RESEARCH REPORT
INTERNATIONAL TRADE

**Title:** Estimation and validation of safe withdrawal times to satisfy export market residue limits (MRLs) for tetracyclines, sulfonamides, and aminoglycosides in target tissues in swine – **NPB #09-256**

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**Institution:** North Carolina State University

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**Industry Summary:**
The objective of this project was to determine whether pigs exposed to water medications can result in stomach tissue levels that exceed safety levels and thus affect export of pork products to countries with safe levels significantly different and set at concentration levels lower than in the US. Commercial sulfamethazine and tetracycline mediations were given to weanling and slaughter weight pigs to determine whether the pig contained the drug at specific withdrawal times for these two drugs. Data from these studies suggest that in spite of the known low bioavailability of these drugs that drug residue levels can persist in several pork tissues as well as stomach tissues for many days beyond the US label withdrawal time for the drug. This is the first report of stomach tissue as the target tissue for tetracycline. These findings are critically important for US export of pork products where the tolerance levels in foreign countries are significantly lower than those in the US. This report provides recommendations based on our experimental findings that will assist swine veterinarians and pork producers in planning treatment protocols and extending withdrawal times for tetracycline and sulfamethazine when used as water additives at the approved label dose.

**Keywords: Sulfamethazine; tetracycline; residues; stomach; withdrawal time.**

**Scientific Abstract:**
The main objective of this pharmacokinetics projects was to assess whether water medications can result in violative residues in blood and tissues of weanling and slaughter weight pigs in a commercial setting. Because of time and cost constraints only sulfamethazine and tetracycline mediations were assessed in these two age classes. Pigs were treated at the recommended dose and dose intervals (4 days) and then slaughtered at specific time periods including the approved withdrawal times and several days beyond the approved withdrawal times. Five (5) time points and 5 replicate pigs for each treatment group were used in this study. Special emphasis was placed on collecting stomach tissue amongst other tissues. Analysis of the tetracycline and sulfamethazine in plasma, muscle, liver, kidney, and stomach tissue data in weanling and slaughter weight pigs demonstrated that these drugs can remain within several tissues, including stomach tissue, beyond the labeled WDT. There were clear differences in withdrawal times between weanling and finisher pigs. Our analyses further demonstrated that WDT should be significantly increased when pork products are exported to jurisdictions with MRLs significantly lower than US tolerances.

**Introduction:**

Pigs medicated with sulfamethazine alone in feed are required to adhere to a 15 day withdrawal time (WDT) and previous work in our laboratory demonstrated why this WDT may be inadequate. Tetracyclines or chlortetracyclines are often given by themselves or in combination with sulfamethazine in feed. When given by itself in feed for finishing pigs the WDT is often zero days while as a water additive the WDT can be 4 days. There are scientifically plausible reasons as outlined below why a residue violation can occur even if the above label WDTs are followed by the producer:

(1) Production practices and/or disease management varies from farm to farm, and this demographic variability could result in drug depletion curves significantly different from those generated from the data used in the initial approval process. There is also intra- and inter-individual variability within a population of pigs. The approval process only requires at a minimum of 25 animals to be slaughtered (5 animals at 5 time points) and the mean depletion curves from these animals do not reflect the true population and associated variance on a livestock farm**.** Our laboratory recently demonstrated that a population approach identified several production variables (e.g., water consumption; barn temperature) which may account for variable depletion curves in some swine operations (Mason et al., 2009, 2010). In summary, our laboratory over the last 10 years have developed both population pharmacokinetics and physiologically-based pharmacokinetic techniques to identify various aspects of swine production that may critically impact the depletion curves of two drugs that are frequently used in swine operations and are the focus for this call for proposals.

(2) The maximum residue levels (MRLs) in many export markets such as the European Union (EU), Russia, and Asia are significantly lower than the US tolerance for many of drugs used in the swine production facilities. Our laboratory reported on this discrepancy in several other livestock drugs as shown by Baynes et al. (1998). By simply requesting that producers target a lower safe level, one strategy could involve withholding the animals for a few additional hours or days before slaughter. Determining what those additional days should be requires integration of all the relevant data sets which this PI has access to via the Food Animal Residue Avoidance Databank (FARAD) and performing the simulations using pharmacostatistical software with which our laboratory has over 12 years experience. Intuitively, this can result in a significantly longer WDT if the MRL is lower than the U.S. tolerance. ***The second objective of this project is therefore to utilize the above data sets generated from the proposed population study to calculate extended WDTs for sulfamethazine, and tetracycline, to ensure that when animals are sent to slaughter there is significant confidence that residue levels in target tissues are below the MRLs in foreign markets.***

**Objectives:**

The main objectives of this project are:

1. To assess the population pharmacokinetics of 3 select drugs (sulfamethazine, tetracycline, and gentamicin) which may be used on swine farms and in so doing identify and quantify variables in a traditional swine facility that influence depletion curves for these 3 drugs. *Because of time and cost constraints, this objective was limited to sulfamethazine and tetracycline water medications and not gentamicin.*

2. To utilize the above data sets generated from the population study to calculate extended WDTs for the 3 selected drugs to ensure that when pigs are slaughtered there is significant confidence that residue levels in target tissues (muscle, liver, kidney, and stomach tissue) are below the MRLs in the EU and Asian countries.

**Materials & Methods:**

**Experimental Design and Animals**:

This project was divided into 5 phases; namely, a pilot study and 4 trials and conducted at the North Carolina State University Swine Education Unit. This farm was selected as it is managed as a typical commercial swine operation and its close proximity to our analytical laboratory facilitated sample collection and processing. The pilot study involved exposing 20 weanling pigs to tetracycline water medication and sampling blood to assess diurnal plasma concentrations as well as stomach tissue concentrations. The next 4 trials involved water medication of weanling (10 - 30 lbs) and slaughter (210 – 250 lb) weight pigs to either tetracycline or sulfamethazine. Blood samples were collected on the $3^{rd}$, $4^{th}$, and $5^{th}$ day of treatment to assess steady state plasma concentrations and animals were slaughtered on the last day of treatment and at 4 later time points to assess drug residue depletion.

**Drugs and Administration**

The water was medicated with HN55 Chemilizer set at an injection Ratio: 1:128. The drugs were used according to label. Tetracycline: TET-SOL 324 (tetracycline hydrochloride) powder (Alpharma, inc. Animal Health) Bridgewater, NJ NADA: #65-140 Purity: 810 g/1134 g in package 71.4% pure. This system allowed for delivery of approximately 10mg/lb BW tetracycline in the drinking water for 4 days as recommended by the label. Sulfamethazine: SMZ-Med 454 (sodium Sulfamethazine) powder (Bimedia, Inc). Le Sueur, MN. ANADA: #200-434 Purity: 100% Sodium sulfamethazine per package. This system allowed for delivery of approximately 247.5 mg/kg BW for day 1 and 123.75 mg/kg BW for day2 – day 4 in the drinking water for swine as recommended by the label. All drug concentrate solutions were made fresh every day so as to minimize the effects of drug degradation in the barn environment.

**Analyses of Tetracycline and Sulfamethazine in Plasma samples**

Tetracycline: Blood was collected from each pig via venapuncture of the anterior vena cava into a 10-mL sodium heparin tube. Plasma was harvested from all blood samples after centrifugation within 2 hours of collection. 200 µl of plasma were added to a Amicon Ultra 0.5 ml Ultracel -10K Membrane filter (Millipore, Billerica MA). 200 µl of the releasing agent (78% water, 20% acetonitrile, 2% o-phosphoric acid) was then added. The Ultracel apparatus was placed in an eppendorf tube and centrifuged at 8500 *g* for 30 min at $4^{o}$C.
HPLC System: Tetracycline was quantified using the 354.4 nm wavelength by the Empower Software (Waters Corporation). The samples were then run on a Waters Atlantis T3 5µm 4.6 x 150 mm and guard column in 25% methanol, 75% .01 M oxalic acid buffer. The run time was 10 min and injection volume was 50 µl. The limit of detection (LOD) was 94 ng/ml and limit of quantification (LOQ) was 187.5 ng/ml.

Sulfamethazine: Blood was collected as described above. One milliliter of plasma was then acidified by the addition of 20 µl o-phosphoric acid . The sample then underwent solid phase extraction. An Oasis MCX 3cc 60 mg (Waters, Milford, MA) cartridge was conditioned with 1 ml of methanol and 1 ml of water. The sample was then added and the cartridge was washed with 1 ml .1N HCL and 1 ml methanol. The cartridges were dried under vacuum for 30 s. Samples were then eluted with 1 ml ammonium hydroxide-acetonitrile (5:95, vol/vol) and the cartridges were then dried again under vacuum. Elution volumes were evaporated to dryness in a Turbo Vap LV evaporated (Zymark; Hopkington, MA) for 15 min at 50°C and 15 mm Hg lb/in$^2$ reagent-grade nitrogen gas. Remaining residue was then reconstituted with .5 ml ammonium acetate buffer (.1 M), vortexed and then injected into the HPLC machine.
HPLC System: The HPLC machine was the waters alliance HPLC. A Waters Atlantis T3 5 µm column (4.6 x 155 mm) was used. All injection volumes for sulfamethazine were 10 µl and the flow rate was 1 ml/min isocratic. Autosampler and column temperature was maintained at 25°C. The wavelength detector was set at

267 nm. The mobile phase conditions for these plasma samples were acetonitrile-water (15:85, vol/vol). All reagents were HPLC grade. The limit of detection (LOD) was 94 ng/ml and limit of quantification (LOQ) was 187.5 ng/ml.

**Analyses of Tetracyline and Sulfamethazine Residues in Stomach Tissues**.

Tetracycline: One gram of tissue was weighed out and 5 ml of McIlvaine EDTA Buffer was added one sample at a time. Each sample was homogenized with a VWR VDI 12 homogenizer for 30 s. The homogenizer tool was rinsed and cleaned between each sample with 25% ethanol, 25% acetone and 50% water mixture. The samples were then centrifuged at 5,000 rpm for 10 min at 4° C. Supernatant was collected and then 5 ml of Mcilvaine was added to the sample. Homogenization was repeated and the sample was then spun again for 5,000 rpm for 10 min at 4°C. Once all of the supernatant was collected, it was centrifuged one more time on the same program and then the supernatant was then processed through solid phase extraction. A mega bond elut plexa 500mg 6ml cartridge (Agilent Technologies) was conditioned with 3 mL of methanol and then 1 ml of pure water. The sample was then filtered through an .8µm Millex-AA (Millipore, Billerica MA) filter. The cartridges were then dried under vacuum. The syringe was then rinsed with 3 ml of water-methanol (95:5, vol/vol) and dried under vacuum again. The samples were then eluted with 2 ml of methanol, dried under vacuum, then 2 more ml of methanol were added and then the cartridges were then dried a final time under vacuum. The elution volumes were then evaporated to dryness in a Turbo Vap LV evaporator (Zymark; Hopkington, MA) for 90 min at 35°C and 15 mm Hg lb/in$^2$ reagent grade nitrogen gas. Residue was then reconstituted with 1ml of oxalic acid-acetonitrile mobile phase (75:25; vol/vol), vortexed and then filtered through a .45 Millex-HV (Millipore, Billerica MA) filter and then injected into the HPLC system. The limit of detection (LOD) was 100 ng/ml and limit of quantification (LOQ) was 500 ng/ml.

Sulfamethazine: One gram minced pig stomach was placed into 15ml disposable centrifuge tube with 2ml of 1.0M perchloric acid (Sigma-Aldrich)and homogenize for 45s on highest speed,6, on VWR VDI12 homogenizer. This was then centrifuged for 10minutes at 1200RPM and 24C (IEC Centra-8Rcentrifuge). The homogenizer tool was cleaned as described above for tetracycline. 500ul of top supernatant layer was removed and place into Millipore Amicon Ultra-0.5ml 10K Ultracel -10K membrane part no. UFC5-010-96) and centrifuged at 15,000xg, 4C for 10min (ThermoFisher Sorvall ST 16R). The filtrate in bottom portion of microcentrifuge tube was removed and place into Waters total recovery vials for HPLC analysis

HPLC Conditions: Waters 2695 Alliance with 2487 dual wavelength UV detector. Wavelength: 267nm; Column temp.: 30C; Sample temp.: 24C Flow rate: 1.0 ml/min. Mobile phase composition: 77% 0.1M ammonium acetate pH 4.5 adjusted with glacial acetic acid: 23% acetonitrile. Column: Waters Atlantis T3 4.6mm x 150mm; 5um particle size plus matching guard column. Retention time of sulfamethazine is around 5.5 minutes. Overall runtime set to 8.00 minutes. Injection volume: 25.0 ul.

**Analyses of Tetracycline and Sulfamethazine Residues in Muscle:**

One gram (1g) minced pig muscle was weighed into 15ml disposeable centrifuge tube. Then added 2ml of 1.0M perchloric acid (Sigma-Aldrich). Homogenize for 45s on highest speed,6, on VWR VDI12 homogenizer. Centrifuge for 10minutes at 1200RPM and 24C (IEC Centra-8Rcentrifuge). Filter about 700ul of the top clear supernatant layer through 0.45um syringe filter (Millipore Millex-HV 13mm PVDF Cat No: SLHVX13NL) directly into HPLC Waters total recovery vials for analysis.

**HPLC Conditions**:

Waters 2695 Alliance with 2487 dual wavelength UV detector was used for HPLC. For tetracycline: Wavelength: 354nm; Column temp.: 35C; Sample temp.: 4C Flow rate: 1.0 ml/min.

Mobile phase composition: 75% 0.05M oxalic acid: 25% acetonitrile. Column: Waters Atlantis T3 4.6mm x 150mm; 5um particle size plus matching guard column. Retention time of tetracycline is around 3.7 minutes. Overall runtime set to 6.00 minutes. Injection volume: 25.0 ul. For sulfamethazine, Wavelength: 267nm; Column temp.: 30C; Sample temp.: 24C Flow rate: 1.0 ml/min. Mobile phase composition: 77% 0.1M

ammonium acetate pH 4.5 adjusted with glacial acetic acid: 23% acetonitrile. Column: Waters Atlantis T3 4.6mm x 150mm; 5um particle size plus matching guard column. Retention time of sulfamethazine is around 5.5 minutes. Overall runtime set to 8.00 minutes; Injection volume: 25.0 ul

**Analyses of Tetracycline and Sulfamethazine Residues in Liver**

The liver was blended and homogenized as described above. Centrifugation was for 10minutes at 5000RPM and 22C (room temp.)(ThermoSci.Sorval ST 16R centrifuge).

For tetracycline, additional cleanup of supernatant with Waters Oasis HLB 3cc (60mg) SPE cartridges (Waters Part No. WAT094226) involved: a. condition HLB cartridge with 2ml MeOH

b. equilibrate HLB cartridge with 2ml Water. c. add sample to cartridge; d. Wash cartridge with 2ml of 95%water:5%methanol; e. Dry cartridge under vacuum for 1 minute; f. Change to new clean labelled 16 x 125mm collection tubes; g. Elute sample with 2ml of methanol; h. apply vaccuum for 1 minute to dry; i. Evaporate elution volumes to dryness in Turbo Vap LV for 45min at 35deg.C; j. Reconstitute residue with 1.0ml of mobile phase (70%-0.05Moxalic acid:30%acetonitrile); k. vortex mix about 30 seconds; l. filter through 0.45um PVDF syringe filters (Millex-HV 13mm cat. No. SLHVX13NL) into HPLC vials for analysis.

For sulfamethazine, additional cleanup of supernatant with Waters Oasis MCX 3cc (60mg) SPE cartridges involved: a. condition MCX cartridge with 1ml MeOH followed by 1ml Water; b. add sample to cartridge; c. Wash cartridge with 1ml of 0.1N HCl (wash 1); d. Wash cartridge with 1ml of Methanol (wash 2); e. Dry cartridge under vacuum for 30s; f. Change to new clean 16 x 125mm collection tubes; g. Elute sample with 1ml of ammonium hydroxide:acetonitrile (5:95;vol/vol); h. dry cartridge again under vacuum; i. Evaporate elution volumes to dryness in Turbo Vap LV for 15min at 50deg.C; j. Reconstitute residue with 0.5ml (500ul) of 0.1M ammonium acetate pH4.5 buffer; k. vortex mix and place into HPLC vials for analysis.

