ABSTRACT

MASON, SHARON ELIZABETH. Comparative Pharmacokinetic Approaches to Estimating the Pharmacokinetics of Water Medications in Swine. (Under the direction of Ronald E. Baynes).

Although water medications have been used in the United States for over 40 years, the pharmacokinetics of these drugs have not been clearly discerned using compartmental pharmacokinetics techniques. No repeated dosing pharmacokinetic studies have been published on water medications in swine. Therefore we proposed to use three proven techniques in veterinary medicine to model water medications in swine.

Non-compartmental modeling, physiologically based pharmacokinetic modeling and population based pharmacokinetic modeling have improved veterinary medicine pharmacology over the last decade. The applications of these techniques to the pharmacokinetics of water medications, however, have not been published. Therefore two in vivo studies were performed to collect pharmacokinetic information on these drugs and then pharmacokinetic modeling was performed to test these techniques and their applications to characterize the disposition characteristics of water medications in swine production settings.

In vivo experiments were performed in pigs based on the age and dosing schedules of those treated in commercial production units with tetracycline and sulfamethazine. Non-compartmental analysis techniques were initially applied to these populations. PBPK and population pharmacokinetic techniques were also applied where relevant to these medications to provide insight into situations that traditional modeling techniques were unable to elucidate. For sulfamethazine, the use of PBPK modeling
proved useful in characterizing the potentially small exposure concentrations that have been documented in the literature for over 25 years.

In contrast to sulfamethazine which is chemically very stable, tetracycline has been shown to degrade over time with exposure to high temperatures and sunlight. Ancillary experiments were performed to characterize the bioactivity of tetracycline water medication as dosed in a production setting. Furthermore, basic pharmacokinetic information on tetracycline administered in water was collected and analyzed.

Finally population based modeling was applied to data collected from commercial farm settings to determine factors that may apply to all water medication administration in the swine industry. This mixed effect modeling technique was able to provide increased support for the non-compartmental pharmacokinetic findings and to identify factors important to plasma concentrations of medications administered in water.

PBPK and population based modeling techniques can be effectively used in modeling water medications in swine. Furthermore, they were able to determine dosing amounts and schedules as well as other factors that affect the concentration of water medications in swine where traditional pharmacokinetic modeling is inadequate.
DEDICATION

L’œur a raison que la raison ne comprend pas.

À ma familia.

Toujours.

The heart has reason that reason does not understand.

To my family.

Always.
BIOGRAPHY

Sharon Mason is a North Carolina native. She completed her bachelors of science at the University of North Carolina at Chapel, a master’s of science degree in Forensic Toxicology at the University of Florida in Gainesville and in 2006 she completed her Doctor of Veterinary Medicine. Sharon has an interest in food safety and the application of pharmacokinetics to protecting the food supply.
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Finally, I would like to acknowledge my fellow graduate students for their input, support, and presence on a daily basis. FARAD funding supported me during this degree process and contributed to some of these findings. The College of Veterinary Medicine Animal Health Fund provided the initial funding for the first study. Finally Dr. Alan Scheidt and Pfizer Animal Health helped to support the in vivo research monetarily and provided ways for it to be publicized.
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INTRODUCTION

Food producing animals are often given medications in formulations or routes that are not typically used in other animal species or in humans. Although these formulations and dosing methods were approved by FDA, available published pharmacokinetic profiles have been of a more observational nature. Over the last few decades, public policy changes have resulted in a shift in the current process for drug approval being significantly different than the methods employed over 30 years ago. However, during this evolution, formulations previously approved have remained the same and bioequivalence methods have allowed the use of these previously approved formulations to serve as guidelines for more recent approvals. For example, chlortetracycline was approved for use in animal feeds in 1963 and sulfamethazine was approved for use in 1975 (Vetgram, www.farad.org/vetgram/). There have been many generic versions of these same medications, approved years after the first drug approval, without having to undergo a full NADA (New Animal Drug Application) process. These medications must meet bioequivalence requirements of the previously approved drugs in order to receive approval (Jackson et al. 2004). However, this does not require that they meet the requirements of the current NADA process. In addition to NADA processes changing, withdrawal time procedures have expanded to better protect the human population; however, older formulations and their approved withdrawal times may not meet the safety level required by newer drugs, but, nonetheless, are still reaching the market today.

Some antibiotic medications, referred to above, were developed and implemented for use in feed and water sources over 40 years ago. Many of the policy changes from FDA in more recent years reflect an increased understanding and implementation of population
pharmacokinetics, but do not require scrutiny of previously approved drugs to meet those standards (FDA 1999; FDA 2003; FDA 2005). As such, some currently approved medications would not be granted approval in today’s climate. This includes very common drugs like aspirin, which has never been official approved for food animal use.

These policy changes have been aided by our improved computational capabilities, which allow more complicated methods of modeling to be applied to both new human and new animal drug approvals, including drug approvals for food producing animals (Martinez et al. 2000). One area that has received minimal scrutiny in the United States, but which has harbored interest in Europe, is the use of pharmacotherapy via water. With the ban in 2006 of all non-therapeutic antimicrobial use in Europe (EC 2003), understanding drugs that are used and assumed to be therapeutic is of greater importance. However, compared to other methods of treating disease, little research has been performed on water medications compared to feed additives and parenteral dosing of these medications. A Pubmed search (on October 27, 2009) for “water medication pharmacokinetics swine” revealed 93 papers, of which only 4 were on water medications given to swine. Overall, only a handful of papers have directly looked at water medications in swine (McCaughey et al. 1991; Agerso et al. 1998; Mason et al. 2008; Dorr et al. 2009; Mason et al. 2009), whereas quite a few more have involved observational studies of these medications and reported plasma levels. Therefore research in the area of water medications is of great importance; however despite the lack of data, much of the pharmacokinetics of these medications was considered axiomatic.

Many of the limitations to analysis of water medications has related to the computational abilities of pharmacokinetic programs using traditional, readily available PK
analyses, when other methods are necessary. One major limitation that is met with water, and feed additives for that matter, is the lack of a known dose of medication taken by animals. This is not a problem often encountered in human drug studies or in small animal medicine because these patients are often dosed directly by clinicians or technical staff, often using parenteral medications. Furthermore, individual animal and client compliance in domestic animals is often good (over 50%) as reported by the American Animal Hospital Association (http://www.aahanet.org/protected/ComplianceExecutiveSummary0309.pdf) summary in 2008. However, in the case of herd health or food animal medicine, an individual animal cannot be medicated several times a day due to labor and animal handling constraints. Therefore, the technique of medicating a whole herd, barn, or pen is commonly employed to avoid individual animal treatments. This ease of treatment, however, may quickly become a pharmacokinetic nightmare. Limitations to pharmacokinetic analysis stem from the way in which many pharmacokinetic programs (WinNonLin, WinBugs, etc) are written. Because most PK programs were originally designed to facilitate quick and easy PK analysis with a limited user interface, typically considered user friendly, these traditional PK programs are exactly the opposite of what is necessary to analyze the data that are acquired in a herd health situation. The largest problem presented by a herd health approach to pharmacokinetics is the unknown dose of the drug. All traditional PK programs the author has encountered allow for multiple dosing regimens; however, a known dose given at a known frequency is required for the program to calculate PK parameters. Even if an approximate dose can be provided, if there is large variability in dosing frequency, as with water medications, the programs are typically unable to account for the large inter-subject
variability, which invariably occurs with water medication dosing. There are other sources of variability including body weights (which cannot always be measured), competition among animals, metabolic differences and sometimes other variables that are drug specific. Fortunately, these populations of food animals, most often are also more homogenous in other ways than clinically presented human or veterinary populations. Some of the relatively uniform variables include clearance, weight ranges, and illness status. Due to the complexity of modeling herds in a traditional manner, we have explored other pharmacokinetic methods of modeling water medications. Three PK approaches (traditional, physiologically based and population based modeling) were employed in this work. Hopefully, their applications to water medications in food animals, which are the focus of this work, will assist in addressing other problems faced by pharmacokineticists and toxicologists. These approaches may be applicable to other animal species, such as wildlife and pet birds (often medicated with water soluble drugs). There is also the potential that toxicologists may be able to use these techniques to model exposure even when the doses are unknown.

**PHARMACOKINETIC BASICS**

Pharmacokinetics (PK) is the science that quantifies and characterizes the way in which drugs are absorbed, distributed, metabolized and finally excreted from the body (referred to as ADME) (Riviere 1997). The effects of a drug on the body, at either the organism or molecular level, are known as pharmacodynamics (PD) (Lees et al. 2004; Rybak 2006). The PK of a drug applies the physiochemical properties of the drug, including (but not limited to) polarity and ionic charges, pH, molecular weight, and stereochemistry, in the
context of absorption, distribution, metabolism and excretion (ADME). PK further defines the duration of a drug’s effect on the body. A drug’s physiochemical properties are defined outside of the body; therefore, the art and interpretation of pharmacokinetics begins when that drug is administered to a subject.

