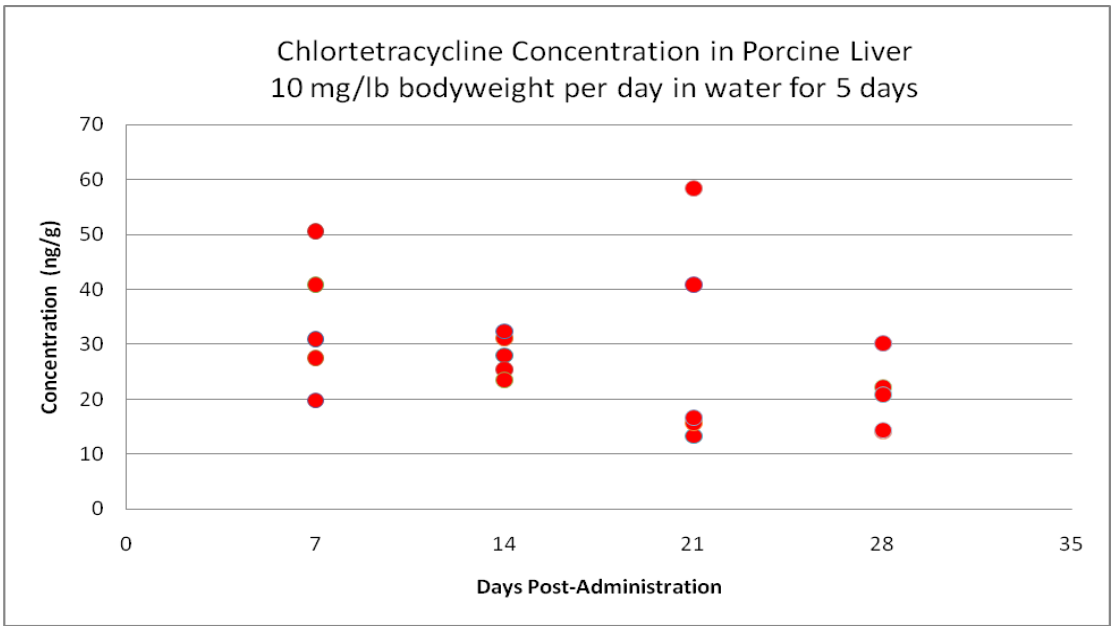


Figure 24:

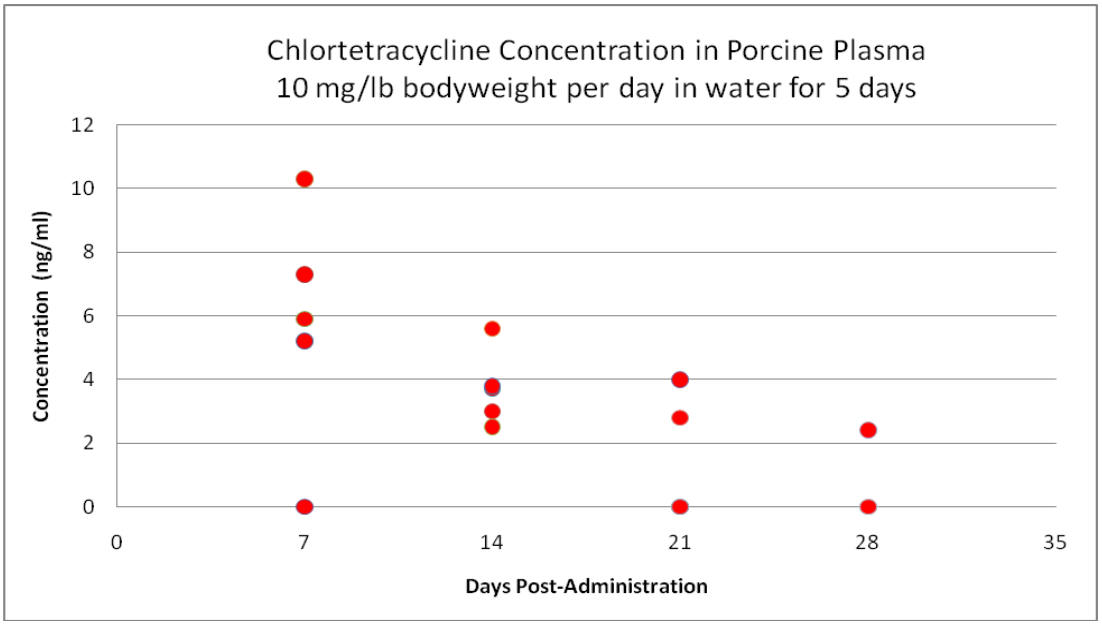


Figure 25:

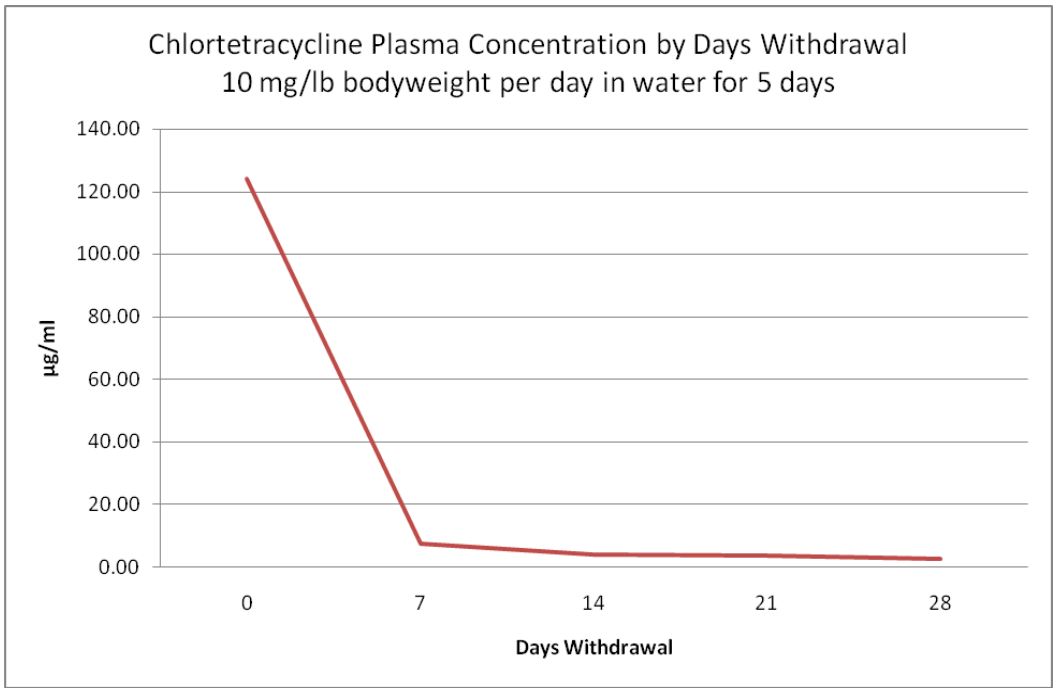


Figure 26:

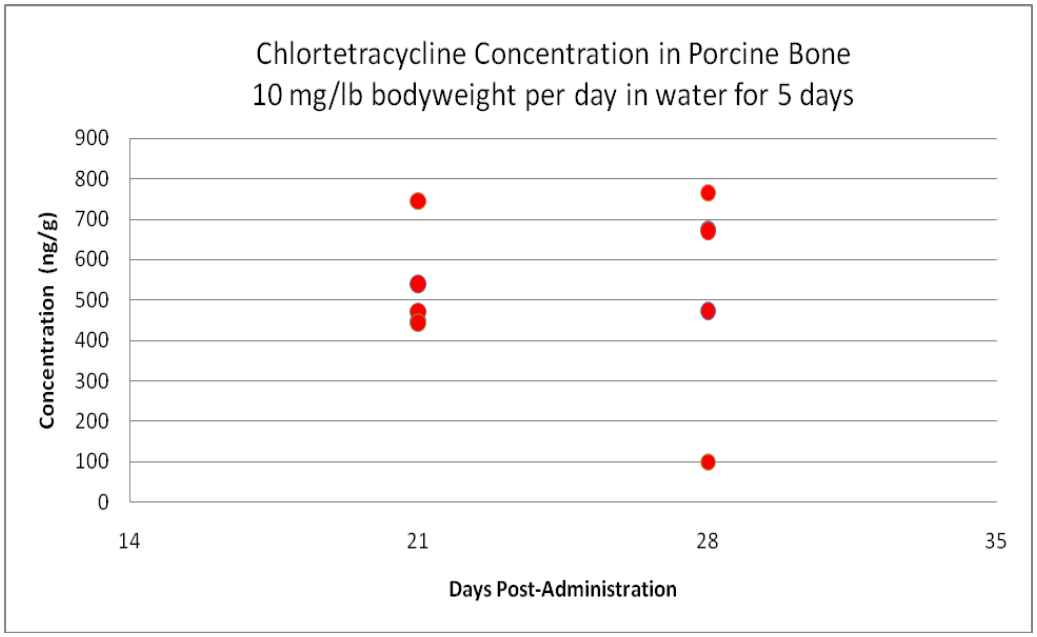


Table 8 - Oxytetracycline 10 mg/lb Per Day in Water for 5 Days

Oxytetracycline in Water at 10 mg/lb Per Day for 5 Days													
Tag	**Sex**	**Pen**	**Slaughter Date**	**Days**	**Muscle CHARM Result**	**Muscle MS (ppb)**	**Kidney (ppb)**	**Liver (ppb)**	**Fat (ppb)**	**Bone (ppb)**	**Colon (ppb)**	**Plasma (ppb)**	**Urine (ppb)**
Level of Detection (LOD)					100 ppb	1.0	1.2	1.0	0.7	2.0	53.7	0.6	2.2
Level of Quantitation (LOQ)					NA	4.0	8.0	8.0	16.0	4.0	74.2	1.0	4.0
74	G	15	4/15/2010	0	Not Found	164.0	768.0	142.0	32.3				
203	B	15	4/15/2010		Not Found	223.0	968.0	261.0	315.0			297.0	
211	G	15	4/15/2010		Not Found	120.0	1040.0	229.0	276.0			176.0	
236	G	16	4/15/2010		Not Found	89.2	647.0	94.2	26.4			138.0	
252	G	16	4/15/2010		Not Found	200.0	1300.0	230.0	123.0			210.0	2228
91	B	17	4/22/2010	7	Not Found	8.5	31.6	D	D			5.6	20
123	G	16	4/22/2010		Not Found	5.2	26.4	D	17.0			3.5	
276	G	15	4/22/2010		Not Found	18.8	120.0	15.7	D			10.4	
284	B	17	4/22/2010		Not Found	4.5	39.1	D	D			6.2	
297	G	15	4/22/2010		Not Found	8.0	74.5	D	16.3			7.2	
54	B	16	4/29/2010	14	Not Found	4.8	36.6	D	D			4.0	190
89	B	16	4/29/2010		Not Found	5.1	35.8	D	D			3.4	
169	G	17	4/29/2010		Not Found	D	46.1	10.1	D			4.8	181
202	G	17	4/29/2010		Not Found	D	65.6	9.8	D			5.4	522
319	G	17	4/29/2010		Not Found	24.7	66.1	17.6	D			6.9	359
6	G	15	5/6/2010	21	Not Found	6.0	41.0	D	D	26.4	574	11.0	
119	G	16	5/6/2010		Not Found	6.4	51.6	D	D	31.9	ND	5.5	
120	G	16	5/6/2010		Not Found	4.6	22.6	D	D	11.2	933	3.4	
311	G	15	5/6/2010		Not Found	D	36.7	D	D	16.3	366	4.1	
315	G	16	5/6/2010		Not Found	4.3	27.0	D	D	23.2	526	5.8	
206	B	15	5/13/2010	28	Not Found	5.1	37.3	D	D	12.7	D	8.6	526
221	B	17	5/13/2010		Not Found	D	30.3	D	D	14.6	ND	4.5	208
254	B	15	5/13/2010		Not Found	D	39.0	D	D	16.0	D	6.0	169
262	G	17	5/13/2010		Not Found	D	25.0	D	D	13.6	ND	5.5	
321	B	17	5/13/2010		Not Found	D	15.9	D	D	15.5	276	1.2	68