**HPLC Conditions:**

Waters 2695 Alliance with 2487 dual wavelength UV detector was used for HPLC analyses. For tetracycline, Wavelength: 354nm; Column temp.: 35C; Sample temp.: 4C Flow rate: 1.0 ml/min. Mobile phase composition: 75% 0.05M oxalic acid: 25% acetonitrile. Column: Waters Atlantis T3 4.6mm x 150mm; 5um particle size plus matching guard column; Retention time of tetracycline was around 3.7 minutes. Overall runtime was set to 6.00 minutes; Injection volume: 25.0 ul. For Sulfamethazine, Wavelength: 267nm; Column temp.: 30C; Sample temp.: 24C Flow rate: 1.0 ml/min. Mobile phase composition: 77% 0.1M ammonium acetate pH 4.5 adjusted with glacial acetic acid: 23% acetonitrile. Column: Waters Atlantis T3 4.6mm x 150mm; 5um particle size plus matching guard column; Retention time of sulfamethazine is around 5.5 minutes. Overall runtime set to 8.00 minutes. Injection volume: 25.0 ul

**Analyses of Tetracyline and Sulfamethazine Residues in Kidney**

One gram of blended pig kidney was placed in 15ml disposeable centrifuge tube. Then added 2ml of 1.0M perchloric acid (Sigma-Aldrich) and homogenized for 45s at 9,000 RPM on OMNI PREP multi-sample homogenizer (OMNI-INC.COM). Centrifuge for 10minutes at 5000RPM and 22C (room temp.)(ThermoSci.Sorval ST 16R centrifuge). Then filter a 250ul aliquot of the supernatant through 0.45um PVDF syringe filters (Millex-HV 13mm cat. No. SLHVX13NL) into HPLC vials for analysis. For sulfamethazine: .the blending, homogenization, and centrifugation steps were similar to above. However, there was an additional cleanup of supernatant with Waters Oasis MCX 3cc (60mg) SPE cartridges as follows: a. condition MCX cartridge with 1ml MeOH followed by 1ml Water; b. add sample to cartridge; c. Wash cartridge with 1ml of 0.1N HCl (wash 1); d. Wash cartridge with 1ml of Methanol (wash 2); e. Dry cartridge under vacuum for 30s; f. Change to new clean 16 x 125mm collection tubes; g. Elute sample with 1ml of ammonium hydroxide:acetonitrile (5:95;vol/vol); h. dry cartridge again under vacuum; i. Evaporate elution volumes to dryness in Turbo Vap LV for 15min at 50deg.C; j. Reconstitue residue with 0.5ml (500ul) of 0.1M ammonium acetate pH4.5 buffer; k. vortex mix and place into HPLC vials for analysis

**HPLC Conditions:**

Waters 2695 Alliance with 2487 dual wavelength UV detector was used for HPLC analyses. For tetracycline: Wavelength: 354nm and 365nm; Column temp.: 35C; Sample temp.: 4C Flow rate: 1.0 ml/min; Mobile phase composition: 75% 0.05M oxalic acid: 25% acetonitrile; Column: Waters Atlantis T3 4.6mm x 150mm; 5um particle size plus matching guard column; Retention time of tetracycline is around 3.7 minutes. Overall runtime set to 6.00 minutes; Injection volume: 25.0 ul. For sulfamethazine: Wavelength: 267nm; Column temp.: 30C; Sample temp.: 24C Flow rate: 1.0 ml/min. Mobile phase composition: 77% 0.1M ammonium acetate pH 4.5 adjusted with glacial acetic acid: 23% acetonitrile. Column: Waters Atlantis T3 4.6mm x 150mm; 5um particle size plus matching guard column; Retention time of sulfamethazine is around 5.5 minutes. Overall runtime set to 8.00 minutes; Injection volume: 25.0 ul

Data Analysis: A population pharmacokinetic program (Phoenix, Pharsight Corp., Mountain View, CA) was used to conduct all of the population PK analyses. This allowed development of nonlinear mixed effects models that will quantify the variability in the depletion kinetics of the 2 drug classes in market weight pigs. Distributions were based on the initial data set and the initial dataset will provide the initial parameter estimate starting values and will help elucidate which of the covariates identified in the swine production facility available provided the most information to the model. Covariates were evaluated based on statistical analysis (ANOVA) and graphical comparisons for residuals, before inclusion in the model. Models were run individually and compared based on graphical fit, Akaike's Information Criteria (AIC), Bayesian Information Criteria (BIC) and the loglikelihood values (LLV). Furthermore the coefficients of variation and standard deviations of the observed values were also taken into consideration of the model fits.

The US FDA tolerance limit method was also used to determine the withdrawal time based on the data collected from this study and to compare these values with the label WDT for these drugs. In the population PK modeling approach, a population PK model was developed for each set of tetracycline and sulfamethazine tissue residue data. Since the tissue residue data were collected from the last phase of depletion curve and it is assumed that the natural log transformed concentration of residue is linear with time, a 1-compartment model with first-order elimination representing the distribution of the drugs in each tissue was used to fit tissue residue data. In this modeling process, the initial amount of tetracycline in the tissue compartment was fixed to 100 unit since the dose to tissue compartment is unknown. Monte Carlo simulations for tissue concentrations of tetracycline were performed based on the developed population PK model and PK parameter estimates from that model. In the Monte Carlo simulations, the initial amount of drug in the tissue compartment was fixed to 100 unit. The Monte Carlo simulation generated concentration-time profiles in the target tissue for 1000 pigs with 100 sets of simulated data for each pig. The simulated tissue concentrations of tetracycline at a given time point were used to estimate the WDI of tetracycline. The WDI was determined when the upper limit of 95% confidence interval of the 99 percentile of tissue concentration-time data was below the target tissue tolerance for the drugs by different regulatory agencies.

**Limit of Detection (LOD) and Limit of Quantification (LOQ).**

Tetracycline: Pk parameters for muscle, liver, kidney, and weanling pig plasma were calculated based on data above LOQs. For finisher pigs plasma and stomach in both weanling and finisher pigs, the calculations were based on data above LODs as majority of data were below LOQ.

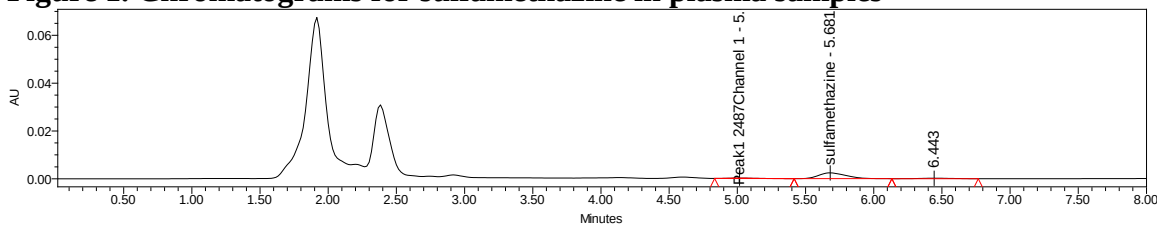
Sulfamethazine: Pk parameters for stomach, muscle, liver, and kidney tissues were calculated based on data above the LOQ. Plasma parameters were calculated based on LOD.

**Results:**

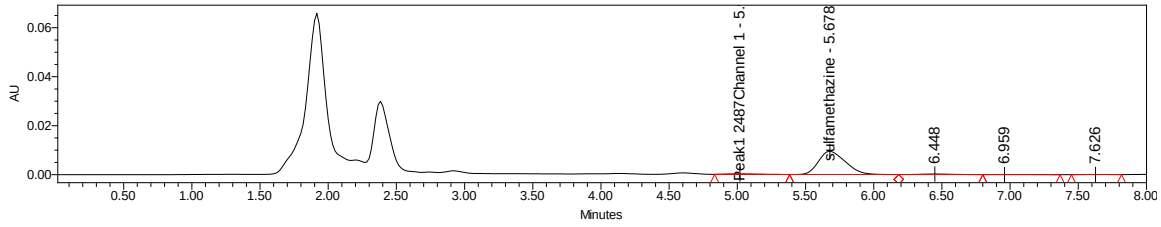
**Analytical Chemistry**:

Our laboratory completed analytical chemistry method development for tetracycline in pig's stomach tissue and had previously developed an assay for measuring this drug in the blood. The assays allow us to analyze stomach tissue with a 78% recovery using an extensive extraction process. Developing these methods was a first priority before we started and therefore took longer than expected. The plasma assay was already developed in our laboratory. Below is an HPLC chromatogram of sulfamethazine in plasma in spiked samples and several animals(**Figure 1**) and tetracycline in plasma and stomach tissue (**Figure 2**).

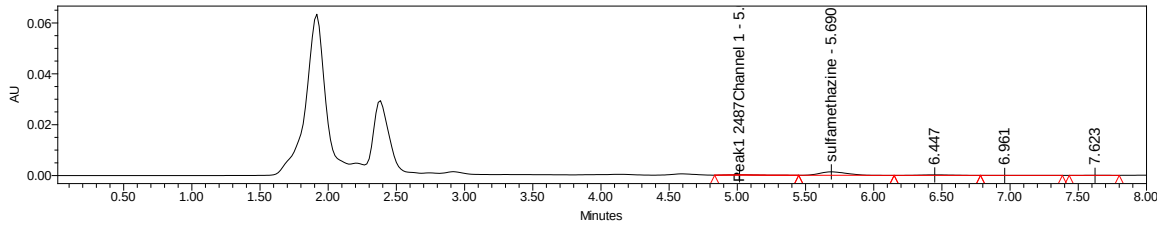
**Figure 1. Chromatograms for sulfamethazine in plasma samples**



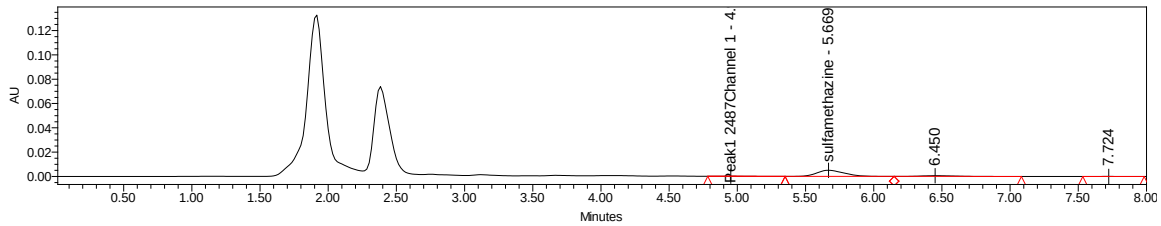

Sulfamethazine-plasma-0.094ppm



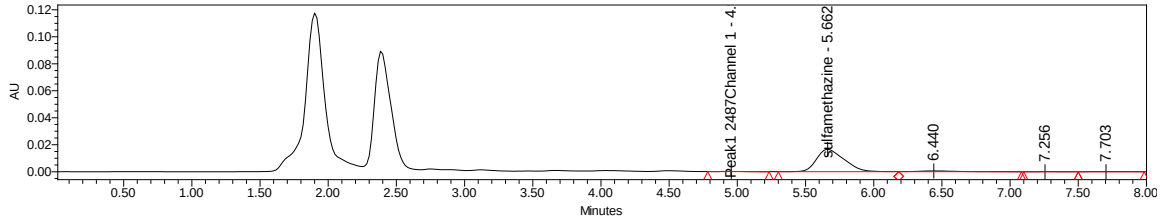

Sulfamethazine-plasma-0.75ppm



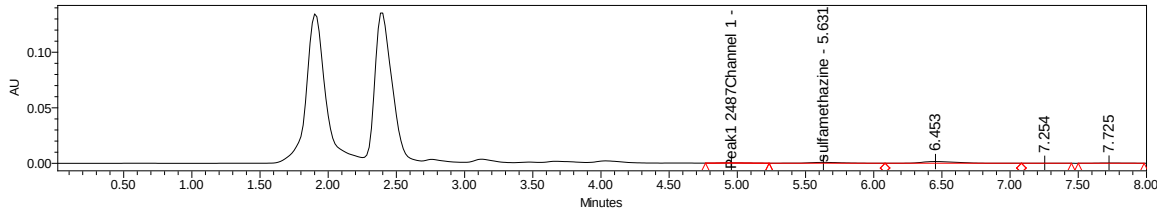

Sulfamethazine-blank plasma



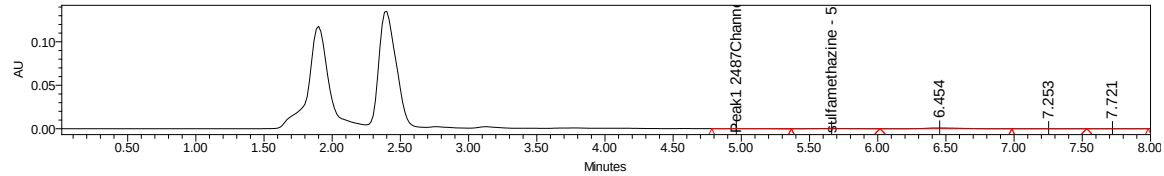

Sulfamethazine-plasma pig 41 W8




Sulfamethazine-plasma pig 58 W8

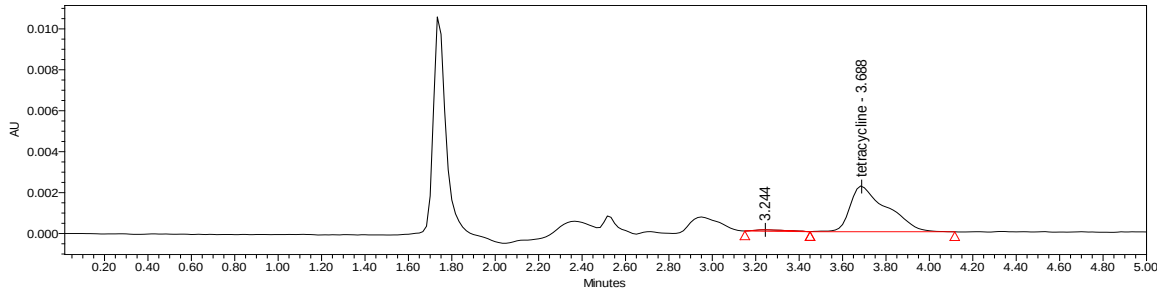



Sulfamethazine-plasma pig 58 W15

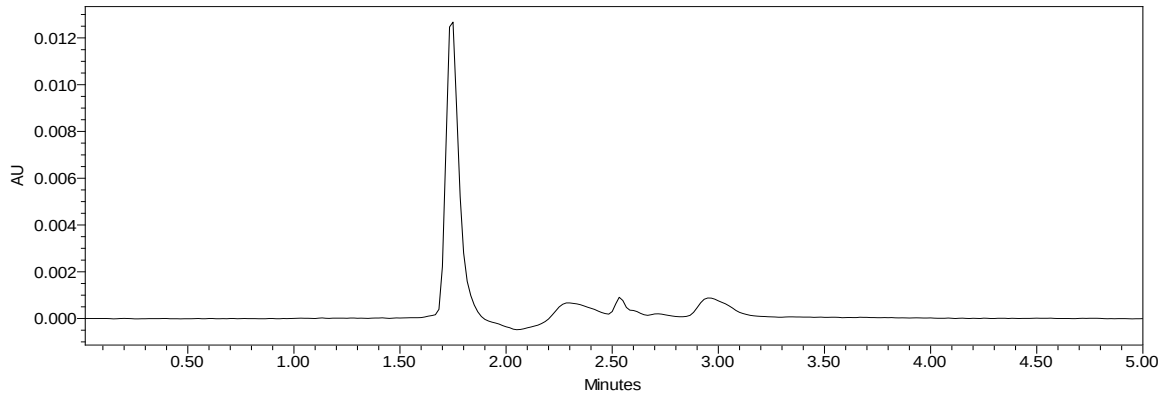



Sulfamethazine plasma pig 58 W22

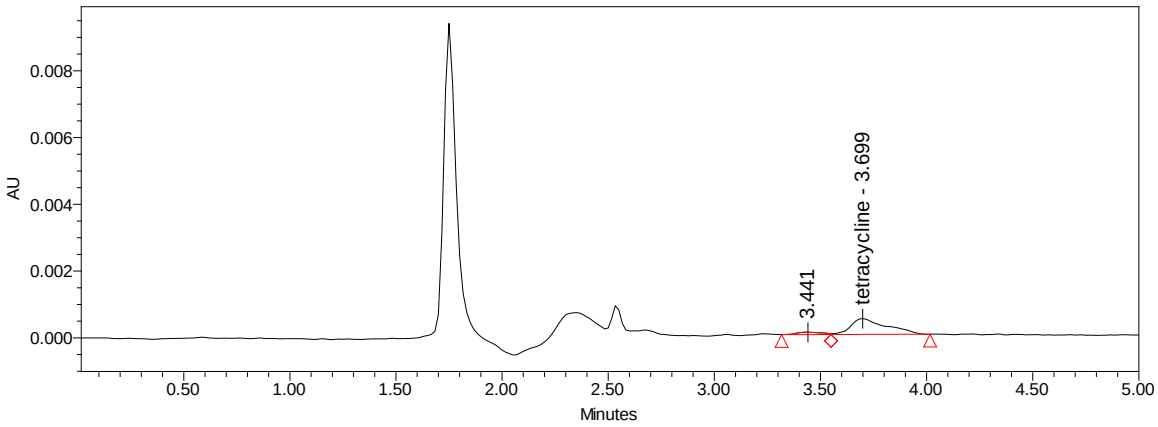
## Figure 2. Chromatograms of tetracycline from plasma and stomach tissues