With all PK modeling, the basis is rates of drug moved or cleared from the body over time. Primary PK parameters are based on the idea of a mass of drug (i.e. the dose in mg, typically) that moves around the body and is removed from the body over a certain period of time (Riviere 1997). This mass over time is the rate and with most drugs at currently approved doses, the clearance remains constant over time. These rates of change can occur between compartments known as micro-constants, as with compartmental PK modeling, or they can refer to the whole body elimination rates or macro-constants. These rates of change within the body are best explained using the mathematical language of calculus through differential equations. Basic pharmacokinetic parameters can be calculated for an individual animal without a physiologic understanding of the processes that are ongoing; this is common with traditional PK modeling. However, because this is a mathematical explanation of physiologic processes, a good understanding of the body as well as the mathematics provides the best situation for application of PK models. This is most important in that PK models can be used to explain drug behavior to clinicians, scientists and pharmacists. And when done properly, PK modeling can both explain and predict what happens to a drug within the body. In the clinical setting, the most useful applications of PK are through models that incorporate drug dosing regimens into the prediction of therapeutic drug levels, with less invasive monitoring of patients (Martin-Jimenez & Riviere, 1998; Wahlby et al.
For research scientists, PK models provide a greater understanding of the drug’s action, disposition and toxic target tissues, as well as providing a method that requires use of fewer experimental subjects (FDA 1999).

Pharmacokinetic models can be, and frequently are, applied to many species; however, the most extensive information available is in humans. Much of the preliminary pharmacokinetic information obtained on drugs is from basic PK studies in animals (FDA 1999). Therefore PK models are key in extrapolating safety in animals to humans prior to testing (Caldwell et al. 1995).

The following review will touch on basic pharmacokinetic modeling principles and design before discussing modeling differences among traditional compartmental models, physiologic-based pharmacokinetic (PBPK) models and population pharmacokinetic models. Understanding PK modeling techniques, their applications and limitations is needed to best understand ADME and then predict drug deposition and elimination in vivo. A full review of ADME is beyond the scope of this work and is best addressed by other reviews (Caldwell et al. 1995; Grossman 2009). Finally, understanding pharmacokinetics (often via modeling) and being able to apply that information to predict dosing regimens, species extrapolations, and toxicity may assist with understanding how targeting a drug’s movement to a certain application will or will not work (Theil et al. 2003; De Buck and Mackie 2007; Yengi et al. 2007; Hou and Wang 2008).
MODELING AND IDENTIFIABILITY

Modeling is a very important aspect of pharmacokinetic analysis. If experiments are crafted in a meaningful and biologically sound way, PK models designed from the measured data provide explanations and predictions for therapeutic levels of drugs and/or clearance rates (Wahlby et al. 2004; Buur et al. 2005). However, models are never one hundred percent correct. In order for that to happen, the model would be infinitely complex to account for every possible outcome (Merle et al. 2004). Parameters (or independent variables) are usually determined from experimental data, but they can be determined by other experiments including ex vivo or in vitro ADME experiments and chemical properties of the drug (Theil et al. 2003; De Buck and Mackie 2007).

Identifiability in modeling is the ability to determine a value to a parameter (Godfrey et al. 1980; Williams 1990). In order for the model to be identifiable, there have to be enough experimental data points from one or more experiment(s) that will help with determining parameters. These parameters can be traditional parameters, population-based parameters with variability as in population PK modeling, or physiologic rates or partition coefficients for PBPK models. Therefore, careful experimental design will shape the type of parameters to be determined and the overall usefulness of the model.

One aspect often overlooked is model structure which plays perhaps the most important role in determining identifiability. Models with unidentifiable parameters or parameters that may be quasi-identifiable (a range can be assigned), often lack enough data due to inability to measure compartments. This is often seen by using a measured compartment to link with unmeasured compartment(s) which leaves some micro-constants
unknown. In this case some of the parameters may not be uniquely identifiable or identifiable only if one or more of the parameters in the measured compartment are known *a priori* (Williams 1990). In traditional PK modeling, the concentration time profile is used to determine values to parameters, but parameters may also be assigned values based on physiologic functions, covariates (inter-individual variability of one or more parameters) or other experimental methods mentioned above. This is important to population based PK modeling because a simple model is usually used in order to allow for identifiability of parameters (Wade et al. 1994). Unless external information is available, these simple models are often used for population PK studies due to the complexity and bias that may occur with more complex models (Ludden et al. 1994). Although models are unable to account for all biological variation, less if the parameters are identifiable, a model may be useful to prediction or explanation of a process (Godfrey et al. 1994; Wahlby et al. 2004). These parameters may be general PK parameters such as Vd (volume of distribution) common to compartmental modeling, or the parameters may be specific to a model, such as a metabolic pathway for a drug in a PBPK model. However, assigning a value to these parameters is necessary to solve the model based on the observed (plasma, tissue, urine concentrations) data (Wahlby et al. 2004).

The parameters discussed above are generally understood to be global parameters (variables). The parameters to which we typically refer are globally identifiable parameters, which have one unique value, or a very small range of values in the case of quasi-identifiable parameters or population models, when calculated under appropriate experimental conditions of low signal to noise ratios for analysis and good experimental design. In some models, in
addition to the global variables, there are local parameters that are often within a programming routine (or compartment) of a model. These locally identifiable parameters can take on various values based on the data that is presented at that time in the program (Godfrey et al. 1994; Evans et al. 2001). These local or unidentifiable parameters lack any specific values and can take on an infinite number of values in the model (Evans et al. 2001). Therefore, when solving or quantifying a model, it is the global variables that we typically refer to needing solutions.

Identifiability in a model can only be determined if the model structure is valid and the experimental design has adequate controllability and observability (Godfrey et al. 1994; Evans et al. 2001). If the experimental designs do not adequately control for changes in response(s) due to parameters or do not allow (direct or indirect) observation of the response of interest, then there will be parameters in the model that are more likely to be unidentifiable or misspecified (Williams 1990). Although a model may have global identifiability, such as clearance in a compartmental PK model, the parameters predicted may be less specific or correct than another model that lacks global identifiability, such as a PBPK model where values are presumptively assigned. With PBPK models, because the physiologic processes are not completely understood or able to be controlled, often assumptions must be made for many of the parameters in the model. These values may be assigned to the model for various organ compartments, including blood flow, metabolic functions and volumes; however, the values may or may not be correct; and in reality we can never know their true values. PBPK models may also contain good local (compartment-based) identifiability. Models with unidentifiable parameters may be more useful or less useful than fully identifiable models
(Godfrey et al. 1994); however, the main assessment of a model is related to its usefulness, not its identifiability, as will be discussed below. If the parameters are not at least quasi-quantifiable, then the model is not solvable, and therefore, it is only a conceptual model.

**ASSUMPTIONS AND MISSPECIFICATION**

With all models, in order to solve the formulae for important parameters, certain assumptions must be made for the mathematical structure to be solved with available data. Generally, more complex models require more assumptions; however even if assumptions are not directly stated, the model type often has certain assumptions that are generally applied whether the modeler knows this or not. Some assumptions that are common to all traditional PK models are compartment homogeneity (Evans et al. 2001) and first order kinetics (Rescigno 2001). Most PBPK models assume a flow limited system (Kawai et al. 1994; Lee et al. 2009), while population based models assume normal distributions for most population-based parameters, unless a non-parametric algorithm is chosen (Lindstrom and Bates 1990; Davidian and Giltinan 1993; Park et al. 1997; Bustad et al. 2006).

Assumptions allow complicated mathematical formulae to be simplified into formulae that can be solved. Based on the number of data points and subjects in the model, modelers are left with a certain amount of room for predicting parameters and solving the proposed model, often discussed in statistics as degrees of freedom. Given an unlimited supply of data and infinite time and computational ability, all models could be solved. However, this would be unreasonable, and therefore, simpler models that explain something
are often used in lieu of unwieldy giant models that explain everything, but cannot be mathematically applied to real world situations (Ludden et al. 1994).

Unfortunately, even with well developed models, based on good experimental design, there will always be model misspecifications (Merle et al. 2004). Misspecifications are errors in the model that affect parameter predictions. They arise from various sources, including falsely acquired variability in error terms, instrument errors and sometimes from bias in the reporting of data. However, major misspecifications occur from making assumptions that are not physiologically or chemically valid. Often this type of misspecification arises from model over-simplification. Unfortunately, many models would not be mathematically calculable without assumptions and simplifications to decrease the number of unknown variables (Evans et al. 2001). Therefore, models often use the fewest number of important parameters to account for the presumed pharmacokinetic processes (FDA 1999; Martin-Jimenez and Riviere 2001; Buur et al. 2005; Buur et al. 2005). These model simplifications are often made via assumptions, such as homogeneity in compartments and instantaneous absorption for intravenous doses (Evans et al. 2001; Merle et al. 2004; Buur et al. 2005; Buur et al. 2005), which may be physiologically equivocal.

For individual-based models, the variability in parameter estimations is assumed to be a random error factor, usually distributed around a mean of zero. This random error should account for both individual differences between subjects and the instrument error. However, when assumptions are made that are not physiologically valid or reasonable to a model or process, this random error term will become very large due to misspecification of the model. One goal in picking a model design or type is to decrease model misspecification in order to
better predict parameter estimates. Also one must know what he is attempting to learn prior to designing an experiment, which should be based on the information needed. Table 1 has an overview of the three types of models to be discussed.

TRADITIONAL MODELING

Traditional pharmacokinetic modeling has typically relied upon intensive-sampling studies, with few to many subjects, with compartmental based results (FDA 1999). However, over the last decade non-compartmental modeling techniques have gained favor. Either of these conventional methods is based upon the idea (even with non-compartmental analysis) of at least a vascular compartment, necessary for the simulation of a concentration-time profile. The idea behind open compartmental analysis is that areas of the body which experience similar disposition and elimination of a drug are lumped together into compartments. These compartments are differentiated by curve fitting of an exponential equation for intravenous data, where drug concentrations are eliminated from the measured (central) compartment at constant rates per unit time \( (k_a) \) of the various exponential curves. For a two compartmental model, the following equation represents two compartments, labeled A and B:

\[
C_p = A e^{-k_a t} + B e^{-k_b t}
\]

(1)

With compartmental models, there are several other assumptions of normality made about the population, including: typically constant, first order processes of elimination and partitioning, no saturation of tissues and absence of covalent/non-competitive binding in tissues or compartments. These assumptions, although broad, also mean that some drugs
cannot be accurately modeled using these techniques because they violate one or more of these assumptions (Riond and Riviere 1988).