Table 9: Mean edible tissue concentrations by days withdrawal after oxytetracycline in the water at 10 mg/lb bodyweight for 5 days.

Oxytetracycline 10 mg/lb Per Day in Water for 5 Days				
Withdrawal Days	**Muscle mean (ppb)**	**Kidney mean (ppb)**	**Liver mean (ppb)**	**Fat mean (ppb)**
0	159.2	944.6	191.2	154.5
7	9.0	58.3	D	16.7*
14	11.5	50.0	12.5	D
21	5.3	35.8	D	D
28	D	29.5	D	D

*Mean of 2 quantifiable samples

Figure 27: Mean edible tissue concentrations by days withdrawal after oxytetracycline in the water at 10 mg/lb bodyweight for 5 days.

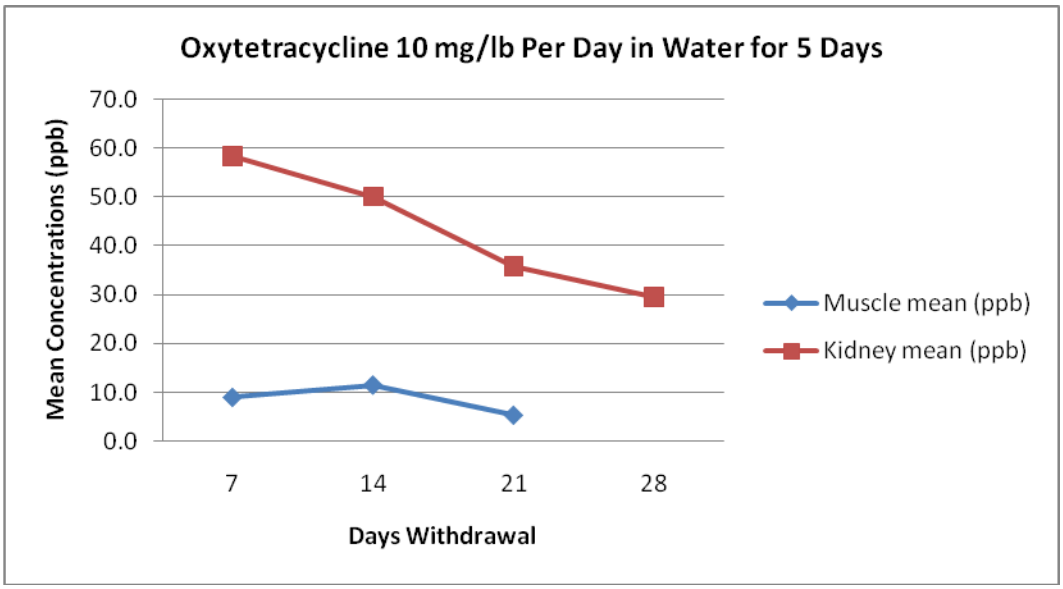
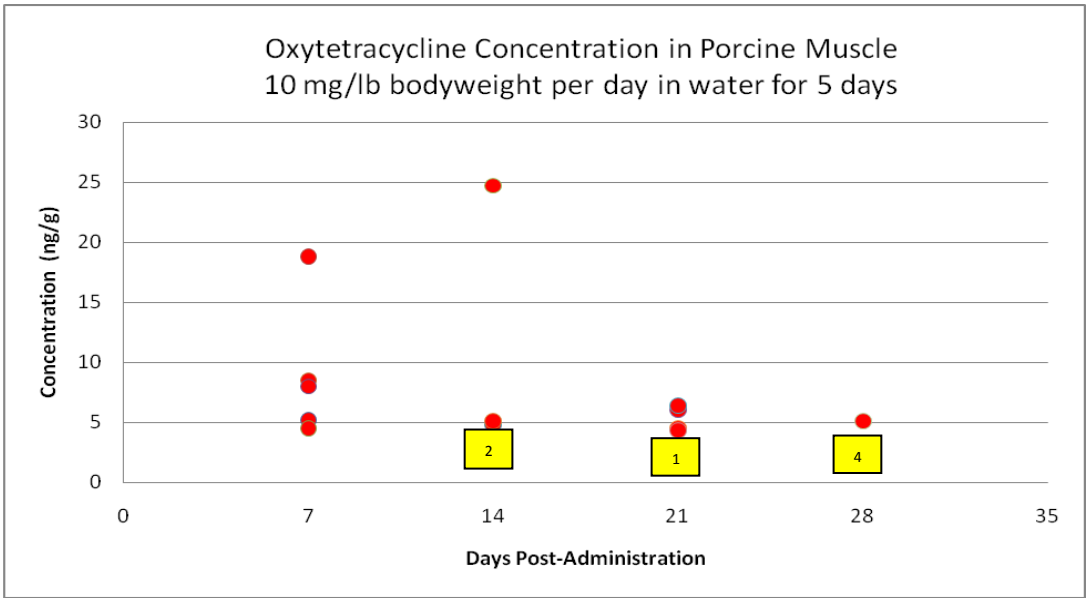


Figure 28:



Yellow boxes indicate number of samples below level of quantitation (LOQ) but above level of detection (LOD)

Figure 29:

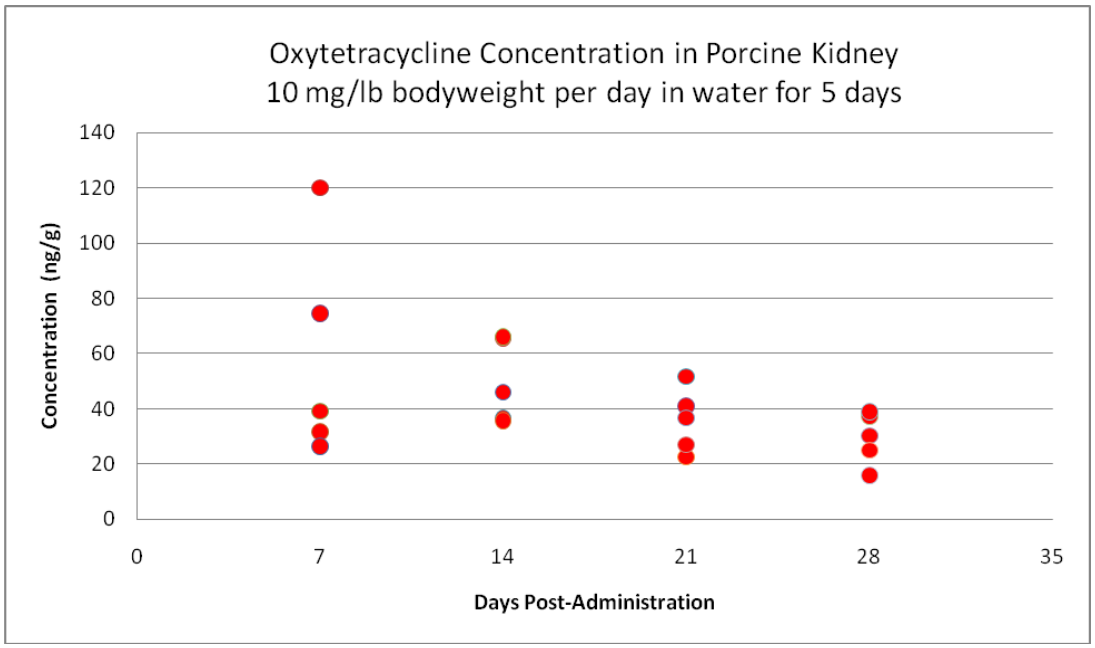
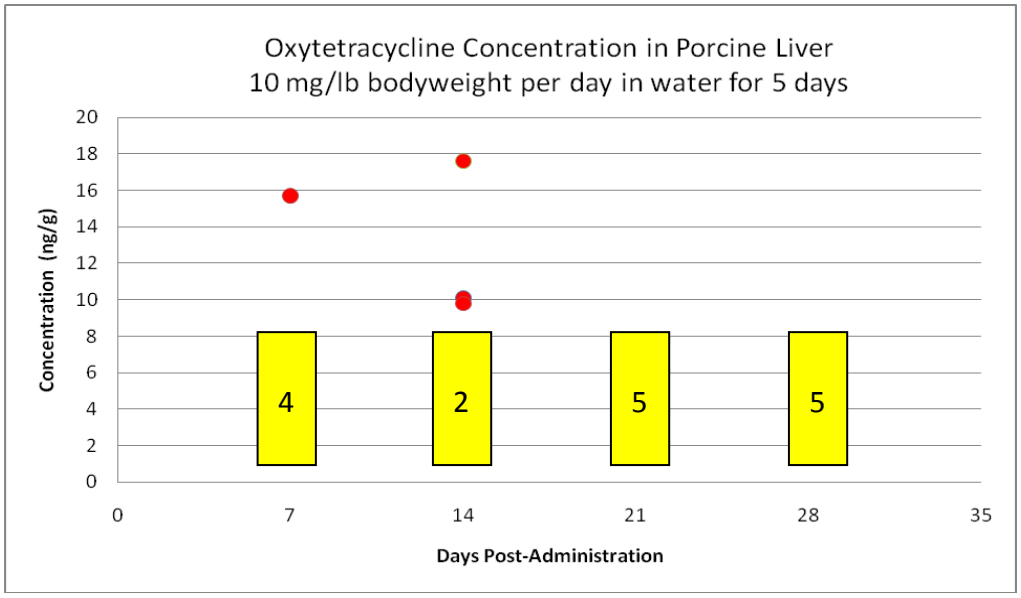