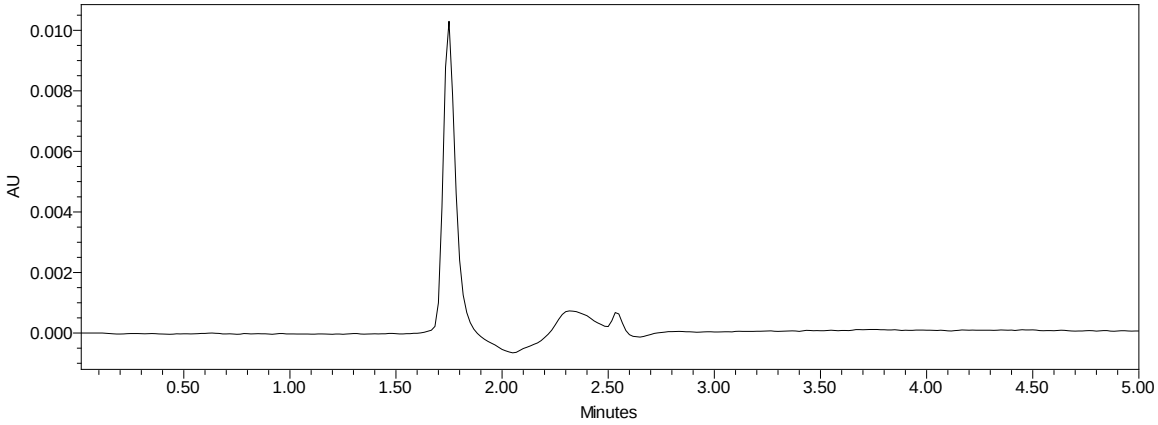

Tetracycline-plasma 0.75ppm



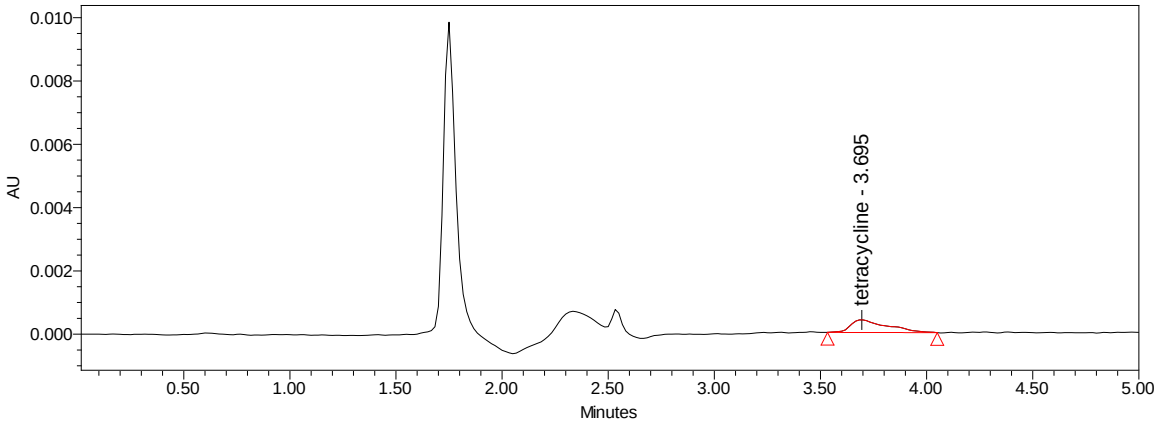

Tetracycline-blank plasma



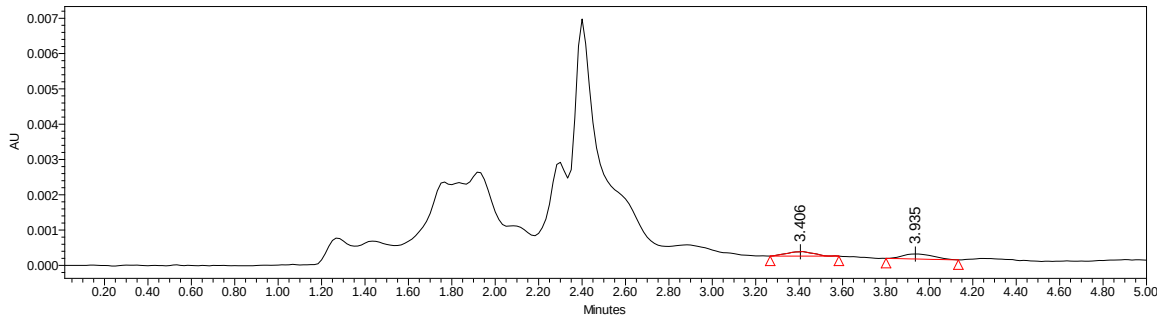

Tetracycline-Pig 18 Day 4



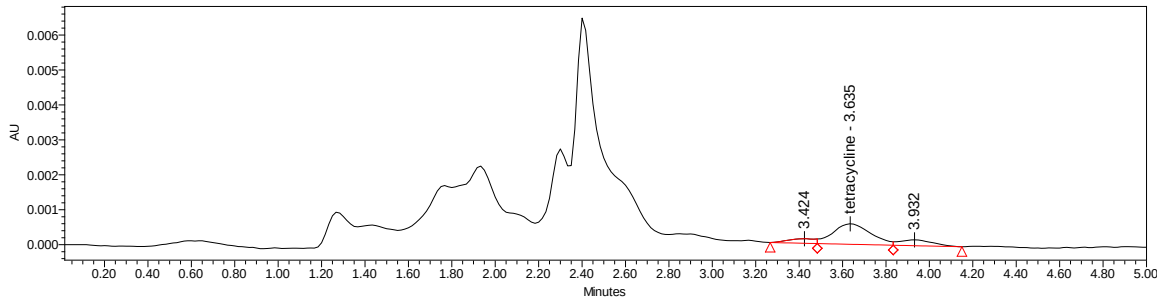

Tetracycline-plasma-pig 19 W8



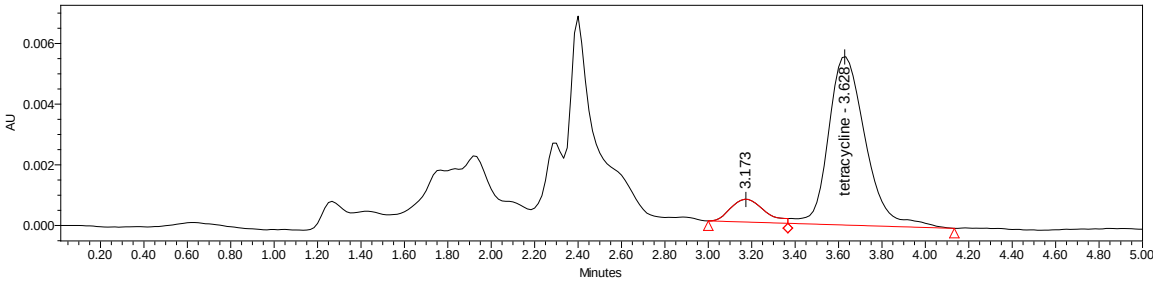

Tetracycline plasma Pig 19 Day4



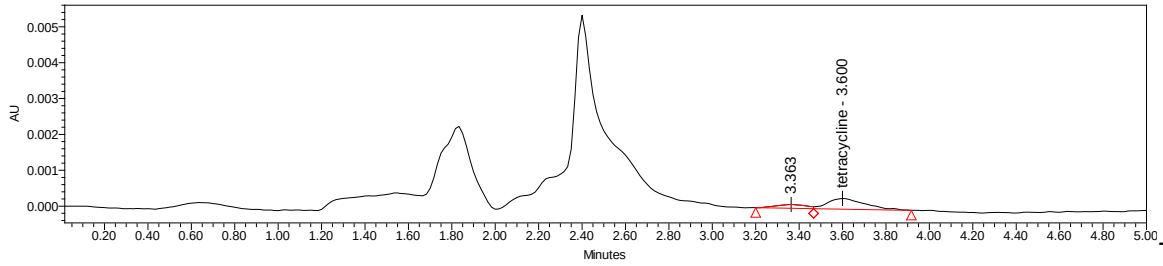

Tetracycline blank stomach




Tetracyclin 0.1ppm spiked stomach




Tetracycline 1.0ppm spiked stomach




Tetracycline pig stomach number 25

## Pharmacokinetic Pilot Studies

The preliminary pilot studies as demonstrated in the figures below depict the achievement of steady-state plasma concentration of tetracycline in weanling pigs exposed to the labeled water medication for 5 days in these preliminary trails. It also shows the diurnal pattern in drug concentrations in these animals. Plasma concentrations of tetracycline hydrochloride were detectable throughout the sampling period starting at 72 hours of addition of the medication in water. The plasma concentrations reached approximately 0.40 ug/mL in the morning and 0.7 ug/mL during the late afternoon in the blood of the pigs (Figures 3A and 3B). These values

were relatively steadily for the 3 measured days of treatment although there is obvious inter-individual variation amongst these pigs. On the 5th day, the medication was stopped and the lines were flushed to prevent unintentional treatment after termination of the medication. Blood samples were taken and showed slow but steady plasma elimination of the medication for the initial 26 hours (Figure 3C). The stomach samples from this pilot experiment showed quantifiable levels of tetracycline hydrochloride after 24 hours of withdrawal. The values from the pharmacokinetic analysis were consistent with previously reported values of a Kel of 0.022 for the first phase and 0.003 for the second phase of the elimination. Volume of distribution was found to be approximately 1.4 L/kg and Clearance for the terminal phase was on average 36.8 L/hr/kg for an assumed 10 mg/kg dose of tetracycline daily and an 8% bioavailability. The initial elimination time had a half-life of 10 hours with a clearance of 31.8 L/kg/hr. These numbers appear consistent with previous values and result in a total body clearance of 0.084 l/kg/h.

**Figure 3A. Individual plasma concentration vs. time plot for tetracycline in weanling pigs from pilot study.**

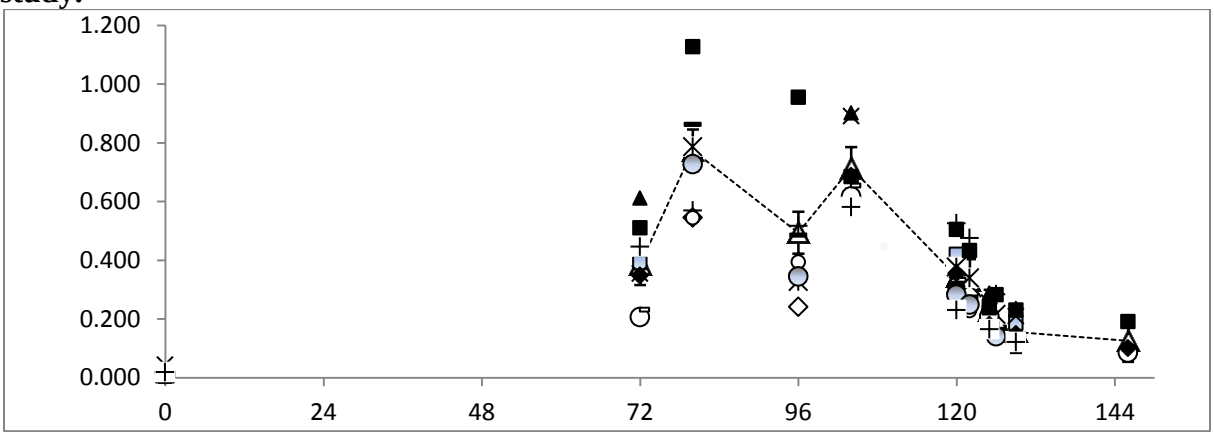



**Figure 3B Mean plasma concentration (+/-SE) vs time plot for tetracycline in weanling pigs from pilot study. The horizontal line was the LOQ.**

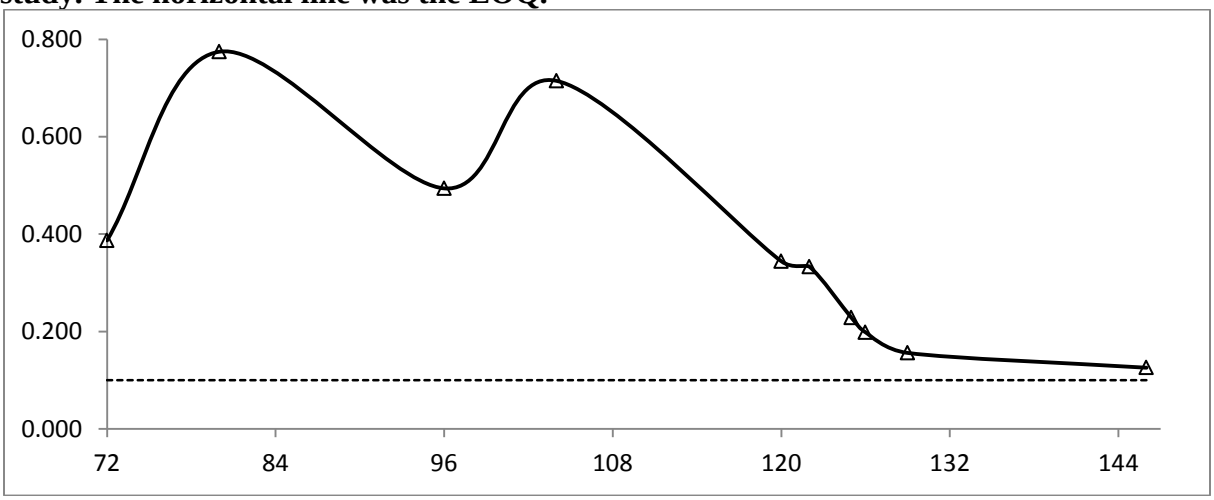
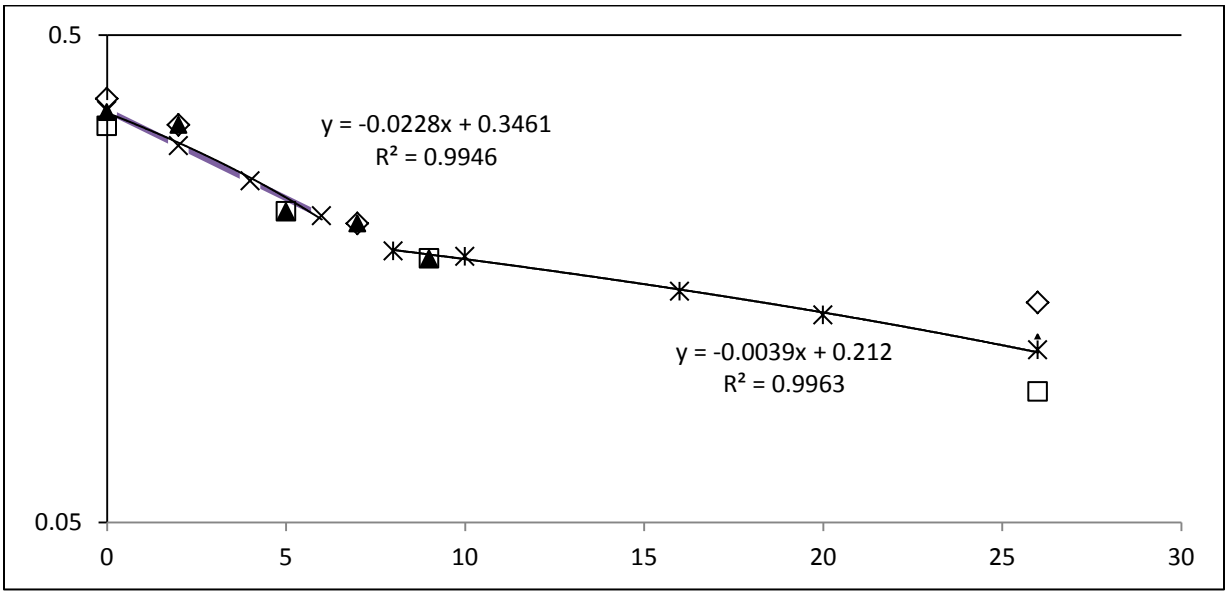



Figure 3C below is the result of pharmacokinetic analysis the elimination times for a two compartment model of the central compartment. The initial elmination phase last approximately 7 hours and is followed by a slower elimination phase that lasts through observed sampling times. The elimination rate equations are included on the graph next to their respective phases.