The most common PK modeling type is compartmental modeling, with one to three compartments (Godfrey et al. 1994; FDA 1999). Once the number of compartments exceeds three, modeling can become overly complex and often other mathematical techniques must be used. Traditional modeling is based on known, directly measured values, usually including the dose (D) and plasma concentrations time profile (Cp). These values are assumed to be precise with little to no variability or error. Primary parameters are calculated from these measured values. These fundamental parameters include the theoretical volume of distribution (Vd) and body clearance (Cl); however, they must be calculated from the concentration time profiles. The following equation illustrates the relationship between these two primary parameters (Riviere 1997):

\[ Cp = \frac{D}{Vd} \]  

(2)

Based on these primary parameters, volume of distribution and body clearance, secondary parameters, not based on physiology, are calculated to represent processes, occurring within the body. These parameters include half-life (T½) and entry and exit rates for each compartment in multiple compartmental models.

Compared to compartmental models, non-compartmental models are usually constructed from sparse data which does not allow a differentiation of compartments from the semi-logarithmic curve or from situations in which inflection points in the elimination curves have inadequate sampling, which would lead to potentially gross inaccuracies in the compartmental rates and half-lives. Non-compartmental modeling is also used for groups of
individuals where there is not one compartment model that fits all individuals (Riviere 1997). Non-compartmental model analysis uses a simple curve or regression line that is often calculated via a process called statistical moment theory (SMT) and referred by other names. SMT uses most of the same assumptions for compartmental analysis, but relies on moments of the concentration-time profile. In SMT, differential equations are often solved with calculus to provide AUC moments per unit time using the trapezoidal rule. Based on the AUC, as the primary moment, the secondary moment, i.e. the differential of the previous curve, reveals a peak, that represents the mean (average) residence time (MRT) for the average molecule. MRT is linked to the half-life of compartmental models by the equation:

\[ T_{\frac{1}{2}} = \ln 2 \times MRT \quad (3) \]

The MRT is the change in the concentration-time curve through time and its peak is therefore equal to the most frequent interval of time that molecules remain in the body. Therefore, non-compartmental analysis can be likened to compartmental analysis with fewer restraints on curve fitting. Traditional parameters can be calculated from SMT as explained elsewhere (Riviere 1997). Furthermore, the same drug over the same time frame may require a three compartment model in one person and a two compartment model in a different subject (Rescigno 2001; Merle et al. 2004). This physiologic variability makes a case for non-compartmental analysis over compartmental analysis and also provides a comparison between individual. Non-compartmental analysis can be used in cases where uncertainty in dose, limitations to modeling or increased variability exist; however, this modeling often requires pharmacokinetic or mathematical programs that do not require dosing schemes. One
The final benefit of SMT over compartmental modeling is the ability to calculate some primary PK parameters without a dosing schedule at all (see Chapter 3 for an example).

With all types of traditional modeling, the need to have adequate sample times is necessary, as can be seen in Figure 1. This figure exhibits the effects of sparse sampling compared to more intensive sampling techniques. Much more information is available from the top panel in Figure 1 than from the lower panel. From Figure 2, one can see the effect of performing logarithmic transformation of the plasma concentrations. The result is that a one-compartment model would fit data in Figure 2. The rate of elimination does not change, but semi-logarithmic transformation of data is best able to demonstrate when no change in the rate is present, compared to a non-transformed plot. The upper panel in Figure 2 shows typical plasma concentration versus time results, while the lower panel shows the transformation, which corresponds with a one compartment model.

For more than a one compartment, the true value of the distribution and elimination rates cannot be directly seen from the curve and data stripping is necessary to differentiate the elimination rates of the two compartments. The process of data stripping will not be discussed further in this review. There are other resources for this and most PK programs can perform data stripping (Riviere 1997; Rescigno 2001; Merle et al. 2004).

Traditional compartmental modeling techniques do not attempt to develop a physiologic rationale to explain the data or even to link the data to compartments if the model contains these. Rather traditional modeling relies upon the data for its constraints, using distribution and elimination rate constants from the data to best fit it to a number of compartments. It is often explained that compartments relate to physiologically related
tissues or areas in the body; however, the time frame of experiments is often a better explanation as to why the same drug requires different structural models most of the time. The importance of the traditional approach is that data-intensive sampling is necessary to avoid missing changes in rate constants as seen in Figure 1 (Riviere 1997; FDA 1999; Green and Duffull 2003). From traditional models, physiology can be super-imposed upon the results to explain what may be happening in the body and assign physiologic functions and rationales to a mathematical process (Rescigno 2001). These approaches are often used for initial studies, when PK parameters are unknown.

Compartmental PK modeling techniques have been used to look at sulfamethazine and tetracycline in swine over the last few decades. However, no approaches used the SMT approach. Pharmacokinetic studies for sulfamethazine have been performed by Nouws et al (1986; 1989); and Sweeney et al (1993). Several compartmental studies have also been performed on tetracycline given intravenous and compared those levels with plasma concentrations attained by feed medicated with tetracycline (Kellaway and Marriot 1975; Kniffen et al. 1989; Nielsen and Gyrd-Hansen 1996). These data can serve as a comparative starting point for modeling these drugs dosed in water in commercially housed swine.

PHYSIOLOGICALLY-BASED PHARMACOKINETICS

Physiologic-based PK (PBPK) modeling requires an understanding of the above concepts in conjunction with specific information about ADME for the drug(s) being modeled. PBPK modeling is a data-intensive modeling system (FDA 1999; Buur et al. 2005; Buur et al. 2005) and is based on heavily sampled individuals. Unlike traditional PK models
that are generally simplified to a log-linear regression model or a compartment model, PBPK modeling uses physiologically important parameters to develop a model that predicts known data (Kawai et al. 1994 (Buur et al. 2005; Buur et al. 2005). In many ways PBPK is similar to compartmental modeling; however, rather than allowing the drug profile to determine the model, in PBPK modeling, the modeler sets up compartments that correspond most often to tissues in the body with known volumes and blood flow rates (Emond et al. 2005). PBPK modeling is therefore an individual-based model. PBPK models attempt to more accurately predict the physiologic processes taking place than compartmental models. This is done by using actual organ or organ systems data, physiologically important to the drug in terms of drug distribution, drug targeting (PD), metabolism and elimination. Defining common parameters such as cardiac output (heart rate and stroke volume), respiration rates, blood flow rates, physiologic effectors (exercise, blood pressure) and metabolic processes are necessary for a functioning model. The major difficulty of PBPK models is appropriately applying the huge amount of physiologic data about organ volumes, blood flow rates and percentages of cardiac output to the model in a meaningful way. Many of these measured values are hard if not impossible to acquire in a valid and physiologically relevant way.

Furthermore, due to the complicated nature of PBPK models, there is a great need for simplification. One major simplification of PBPK modeling has been and remains the need for the model to be flow-limited (Kawai et al. 1994). Without the model being flow limited, the model would be completely unsolvable without protein-binding/dissolution rates and transporter processing rates. However, when making a PBPK model flow limited, one must assign partition or diffusion constants that determine the rate or portion of drug in the blood
that will enter the compartment or organ being modeled. The partition coefficient is often very difficult to determine *in vivo* and model optimization is often performed to determine partition coefficients. Finally, PBPK models assume instantaneous diffusion with homogenous compartmental mixing. These simplifications ignore the effect of cells and various cell types within compartments that represent organs, but often work to make the model meaningful without being overly complex.

Because PBPK models are individual models, most variation in PBPK models is intra-individual error (Evans et al. 2001; Buur et al. 2005; Buur et al. 2005). Intra-individual error is found within an individual, often tied to physiologic processes discussed above. Due to their individual nature, PBPK models often lump intra-individual error with measurement error from variability in the analytical process which is up to 20% in some validated assays (Bressolle et al. 1996). Measurement error is often assumed to be larger than the physiologic error, and thus intra-individual error is often ignored. However, with some assays measurement error is very small and therefore, assuming measurement error is the total error in PBPK models can lead to misspecification of the model. This misspecification is especially troublesome when developing PBPK models for application to non-homogenous or genetically polymorphous populations, especially if differences in metabolic or elimination processes are unknown between the model population and a genetically different or diseased population. Model misspecification often cannot be differentiated from large intra-individual errors. Crucial to proper PBPK model development is a good understanding of ADME and its proper application. Based on the potential for misspecification, when variability is large, validation of PBPK models can be difficult.
The greatest limitation of PBPK modeling is its lack of certainty for many physiologic parameters. This unfortunately provides a sizeable and unquantifiable error, which makes model validation difficult. If two populations are known to be similar it may be safe to make population predictions with PBPK models. However, populations must be similar as the model may lack external validity in a target population due to variability not represented in the model (FDA 1999). One example of this type of variability occurs with genetic polymorphisms in populations. These polymorphisms result in a ‘high’ metabolic status and a ‘low’ metabolic status (Clewell and Andersen 1996). Some modeling programs allow optimization of models based on minimizing sum of squares of the residuals and allow comparison of the model output to the observed data. Model optimization algorithms predict the best parameter values based on the designed model, and they often allow other data sets to be included for model comparison. However, with PBPK models, due to the variability in sources of data for model development (including PK studies for comparable data, metabolism data, etc), methods of model comparison are not easily performed. It may be possible to use statistical programs to perform residual sum of squares comparisons between models for a single data set in addition to graphical methods.