Figure 30:



Yellow boxes indicate number of samples below level of quantitation (LOQ) but above level of detection (LOD)

Figure 31:

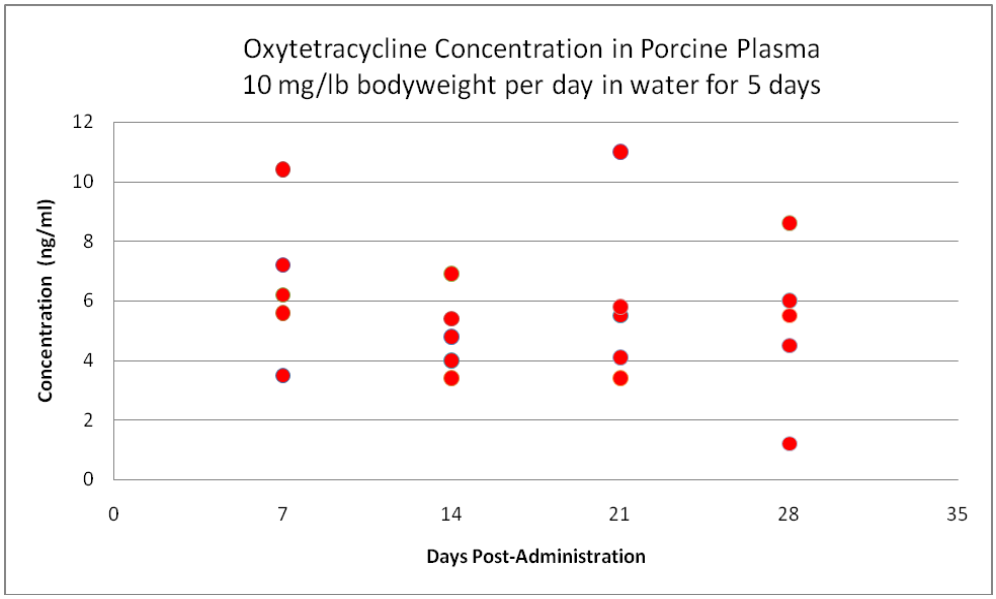


Figure 32:

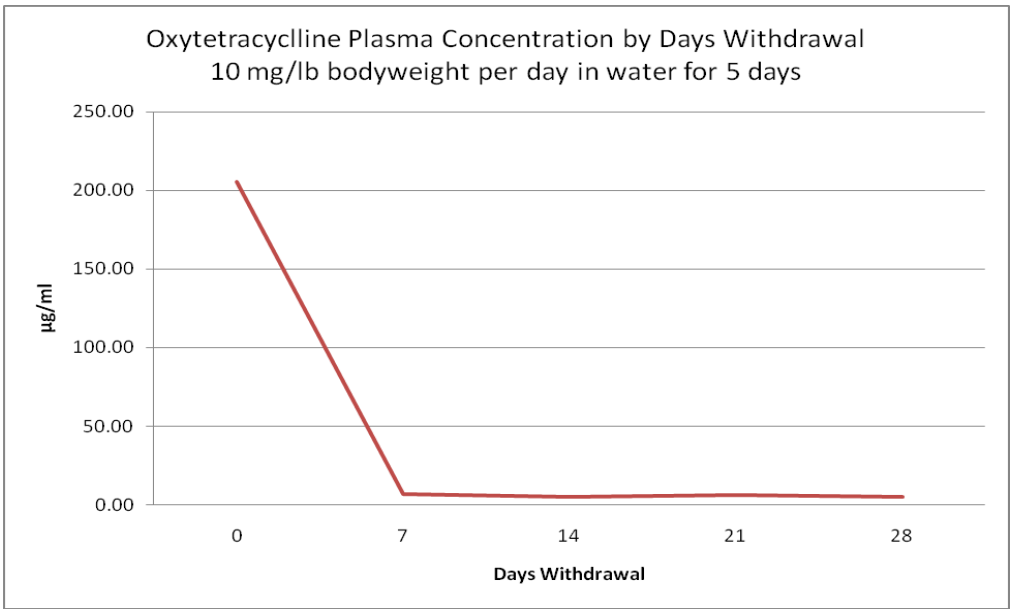


Figure 33:

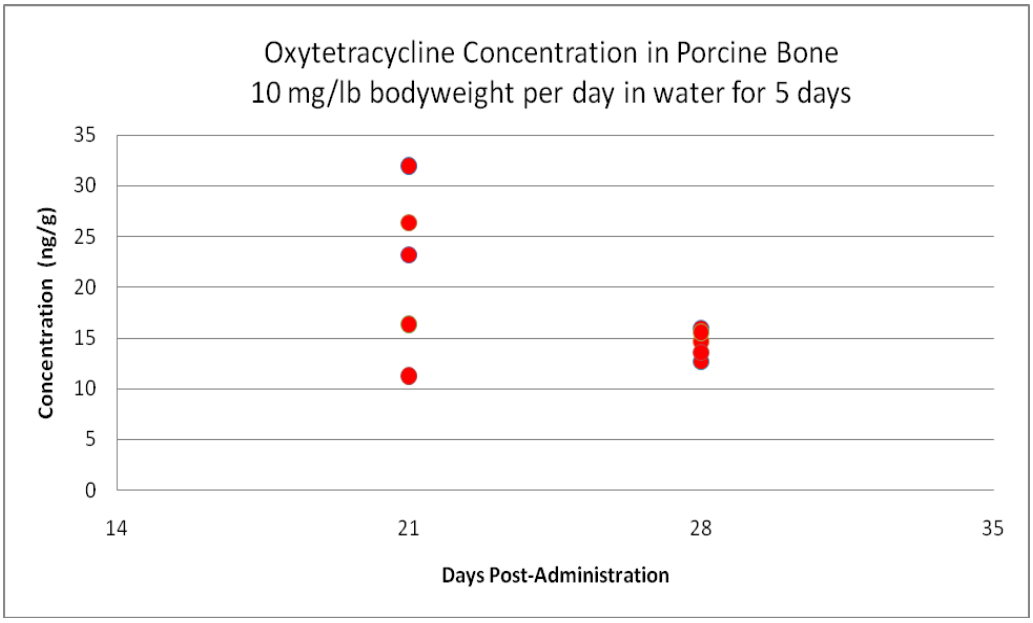


Table 10 - Tetracycline 10 mg/lb Per Day in Water for 5 Days

Tetracycline in Water at 10 mg/lb Per Day for 5 Days														
Tag	**Sex**	**Pen**	**Slaughter Date**	**Days**	**Muscle CHARM Result**	**Muscle MS (ppb)**	**Kidney (ppb)**	**Liver (ppb)**	**Fat (ppb)**	**Bone (ppb)**	**Colon (ppb)**	**Plasma (ppb)**	**Urine (ppb)**
Level of Detection (LOD)					25 ppb	0.5	0.6	0.2	0.4	3.6	6.4	2.0	9.5
Level of Quantitation (LOQ)					NA	4.0	4.0	1.0	4.0	8.0	16.0	4.0	16.0
69	G	13	4/15/2010	0	Suspect	161.0	496.0	163.0	4.2			155.0	
78	G	13	4/15/2010		Suspect	899.0	1500.0	723.0	28.2			372.0	
112	B	12	4/15/2010		Suspect	285.0	449.0	139.0	6.0			213.0	668
235	G	12	4/15/2010		Suspect	668.0	1430.0	363.0	40.7			531.0	
287	G	12	4/15/2010		Suspect	375.0	894.0	316.0	7.9			363.0	
4	B	14	4/22/2010	7	Suspect*	18.0	66.7	24.6	D			16.1	626
81	G	12	4/22/2010		Not Found	22.3	85.9	19.3	ND			14.9	
93	B	14	4/22/2010		Not Found	16.6	59.6	17.2	D			14.7	403
103	G	13	4/22/2010		Not Found	24.1	61.8	15.1	D			19.5	
127	G	12	4/22/2010		Suspect	21.8	48.1	19.5	D			18.0	
19	B	13	4/29/2010	14	Not Found	9.8	33.6	9.0	D			6.1	251
24	B	13	4/29/2010		Suspect	36.2	142.0	34.1	D			23.6	
128	G	14	4/29/2010		Not Found	17.6	74.6	19.7	ND			9.8	
133	G	14	4/29/2010		Not Found	15.0	58.0	15.5	ND			7.0	
201	G	14	4/29/2010		Not Found	14.2	50.5	10.7	D			9.3	171
110	G	12	5/6/2010	21	Not Found	17.7	60.0	10.7	D	86.0	606	14.9	335
115	G	13	5/6/2010		Suspect	22.7	72.7	19.8	D	89.5	184	15.0	
157	G	12	5/6/2010		Not Found	14.0	44.3	7.6	D	42.9	235	10.5	
207	G	13	5/6/2010		Not Found	15.9	66.4	11.6	D	79.5	156	21.5	
250	G	13	5/6/2010		Not Found	18.6	55.5	14.4	D	56.5	D	11.9	
23	B	12	5/13/2010	28	Not Found	19.0	36.8	8.5	D	44.6	ND	5.7	198
51	B	14	5/13/2010		Not Found	12.5	40.2	10.2	D	60.5	ND	6.1	512
153	B	12	5/13/2010		Not Found	12.9	50.9	10.4	ND	71.5	129	8.9	284
162	G	14	5/13/2010		Not Found	19.9	76.1	12.7	D	57.0	ND	9.9	426
304	B	14	5/13/2010		Not Found	26.9	84.0	19.0	D	75.0	ND	11.7	424

Table 11: Mean edible tissue concentrations by days withdrawal after tetracycline in the water at 10 mg/lb bodyweight for 5 days.

Tetracycline 10 mg/lb Per Day in Water for 5 Days				
Withdrawal Days	Muscle mean (ppb)	Kidney mean (ppb)	Liver mean (ppb)	Fat mean (ppb)
0	477.6	953.8	340.8	17.4
7	20.6	64.4	19.1	D
14	18.6	71.7	17.8	D
21	17.8	59.8	12.8	D
28	18.2	57.6	12.2	D

Figure 34: Mean edible tissue concentrations by days withdrawal after tetracycline in the water at 10 mg/lb bodyweight for 5 days.