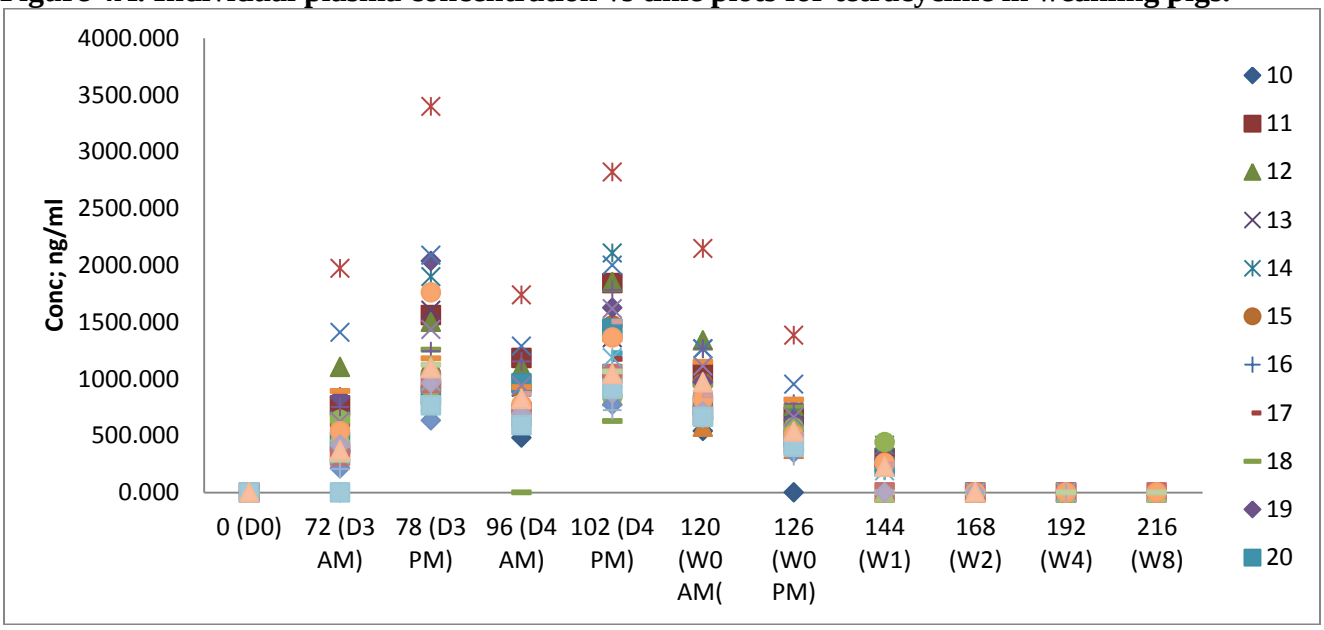
**Figure 3C. Depletion of tetracycline in plasma from pilot study.**



## Results from Tetracycline Pharmacokinetic Trials.

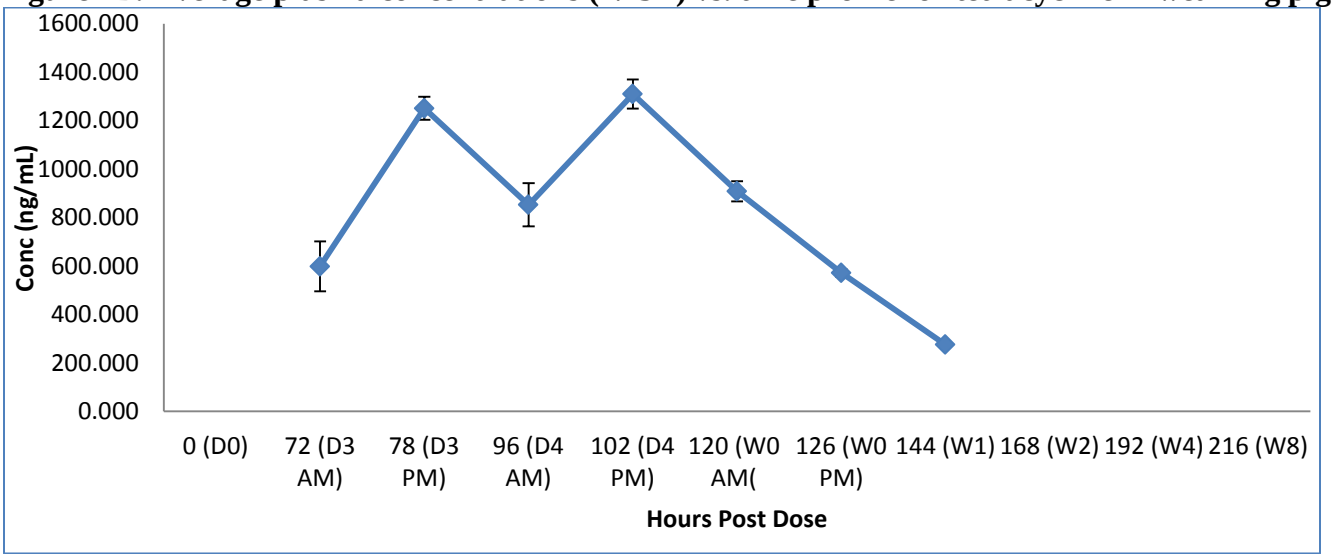
Figures 4A and 4B below depict the individual animal (A) and the average (B) plasma concentration depletion during the dosing (D0, D3, D4) and drug withdrawal (W0, W1, W4, W8) of the medicated water. The label withdrawal time is 4 days (W4), but there are clearly residues of tetracycline in the blood of some pigs beyond this withdrawal time.

**Figure 4A. Individual plasma concentration vs time plots for tetracycline in weanling pigs.**




As demonstrated in our preliminary studies, there were apparent maximum and minimum steady state plasma concentrations with the former occurring in the afternoon and the latter in early morning . The Cmax and half-life data are very similar to what was reported in the literature for weanling/grower pigs.

**Figure 4B. Average plasma concentrations (+/-SE) vs. time profile for tetracycline in weanling pigs.**




The depletion profiles (Figures 5A and B) for adult finisher pigs were slightly different from those seen in the weanling pigs. In these studies, AM blood samples were not collected as was done with the weanling pigs and Cmax did appear to be achieved until D4. Depletion appears to be slower in these pigs than in weanling pigs although we only have 3 data time points from which to make this assessment. Interestingly, there was one pig with detectable drug levels at W4. Recall that this is the label WDT for this drug.

**Figure 5A. Individual plasma concentration vs time plot for tetracycline in finisher pigs.**

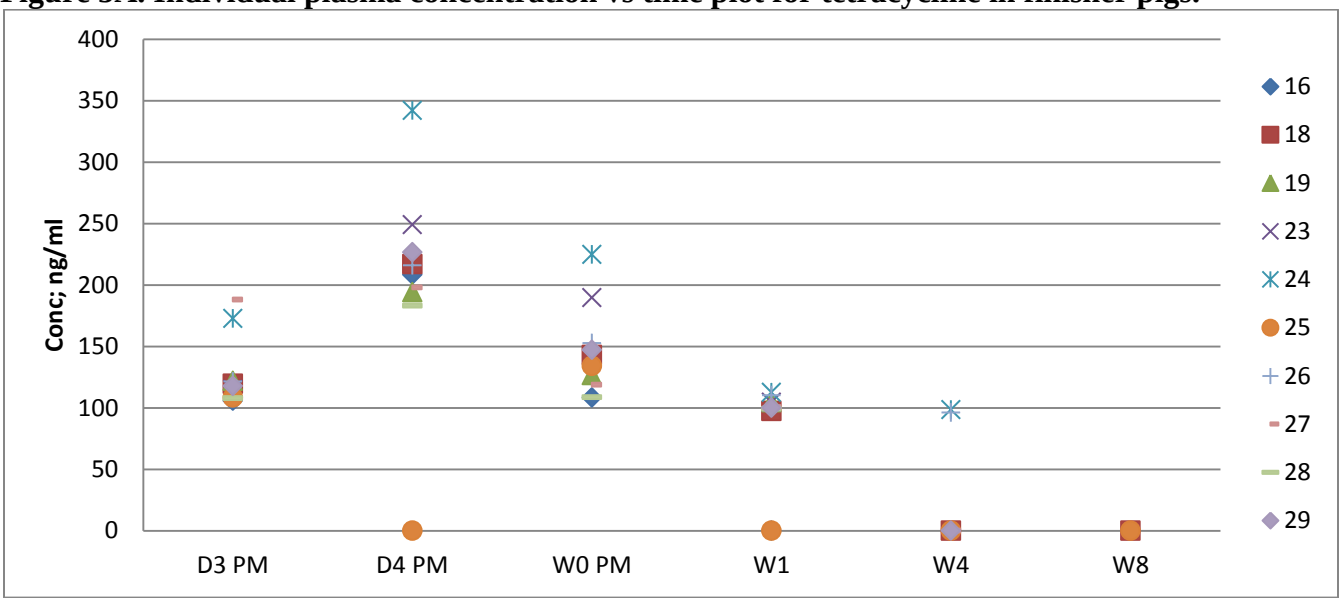



**Figure 5B. Average (+/-SE) plasma concentration vs. time profile for tetracycline in finisher pigs. The horizontal is the LOQ for tetracycline in plasma.**

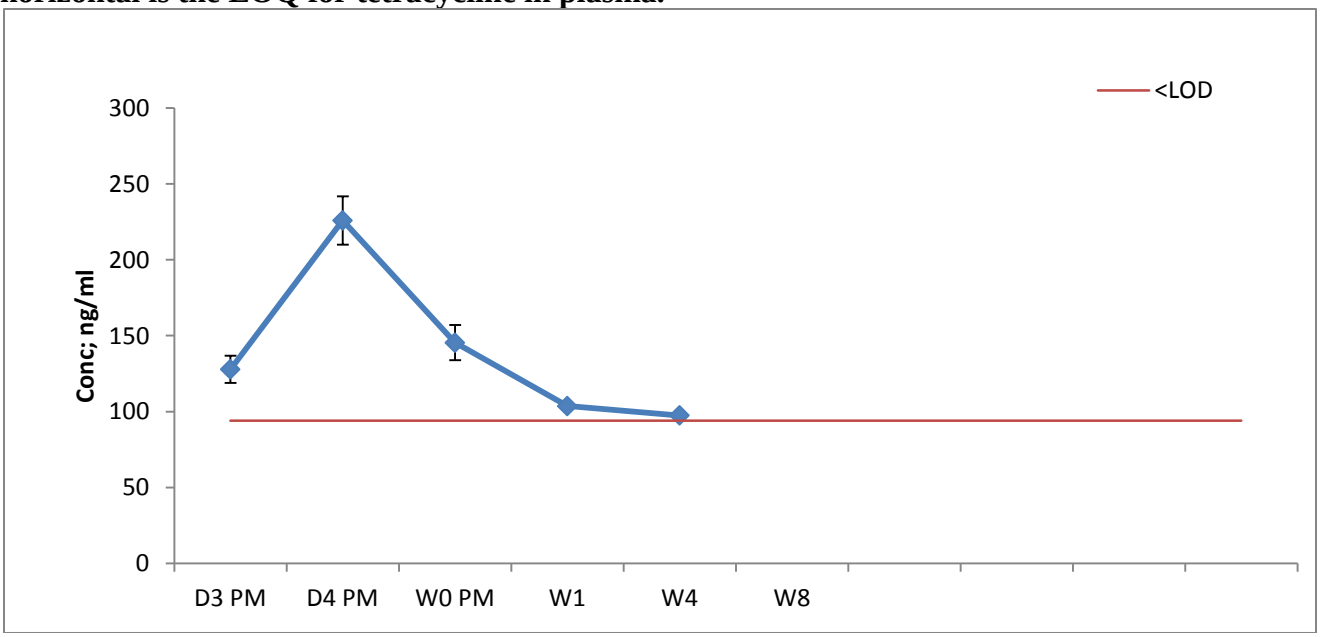



**Table 1** below is a summary of the non-compartmental pharmacokinetic analyses of the plasma data from weanling and finisher pigs. Several of these data are comparable with the literature. The half-life for the finisher pigs is extremely long and may be a product of fewer pigs with detectable plasma concentrations at later time points which skewed the depletion slope.

**Table 1. Summary of the noncompartmental pharmacokinetic analysis of the plasma data from weanling and finisher pigs.**

| | Tlast (h) | AUCinf (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| **Weanling Plasma (Based on individual data)** | **135.1 ± 9.4 (n = 28)** | **77,000 ± 36,580 (n = 22)** | **1324.0 ± 549.0 (n = 28)** | **90.8 ± 12.1 (n = 28)** | **10.3 ± 2.9 (n = 22)** |
| **Finisher Plasma (Based on individual data, n = 27)** | **112.0 ± 12.2** | **9,739 ± 4,273*** | **168.8 ± 58.7** | **102.8 ± 4.6** | **Not estimable** |

Tetracyline residues in pig stomach were detectable up to 8 days (W8) in weanling and adult pigs as shown in **Figure 6 below**. This is surprising as the WDT for this drug is 4 days.