PBPK models have been used in various ways in the last 2 decades. First, they can be used to extrapolate to situations of unknown dosing (e.g. (Mason et al. 2008), potential changes in pharmacokinetic parameters or rates of elimination (Emond et al. 2005; Emond et al. 2006) and extrapolation across species from those previously characterized (Kirman et al. 2003; De Buck and Mackie 2007). This techniques provides a way to scale physiologically rather than on traditional allometric (body weight), surface area or other scaling factors
(Kirman et al. 2003). This flexibility is a great asset and allows PK modeling of situations not feasibly modeled via traditional methods, especially where physiologic processes are known but there is limited data for that species (Emond et al. 2005). Chapter 2 of this work compares a traditional model to a previously validated PBPK model (Buur et al. 2005) to determine what might be happening in a production setting. The flexibility of PBPK modeling is the only technique discussed in this work that allows the aforementioned situation to be elucidated.

Finally, PBPK modeling can be combined with many other techniques including Monte Carlo analysis, covariate modeling, Bayesian forecasting and statistical techniques to provide a population approach to pharmacokinetics (Clewell and Andersen 1996; Sproule et al. 1997; Jonsson and Johanson 2003; Buur et al. 2006; Sprandel et al. 2006). Typically, since PBPK modeling is an individual-based modeling approach, the use of these population methods are applied on top of the developed model. These population modeling techniques are based on statistical analysis and typically apply normal distributions over parameter values to account for physiologic variability and error in the model. By running a PBPK model many hundreds of times using these distributions, one can develop a sense of the likelihood of what may be happening in a population of animals or humans exposed to a drug at a given route and dose. Buur et al (2006) applied this technique to residue analysis for veterinary medicine recently. The aforementioned techniques have been used in conjunction with PBPK models to determine population and pharmacodynamic effects (el-Masri et al. 1995; Gueorguieva et al. 2007) and should be reviewed for more information on PD.
POPULATION PHARMACOKINETICS

Population PK modeling is a statistically based pharmacokinetic approach to modeling sparse data of a large numbers of subjects. This techniques uses the variability inherent to the population for better modeling sub-groups that cannot be addressed fully by traditional PK models (del Castillo et al. 2006). The covariate structures, or subject specific groupings, in a system of equations is overlaid on a traditional compartmental pharmacokinetic model. The statistical components of the modeling allow for a normal distribution for the error terms to account for intra-subject variability and variability in pharmacokinetic parameter distributions. These parameter distributions can be parametric, semi-parametric or non-parametric, but most modeling algorithms employ parametric distributions. These distributions are applied to the PK parameters population mean by error terms or equations based on multiplicative, additive or exponential relationships. The determination of the error modeling scheme is best determined by the nature of the data and is beyond the scope of this review (Davidian and Gallant 1992; Davidian and Giltinan 1993; Park et al. 1997; Parke et al. 1999).

The benefit of this type of modeling is evidenced when data is scarce for individuals and there are differences in pharmacokinetic parameters or measured outcomes across groups of individuals based on factors, typically referred to as covariates. In population PK models, it is useful to understand the physiology of the drug, as with PBPK (Karlsson and Sheiner 1994; Mager and Goller 1995; Martin-Jimenez and Riviere 2001; Martin-Jimenez and Riviere 2002; Vinks 2002; Wahlby et al. 2004). However, population PK modeling is not based on a physiologic model but rather statistical theory of population distributions with an
assumed variability (set by the modeler). In order to help characterize or define the
dividual variability seen in PK models, covariates are used to address this inter-individual
(or between subject) variability (FDA 1999; Merle et al. 2004; del Castillo et al. 2006).
Emphasis is often placed on covariates that provide information on differences in
pharmacokinetic parameters among subgroups. These differences typically relate to
physiologic parameters of metabolism or clearance of a drug (such as enzyme
polymorphisms or disease states), as well as other changes in basic physiologic parameters
(Martin-Jimenez and Riviere 2001; Wahlby et al. 2004) including volume of distribution and
protein binding. Population PK covariates may include physiologic functions, such as serum
creatinine, liver-specific enzyme levels, age, blood pressure, or body condition score or
weight; or they may be categorical variables, such as gender or health status (Martin-Jimenez
and Riviere 2001; Mager 2004; Wahlby et al. 2004). These covariates are used to provide
insight into the pharmacokinetics related to a drug by providing a physiologic basis for
mimicking variability present in the population. Because this technique is based in statistics,
not a physiologic-based technique, population PK experiments can use less intensive data
collection than PBPK models while maintaining a robust ability to predict population
parameters across subgroups (FDA 1999; Wahlby et al. 2004).

The strength to population PK modeling lies in its ability to account for both intra-
individual and inter-individual errors (Martin-Jimenez and Riviere 2001; del Castillo et al.
2006). The intra-individual error has to be set before the inter-individual error is addressed.
Once intra-individual variability is specified, often by repeated measures, the inter-subject
error is set using the covariate distributions in the model. Finally, undefined
‘interoccasional’ or random error ($\varepsilon$) accounts for other variability (Wahlby et al. 2004). Therefore, we can say that the identifiable sources of variability (parameter variability, covariate effects, and measurement error) for the model are what allow this method to work. These are notated as follows:

- $\eta$ for intersubject error (i.e. individual differences)
- $\omega^2$ for intersubject variability.
- $\varepsilon$ for random error for each observed $\eta$, which may contain variability in intra-individual error depending upon how the model is specified.

Each of these types of errors is set for the parameters involved in the model in order to simulate the concentration-time profiles and other directly measured data. With population PK modeling, it is essential that differences among these error terms be specified. Without defining the error types, model covariates or bias may not be fully elucidated. It is possible to develop a model without defining each error type; however, the coefficient of variation across the PK parameters in the model would undoubtedly be very high or incalculable. Therefore the accuracy and precision of the PK parameters would be poor because of undefined error rates, which increase model uncertainty.

Because this is a statistical technique, one of the benefits of mixed effects population modeling is the ability to employ bootstrapping, re-sampling, and data splitting to the original data sets, if necessary, to increase sample population sizes. The above techniques along with varying the type of parameter distributions can help modelers avoid misspecification of parameter variability and improve model fit (Davidian and Gallant 1992; Park et al. 1997; Parke et al. 1999; Oberg and Davidian 2000; Bustad et al. 2006; Sprandel et
Various studies have been performed in the literature in both human and veterinary medicine applying various programs including NonMEM, WinBugs and WinNonMix to population pharmacokinetic modeling. Further review is directed to the following sources (Davidian and Gallant 1992; Karlsson and Sheiner 1994; Yuh et al. 1994; Vinks et al. 1996; Concordet and Toutain 1997; Martin-Jimenez and Riviere 1998; Gupta et al. 2001; Martin-Jimenez and Riviere 2001; Martin-Jimenez and Riviere 2002; Peyrou et al. 2004; Anderson et al. 2006; del Castillo et al. 2006; Chervoneva et al. 2007).

**MIXED EFFECTS MODELING OF WATER MEDICATIONS IN HERDS**

The above population modeling techniques have been applied to veterinary medicine very effectively. However, when attempting to model medications administered via water, there are several unique concerns related to the method as well as to treating swine in commercial facilities. Therefore, many of the premises in previous studies may not be feasible in commercial settings. The largest aforementioned problem is the fact that there is a variable and unknown dose for each animal. Using deconvolution techniques may be laborious, unfeasible, or provide large ranges of doses based upon the structural PK model used. Deconvolution techniques also require prior information relative to the PK parameters for the model structure chosen as well. *A priori* information for PK parameters is helpful but not necessarily required for a population model. Whereas for a traditional deconvolution techniques, clearance or elimination rates must be known in order for a dose to be calculable. With some types of population pharmacokinetic modeling one can actually bypass
calculating the actual dose if known covariates and fixed variables are available to help determine a dose. This technique is discussed further in Chapter 5.

Although the statistical portion of the model is defined by parameter distributions, a traditional PK structural (i.e. compartmental) model is still the basis of the design. In determining which model type to use (compartmental type (micro vs. macro) and number, sampling schemes, etc.), there are many approaches in the literature and often involve different algorithms for crafting studies including D-optimal design (Tod et al. 1998; Green and Duffull 2003; Gueorguieva et al. 2006) and two stage model approaches (Yeap and Davidian 2001; Tam et al. 2003). There are also pharmacoepidemological (Clewell and Andersen 1996; Dorr et al. 2009) and pharmacogenetic approaches (Borges et al. 2006) that may be able to provide PK data for analysis. In Chapter 5, the final model chosen was an open one-compartment traditional PK model. However, a two compartment model was also considered as an option.

Due to the flexibility of pharmacostatistical models and the ability to use Bayesian forecasting, some of the limitations to traditional modeling, such as needing a dose, can be avoided. The model types chosen were based upon the data from the initial tetracycline study. The data was collected from a two-stage modeling scheme, with relatively data rich sampling in few animals over the dosing period. Much individual subject data was collected and calculated in this process in addition to plasma concentration-time profiles, including body weight, daily weight gain, water use, water concentration, and serum creatinine.

The two stage model allowed the data to be collected and interpreted by a traditional SMT technique that gave estimates of apparent bioavailability and clearance for the groups.
of animals (discussed further in Chapter 3). This two stage method allowed individual PK parameters to be calculated first and then group parameters were calculated. This initial data was applied to the population mixed effects model to determine factors important to plasma concentration variability for a larger population (Jonsson et al. 2000; Bustad et al. 2006). This method also provided a priori parameter estimates of the group PK parameters and served as a later comparison to the final model parameters.