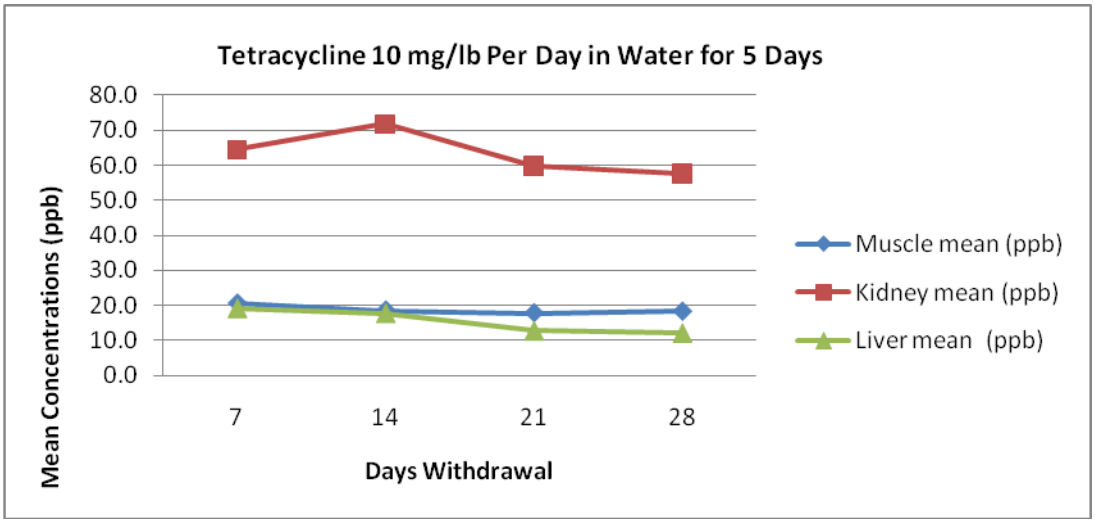


Figure 35:

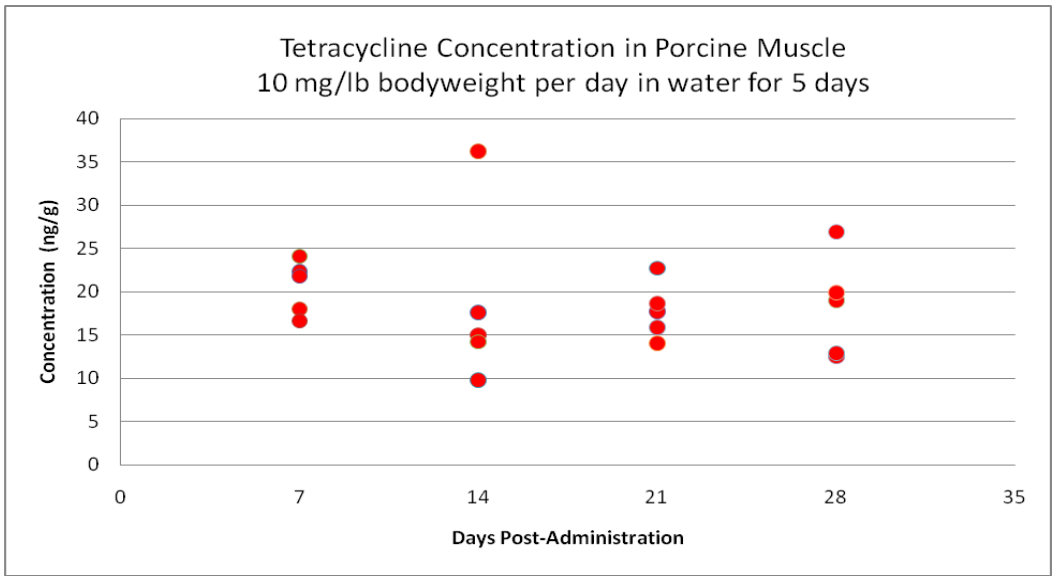


Figure 36:

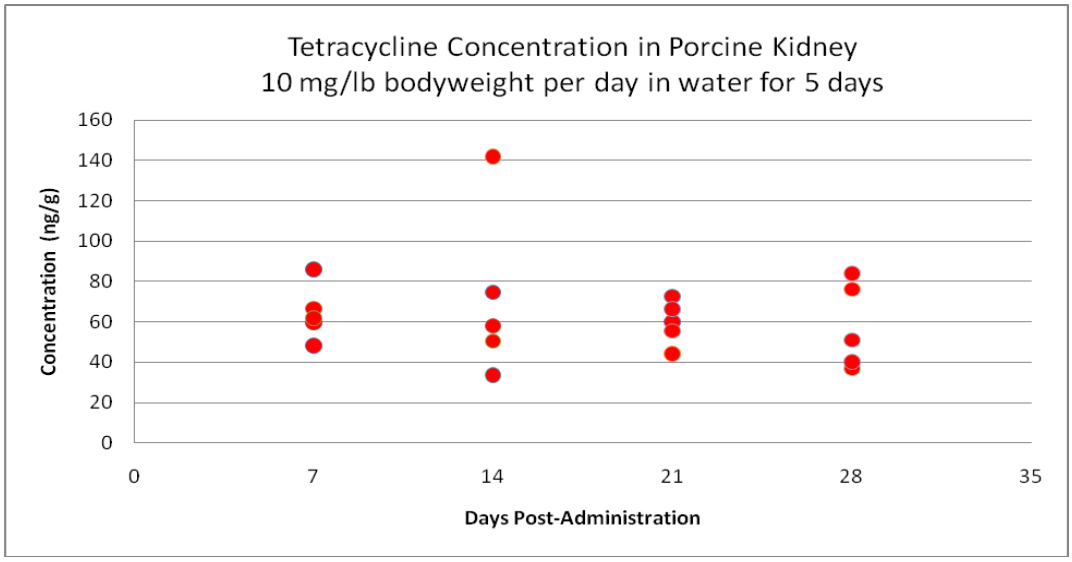


Figure 37:

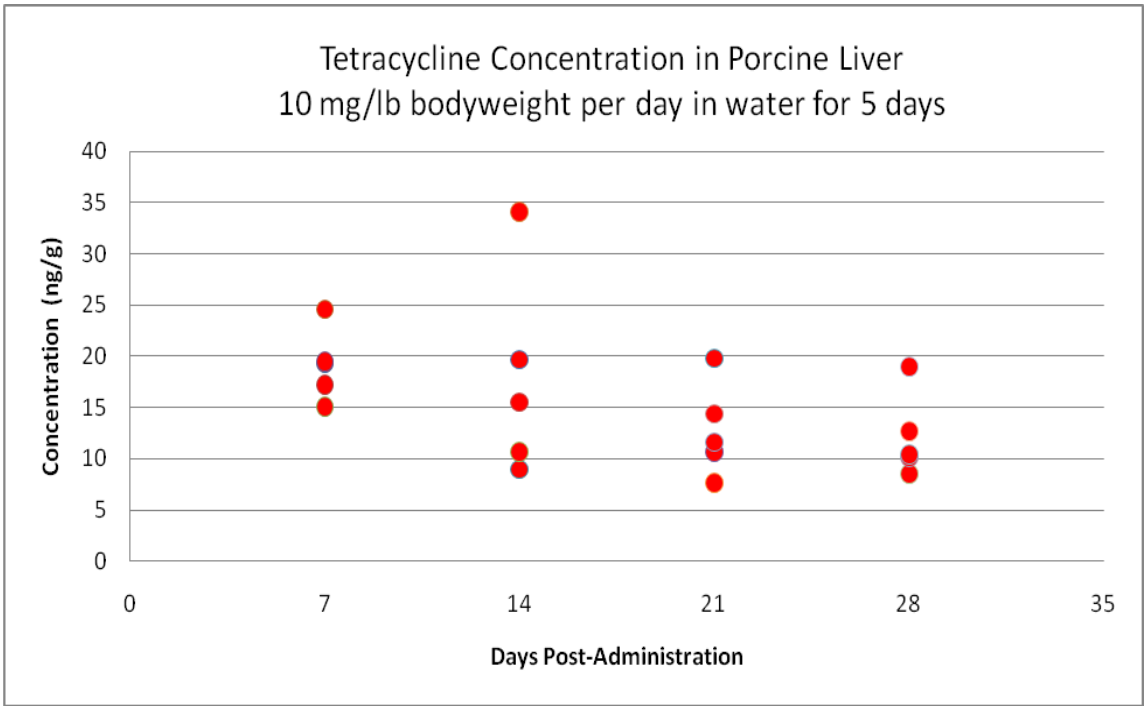


Figure 38:

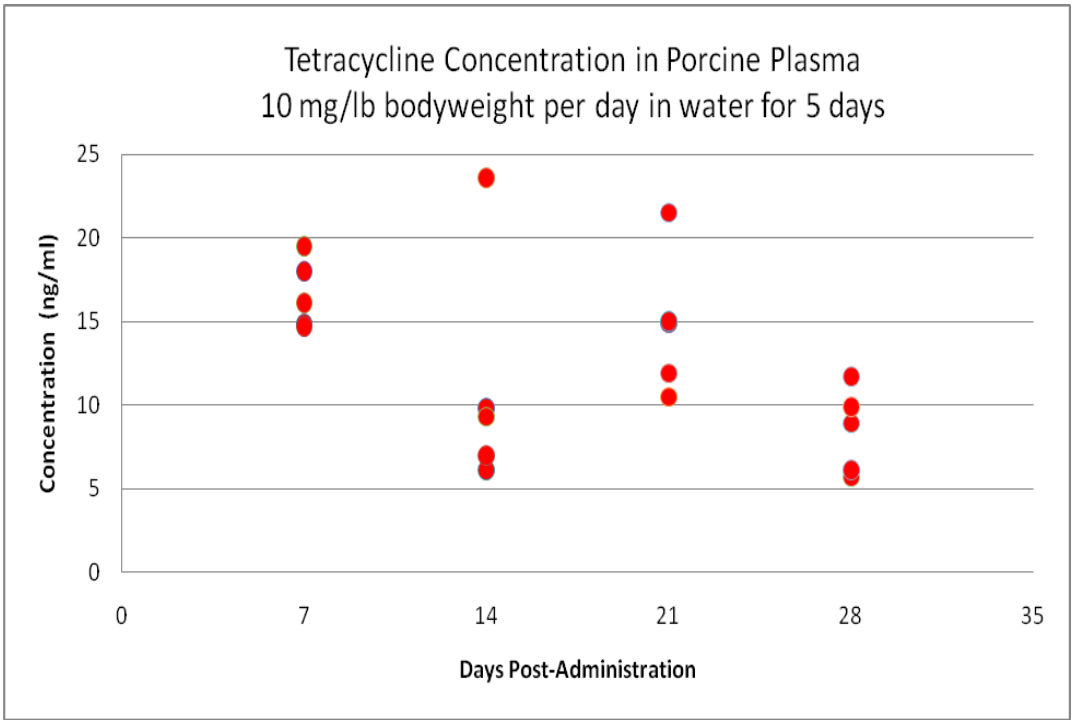


Figure 39:

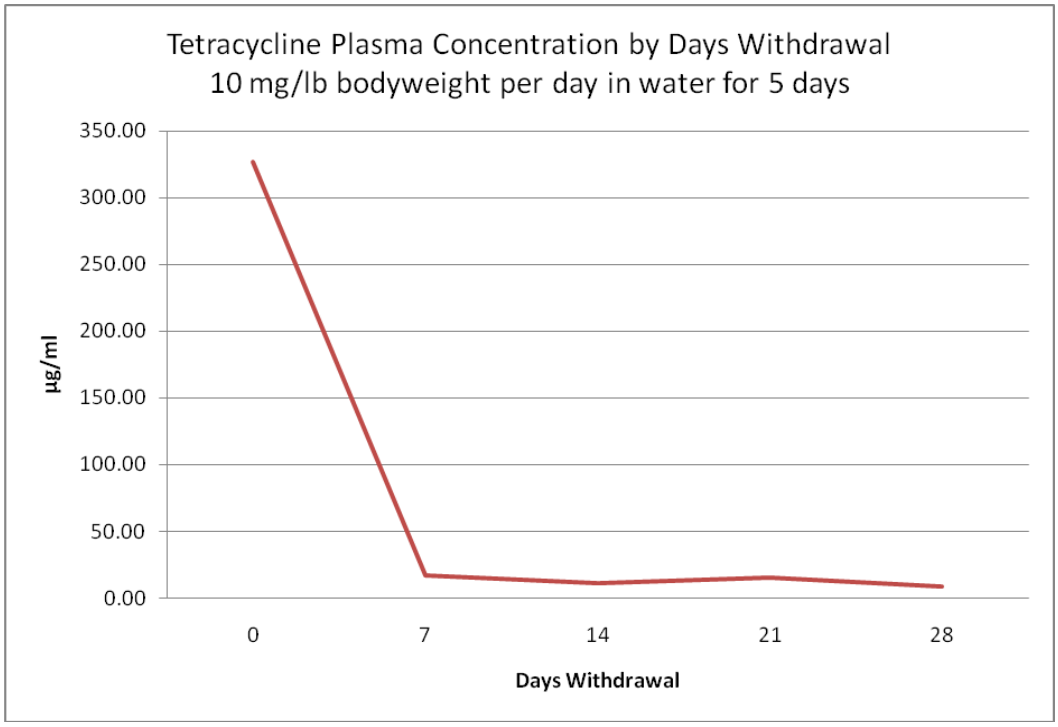


Figure 40:

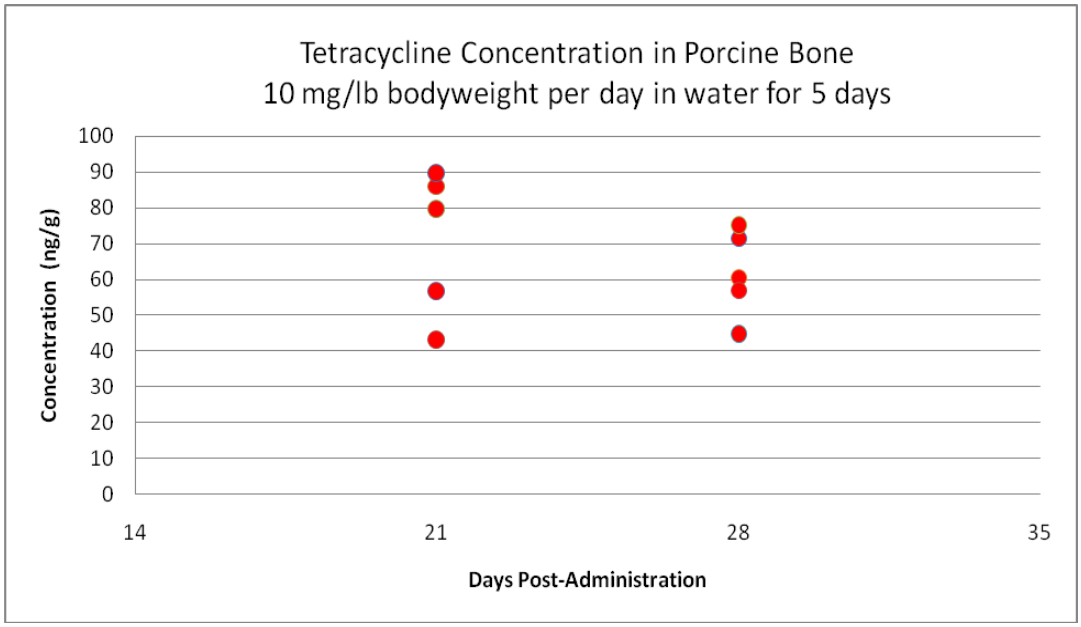
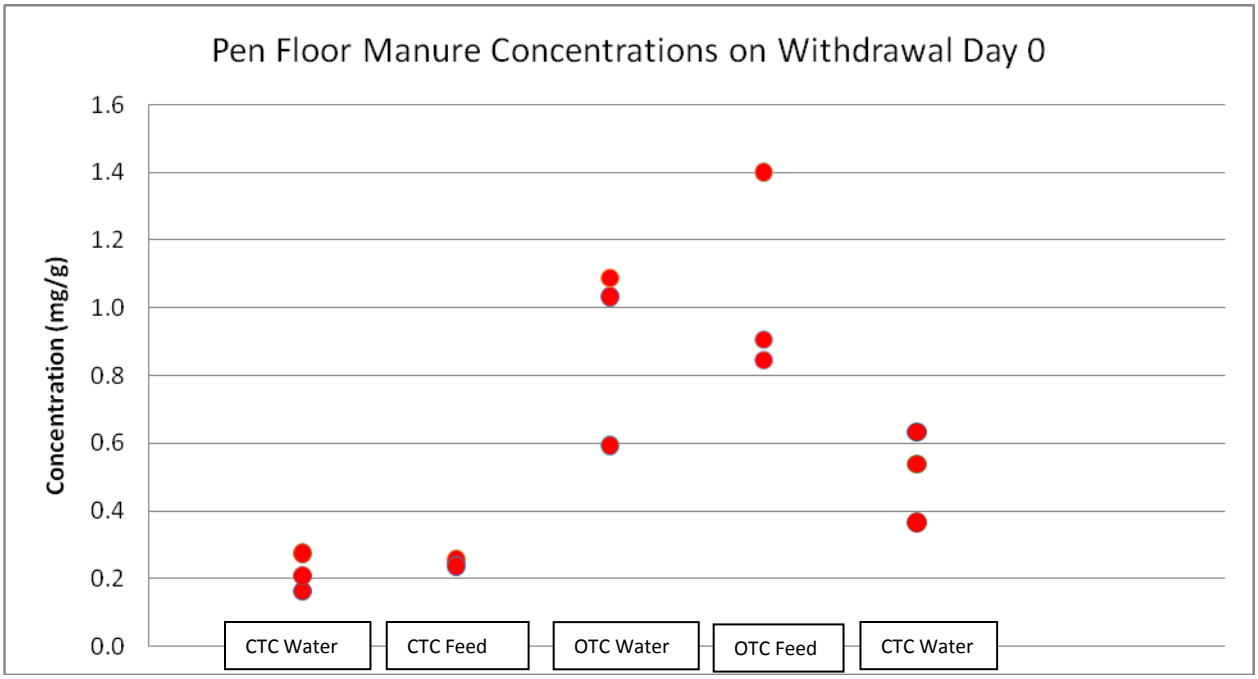


Table 12 - Concentrations of chlortetracycline, oxytetracycline, and tetracycline in manure from the pen floor with zero withdrawal on the day of cessation of drug administration

Treatment	Pen	Concentration (ng/g or ppb dry weight)
Chlortetracycline 10 mg/lb in water for 5 days	**3**	274,903
	4	163,103
	5	207,911
Chlortetracycline 10 mg/lb in feed for 14 days	**6**	248,287
	7	254,459
	8	236,667
Oxytetracycline 10 mg/lb in feed for 14 days	**9**	1,400,000
	10	844,687
	11	906,404
Oxytetracycline 10 mg/lb in water for 5 days	**15**	1,033,846
	16	593,857
	17	1,087,719
Tetracycline 10 mg/lb in water for 5 days	**12**	632,509
	13	364,548
	14	538,206

Figure 41: Scatter plot presentation of withdrawal day 0 pen floor manure concentrations. Note that the concentrations in this figure are now mg/g.



VII.b: Results - Bacitracin Residue Study

Table 13 - Bacitracin Concentrations in Tissues from Sows by Days Withdrawn after Feeding 750 g/ton BMD.

		Tissue bacitracin concentration (ppb or ng/g)			
LOD (ng/g or ppb)		38	97	75	17
LOQ (ng/g or ppb)		200	292	225	100
Withdrawal (days)	Sow	Muscle	Liver	Kidney	Fat
0	4	0.0	485	0.0	0.0
	6	<LOQ	0	0.0	0.0
	8	0.0	0	0.0	0.0
	10	0.0	<LOQ	0.0	0.0
	12	0.0	0	0.0	0.0
7	1	<LOQ	0	0.0	0.0
	5	0.0	612	0.0	<LOQ
	7	0.0	671	0.0	0.0
	9	0.0	435	0.0	<LOQ
	11	<LOQ	466	0.0	<LOQ

IX. Discussion

Tetracyclines: This study represents the first time that residues of the tetracyclines have been studied in pigs for an extended period with analytical equipment capable of detecting low-level residues. In the United States, the current regulatory methods for the tetracyclines are microbiological assays, where the concentration of the parent compound (the original compound injected) and any microbiologically active metabolites (whether identified or not) are determined by activity against a test bacterial organism. In contrast, this study utilized High-Performance Liquid Chromatography (HPLC) coupled with a triple quad mass spectrometer (MS/MS). The mass spectrometer was an API 4000, one of the most advanced units commonly in use in laboratories today. There are other units, such as the API 5000, which may provide even greater sensitivity. It is also possible for other laboratories to develop extraction and analytical methods which would provide greater sensitivity. If a government or trade group wishes to regulate on the basis of no detectable residues, then this threshold will be an ever shrinking target as technology advances.

The results of this study are not intended to replicate the regulatory methods of the United States, nor any other regulatory jurisdiction. Rather, the extraction and analytical methods were developed

to attain the lowest possible levels of detection (LOD) and quantification (LOQ) using the equipment in this laboratory. These methods were sufficient to detect prolonged, low-level concentrations of chlortetracycline, oxytetracycline, and tetracycline in edible tissues as well as colon contents, urine, plasma, and bone.