**Figure 6. Individual stomach tissue concentration vs. time plots for tetracyline in weanling and finisher pig stomachs.**

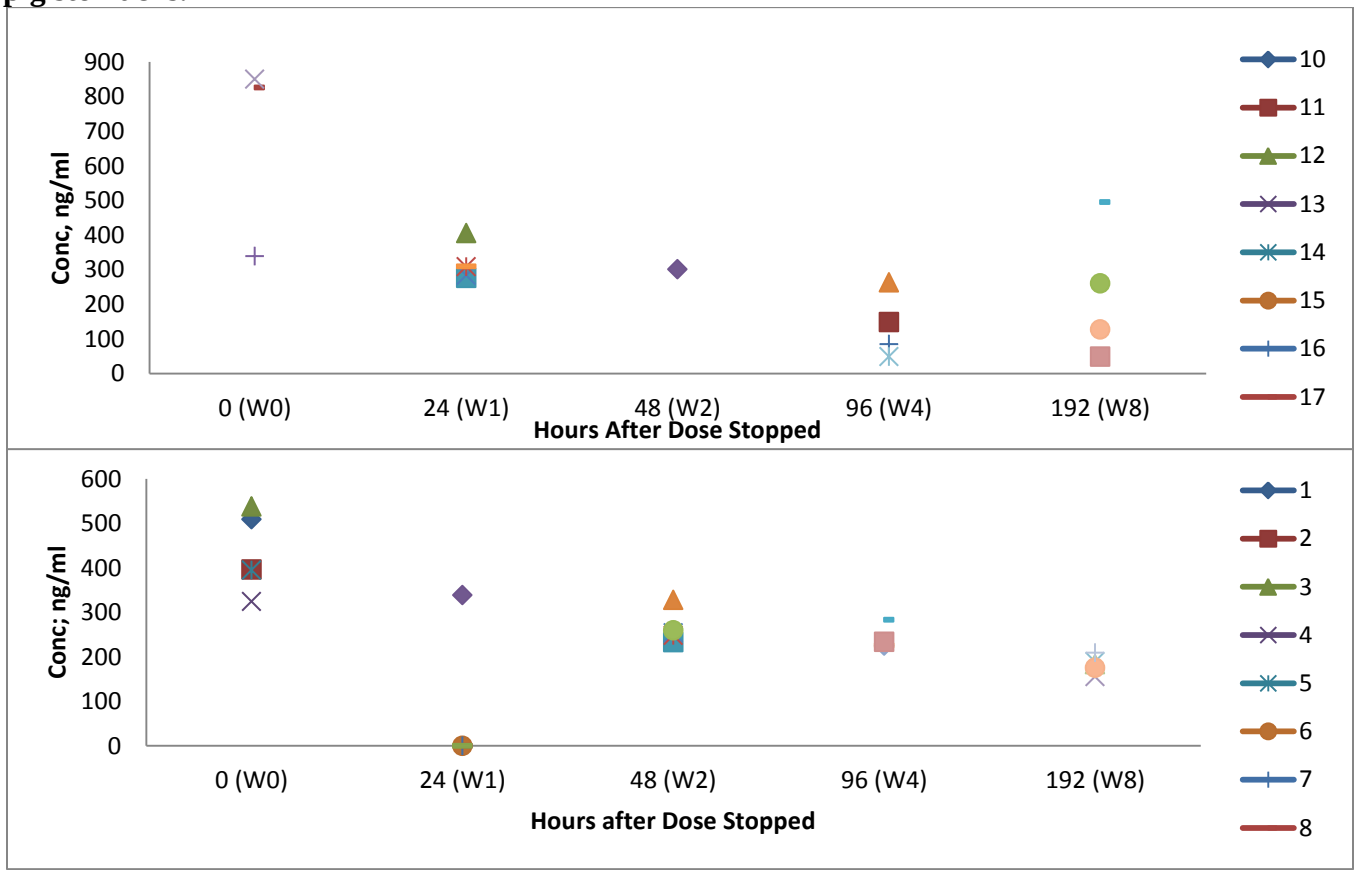



Table 2 below is a summary of the pharmacokinetic analysis of the data to generate PK parameters useful for estimation of a withdrawal time for stomach tissue. These half-lives are long compared to those reported for other tissues in pigs for this drug. There is only one study (Mercer et al., 1978) in the literature that estimated stomach half-lives; however, they captured only early points (< 4hrs) after an IV dose (11 mg/kg) with a half-life of 7.22 hrs. Estimates from other studies where tissues depletion was assessed estimated half-lives as long as 24 - 72 hrs.

**Table 2. Summary of the noncompartmental pharmacokinetic analysis of the stomach tissue data from weanling and finisher pigs**

| | Tlast (h) | AUClast (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Stomach (W0 to W4 for estimation of t1/2) (n = 11) | 192 | 55,034* | 671.7 | 0 | 62.4 (R2 = 0.847) |
| Finisher Stomach (W0 to W8 for estimation of t1/2) (n = 21) | 192 | 48,531* | 431.9 | 0 | 169.6 (R2 = 0.883) |

* AUClast was calculated for these data as the extrapolation of AUCinf is above 20%.

**Tetracycline Residues in Liver, Muscle, and Kidney**

The following three figures and three tables summarize the pharmacokinetic parameters for tetracycline in three tissues; liver, muscle, and kidney. Determination of several PK parameters were limited by residue levels below the LOD and LOQ for several animals especially at later time points.

**Figure 7. Individual liver concentration – time profiles for tetracycline in weanling and finisher pigs.**

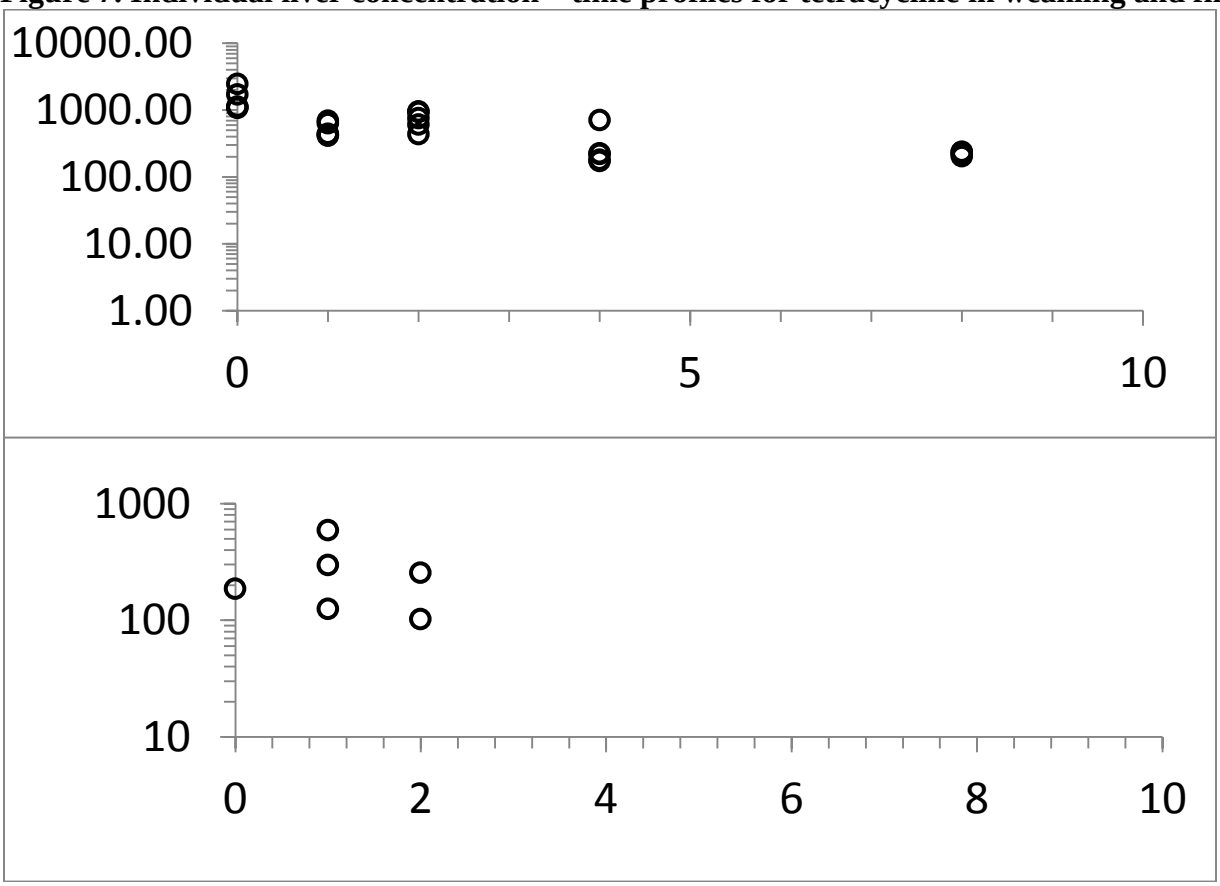



**Table 3. Summary of the noncompartmental pharmacokinetic analysis of the liver tissue data from weanling and finisher pigs**

| | Tlast (h) | AUClast (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Liver (W0 to W4 for estimation of t1/2). (n = 24) | 192 | 85,684.9 | 1,494.2 | 0 | 48.8 (R2 = 0.757) |
| Finisher Liver (W1 to W2 for estimation of t1/2). (n = 5) | 48 | 10,082.0 | 339.0 | 24 | 26.0 |

**Figure 8. Individual muscle concentration – time profiles for tetracycline in weanling and finisher pigs.**

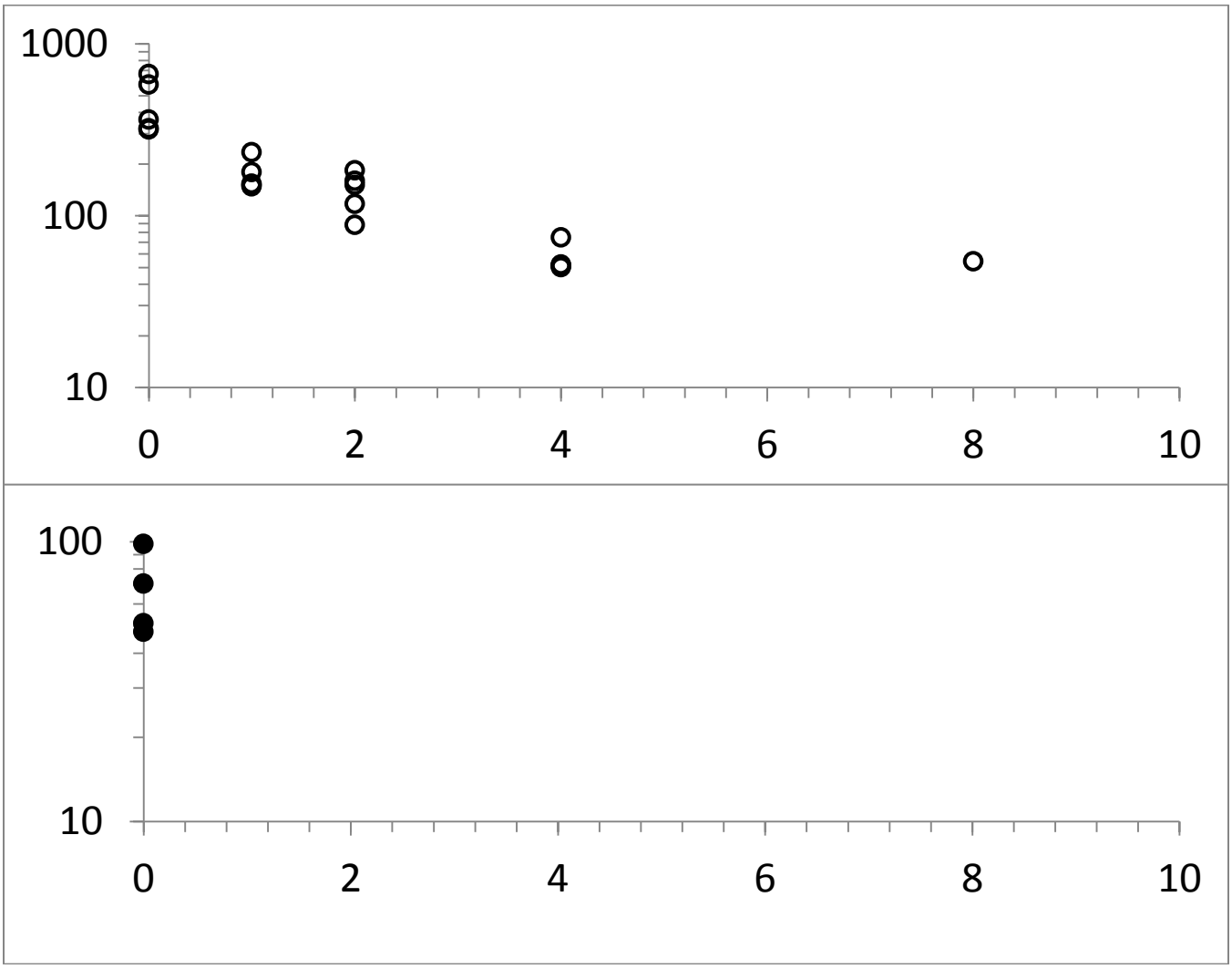



**Table 4. Summary of the noncompartmental pharmacokinetic analysis of the muscle tissue data from weanling and finisher pigs**

| | Tlast (h) | AUCinf (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Muscle (W0 to W4 for estimation of t1/2) | 96 | 17,850 | 450.2 | 0 | 34.1 |
| Finisher Muscle | 0 | - | 66.9 | 0 | - |

AUC and t1/2 for Finisher muscle cannot be estimated due to the limited data

**Figure 9. Individual kidney concentration – time profiles for tetracycline in weanling and finisher pigs.**

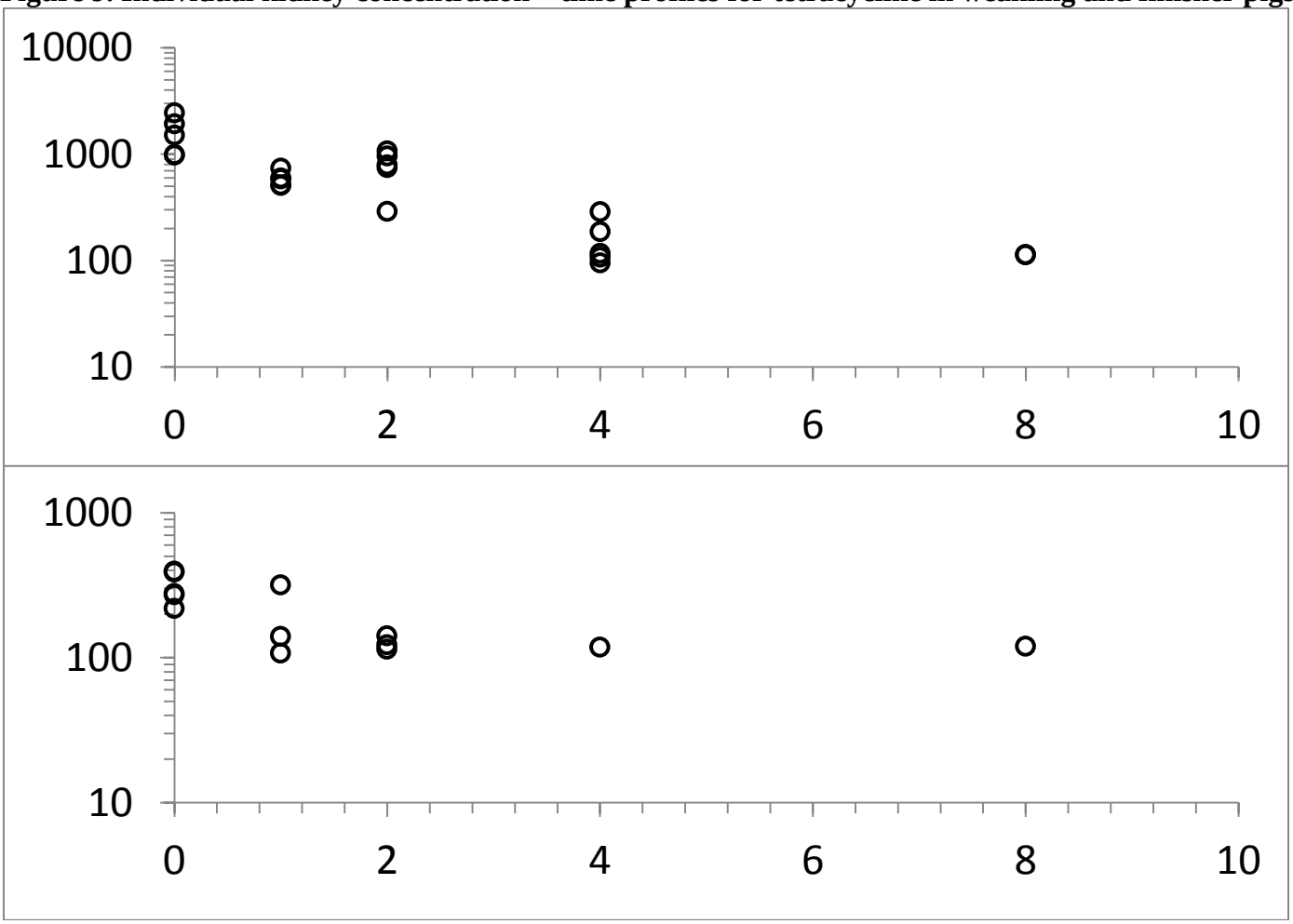



**Table 5. Summary of the noncompartmental pharmacokinetic analysis of the kidney tissue data from weanling and finisher pigs**

| | Tlast (h) | AUClast (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Kidney (W0 to W8 for estimation of t1/2) | 192 | 80,534 | 1,573.3 | 0 | 52.2 |
| Weanling Kidney (W0 to W4 for estimation of t1/2) | 192 | 77,161 | 1,573.3 | 0 | 31.7 |
| Finisher Kidney (W0 to W2 for estimation of t1/2) | 48 | 9,613.9 | 311.5 | 0 | 36.9 |

## Results from Sulfamethazine Pharmacokinetic Trials

Plasma concentration profiles as depicted in figure 10 below demonstrate the same diurnal plasma concentration profile as depicted for the tetracycline studies in weanling pigs. Plasma concentrations were significantly higher than that for tetracycline. This was not surprising as the sulfamethazine dose was significantly higher (5-10 fold) than tetracycline dose.