In the initial model development, after this two-stage modeling was finished, a large amount of variability was left among individual animal concentrations. Furthermore, two of the treatment group’s steady state concentrations were not able to be differentiated. A basic linear model was developed that related steady state plasma concentrations to the water concentrations to which the animals were exposed (Figure 3). The reasons behind this large variability were unknown in the initial study.

In creating the mixed effects model, potential covariates were selected from collected data as well as from published covariates commonly found in population modeling (Mager 2004). The major covariates considered were serum creatinine as a marker for clearance, body weight as a surrogate for volume of distribution and bioavailability. Other factors that were measured and considered for inclusion in the model were water consumption and the ambient temperature. The final covariates chosen for the model should account for inter-individual variability in the model and decrease the overall intra-subject and random error significantly.

However, not all covariates that seem important will be useful. Therefore a selection process is necessary. The basic ANOVA (linear) model above that characterizes
concentration–time profiles related to the potential covariates was considered first. By graphically plotting residuals of this linear model as a function of the covariate values for each subject, a wealth of information on that covariate and its relationship to the data surfaced. The graphical method allows one to determine the effects of each variable to the results of a either a linear or compartmental model (Davidian and Gallant 1992). This is analogous to multiple linear regression analysis of data to compare model fits. The graphical technique, if used properly, can also reveal non-linear relationships of covariates that ANOVA methods cannot detect. The residuals’ plot also helps explain the relationship of these covariates. When no relationship or bias to the model is present from the covariate, the residuals are evenly spaced above and below the estimated value for the model. However, if covariates skew the residuals in non-random patterns, then this covariate should be considered in the model design. This technique is often used with linear regression model evaluations. This highly useful tool provides some insight into variables of importance and can also suggest the type of relationship (linear, exponential, polynomial, etc.) that the covariate may have to the model.

In Figure 3, the ANOVA model for predicting tetracycline concentrations in swine was used as a predictor of data for Dorr et al (2009). Based on the plotted residuals, it is clear that there is some model misspecification that over-predicts most of the time points. This supported the need for a population model because the assumed linear model was inadequate to predict across the 2 populations.

Another method of selecting covariates is to use step-wise additions of suspected covariates to a population compartmental analysis (Gobburu and Lawrence 2002). Most
modeling programs will show plot graphical relationships between PK parameters and the covariate being tested across the populations, so differences can be detected. This is another method for selecting covariates without performing statistical assessments prior to model inclusion (Phoenix WinNonMix 6.0, Pharsight Co., Mountain View, CA).

Once the basic model structure is selected, many programs include a list of different modeling algorithms. The algorithm selection allows one to compare the model results using various model algorithms to determine the best algorithm to use. However, there are also various published comparisons among algorithms and types of modeling methods to use. Different algorithms include differences in the assumptions on normality, independent of samples, and use of previous data in determining PK parameters. A few algorithms are implemented in most programs, including first order, first order conditional estimation and extended least squares (or other algorithm that is semi-parametric). With population studies, repeated measures from some individuals are present and therefore, intra-individual variability can be easily calculated with an algorithm that handles repeated measures.

The first order (FO) sum of squares is commonly used and typically assumes independence of samples with a normal (Gaussian) distribution of error terms (Tanigawara et al. 1994; Jonsson et al. 2000; Bustad et al. 2006). It may stray more for data samples with intensive sampling from fewer individuals than other modeling algorithms. The First Order Conditional Estimate (FOCE) model which includes Bayesian forecasting, does assume normality, but with a Lindstrom-Bates algorithm, it includes a correction for repeated measures (Lindstrom and Bates 1990; Yuh et al. 1994). Bayesian forecasting allows the use of previous data points and parameter estimates to help determine the next estimate for the
model when used for each individual. This method can be more accurate for sparse population sampling where the individual parameters would normally contain greater error than the typical FO or least squares techniques (Tanigawara et al. 1994; Jonsson et al. 2000). Finally, the FOCE-Extended Least Squares or semi-parametric approach does not require normality and uses Bayesian forecasting as does FOCE to determine the parameters (Bustad et al. 2006). This method is best used to determine bimodal or populations with non-normal distributions of one or more parameters. However, when this type of algorithm is used for populations with normal parameter distributions, parameter skewing is more common (Bustad et al. 2006).

Differences in algorithms can only be appreciated by running a model with known parameters and comparing algorithm outputs directly. In general, for normal or fairly normal populations, FOCE –LB and FO work well (Gobburu and Lawrence 2002; Bustad et al. 2006). However, if there is a bimodal or non-normal population parameter, algorithms that do not assume normality and non-parametric techniques are better at fitting the data (Bustad et al. 2006). The algorithm may be chosen based on expected outcome or on model simulations. Evaluating the differences between models with covariate or PK structural differences can be very difficult. Some of the means to do this are discussed below.

VALIDATION AND COMPARISONS

Determining the validity of a model is quite subjective. Unfortunately when a model is developed, there is no definitive way to know that a model is valid or useful; and models can only be disproven, never proven true. However to determine a model’s validity, it may
be good to ask whether the model needs to be useful within only a limited context, such as in
your laboratory among technicians, or whether validity is required in other contexts. This is
a commonality in laboratory testing, where most sample ranges are laboratory specific.

This same concept can be applied to models. A model can be evaluated on several
fronts. Firstly, a model that explains a process may not actually need to be validated. This
would be a conceptual model that cannot be quantified. However, with pharmacokinetics,
models need to often be applied to other similar situations. Applications of PK models to
other situations is of greater importance than conceptual models because applications
determine if the techniques used or methods employed are useful beyond the conditions of
initial development.

A model’s applicability beyond its development conditions is often referred to as its
overall validity. There are two types of validity: internal and external. If experiments are
designed and performed correctly, a model should have internal validity. Method validation
is especially important for PBPK and population PK analyses because the method ensures
internal validity (Bressolle et al. 1996; FDA 1999). However, external validity must be
determined from a separate validation process. Validation of models has been discussed via
FDA in its Guidance for Industry (2003). External validation is best done via at least two
data sets. The first set is needed to develop the model while the second set is used to validate
it. The validation set data are entered into the model and the model’s predictions are
compared to the response data. Model validation suggests that a model can predict data
outside of its sample population but within some constraints such as population type or
dosing range (FDA 1999; Wahlby et al. 2004; Buur et al. 2005; Buur et al. 2006).
There are several model selection criteria described in the literature, but only three will be mentioned here. Typically modeling programs provide at least one type of selection criterion with model runs. These criteria and the graphical results of nested models with progressive additions of covariates, including different relationships of the covariates, are a helpful way to develop a model. In developing population PK models, using two or more data sets collected from different studies provides for a more robust model design. However, this approach may omit certain covariate data for some inter-study comparisons, which may increase the total model score (show a decreased fit). Some of the typical model comparison tests for non-linear models are the loglikelihood ratio, Akaike’s Information Criteria and Bayesian Information Criteria. There are many other methods that are employed, but these criteria are traditional recognized by many programs and are readily calculated.

The log likelihood ratio (LLR) is a test statistic that uses a value assigned to a model based on a chi square distribution. This distribution assumes statistically independent variables with a Gaussian distribution. If this assumption of independent variables or covariates is violated then the LLR may be invalid. This ratio is based on the comparison of two or more “nested” or similar models. When models are “nested”, they contain the same basic structure and often additional variables are added one at a time to the model. When comparing the ratio of two LLR with different degrees of freedom (different numbers of parameters), the ratio is accessed based on the Type I error rate (alpha), which is set by the modeler. If the test statistic is significant, then the new model is better than the previous model. If the LLR is not significant then the model with fewer parameters is a better fit even thought the likelihood value is smaller. Whenever more parameters are added to any model,
the fit of the model based on a sum of squares estimate will increase because the random error term of the model will decrease. This test provides a way to statistically compare models based on a p-value statistic. However, this statistic often exhibits a bias towards a simpler model and is considered biased when used with sparse sampling techniques (Ludden et al. 1994).

Akaike’s information criterion (AIC) is one of the most popular and often used methods for both linear and non-linear model comparisons. The AIC is an algorithm that can also be used to compare similar models, but the models do not necessarily have to be nested for comparison. However, the use of AIC across datasets should be approached with care as this value tends to be affected by the sample size (Burnham and Anderson 2004). The AIC values can be directly compared, but the total value is affected by the data as well as the model itself which and therefore, changing only one at a time is the best practice for comparison. The lower the AIC value, the better the model fit. The following equation represents the AIC calculation:

\[ AIC = 2k - 2 \ln (L), \]  

(5)

Where, \( k \) is the number of parameters and \( L \) is the maximized value of the likelihood function (or sum of squares) for the model in question (Ludden et al. 1994). This formula is based on the residual sum of squares for all observations (i.e. just the random error term variance). This value does not have to be compared to another model by a statistic like the LLR because it includes a small penalty, an increase in the AIC value, based on 2 times the number of parameters in the model. Therefore when AIC values are compared between two models, the modeler does not have to determine if over-fitting of the model is occurring but
can rather directly compare the AIC values. The AIC is more predictive with complex models and with sparse sampling methodologies than the LLR or F-tests. This criterion is better for selecting complex models compared to either the LLR or the Bayesian information criterion (Ludden et al. 1994; Vonesh et al. 1996; Burnham and Anderson 2004). There is also a corrected AIC value, which has a modified equation to prevent bias when sample size is small. With large sample sizes it converges to the original AIC value, but some scholars suggest it always be used in place of the original AIC value (Burnham and Anderson 2004).