The prolonged concentrations in edible tissues detected in this study are very small in relation to the U.S. tolerance. The acceptable U.S. daily intake (ADI) for total residues of chlortetracycline, oxytetracycline, and tetracycline is 25 micrograms/kg of body weight (21 CFR Part 556, 2011). In the standard 60 kg regulatory human, this equates to 1,500 micrograms, or 1,500,000 nanograms per day. The ADI is the amount of drug that may be consumed daily throughout life with no adverse effects, based on toxicology tests combined with safety factors in the calculations.

The consumption estimates for edible tissues in effect at the time that the tolerances for total residues of chlortetracycline, oxytetracycline, or tetracycline were established resulted in U.S. tissue tolerances of 2 ppm (2,000 ppb) in muscle, 6 ppm (6,000 ppb) in liver, and 12 ppm (12,000 ppb) in fat and kidney (21 CFR Part 556, 2011).

The Food and Agricultural Organization of the United Nations and World Health Organization (FAO/WHO) food standards for veterinary drug residues in food are listed as maximum residue limits (MRLs) in the Codex Alimentarius (food code). The reported MRLs for chlortetracycline/ oxytetracycline/tetracycline are 200 ppb for muscle, 600 ppb for liver, and 1200 ppb for kidney (Codex, 2011). Methods of analysis in support of these MRLs are also available on the Codex Alimentarius web site.

While it is again pointed out that this study did not incorporate regulatory methods, the results indicate edible tissue residues well below the U.S. tolerances for all edible tissues at zero days, and below the Codex Alimentarius MRLs at 7 days withdrawal time (the next withdrawal time tested after 0 withdrawal). When the values of muscle residues were evaluated from withdrawal days 7 to 28, the maximum observed residues were 29.0 ppb for CTC (both feed and water groups), 24.7 ppb for oxytetracycline (both feed and water groups), and 36.2 for tetracycline. However muscle residues continued to be detected (LOD of 1 ppb for chlortetracycline and oxytetracycline, LOD of 0.5 ppb for tetracycline) and quantified (LOQ of 4 ppb for all compounds) for the entire test period out through 28 days. Calculated muscle residue elimination half-lives from withdrawal day 7 to withdrawal day 28 were 18.0, 31.6, and 118.5 days for chlortetracycline, oxytetracycline, and tetracycline respectively. Continued presence of residues were also confirmed in kidney, liver, plasma, urine, and colon contents, although the magnitude and extent of residues varied by tissue.

It is important to understand the concept of epimerization of the tetracycline compounds in relation to the concentrations reported here. Degradation of chlortetracycline and tetracycline in the animal and possibly during the extraction process have been reported. In the United States, the regulatory method remains a microbiological assay, which would detect any active forms of these compounds. In other countries, methods of analysis may include techniques specifically detecting parent compound and one or more of the primary breakdown products. As described in the methods section of this report, it was confirmed that the analytical method reported here included the primary epimer of both chlortetracycline and tetracycline as indistinguishable from the parent compound. Therefore,

analytical methods detecting these compounds separately, due to chromatographic separation, would not be expected to report higher concentrations than reported here when the parent compound and the primary epimer concentrations were added together for an estimation of total residues.

Prolonged presence of biologically active chlortetracycline in the feces of pigs has been previously reported by Hansen, et al (2002) utilizing biosensors. Chlortetracycline was quantified in the feces of one pig in excess of 30 days after cessation of drug administration through the feed (LOD 30 ppb). In the study reported here, no CTC residues were detected in colon contents at 28 days (LOD 19.1 ppb), but multiple residues were quantified at 21 days well in excess of the 32.0 ppb LOQ.

The discovery of prolonged, low-level residues of the tetracyclines in swine tissues suggests some type of reservoir from which the drug is slowly released. The prolonged residues in bone in this study at concentrations in excess of other tissues suggest that bone serves as a reservoir. Calcium binding of the tetracyclines has been well established, as has persistence in bone. Estimates for tetracycline of 0.1% of an oral dose and 10% of an intravenous dose remaining in the skeleton after one week were reported by Buyske, et al.,(1960). In the Buyske, et al., study, a single 250 mg/kg oral dose of either chlortetracycline or tetracycline in rats resulted in bone concentrations of 2,200 to 2,400 ppb and 300 to 500 ppb, respectively, 4 weeks after administration.

In comparison, after the administration regimens in the study reported here, 28 day withdrawal chlortetracycline concentrations in bone ranged from 375 to 1120 ppb in the feed group and from 100 to 766 ppb in the water group. For tetracycline, 28 day bone concentrations ranged from 44.6 to 75.0 ppb. Oxytetracycline 28 day withdrawal concentrations in bone ranged from 54 to 117 ppb in the feed group and from 12.7 to 16.0 ppb in the water group. In all cases, the 21 day withdrawal bone concentrations closely approximated the 28 day concentrations, suggesting a slow release from the bone.

Elimination half-life values for muscle also suggested slow elimination at the low concentrations detected from withdrawal days 7 to 28, with chlortetracycline, oxytetracycline, and tetracycline values estimated as 18.0, 31.6, and 118.5 days, respectively.

Extrapolation beyond results reported in this study may lead to incorrect conclusions as to residue characteristics beyond 28 days withdrawal time due to changes in elimination characteristics, such as demonstrated between the withdrawal periods of zero to 7 days, and 7 to 28 days. For example, chlortetracycline demonstrated the highest bone concentrations on withdrawal day 28, yet also had the shortest estimated muscle half-life. Other considerations in evaluating this relationship include the relative recovery of the different tetracycline compounds from bone, the rate of release of residues from the bone for each compound, and the contribution of other reservoirs in the body to the extended low-level residues.

Bacitracin: The U.S. tolerance for bacitracin from zinc bacitracin or bacitracin methylene disalicylate in uncooked edible tissues of swine is 0.5 ppm (500 ppb). The non-regulatory method used in this study detected, but was not able to quantify concentrations in 3 of 10 muscle samples, in no kidney samples, and in 3 of 10 fat samples (including both zero and 7 day withdrawals after feeding).

Residues were detected but not quantified in one of the ten liver samples, and were quantified at concentrations near the tolerance in 5 of the 10 samples. It is not clear why 4 of these quantified samples were at 7 days withdrawal and only one was at zero days withdrawal. The sows were positively identified at all stages of the study and the number sequences were confirmed in relation to the sampling protocol.

Discussion References:

21 CFR Part 556 Title 21 (2011) – Food and Drugs, Chapter I Food and Drug Administration, Department of Health and Human Services, Subchapter E – Animal Drugs, Feeds, and Related Products. Part 556 Tolerances for Residues of New Animal Drugs in Food. Accessed 10-15-2011 at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=556.

Buyske, DA, Eisner HJ, Kelly RG. Concentration and persistence of tetracycline and chlortetracycline in bone. Journal of Pharmacology and Experimental Therapeutics 130:150-156, 1960.

Codex alimentarius Veterinary Drug Residues in Food Maximum Residue Limits (2011). Accessed 10-15-2011 at http://www.codexalimentarius.net/mrls/vetdrugs/jsp/vetd_q-e.jsp.

Hansen LH, Aarestrup F, SØrensen SJ. (2002) Quantification of bioavailable chlortetracycline in pig feces using a bacterial whole-cell biosensor. *Veterinary Microbiology* 87:51-57, 2002.

Appendix A: History of feed mill activity prior to mixing chlortetracycline and oxytetracycline-containing feed delivered to pigs in this study.

Other than the feed mixed for our study and bagged, there had been no CTC in this feed mill for well over a year. There are no tetracycline products available for feed inclusion.

Pre-study activity at the feed-mill is as follows:

Last load of feed delivered to the study room which would have been in the bin when pigs were placed in the study room.

On February 1st, 2 ton of ration 205 went to Bin 13 (the bin for the pigs in this study). Immediately prior to this mix in the feed mill, a batch of non-medicated sacked gilt feed was mixed. Prior to this there was 3 ton of non-medicated pig grower that was mixed and went on the truck. This would have come through the truck auger immediately prior to the ration 205. No medicated feed was mixed that day (2/1/2010). The last day prior to this that something was on the truck was January 26th, the diets on the 26th were all non-medicated.

Rations delivered while the pigs were in the study room

February 10th, study pigs were delivered to the barn in which the study was conducted. They were approximately 63 pounds at 70 days of age. They were started with non-medicated starter feed from another trial which was confirmed to be non-medicated.

On March 11, a ton of ration 205 was mixed for bin 13 (first batch of the day). Everything mixed and delivered that day was non-medicated swine feed.
The batch before the bin 13 ration mix on the previous day in the feed mill (March 10th) was horse feed with a lot of molasses (bagged). Also on the 10th, a non-medicated swine test diet was mixed and this was the last thing on the truck delivered on the 10th prior to the 11th.

On March 26th, a batch of phase II swine starter with neo/terra was mixed and bagged (did not go on truck). Three batches of non-medicated cow feed went through the mixer immediately after this batch.

The bagged feed for the study was mixed March 30th, it did not go on the truck. After this ration being bagged, no other oxytetracycline or chlortetracycline-containing feeds were mixed in the mill prior to the mixing of two loads delivered to Bin 13 during the study.

Bin 13 mixed and delivered on April 7th,
Previous day (6th), 5 rations mixed, the middle one was a lamb finish ration with bovatec, otherwise no medications.
Bin 13 ration was the first mixed on the 7th. This ration was on the back bin of the truck and would have come off first, the second bin that day was a mix with neo/terra but was mixed after bin 13 ration and unloaded after bin 13 ration.

Bin 13 - 2 ton mixed and delivered on April 20th

Six rations mixed the previous day, the first one had Rumensin, second was bull feed, rations 4-6 were swine rations with no medication. All feed through truck on the 19th was non-medicated. On the 20th, the first 3 bins on the truck were non-medicated swine rations. This is the load that would have gone to the swine farm.

Appendix B: Standard curves and quality control results for each tissue within treatment.

Standard curve and quality control (QC) sample results for analytical runs are presented here. Acceptance of standards for the standard curve is at ± 15% for all points to be included, except for the lowest concentration, which may vary ± 20%. Acceptance of quality control sample results is at ± 20% for the lower QCs set near the bottom of the curve and ± 15% for the higher standards. Three of 4 QC samples are required to meet acceptance requirements for the analytical run to be accepted.

In some cases the standard curve and quality control results may be the same for a tissue related to different routes for the same drug. When smaller numbers of samples were analyzed for a drug (e.g., plasma and bone), the samples from both routes of administration for either chlortetracycline or oxytetracycline may have been analyzed as one analytical run.