**Figure 10. Individual plasma concentration vs. time plots of sulfamethazine in weanling and finisher pigs**

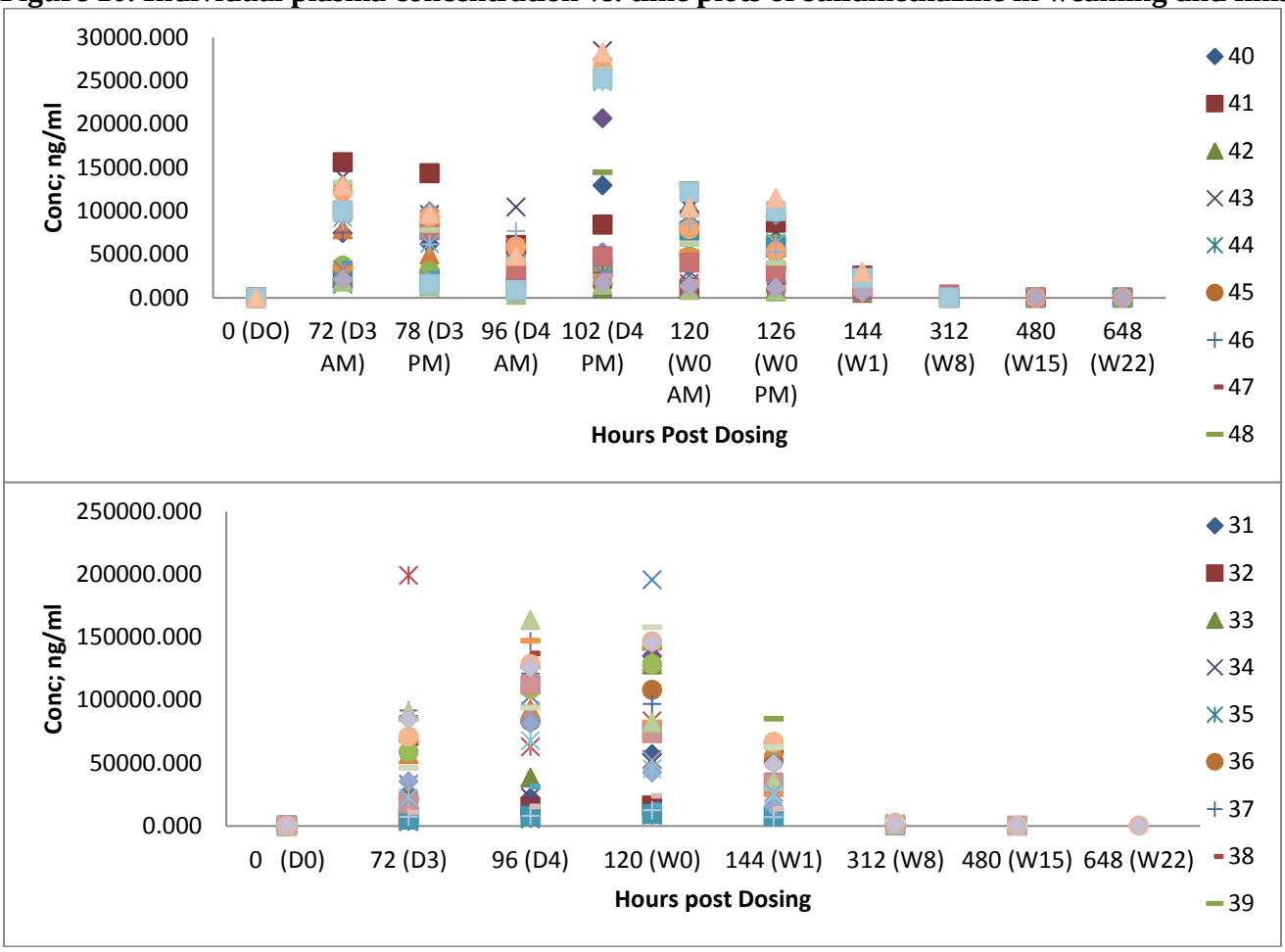



**Table 6. Summary of the noncompartmental pharmacokinetic analysis of the plasma tissue data from weanling and finisher pigs**

| | AUCinf (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|
| Weanling Plasma (based on individual data) | 473,469 ± 377,404 (n = 24) | 8,067 ± 9,529 (n = 30) | 95.8 ± 18.7 (n = 30) | 11.6 ± 5.2 (n = 24) |
| Adult Plasma (based on individual data) | 7,010,990 ± 4,468,407 (n = 20) | 76,879 ± 57,620 (n = 28) | 108.8 ± 13.7 (n = 28) | 27.0 ± 7.5 (n = 23) |

**Figure 11. Individual stomach concentration vs. time plots for sulfamethazine in weanling and finisher pigs.**

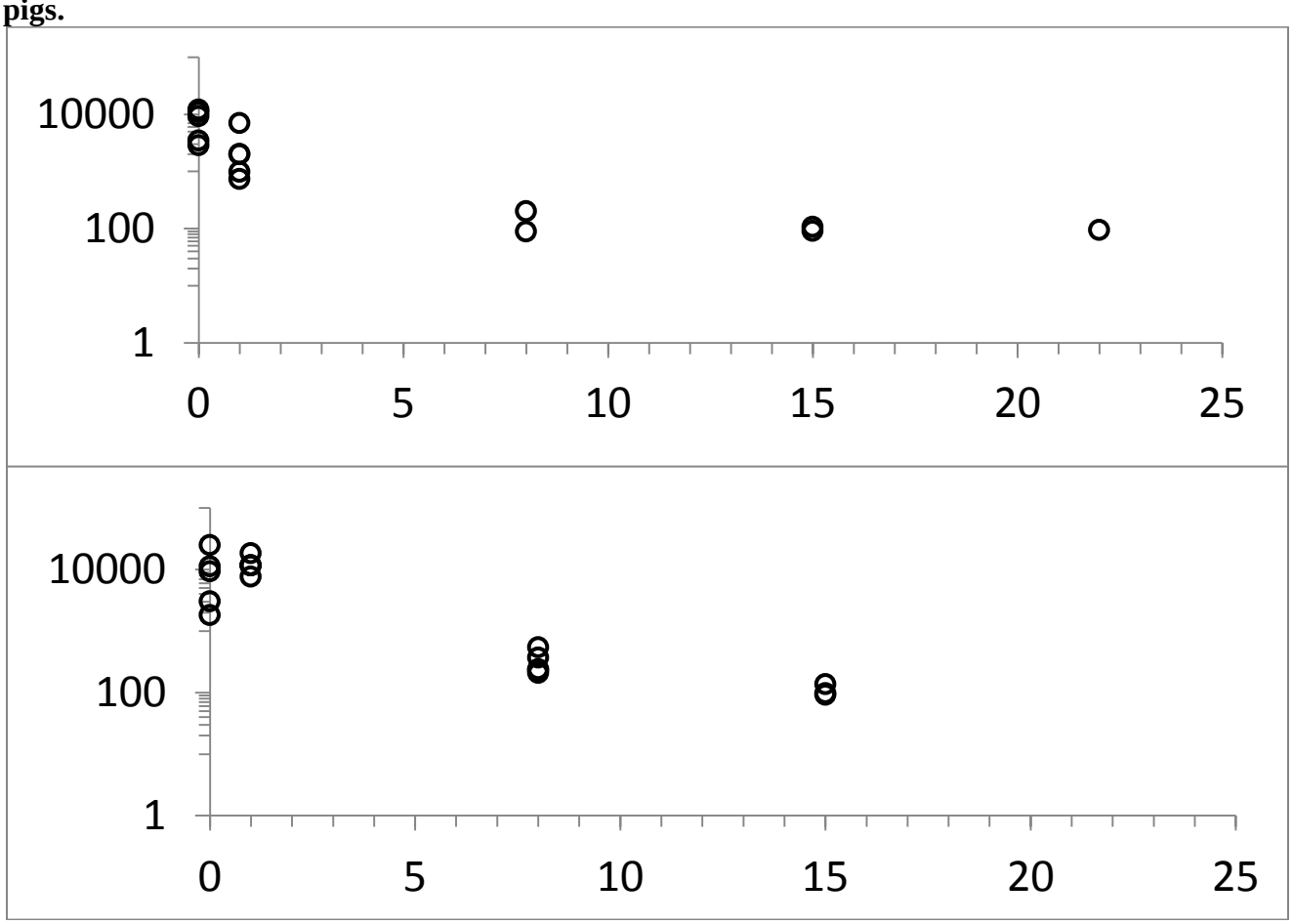



**Table 7. Summary of the noncompartmental pharmacokinetic analysis of the stomach tissue data from weanling and finisher pigs**

| | Tlast (h) | AUCinf (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Stomach (W0, W1 and W8 for estimation of t1/2) | 354 | 250,840 | 7,712.5 | 0 | 35.4 |
| Adult Stomach (W1 to W8 for estimation of t1/2) | 360 | 857,554 | 12,256 | 24 | 32.0 |

**Figure 12. Individual muscle concentration vs. time plots for sulfamethazine in weanling and finisher pigs.**

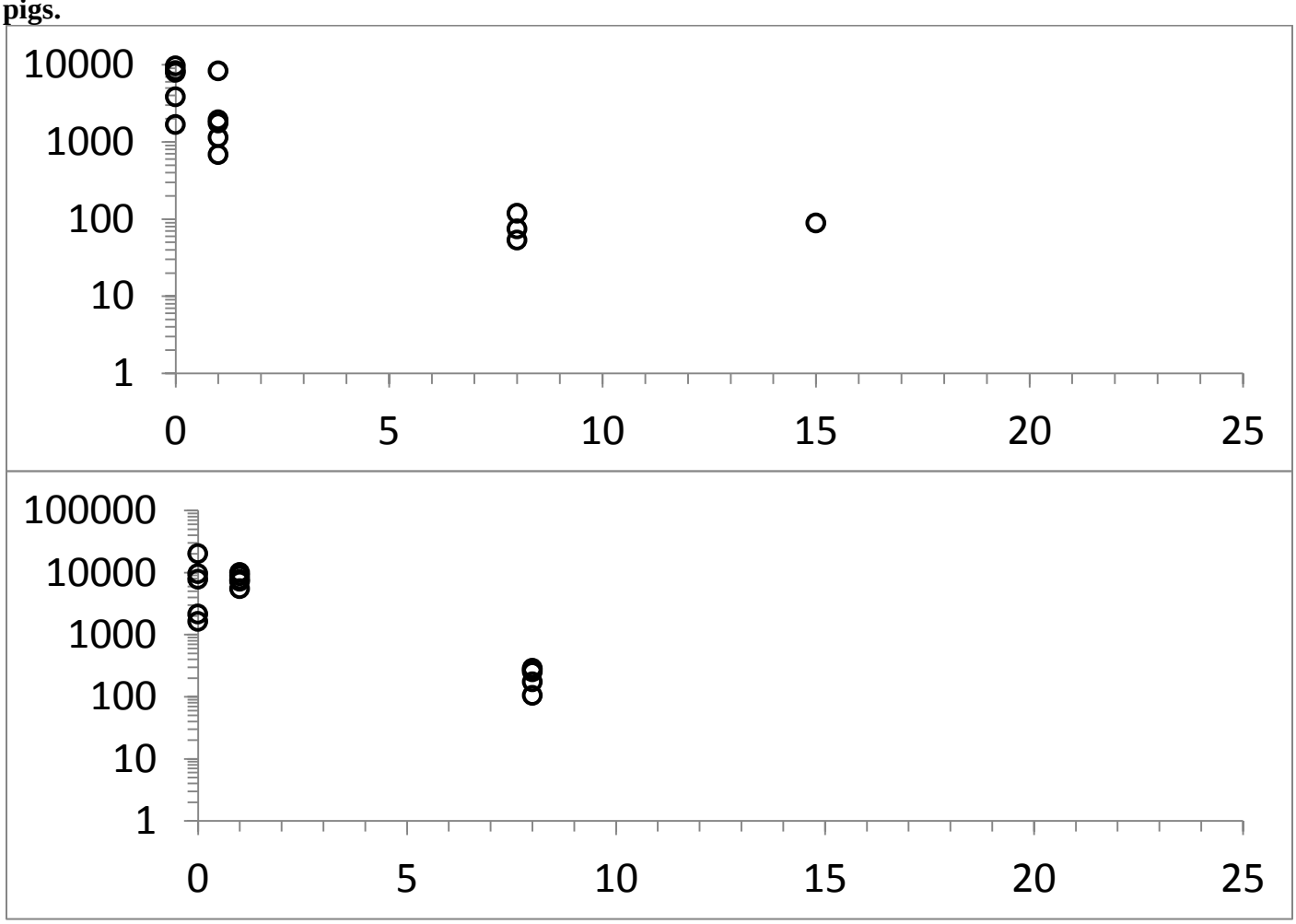



**Table 8. Summary of the noncompartmental pharmacokinetic analysis of muscle tissue data from weanling and finisher pigs**

| | Tlast (h) | AUCinf (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Muscle (W0, W1 and W8 for estimation of t1/2) | 186 | 208,409 | 6,280.1 | 0 | 31.0 |
| Adult Muscle (W1 to W8 for estimation of t1/2) | 192 | 563,083 | 8,296.0 | 24 | 32.5 |