Bayesian information criterion (BIC), also called the Schwartz criterion, is another method of model comparison that has gained favor in the last decade as well. It is based on a similar equation as the AIC, residual sum of square (RSS) error with a penalty for over-specifying the model. The main difference between the BIC and the AIC, besides the philosophy of their bases, is the size of the penalty for adding parameters. The following equation describes the BIC:

\[-2 \ln p(x|k) = -2 \ln L + k \ln (n); \quad (6)\]

where \( p(x|k) \) is the likelihood of the observed data \((x)\) given the number of parameters, \( k \); \( L \) is the likelihood function for the estimated model and \( n \) is the number of observations. The value of \(-2 \ln L\) is equal to \(\text{RSS}/(\text{variance of error terms})\) and is therefore dependent on the number of observations \((n)\). The statistics are more complicated with repeated dosing regimens, but the basic premise is that the model is based on decreasing the error term and avoiding an overfitted model. The BIC has been found to be less likely to correctly select more complex models compared to the AIC (Ludden et al. 1994; Burnham and Anderson 2004). However, overall, the BIC is good at selecting the correct model in
sparse sampling situations (Markon and Krueger 2004; Link and Barker 2006). With the
BIC, a smaller model number is a better fit, as with the AIC and LLR. It is likely that
evaluating a model in light of these three criteria will give a better estimate than just using
one alone. By using two or more, some of the biases will be removed and the models can be
viewed in light of the limitations of each method.

Once a group of models is selected, one or more of the above criteria should then be
used to assess an independently collected data set when run on the models. If one of the
models represents a “good fit”, then that model may be valid for multiple datasets. The
model should then be applied to at least another population or dataset to determine its
validity. With a robust enough model design and analysis, a model that appears valid for
more than one dataset would appear to be potentially externally validated and should be
tested across various populations.

However, one reported drawback for population modeling is the accuracy to
individual patients (Vinks 2002). Using some traditional modeling techniques and known
covariates, a tighter range for the individual may be conceivable; however, the goal of
population pharmacokinetic modeling is not to model one person but to provide a way to
determine what doses of a drug should be applied to a group of individuals with known
disease (or other covariates), or conversely, to predict likely plasma levels of a drug when a
person within a covariate group is in need of a specific treatment. The population model will
determine a regimen that will work for a population of individuals; however, there are still 5
to 10 people per 100 that may not fit that population model. This lack of individuality is the
reported drawback to population modeling. However, if the model is well designed and
enough information is known about a subject, population PK models should provide a range into which most subjects will fall. In many respects this is better for most subjects as an individual PK model cannot be made for individuals during a treatment period due to the intensive sampling. This concept is beneficial in a clinical setting where ill patients cannot be monitored as closely as experimental subjects. Using population modeling techniques can also be applied as described above for water medication dosing because the average or common levels of a drug within the herd are more important than any individual animal’s plasma concentration of that drug (FDA 1999; Wahlby et al. 2004).

VETERINARY MEDICAL APPLICATIONS OF THIS RESEARCH

The application of models in pharmacokinetic analysis is important for explanations and predictions of therapeutic drug concentrations in veterinary patients. Previous studies have applied population pharmacokinetic modeling to veterinary medicine and specifically to food animal medicine (Martin-Jimenez and Riviere 2001; Martin-Jimenez et al. 2002; Martin-Jimenez and Riviere 2002). Traditional modeling has been used extensively in veterinary medical research. PBPK models have used less frequently in veterinary medicine; however, this technique has been found useful in predicting population based drug elimination and withdrawal times in pigs (Buur et al. 2005). Finally, population based pharmacokinetics began being used in veterinary medicine in the 1990s and has been used by others (del Castillo et al. 2006) to better characterize observed concentrations in animal populations. Therefore these kinetic techniques have already been described and applied in veterinary medicine to oral and intravenous medications.
The previous comparisons of traditional, PBPK and population PK models illustrate that models can vary greatly and yet they can all be applied to veterinary medicine. Models are only as useful as their ability to complete the tasks for which they are employed. Any of the three model types can be used, but one model type may be better suited for one task over another. The usefulness of these models lies in their application to better explain real life circumstances. PBPK modeling used in Chapter 2 helps to better model a contamination situation which cannot be adequately addressed by traditional modeling. In Chapter 3, statistical moment theory is used to illuminate the pharmacokinetic parameters of tetracycline water medication, when an unknown dose is drunk by animals. However, these kinetics may only be dubiously applied to a population with various doses and situations. Therefore, Chapter 5 describes a non-linear mixed effects model that was developed to handle an unknown dose, with known exposure levels to three populations of animals. From this population based modeling technique, the role of covariates, both internal and external to the pig, are used to predict plasma levels that might be seen in swine operations. From this model, a 90% confidence interval for a population of animals with similar characteristics can be discerned. Finally Chapter 4 discusses the role of tetracycline water medication from the perspective of stability and it biological activity to round out the usefulness of this medication in industry settings.

These various PK techniques can add to the body of knowledge available on how to creatively approach problems that have been avoided due to their complexity and difficulty. These techniques may help with residue avoidance in populations of food animals (Martin-Jimenez and Riviere 1998; FDA 1999), toxicology dosing dilemmas in back-calculating...
potential exposure (el-Masri et al. 1995; Jonsson and Johanson 2003; Mason et al. 2008) and in other variable dosing situations such as in treating wildlife, pet birds and rabbits. The applications of these methods to veterinary medicine in general and to herd medicine specifically are the focus of this work.
Table 1: Generalized Characteristics of the Three Model Types

<table>
<thead>
<tr>
<th>Model Type</th>
<th>Error</th>
<th>Approach</th>
<th>Direct Application</th>
<th>Sampling</th>
<th>Extended Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compartment</td>
<td>Random</td>
<td>Curve fitting</td>
<td>Individual</td>
<td>Few subjects, many time points</td>
<td>Averaging of PK parameters is used to apply to populations</td>
</tr>
<tr>
<td>PBPK</td>
<td>Random</td>
<td>Mechanistic</td>
<td>Individual</td>
<td>Many time points from few to many subjects</td>
<td>Monte Carlo, Bayesian and statistical techniques can be applied over model to extend it to a population</td>
</tr>
<tr>
<td>Population PK</td>
<td>Random</td>
<td>Curve Fitting</td>
<td>Population</td>
<td>Few subjects with many time points to many subjects with few time points</td>
<td>Difficult to apply to individuals. May apply to covariate specific populations</td>
</tr>
<tr>
<td></td>
<td>Intraindividual</td>
<td>Statistical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interindividual</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: The top panel shows a more intensive sampling regimen of the plasma compartment than the lower panel. The compartmental PK analysis from the sparse sampling scheme will not detect the distribution phase from 0 to 5 hours in this example.
Figure 2: Data transformation of pharmacokinetic profiles by logarithmic transformation of the y-axis is frequently performed prior to compartmental PK analysis. The top panel exhibits untransformed data, while the lower panel shows the transformed data.
Figure 3: Plot of residuals versus time for a model applied to validation data. The residuals from this model are systematically too low for the data, suggesting that the model is overpredicting values, and is likely misspecified. As with many pharmacokinetic models, the early time points, where concentrations are higher, tend to exhibit greater variation than later time points. This bias can be corrected by weighting the terminal time points.
References


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Gupta, S., C. Banfield, B. Kantesaria, M. Marino, R. Clement, M. Affrime and V. Batra (2001). "Pharmacokinetic and safety profile of desloratadine and fexofenadine when


of varying doses of intravenous metronidazole."


Sulfamethazine Water Medication Pharmacokinetics and Contamination in a Commercial Pig Production Unit

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Sulfamethazine Water Medication Pharmacokinetics and Contamination in a Commercial Pig Production Unit

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ABSTRACT

Sulfamethazine is often used to treat disease in the swine industry. Sulfamethazine is available as a water or feed medication and historically (over the past 40 years) has been associated with residue violations in both the United States and Europe. Despite sulfamethazine's approval for use as a water medication, little research on the pharmacokinetics of the water formulation is available. Therefore, a pilot study was performed to determine the plasma levels of an approved sulfamethazine water medication. Plasma levels in pigs treated with an oral bolus (25 mg/kg), which is equivalent to the total drug consumed within a 24-h period, achieved therapeutic concentrations (50 μg/ml). Nontoxicokinetic-based pharmacokinetic model parameters for clearance, half-life, and volume of distribution were consistent with previously published values in swine. However, the above treatment resulted in exposure of pen mates in sulfamethazine at levels currently above tolerance (114 ppm). Using a physiologically based pharmacokinetic model, the treatment dose simulation was compared with observed plasma levels of treated pigs. Flexibility of the physiologically based pharmacokinetic model also allowed simulation of contaminating plasma levels to estimate contamination exposure. A simulated exposure of 0.15 mg/kg twice within approximately 8 h resulted in detectable levels of sulfamethazine in the control pigs. After initial exposure, a much lower dose of 0.059 mg/kg maintained the contamination levels above tolerance for at least 3 days. These results are of concern for producers and veterinarians, because in commercial farms, the entire barn is often treated, and environmental contamination could result in residues of an unknown duration.

Over the past decade, the concern of antimicrobial resistance has increased the scrutiny on veterinary medications, routes of administration, and dosages administered to food animals (1–3, 19, 172). In 1996, as a direct result of the Animal Medicinal Drug Use Clarification Act of 1994 (29), the prescribing power of veterinarians increased dramatically. The Act also included stringent criteria concerning the administration of antibiotics to food-producing animals. Despite this step to increase veterinarians' abilities to combat animal disease, human health advocacy and regulatory groups have become more vigilant in monitoring antimicrobial use, emerging resistance, and its potential impact on public health. In the last decade, there have been five federal notices concerning antibiotic resistance that restricted the use of certain classes of antibiotics in food animals (3). In order to protect human health, withdrawal times must be provided for all medications prescribed to food animals. Withdrawal times are set by the U.S. Food and Drug Administration (FDA), based on the 95th confidence interval of the 99th percentile of the population, as described in their Guidance for Industry (2).