Chlortetracycline administered through the feed at a target of 10 mg/lb bodyweight for 14 days: Standard curve and QCs. (CTC Feed)

CTC Feed – Muscle analysis

Calculated Limit of Detection: (LOD) 1.0 ng/g
Calculated Limit of Quantitation (LOQ): 3.1 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	3.8	94
Standard	8	6.8	85
Standard	16	14.8	92
Standard	32	30.9	96
Standard	64	63.7	100
Standard	128	130	101
Standard	256	256	100
Standard	512	588	115
Standard	1024	984	96
QC	8	7.7	96
QC	8	7.1	88
QC	64	66.8	104
QC	64	71.8	112

CTC Feed – Kidney analysis

Calculated Limit of Detection: (LOD) 1.4 ng/g
Calculated Limit of Quantitation (LOQ): 4.1 ng/g
Lower quantitation limit of standard curve: 8 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	7.7	97
Standard	16	15.9	99
Standard	32	27.9	87
Standard	64	59.0	92
Standard	128	102.0	79-out
Standard	256	262.0	102
Standard	512	513.0	100
Standard	1024	1030.0	100
Standard	2048	2020.0	99
Standard	4096	4060.0	99
QC	8	8.7	108
QC	8	6.8	86
QC	64	68.4	107
QC	64	59.8	94
QC	512	521.0	102
QC	512	535.0	105

CTC Feed – Liver Analysis

Calculated Limit of Detection: (LOD) 0.3 ng/g
Calculated Limit of Quantitation (LOQ): 0.9 ng/g
Lower quantitation limit of standard curve: 2 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	2	2.3	115
Standard	4	4.0	99
Standard	8	7.6	95
Standard	16	15.3	96
Standard	32	28.3	88
Standard	64	59.0	92
Standard	128	107.0	83-out
Standard	256	292.0	114
Standard	512	511.0	100
Standard	1024	1020.0	100
QC	8	8.6	107
QC	8	9.0	113
QC	64	66.6	104
QC	64	63.3	99

CTC Feed – Fat Analysis

Calculated Limit of Detection: (LOD) 0.1 ng/g
Calculated Limit of Quantitation (LOQ): 0.3 ng/g
Lower quantitation limit of standard curve: 1 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	1	1.1	108
Standard	2	2.1	107
Standard	4	5.3	131-out
Standard	8	8.8	110
Standard	16	14.6	91
Standard	32	27.2	85
Standard	64	58.0	91
Standard	128	138.0	108
Standard	256	226.0	88
Standard	512	559.0	109
Standard	1024	1010.0	98
QC	8	7.7	96
QC	8	7.4	93
QC	64	68.8	108
QC	64	58.6	92

CTC Feed – Bone Analysis

Calculated Limit of Detection: (LOD) N/A
Calculated Limit of Quantitation (LOQ): 32 ng/g

Since a non-matrix curve was used with diluted samples, the blank is solvent and the LOD is essentially zero. The LOQ is therefore the lowest linear standard.

	Standard	Calculated	
	Concentration	Concentration	Accuracy
Tag	ng/g	ng/g	%
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	32	28.8	90
Standard	64	57.5	90
Standard	128	121	94
Standard	256	255	100
Standard	512	449	88
Standard	1020	1190	116-out
Standard	2050	1980	97
QC	256	260	102
QC	256	263	103
QC	2050	2040	100
QC	2050	1970	96

CTC Feed – manure/colon content analysis

Calculated Limit of Detection: (LOD) 19.1 ng/g
Calculated Limit of Quantitation (LOQ): 32 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, the LOD and LOQ were applied, and then corrections to dry weight were made for samples >LOQ.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	32	27.2	85
Standard	64	67.3	105
Standard	128	144	113
Standard	256	256	100
Standard	512	493	96
Standard	1020	993	97
Standard	2050	1810	88
QC	128	140	109
QC	128	128	100
QC	512	506	99
QC	512	492	96

Oxytetracycline administered through the feed at a target of 10 mg/lb bodyweight for 14 days: Standard curve and QCs. (OTC Feed)

OTC Feed - Muscle

Calculated Limit of Detection: (LOD) 1.0 ng/g
Calculated Limit of Quantitation (LOQ): 2.9 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	3.3	82
Standard	8	7.3	91
Standard	16	16.3	102
Standard	32	32.6	102
Standard	64	63.7	100
Standard	128	130	101
Standard	256	255	100
QC	8	6.3	79 - out
QC	8	7.0	87
QC	64	71.0	111
QC	64	70.5	110

OTC Feed - Kidney

Calculated Limit of Detection: (LOD) 1.2 ng/g
Calculated Limit of Quantitation (LOQ): 3.5 ng/g
Lower quantitation limit of standard curve: 8 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	7.6	95
Standard	16	17.2	107
Standard	32	34.7	108
Standard	64	66.2	103
Standard	128	147.0	115
Standard	256	209.0	82-out
Standard	512	541.0	106
Standard	1024	1020.0	100
Standard	2048	1920.0	94
QC	8	7.6	96
QC	64	68.5	107
QC	64	58.2	91
QC	512	546.0	107
QC	512	588.0	115

OTC Feed - Liver

Calculated Limit of Detection: (LOD) 1.0 ng/g
Calculated Limit of Quantitation (LOQ): 3.1 ng/g
Lower quantitation limit of standard curve: 8 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	7.7	96
Standard	16	16.2	101
Standard	32	37.0	116-out
Standard	64	65.0	102
Standard	128	120.0	94
Standard	256	259.0	101
QC	8	7.9	98
QC	8	6.7	83-out
QC	64	66.9	105
QC	64	72.6	113

OTC Feed - Fat

Calculated Limit of Detection: (LOD) 0.7 ng/g
Calculated Limit of Quantitation (LOQ): 2.2 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	4.1	101
Standard	8	6.3	78-out
Standard	16	14.1	88
Standard	32	31.4	98
Standard	64	66.4	104
Standard	128	127.0	99
QC	8	8.7	109
QC	8	7.9	98
QC	64	69.9	109
QC	64	56.7	89

OTC Feed – Bone Analysis

Calculated Limit of Detection: (LOD) 2.0 ng/g
Calculated Limit of Quantitation (LOQ): 4 ng/g

Since a non-matrix curve was used with diluted samples, the blank is solvent and the LOD is essentially zero. The LOQ is therefore the lowest linear standard.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	4.1	103
Standard	8	9.7	121-out
Standard	16	14.7	92
Standard	32	34.7	108
Standard	64	58.3	91
Standard	128	126	98
Standard	256	270	105
QC	16	18.0	112
QC	16	16.9	105
QC	64	57.8	90
QC	64	66.8	104

OTC Feed – manure/colon content analysis

Calculated Limit of Detection: (LOD) 53.7 ng/g
Calculated Limit of Quantitation (LOQ): 74.2 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, the LOD and LOQ were applied, and then corrections to dry weight were made for samples >LOQ.

	Standard	Calculated	
	Concentration	Concentration	Accuracy
Tag	ng/g	ng/g	%
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	64	74.2	116
Standard	128	145	113
Standard	256	255	100
Standard	512	526	103
Standard	1020	1020	100
Standard	2050	1850	90
QC	128	115	90
QC	128	114	89
QC	512	535	105
QC	512	513	100

Chlortetracycline administered through the water at a target of 10 mg/lb bodyweight for 5 days: Standard curve and QCs. (CTC Water)

CTC Water - Muscle

Calculated Limit of Detection: (LOD) 1.0 ng/g
Calculated Limit of Quantitation (LOQ): 3.1 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	4.1	101
Standard	8	7.1	89
Standard	16	16.5	103
Standard	32	32.3	101
Standard	64	51.2	80-out
Standard	128	134	105
Standard	256	292	114
QC	8	8.3	103
QC	8	7.7	96
QC	64	64.6	101
QC	64	62.8	98

CTC Water - KIdney

Calculated Limit of Detection: (LOD) 1.4 ng/g
Calculated Limit of Quantitation (LOQ): 4.1 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	3.8	94
Standard	8	7.6	95
Standard	16	13.8	86
Standard	32	33.8	106
Standard	64	45.1	70-out
Standard	128	120.0	94
Standard	256	260.0	102
Standard	512	515.0	101
Standard	1024	934.0	91
Standard	2048	2080.0	102
Standard	4096	4100.0	100
QC	8	7.5	93
QC	8	6.7	83
QC	64	61.2	96
QC	64	67.1	105
QC	512	531.0	104
QC	512	466.0	91

CTC Water - Liver

Calculated Limit of Detection: (LOD) 0.3 ng/g
Calculated Limit of Quantitation (LOQ): 0.9 ng/g
Lower quantitation limit of standard curve: 8 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	8.6	108
Standard	16	18.1	113
Standard	32	42.2	132-out
Standard	64	72.6	113
Standard	128	66.0	52-out
Standard	256	238.0	93
Standard	512	458.0	90
Standard	1024	1050.0	103
QC	8	8.8	110
QC	8	8.9	111
QC	64	68.9	108
QC	64	75.8	118

CTC Water - Fat

Calculated Limit of Detection: (LOD) 0.1 ng/g
Calculated Limit of Quantitation (LOQ): 0.3 ng/g
Lower quantitation limit of standard curve: 8 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	8.0	100
Standard	16	18.7	117-out
Standard	32	28.2	88
Standard	64	52.1	81-out
Standard	128	128.0	100
Standard	256	279.0	109
Standard	512	461.0	90
Standard	1024	1040.0	102
QC	8	9.0	113
QC	8	8.3	104
QC	64	60.4	94
QC	64	61.9	97

CTC Water – Bone Analysis

Calculated Limit of Detection: (LOD) N/A
Calculated Limit of Quantitation (LOQ): 32 ng/g

Since a non-matrix curve was used with diluted samples, the blank is solvent and the LOD is essentially zero. The LOQ is therefore the lowest linear standard.

	Standard	Calculated	
	Concentration	Concentration	Accuracy
Tag	ng/g	ng/g	%
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	32	28.8	90
Standard	64	57.5	90
Standard	128	121	94
Standard	256	255	100
Standard	512	449	88
Standard	1020	1190	116-out
Standard	2050	1980	97
Standard	4100	3280	80-out
QC	256	260	102
QC	256	263	103
QC	2050	2040	100
QC	2050	1970	96

CTC Water – manure/colon content analysis

Calculated Limit of Detection: (LOD) 19.1 ng/g
Calculated Limit of Quantitation (LOQ): 32 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, the LOD and LOQ were applied, and then corrections to dry weight were made for samples >LOQ.