**Figure 13. Individual liver concentration vs. time plots for sulfamethazine in weanling and finisher pigs.**

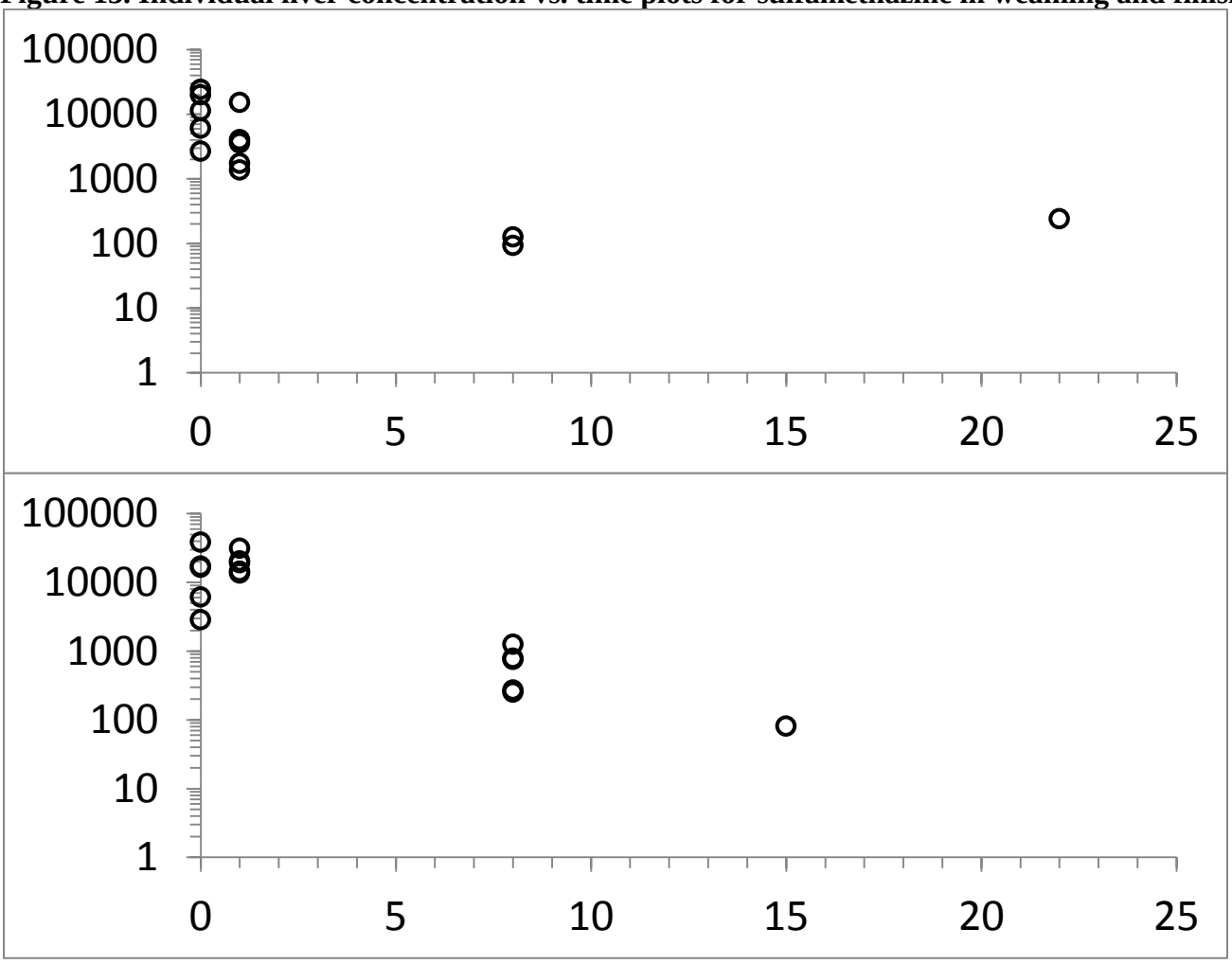



**Table 9. Summary of the noncompartmental pharmacokinetic analysis of liver tissue data from weanling and finisher pigs**

| | Tlast (h) | AUCinf (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Liver (W0, W1, and W8 for estimation of t1/2) | 186 | 377,742 | 12,840.7 | 0 | 28.2 |
| Adult Liver (W1 to W8 for estimation of t1/2) | 192 | 1,416,763 | 19,903.9 | 24 | 34.3 |

**Figure 14. Individual kidney concentration vs. time plots for sulfamethazine in weanling and finisher pigs.**

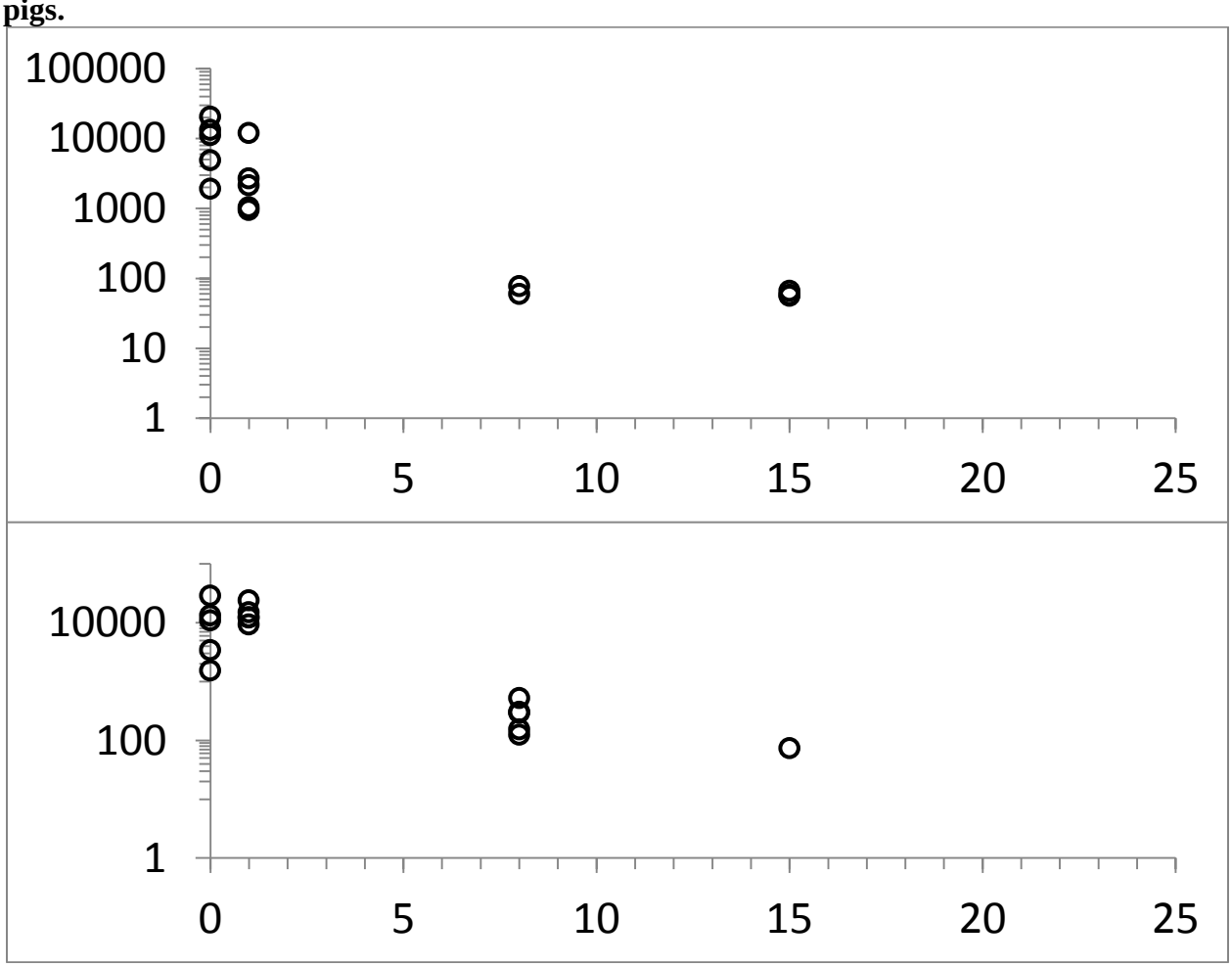



**Table 10. Summary of the noncompartmental pharmacokinetic analysis of kidney tissue data from weanling and finisher pigs**

| | Tlast (h) | AUCinf (ng/ml*h) | Cmax (ng/ml) | Tmax (h) | T1/2 (h) |
|---|---|---|---|---|---|
| Weanling Kidney (W0, W1, and W8 for estimation of t1/2) | 354 | 284,937 | 10,370.8 | 0 | 26.9 |
| Adult Kidney (W1 to W8 for estimation of t1/2) | 192 | 936,048 | 14,616.6 | 24 | 29.5 |

## Determination of Withdrawal Intervals (WDI) using Tolerance Limit Method and Population pharmacokinetics (PopPK) plus Monte Carlo Simulation

Both methods were use to determine the WDI for both drugs in weanling and finisher pigs. The WDI was determined for each tissue of 4 tissues for various jurisdictions such as Russia, EU, and the U.S. The figures below demonstrate the WDI determination by PopPK method and why the WDI will be vastly different in these jurisdictions for stomach tissues. The horizontal lines depict the safety level, tolerance or MRL in that jurisdiction.