U.S. Department of Agriculture surveillance programs monitor carcasses for drug residues. Over the past several years, sulfamethazine use has resulted in multiple residue violations in swine. These violations may be due to an inaccurate withdrawal time (5), inaccruate medicating of animals, or from environmental contamination of the drug (7, 10–12). There is evidence that the current FDA withdrawal time of 15 days (set in 1982) is inadequate for an oral loading dose of (247 mg/kg) and daily dose of 125 mg/kg for 4 consecutive days (5, 14). In order to prevent violations, due to increased imported restrictions, many swine production units implement an 80 day withdrawal time for sulfamethazine water medication in treated barns (122).

With heightened scrutiny on food animal antimicrobial use and legislators encouraging a ban on all nontherapeutic use of antibiotics, the food production industry needs effective and easily administered medications. Since no extra-label medicated feeds can be prescribed (29), drugs are often administered by water. To our knowledge, there are no published reports on therapeutic concentrations of sulfamethazine water medications and their potential effect on the animal environment.

This study used both traditional nontoxicokinetic (PK) modeling analysis and a previously validated physiologic-based pharmacokinetic (PBPK) model (13, 6) to validate this oral gavage study to predict exposure in pen mates. PBPK has been used most in toxicology to explain dosing ranges in humans. Compared with
traditional PK modeling, PBPK modeling uses parameters based on animal physiology to model drug deposition. Some parameters used are organ perfusion, metabolic pathways, and excretion mechanisms. These parameters allow a validated model to use sparse time points, from which a traditional model would not be able to predict. Bau et al. (S6) have shown that PBPK modeling can predict withdrawal times for drugs. The U.S. Food Animal Residue Avoidance Databank project has used PBPK models to calculate withdrawal intervals for tetracycline in sheep and sulfamethazine in swine. Therefore, we extended this model to predict oral exposure in nursery age pigs and pen mate exposure to sulfamethazine.

Because sick pigs do not drink as much as healthy pigs (9, 10), this pilot study modeled the pharmacokinetic PK parameters when healthy pigs were orally gavaged with a currently approved sulfamethazine product (Sulfamethizole, Fort Dodge Animal Health, Madison, N.J.). This provides maximum plasma concentration levels that can be attained by oral sulfamethazine. Furthermore, we compared traditional PK modeling with a PBPK model to mimic the treated-pig levels. The purpose of this study was to validate our PBPK model for oral data, by comparing the data obtained from a known dose of sulfamethazine in an all-in-all-out production system to previously obtained research data (5). Furthermore, we used this information to model the contamination seen in the control pigs housed with the treated pigs, an unexpected finding, and to make predictions on the magnitude of contamination that was seen.

**MATERIALS AND METHODS**

High-performance liquid chromatography methods. Sample preparation and processing were based on the method published by Bau et al. (S5, 6). Nine standard solutions containing sulfamethazine (Spectrum Chemical Manufacturing Company, New Brunswick, N.J.) were prepared (0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, and 20 ppm or μg/mL from a 1,000-ppm stock solution) in both double deionized water (MilliQ) and plasma, using no more than 5% (volume) stock. Plasma and water standards produced calibration curves consistent and accurate, with variability less than 2% and placenta recovery of 93 to 98%. Plasma samples were filtered with a 3-cm VICI solid-phase extraction column (Waters, Milford, Mass.) before injection (5).

All samples were run on an Atlantis C18 column (C18 column, 4.6 mm 250 mm, 5 μM; Waters) in 77% ammonium acetate buffer, pH adjusted to 4.3, and 25% acetonitrile on a liquid chromatograph (Waters 2696 with autosampler) at 1-mm flow rate. Detection and quantification were performed on a Waters 990 photoelectric array detector at 266 nm. Prior to analysis, samples were held at 24°C before each run while the column was heated to 30°C for 6.5 min run times, with peak elution occurring at 5.1 min. Peaks were symmetrical, sharp, and crisp. Limit of quantification was at the current FDA tolerance level of 0.1 ng/mL and limit of detection was set at 0.05 ng/mL.

Animals. Pigs at 4 to 5 weeks of age were housed at the North Carolina State University Teaching Animal Unit (TAU) swine facility nursery on in-floor floors. The room is removed via a wash system that runs under the pens every hour. The TAU acts as a commercial all-in-all-out one-site production facility. University Institutional Animal Care and Use Committee approval was obtained for the animal study. Fourteen pigs between 11.5 and 15 kg from the TAU nursery were chosen in a complete randomized block design (see Table 1). At 21 days of age, pigs were weaned and moved to the nursery. They were allowed 12 days of acclimation to the facility, during which they were fed a medicated starter ration containing penicillin for 4 days, and were provided a 20% protein, nonmedicated diet for the remainder of the study. On the first day of the study, animals were weighed, had initial blood samples obtained, and were ear tagged for identification. Six animals were used as controls. Of the 14 pigs, 8 were chosen at random of both male and female sex for the gavage treatment. A metal speculum was used as a gag and a polyethylene Foley catheter was inserted into the meatus of each pig. Sulfamethazine (Sulfamethizole, Fort Dodge Animal Health) was given orally at approximately 217.5 mg/kg (actual amount as dosed was 200 mg/kg), as per the labeled initial-day water medication dose. Due to the volume that had to be administered, the medication could not be diluted. No gastrointestinal or other side effects were observed from the treatment. Approximately 5 mL of whole blood was drawn from each animal at 0, 2, 4, 6, 8, 12, 24, 48, and 72 h postgavage. Blood was centrifuged at 3,000 rpm for 15 min, and the plasma was spinepar and frozen at −80°C until samples were processed, within 6 weeks of the study being completed.

**PK analysis.** Conventional PK analysis was performed using WinNonlin (Pharsight, Mountain View, Calif.), which included a noncompartmental analysis on data from each pig. The terminal slope from plotting the plasma concentrations over time was used to calculate half-life. As, clearance, volume of distribution (V) and area under the curve (AUC) for each of the treated pigs. The above parameters except for V and AUC were calculated for the control pigs as well. We compared these values with the literature to validate the study. A previously validated PBPK model (5, 6) allowed analysis of both sets of pig data using Analyst software (AB Sciex Technologies, Huntsville, Ala.). The model was optimized using physiologic parameters from previously published data (14, 15). Treatment-pig data were modeled and compared to this optimization. In order to analyze the control-pig data, a dosing portal was written to include an adjustable lag time and flexible dosing schedule (in both time and dose). This optimized model, including the new dosing portal with lag time, was used to determine the control-pig exposure. These data were compiled with averages and standard deviations using Excel (Microsoft Corp., Redmond, Wash.).

**RESULTS**

All treated pig plasma sulfamethazine concentrations rose during the first 2 to 12 h after oral gavage, and then began to drop from peak levels between 400 and 700 μg/mL (ppm) (Fig. 1A). Within 24 h of the gavage dose, sulfamethazine was detectable in the plasma of untreated pigs.

<table>
<thead>
<tr>
<th>TABLE 1.</th>
<th>Spinal layout of animal pens</th>
<th>Pen A</th>
<th>Pen B</th>
<th>Pen C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>T or C</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
</tr>
<tr>
<td>Animal</td>
<td>T or C</td>
<td>A4</td>
<td>A5</td>
<td>A6</td>
</tr>
<tr>
<td>Animal</td>
<td>T or C</td>
<td>C11</td>
<td>C12</td>
<td>C13</td>
</tr>
<tr>
<td>Animal</td>
<td>T or C</td>
<td>C14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal</td>
<td>T or C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* T: treatment pigs; C: control.
and varied between 0.20 to 0.71 \( \mu \text{g/ml} \). The average apparent maximal concentration \( C_{\text{max}} \) in untreated control pigs was 0.573 \( \mu \text{g/ml} \) (Fig 1B). Note that plasma levels were above tolerance in untreated pigs by 8 h after gavage of treatment pigs.

Noncompartmental analysis using WinNonlin of the eight treated pigs and six control pigs led to calculation of conventional PK parameters (Tables 2 and 3). Average half-life for the treated pigs was 11.4 h, with an apparent average time to maximal concentration of 5.75 h. Because no intravenous data are available, bioavailability could not be measured and therefore, PK parameters are confounded by unknown bioavailability.

In addition to performing a traditional PK analysis, we also used a previously validated PBPK model (5, 60) in an attempt to improve modeling of observed data. Using the original PBPK system, we optimized the model using published data, extending past 72 h (10). Using the optimized model at a dosage of 250 mg/kg, the simulation predicted actual treated pig plasma concentrations (Fig 2A). A regression line of observed data versus predicted data yields a correlation coefficient of 0.71 (Fig 2B), and the residuals suggest no systematic bias in the results (Fig 2C).

We modeled control pig exposure and determined an estimated oral exposure dose and consistent time interval of exposure. The data in Figure 3A is a PBPK model simulation, which is a more accurate representation of continual oral drug exposure than is the model in Figure 1B. Figure 3 shows the simulation model compared with the control pig data. The residual graph indicates constant variance. From PBPK simulation of the control pig data, it appears that pigs were exposed to the equivalent of two initial doses of 0.15 mg/kg at hours 4 and 8 on day 1. Every 4 h subsequently, the pigs had only to consume approximately 0.089 mg/kg sulfamethazine to maintain plasma levels above tolerance. The model suggests this approximate exposure level until 56 h after the initial treatment.