	Standard	Calculated	
	Concentration	Concentration	Accuracy
Tag	ng/g	ng/g	%
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	32	27.2	85
Standard	64	67.3	105
Standard	128	144	113
Standard	256	256	100
Standard	512	493	96
Standard	1020	993	97
Standard	2050	1810	88
QC	128	140	109
QC	128	128	100
QC	512	506	99
QC	512	492	96

Oxytetracycline administered through the water at a target of 10 mg/lb bodyweight for 5 days: Standard curve and QCs. (OTC Water)

OTC Water - Muscle

Calculated Limit of Detection: (LOD) 1.0 ng/g
Calculated Limit of Quantitation (LOQ): 2.9 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	3.5	88
Standard	8	7.8	97
Standard	16	13.3	83 - out
Standard	32	28.0	88
Standard	64	63.0	99
Standard	128	130	102
Standard	256	265	104
Standard	512	510.0	100
Standard	1024	1020.0	100
QC	8	6.7	84
QC	8	8.6	107
QC	64	61.7	96
QC	64	64.8	101

OTC Water - Kidney

Calculated Limit of Detection: (LOD) 1.2 ng/g
Calculated Limit of Quantitation (LOQ): 3.5 ng/g
Lower quantitation limit of standard curve: 8 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	8.7	109
Standard	16	16.8	105
Standard	32	33.6	105
Standard	64	64.2	100
Standard	128	129.0	101
Standard	256	278.0	109
Standard	512	522.0	102
Standard	1024	948.0	93
Standard	2048	2080.0	102
QC	8	9.1	113
QC	64	62.8	98
QC	64	61.5	96
QC	512	509	100
QC	512	503	98

OTC Water - Liver

Calculated Limit of Detection: (LOD) 1.0 ng/g
Calculated Limit of Quantitation (LOQ): 3.1 ng/g
Lower quantitation limit of standard curve: 8 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	0.5	< 0	N/A
Standard	8	8.0	100
Standard	16	17.2	108
Standard	32	35.6	111
Standard	64	81.4	127-out
Standard	128	114.0	89
Standard	256	248.0	97
Standard	512	514.0	100
Standard	1024	1030.0	100
QC	8	7.7	96
QC	8	6.9	86
QC	64	68.0	106
QC	64	58.7	92

OTC Water - Fat

Calculated Limit of Detection: (LOD) 0.7 ng/g
Calculated Limit of Quantitation (LOQ): 2.2 ng/g
Lower quantitation limit of standard curve: 16 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	9.8	122-out
Standard	16	18.7	117
Standard	32	28.5	89
Standard	64	66.9	105
Standard	128	115.0	90
Standard	256	262.0	102
Standard	512	512.0	100
QC	8	8.5	106
QC	8	10.0	125-out
QC	64	58.7	92
QC	64	56.6	89

OTC Water – Bone Analysis

Calculated Limit of Detection: (LOD) 2.0 ng/g
Calculated Limit of Quantitation (LOQ): 4 ng/g

Since a non-matrix curve was used with diluted samples, the blank is solvent and the LOD is essentially zero. The LOQ is therefore the lowest linear standard.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	4.1	103
Standard	8	9.7	121-out
Standard	16	14.7	92
Standard	32	34.7	108
Standard	64	58.3	91
Standard	128	126	98
Standard	256	270	105
QC	8	18.0	112
QC	8	16.9	105
QC	64	57.8	90
QC	64	66.8	104

OTC Water – manure/colon content analysis

Calculated Limit of Detection: (LOD) 53.7 ng/g
Calculated Limit of Quantitation (LOQ): 74.2 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, the LOD and LOQ were applied, and then corrections to dry weight were made for samples >LOQ.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	64	74.2	116
Standard	128	145	113
Standard	256	255	100
Standard	512	526	103
Standard	1020	1020	100
Standard	2050	1850	90
QC	128	115	90
QC	128	114	89
QC	512	535	105
QC	512	513	100

Tetracycline administered through the water at a target of 10 mg/lb bodyweight for 5 days: Standard curve and QCs. (TET Water)

TET Water - Muscle

Calculated Limit of Detection: (LOD) 0.5 ng/g
Calculated Limit of Quantitation (LOQ): 1.4 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	4.2	106
Standard	8	7.8	98
Standard	16	12.1	76 - out
Standard	32	28.5	89
Standard	64	76.0	119 - out
Standard	128	124.0	97
Standard	256	249.0	97
Standard	512	515.0	101
Standard	1024	896.0	88
QC	8	6.0	75 - out
QC	8	6.7	83
QC	64	65.9	103
QC	64	67.3	105

TET Water - Kidney

Calculated Limit of Detection: (LOD) 0.6 ng/g
Calculated Limit of Quantitation (LOQ): 1.9 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	4.7	117
Standard	8	7.3	91
Standard	16	12.9	81-out
Standard	32	26.1	82-out
Standard	64	40.6	63-out
Standard	128	141.0	110
Standard	256	273.0	107
Standard	512	488.0	95
Standard	1024	1050.0	103
Standard	2048	3090.0	151
Standard	4096	4090.0	100
QC	8	7.7	97
QC	8	8.5	106
QC	64	77.6	121-out
QC	64	69.7	109
QC	512	500.0	98

TET Water - Liver

Calculated Limit of Detection: (LOD) 0.2 ng/g
Calculated Limit of Quantitation (LOQ): 0.7 ng/g
Lower quantitation limit of standard curve: 1 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	1	1.1	110
Standard	2	2.0	100
Standard	4	2.5	63-out
Standard	8	7.1	89
Standard	16	11.7	73-out
Standard	32	29.3	92
Standard	64	47.8	75-out
Standard	128	138.0	108
Standard	256	286.0	112
Standard	512	497.0	97
QC	8	5.2	64-out
QC	8	7.8	97
QC	64	60.2	94
QC	64	54.4	85

TET Water - Fat

Calculated Limit of Detection: (LOD) 0.2 ng/g
Calculated Limit of Quantitation (LOQ): 1.2 ng/g
Lower quantitation limit of standard curve: 4 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	4	4.3	107
Standard	8	7.4	93
Standard	16	16.3	102
Standard	32	31.3	98
Standard	64	68.1	106
Standard	128	126.0	98
Standard	256	235.0	92
Standard	512	523.0	102
Standard	1024	999.0	98
QC	8	6.6	83
QC	8	9.1	113
QC	64	63.2	99
QC	64	62.1	97

TET Water – Bone Analysis

Calculated Limit of Detection: (LOD) 3.6 ng/g
Calculated Limit of Quantitation (LOQ): 8 ng/g

Since a non-matrix curve was used with diluted samples, the blank is solvent and the LOD is essentially zero. The LOQ is therefore the lowest linear standard.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	8	8.3	104
Standard	16	17.2	107
Standard	32	33.4	104
Standard	64	63.2	99
Standard	128	131.0	102
Standard	256	274.0	107
QC	8	14.3	89
QC	8	16.6	104
QC	64	66.4	104
QC	64	62.6	98

TET Water – manure/colon content analysis

Calculated Limit of Detection: (LOD) 6.4 ng/g
Calculated Limit of Quantitation (LOQ): 16 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, the LOD and LOQ were applied, and then corrections to dry weight were made for samples >LOQ.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	16	16.2	101
Standard	32	27.3	85
Standard	64	60.2	94
Standard	128	125	98
Standard	256	346	135
Standard	512	516	101
Standard	1020	949	93
QC	128	130	102
QC	128	134	105
QC	512	484	95
QC	512	478	93

Pen Floor Manure Analysis Standard Curves and Quality Control Samples

Chlortetracycline in Manure

Calculated Limit of Detection: (LOD) : Since a non-matrix standard curve was used with diluted samples, the blank is solvent and the LOD is essentially zero.

Calculated Limit of Quantitation (LOQ): 16 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, and then corrections to dry weight were made.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	32	28.8	90
Standard	64	57.5	90
Standard	128	121	94
Standard	256	255	100
Standard	512	449	88
Standard	1020	1190	116
Standard	2050	1980	97
QC	256	260	102
QC	256	263	103
QC	2050	2040	100
QC	2050	1970	96

Oxytetracycline in Manure

Calculated Limit of Detection: (LOD) : Since a non-matrix standard curve was used with diluted samples, the blank is solvent and the LOD is essentially zero.

Calculated Limit of Quantitation (LOQ): 64 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, and then corrections to dry weight were made.

	Standard	Calculated	
	Concentration	Concentration	Accuracy
Tag	ng/g	ng/g	%
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	64	65.8	103
Standard	128	131	103
Standard	256	264	103
Standard	512	547	107
Standard	1020	1020	99
Standard	2050	2040	100
QC	256	288	112
QC	256	267	104
QC	2050	1980	97
QC	2050	2060	100

Tetracycline in Manure

Calculated Limit of Detection: (LOD) : Since a non-matrix standard curve was used with diluted samples, the blank is solvent and the LOD is essentially zero.

Calculated Limit of Quantitation (LOQ): 64 ng/g wet weight (lowest linear standard)

Note: Determinations were made on a wet weight basis, and then corrections to dry weight were made.

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank	0	N/A	N/A
Blank	0	N/A	N/A
Standard	64	66.0	103
Standard	128	140	110
Standard	256	267	104
Standard	512	495	97
Standard	1020	1050	102
Standard	2050	2040	100
QC	256	256	100
QC	256	272	106
QC	2050	2040	100
QC	2050	2090	102

Bacitracin Standard Curves and Quality Control (QC) Samples

Bacitracin - Muscle: LOD 38 ng/g, LOQ 200 ng/g (lowest linear standard)

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank		N/A	
Blank		N/A	
Standard	200	197	98
Standard	400	345	86
Standard	700	529	76-out
Standard	1000	1190	119
Standard	2000	2180	109
Standard	4000	3460	86
Standard	7000	7250	104
QC	200	211	105
QC	200	212	106
QC	4000	4110	103
QC	4000	3840	96

Bacitracin - Liver: LOD 97 ng/g, LOQ 292 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank		N/A	
Blank		N/A	
Standard	200	231	116
Standard	400	351	88
Standard	700	807	115
Standard	1000	1060	106
Standard	2000	1980	99
Standard	4000	3840	96
Standard	7000	7080	101
QC	400	462	116
QC	400	536	134-out
QC	2000	2150	107
QC	2000	2180	109

Bacitracin – Kidney: LOD 75 ng/g, LOQ 225 ng/g

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank		N/A	
Blank		N/A	
Standard	200	191	95
Standard	400	436	109
Standard	700	467	67-out
Standard	1000	960	96
Standard	2000	1990	100
Standard	4000	4260	106
Standard	7000	6860	98
QC	400	436	109
QC	400	567	142-out
QC	2000	1910	96
QC	2000	1930	97

Bacitracin – Fat: LOD 17 ng/g, LOQ 100 ng/g (lowest linear standard)

Tag	Standard Concentration ng/g	Calculated Concentration ng/g	Accuracy %
Double Blank		N/A	
Blank		N/A	
Standard	100	90	90
Standard	200	191	95
Standard	400	407	102
Standard	700	628	90
Standard	1000	762	76 - out
Standard	2000	2100	105
Standard	4000	4020	101
Standard	7000	6970	100
QC	400	413	103
QC	400	417	104
QC	2000	2100	105
QC	2000	2150	108