**Figure 14. WDI for Tetracycline in Finisher Using Russia MRL (10 ppb) based on export verification program.**

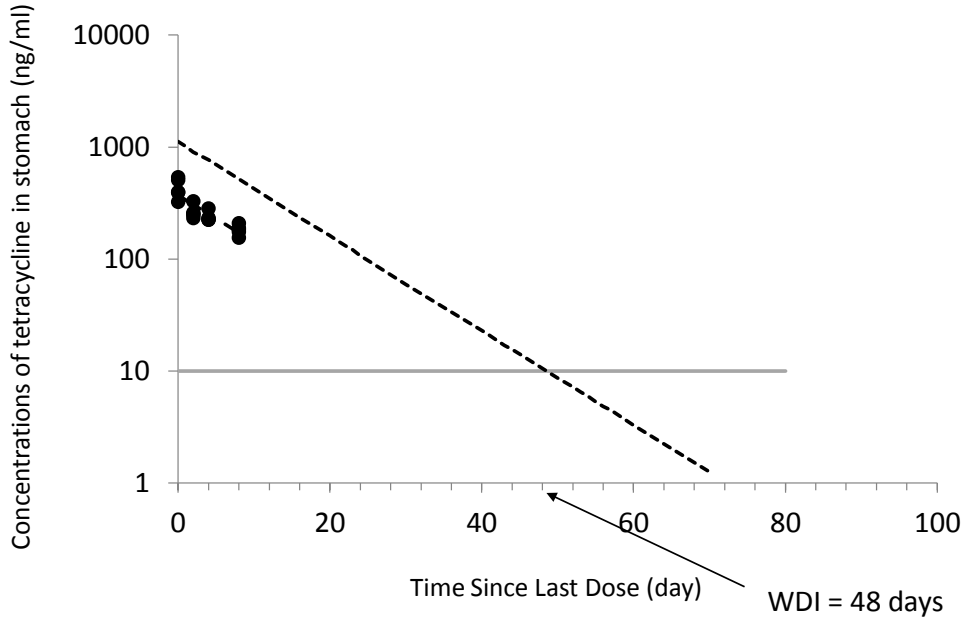


**Figure 15. WDI for tetracycline in finisher pigs using EU MRL for muscle (100 ppb).**

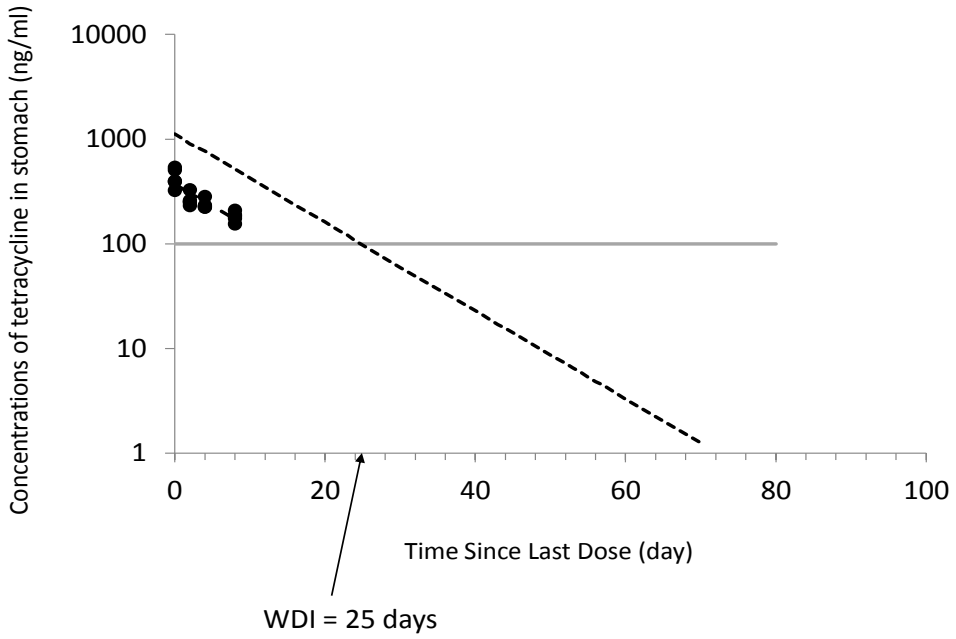



**Figure 16. WDT for tetracycline in finisher pigs using U.S. tolerance for muscle (2000 ppb).**

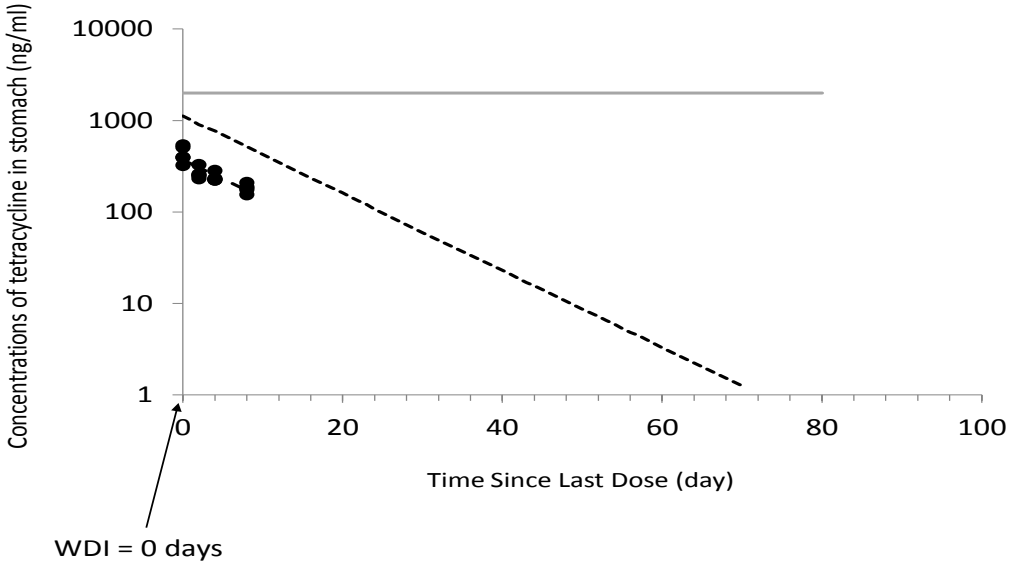


**Figure 17. WDT for tetracycline in finisher pigs using U.S. tolerance for kidney (12,000 ppb).**

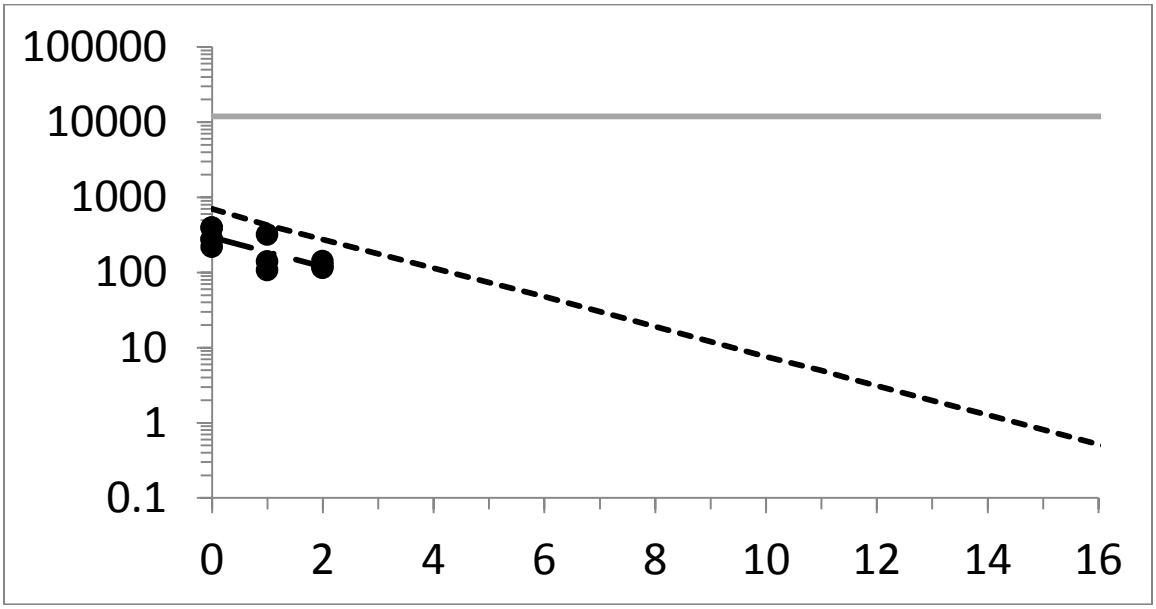



**Figure 18. WDT for tetracycline in finisher pigs using EU MRL for kidney (600 ppb).**

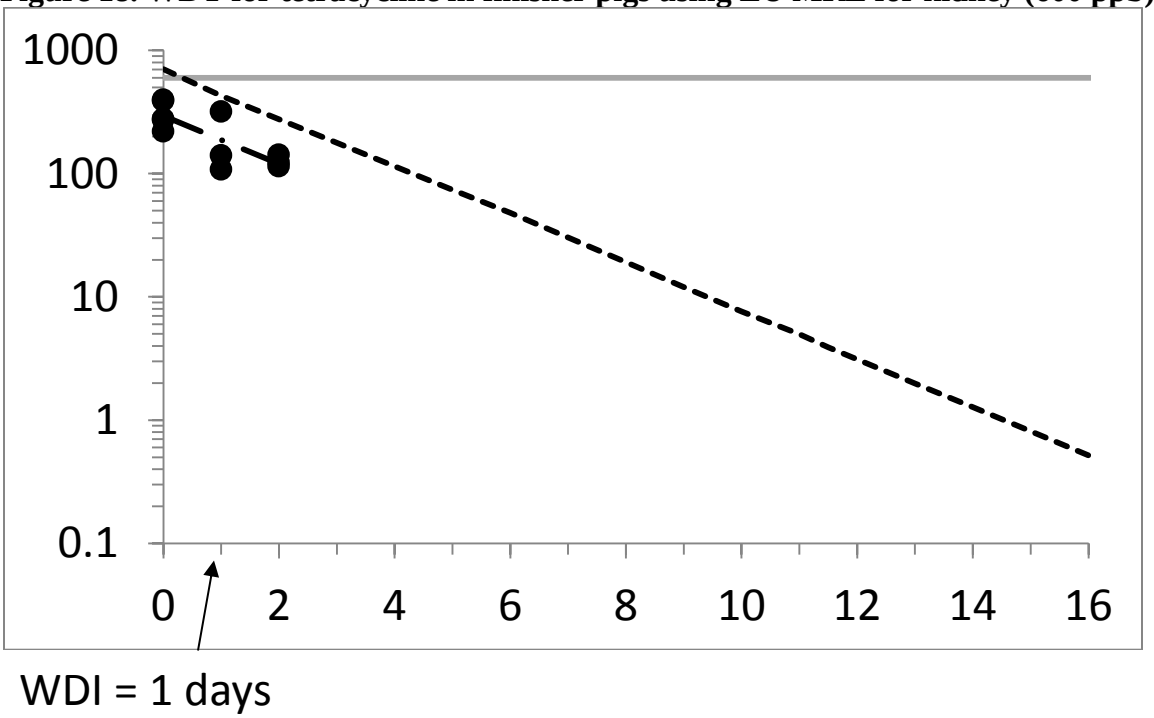


**Figure 19. WDT for tetracycline in weanling pigs using previous tolerance for kidney (250 ppb)**

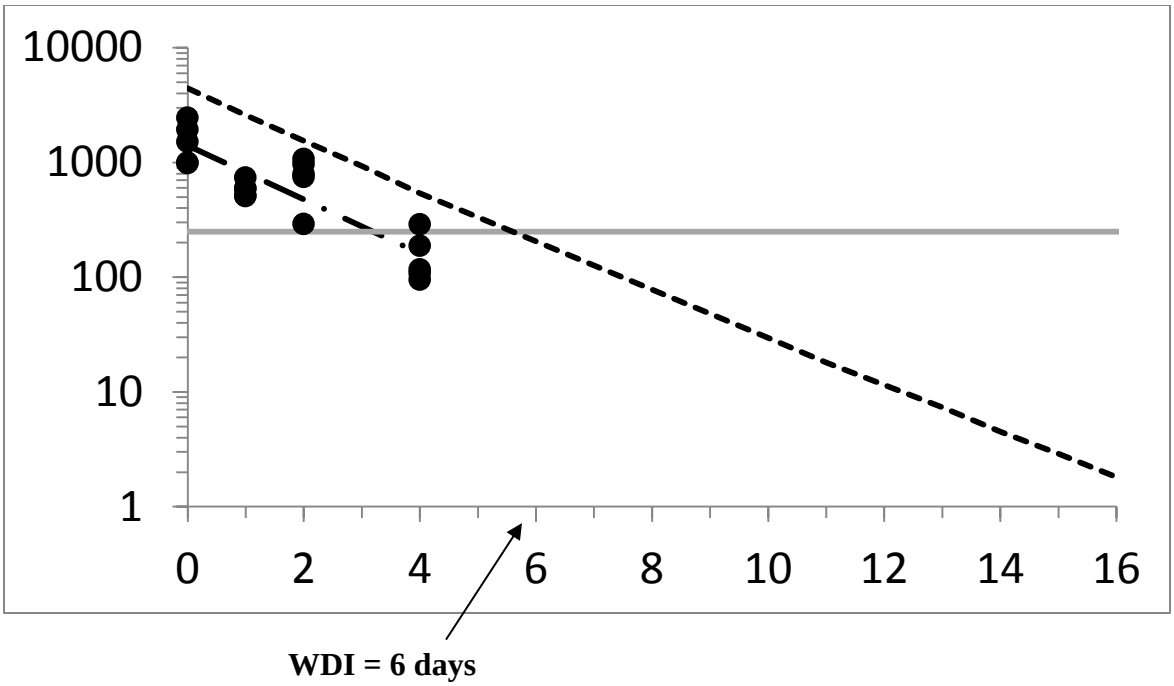
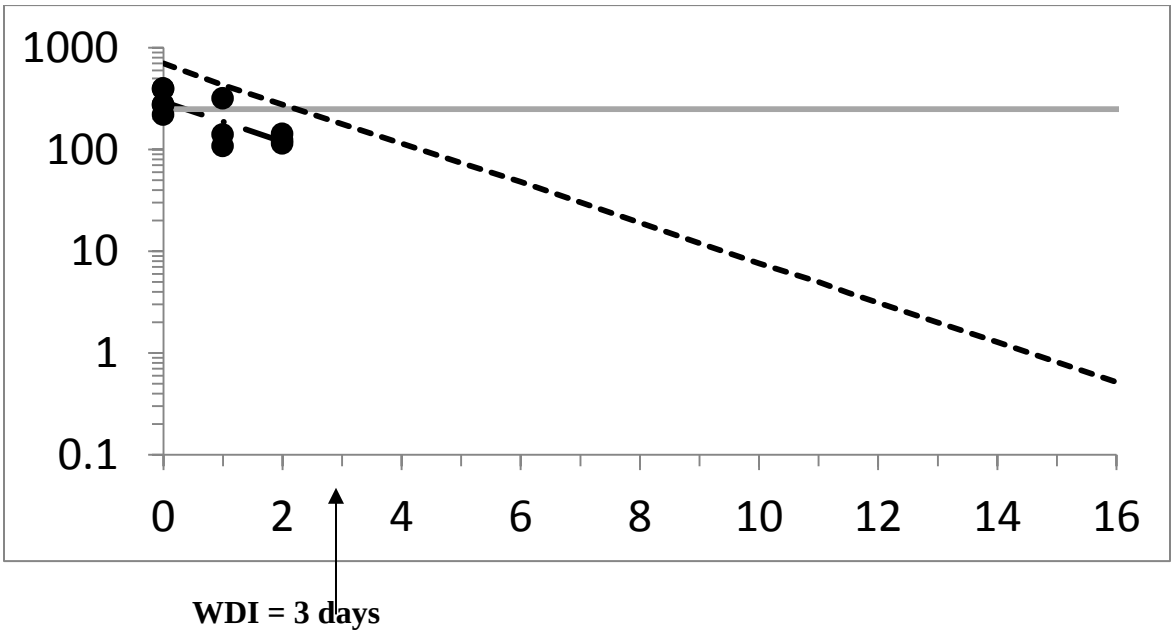



**Figure 20. WDT for tetracycline in finisher pigs using previous tolerance for kidney (250 ppb)**



Based on these analyses (Figures 10-20), tables 11-13 below best summarizes the estimated WDIs for these drugs in various jurisdictions, US, EU, or Russia.

**Table 11. Estimated WDIs (days) for Tetracycline using tissue depletion data.**

| | US[1] | EU[1] | Russia[1] | | US[2] | EU[2] | Russia[2] | |
|---|---|---|---|---|---|---|---|---|
| Stomach (W)<br>Stomach (F) | 1<br>0 | 19<br>31 | 36<br>86 | | 1<br>0 | 11<br>25 | 20<br>48 | |
| Liver (W)<br>Liver (F) | 0<br>* | 10<br>* | | | 0<br>0 | 12<br>4 | | |
| Muscle (W)<br>Muscle (F) | 0<br>* | 5<br>* | | | *<br>* | 5<br>* | | |
| Kidney (W)<br>Kidney (F) | 0<br>0 | 5<br>2 | | | 0<br>0 | 4<br>1 | | |

W = weanling pigs; F = finisher pigs.
1 = WDI from Tolerance limit method; 2 = WDI from PopPK method
* = WDI cannot be estimated due to high variability, limited data, or data below LOQ.

**Table 12. Estimated WDIs (days) for Tetracycline using previous tolerance (250 ppb).**

| | US[1] | | US[2] | |
|---|---|---|---|---|
| Kidney (W)<br>Kidney (F) | 7<br>5 | | 6<br>3 | |

1 = WDI from Tolerance limit method; 2 = WDI from PopPK method

**Table 13. Estimated WDIs (days) for Sulfamethazine using tissue depletion data.**

| | US, EU[1] | | US, EU[2] | |
|---|---|---|---|---|
| Stomach (W)<br>Stomach (F) | 20<br>14 | | 13<br>13 | |
| Liver (W)<br>Liver (F) | 16<br>16 | | 12<br>19 | |
| Muscle (W)<br>Muscle (F) | 16<br>13 | | 12<br>12 | |
| Kidney (W)<br>Kidney (F) | 15<br>14 | | 20<br>13 | |

W = weanling pigs; F = finisher pigs
1 = WDI from Tolerance limit method; 2 = WDI from PopPK method
Note: The US tolerances and EU MRLs for sulfonamides are 100ppb or 0.1ppm.

**Discussion & Conclusions:**

The above results demonstrate that drug medications in water can result in plasma, tissue and even stomach tissue drug concentrations for time periods beyond the withdrawal of the medication in some animals within a given herd. The pilot study demonstrated that there were diurnal patterns to the disposition of drugs given via water medications and this reflects the diurnal drinking habits of pigs. This can result in Cmax and Cmin steady state values that may be below the minimum inhibitory concentration (MIC) for several bacterial pathogens. Whereas this may not be important for gastrointestinal pathogens, it may be important for systemic treatment of other organs and thus plasma concentrations may be an indicator of whether a therapeutic level is achieved in those organs. It can be argued that from several of our observations that this may be associated with the emergence of antimicrobial resistance on some swine farms. This may be relevant for treatment of some respiratory pathogens and where lung tissue often reflects plasma concentrations that do not approach the MIC. Lung tissue and plasma concentrations for tetracycline are often equivalent at steady state plasma concentration.

Sampling of stomach tissue, analytical chemistry of stomach tissues, and pharmacokinetic analyses provided the first reported evidence that drug residues can persist in the stomach tissue for several days after the drug has been removed from the water supply to the pig barns. The data suggest that there is some variability across pigs and that not all pigs had detectable tetracycline residues in the stomach tissue at 8 days after withdrawal of the drug. Pharmaockinetic analysis of this data is difficult as there is very limited information about daily drug intake for each pig and this limits PK modeling of the data. Our modeling exercises however utilized the statistical tolerance limit method used by US FDA and most other jurisdictions to determine the withdrawal interval for pigs if they were exposed to tetracycline or sulfamethazine as a water additive according to label. It is clear from our analyses that if the US tolerance for pig muscle was used as a point of departure (POD), then there will be no need for a withdrawal time if stomach tissue was the target organ. However, if an EU MRL or Russian MRL was used as the POD, then the WDI will need to be significantly extended as indicated in the tables and figures provided. The PopPK method used to derive the WDI is a sound pharmacostatistical approach but was not as conservative as the tolerance limit method guidelines as established by US FDA CVM. In spite of this, the WDIs for the various tissues were comparable using US tolerances. It should also be noted that the current US WDT of 4 days was established several decades ago when the US tolerance for tetracycline was 250 ppb. The US tolerance was significantly increased for the various tissues outlined in the table 14 below. It should be noted that when the US tolerance of 250 ppb is used the WDI approaches the approved WDT of 4 days. If the current US tolerance was applied the WDI approaches zero.

**Table 14. Comparison of tolerances and MRLs across jurisdictions for tetracyclines.**

| | U.S. *Tol. | U.S. Tol. | EU MRL | JECFA MRL |
|---|---|---|---|---|
| **Muscle** | **0.25** | **2** | **0.1** | **0.2** |
| **Liver** | **0.25** | **6** | **0.3** | **0.6** |
| **Kidney** | **0.25** | **12** | **0.6** | **1.2** |
| **Fat** | **0.25** | **12** | **NA** | **NA** |
| **Milk** | **0.08** | **0.3** | **0.1** | **0.1** |

*Tol = US tolerance prior to 1997. CFR (1997*). Food and Drugs, 21 Parts 500 to 599, revised as of April 1, 1997. Code of Federal Regulations, 21 CFR Ch1, (4-1-97 ed.).*

The data also demonstrated for the most part that there are significant differences in WDIs when you compare weanling and finisher pigs. This is not surprising drug clearance varies across age classes and often an important population variable if ignored can result in violative residues. The exception to this rule was the stomach tissue where finisher pigs had longer WDIs than weanling pigs. Physiological and anatomical differences as well as eating habits could be reasonable explanations for these significant differences.

Sulfamethazine plasma concentrations in finisher pigs were significantly higher than that in weanling pigs. This could be a function of water intake differences across age groups although this trend was reversed

with tetracycline. The very long half-life could be as a result of limited time points to adequately capture the tail of the depletion curve which consist in this case of only 1 pig at W8 which may be skewing the analysis. Use of earlier time points suggests that the half-life is closer to 20 hrs which is consistent with other oral gavage studies. Sulfamethazine WDIs were similar to the US FDA approved WDT of 15 days with several exceptions where the WDI was as much as 20 days for weanling stomach and weanling kidney. All jurisdictions use 100ppb sulfamethazine as the MRL or tolerance for all tissues so no WDI estimates were determined for MRLs lower than 100ppb; however, this can be easily estimated from this data set. It is no surprise that several tissues exceeded the label 15 day WDT for sulfamethazine as several of PBPK modeling exercises in our laboratory for this drug in pigs suggested that the WDT should be extended to as much as 21 days (Buur et al., 2006).

In summary, these series of studies clearly demonstrated that water medication does not always achieve therapeutic plasma concentrations in all pigs whether they are weanling or finisher pigs. There is considerable variability in plasma and tissue concentrations of both drugs which could be attributed to environmental conditions as well as daily water intake. Drugs in water medications can persist in stomach and other tissues for extended time periods and U.S WDT of 4 days for tetracycline and 15 days for sulfamethazine may be insufficient to prevent residue violations in overseas markets.

**Future research questions**

The data from this research did reveal several unexpected yet surprising findings and generated the following research questions that should be addressed within the context of our findings:

- Question the effectiveness at the label dose as plasma and tissue levels at steady state are often below the MICs for most microorganisms. Should these water medications be limited to some disease indications and not those that require significant bioavailability.
- Does dosing pigs via water medications at these sub-therapeutic concentrations encourage the emergence of resistant microorganisms. Faulty chemolizers were discovered in this project prior the trials and were corrected. This as well as inaccurate mixing my play a significant role in poor medication of herds.
- Research is needed to establish a more appropriate and thereby effective therapeutic regimen. This may require (1) higher doses for shorter durations, (2) increased bioavailability of novel water medications, and (3) better understanding of whether the sporadic emergence of residue and/or resistance and thus variability in drug disposition in a large herd may be related to genetic polymorphism currently being observed for some drugs in several livestock species including pigs.