**DISCUSSION**

Traditional PK values obtained from this animal study are consistent with PK data previously reported in the literature for administration of sulfamethazine (13–15, 21). Available papers typically report plasma concentrations of greater than 50 \( \mu \text{g/ml} \) for conventional therapeutic dosages (13–15, 21). The treatment pig data verify that therapeutic concentrations of sulfamethazine were achieved through water medication. This study did not investigate the ability of sulfamethazine as dosed in drinking water to achieve therapeutic concentrations. Instead, a total daily dose was given in one bolus in order to determine the highest plasma concentrations of oral sulfamethazine and to obtain relevant PK parameters. Appropriate dosing of water medications to achieve therapeutic concentrations is a subject of future research.

All pigs, except for pig 12 (treatment group), had initial sulfamethazine levels below the detection limit. Although pig 12 had 0.09 \( \mu \text{g/ml} \) sulfamethazine at the start of the study, this value is below the tolerance limit. This does bring into question if the pigs were previously exposed to sulfamethazine. Exposure could have occurred in the farrowing barn through milk or creep feed; however, the 12-day washout period should have been adequate to remove any sulfamethazine contamination, as the pigs were not intentionally exposed to sulfamethazine. Studies have reported plasma samples containing trace amounts of sulfamethazine in pigs that were not previously treated (12). Interspecies variability in metabolism is another possible cause of the trace amounts of sulfamethazine present (12). There may also be unrecognized age differences in pig acetylation metabolism (38). Finally, there was a wide range of \( C_{\text{max}} \) values (413 to 677 \( \mu \text{g/ml} \)), which were not associated with sex, age, or size. Acetylation differences (although never documented in pigs) could explain the variability of peak plasma levels in treated pigs. Due to the large dose of sulfamethazine given to pig 12 (a treatment pig), this trace level would not significantly alter the peak plasma levels.

Environmental contamination was shown to be a significant concern in this study. Control pigs showed plasma sulfamethazine levels above tolerance by 8 h after gavage treatment of pen mates. Thus, it is possible to contaminate
TABLE 2. Pharmacokinetic parameter estimates for treatment pigs<sup>a</sup>

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>$\lambda_t$ (days)</th>
<th>Oral half-life (h)</th>
<th>$C_{max}$ (ng/ml)</th>
<th>$T_{max}$ (hr)</th>
<th>Clearance oral/kg/day&lt;sup&gt;b&lt;/sup&gt;</th>
<th>V oral/kg&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MCL range (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0583</td>
<td>12.651</td>
<td>641</td>
<td>2</td>
<td>18.562</td>
<td>0.34</td>
<td>11.57</td>
</tr>
<tr>
<td>2</td>
<td>0.0514</td>
<td>11.388</td>
<td>471</td>
<td>4</td>
<td>15.472</td>
<td>0.22</td>
<td>13.18</td>
</tr>
<tr>
<td>3</td>
<td>0.0563</td>
<td>10.907</td>
<td>618</td>
<td>6</td>
<td>10.753</td>
<td>0.69</td>
<td>13.01</td>
</tr>
<tr>
<td>4</td>
<td>0.0637</td>
<td>10.183</td>
<td>677</td>
<td>4</td>
<td>9.738</td>
<td>0.82</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>0.0562</td>
<td>12.386</td>
<td>938</td>
<td>6</td>
<td>8.715</td>
<td>0.33</td>
<td>10.07</td>
</tr>
<tr>
<td>6</td>
<td>0.0663</td>
<td>7.187</td>
<td>296</td>
<td>8</td>
<td>8.593</td>
<td>0.33</td>
<td>9.04</td>
</tr>
<tr>
<td>7</td>
<td>0.0776</td>
<td>10.909</td>
<td>368</td>
<td>12</td>
<td>12.396</td>
<td>0.19</td>
<td>15.17</td>
</tr>
<tr>
<td>Avg</td>
<td>0.0643</td>
<td>11.382</td>
<td>552</td>
<td>5.33</td>
<td>20.407</td>
<td>0.29</td>
<td>10.88</td>
</tr>
<tr>
<td>SD</td>
<td>0.0165</td>
<td>2.839</td>
<td>100</td>
<td>3.11</td>
<td>8.74</td>
<td>0.08</td>
<td>3.52</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pharmacokinetic parameters for treated pigs given a 250-mg/kg dose of sulfamethazine by oral gavage. All parameters were calculated in WinNonlin (Pharsight) and reflect predictions until the plasma levels are no longer detectable. $\lambda_t$, elimination constant (also known as $k_{e}$); half-life, the time it takes clear one-half of the concentration of the drug from the body; $C_{max}$, the maximum plasma concentration; $T_{max}$, the observed time of peak plasma concentration; $V$, the volume of distribution and can be related to the level of sulfamethazine within body compartments; clearance, total body clearance; MCL, at a level under the curve.

<sup>b</sup> Both clearance and $V$ have been corrected for bioavailability when the mono- or compartmental values were calculated.

The pig enclosure and achieve plasma levels of antibiotic in non-treated animals that are of regulatory concern. To study the effects of this contamination, a PBPK model was adopted and used to estimate possible oral exposure scenarios. The model simulation (Fig. 3A), developed to represent the control pigs, uses physiology consistent with pig behavior: Pigs between 1 and 2 weeks of age suckle every hour (13). Their frequency of drinking decreases gradually from weaning at 21 days until it mirrors adult drinking patterns. Adults drink approximately 75% of their water between 800 a.m. and 800 p.m. Based on the age of these pigs, the model is consistent with pig behavior (13).

Based on the PBPK simulation, we hypothesize that control pigs were being exposed from the elimination waste of the treated pigs until approximately 56 h after initial exposure of the treated pigs (Fig. 3A). This is of concern because kidney and muscle levels of the antibiotic are at least one-third of the peak plasma levels. Therefore, kidney and possibly muscle could be contaminated by sulfamethazine. After approximately 56 h, the amount of excretion was low enough that it could be eliminated as fast as it entered the body. Therefore, traditional PK parameters for control pigs, including clearance and half-life, are not accurate for sulfamethazine because of flip-flop kinetics, where kinetics absorption of the drug influences the plasma concentration. Our model is based on intermittent exposure from eating or drinking, although pigs may be continuously exposed to low levels of sulfamethazine from urine and/or feces (7, 10-12).

We do not know how much contamination is now occurring in large production systems from water medications. However, the apparent ease of transfer of antibiotic from treated to untreated pigs can become an issue. The method of housing has been drawn into question with other studies (7, 10, 11). McCauley (10-12) demonstrated pen, food, and water contamination of pig housing at production and slaughter facilities when floors were solid or bedding was not changed daily. However, our observations are significant because there are no published reports in the literature that have reported detectable levels of contamin-

TABLE 3. Selected pharmacokinetic parameters for control pigs<sup>c</sup>

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>$\lambda_t$ (days)</th>
<th>Oral half-life (h)</th>
<th>$C_{max}$ (ng/ml)</th>
<th>$T_{max}$ (hr)</th>
<th>Clearance oral/kg/day&lt;sup&gt;d&lt;/sup&gt;</th>
<th>V oral/kg&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0172</td>
<td>40.216</td>
<td>0.273</td>
<td>7</td>
<td>7.792</td>
<td>0.49</td>
</tr>
<tr>
<td>2</td>
<td>0.0213</td>
<td>29.6853</td>
<td>0.702</td>
<td>27</td>
<td>5.782</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>0.0157</td>
<td>44.6094</td>
<td>0.606</td>
<td>48</td>
<td>3.982</td>
<td>0.49</td>
</tr>
<tr>
<td>4</td>
<td>0.0175</td>
<td>39.5412</td>
<td>0.657</td>
<td>48</td>
<td>4.192</td>
<td>0.28</td>
</tr>
<tr>
<td>5</td>
<td>0.0086</td>
<td>80.9163</td>
<td>0.5448</td>
<td>8</td>
<td>3.972</td>
<td>0.28</td>
</tr>
<tr>
<td>6</td>
<td>0.0293</td>
<td>47.4441</td>
<td>0.448</td>
<td>48</td>
<td>3.982</td>
<td>0.28</td>
</tr>
<tr>
<td>7</td>
<td>0.0237</td>
<td>46.972</td>
<td>0.573</td>
<td>32.33</td>
<td>6.282</td>
<td>0.28</td>
</tr>
<tr>
<td>Control</td>
<td>0.0167</td>
<td>17.608</td>
<td>0.103</td>
<td>17.1</td>
<td>1.211</td>
<td>0.28</td>
</tr>
</tbody>
</table>

<sup>c</sup>The pharmacokinetic parameters for control pigs exposed to environmental contamination of a 250-mg/kg dose of sulfamethazine by oral gavage. All parameters were calculated in WinNonlin (Pharsight) and reflect predictions until the plasma levels are no longer detectable. $\lambda_t$, elimination constant (also known as $k_{e}$); half-life, the time it takes clear one-half of the concentration of the drug from the body; $C_{max}$, the maximum plasma concentration; $T_{max}$, the observed time of peak plasma concentration; clearance, total body clearance.

<sup>d</sup>Clearance was corrected for bioavailability by WinNonlin.

<sup>e</sup> One data point was removed because of inconsistency with the rest of the data and inability to verify that it had not been mislabeled.
FIGURE 2. (A) Simulation data shows the physiology-based pharmacokinetic model (line) is a good representation of the individual points, which are treatment pig plasma levels. (B) Regression line is a good fit given that this model is optimized to intravenous instead of oral data. (C) Residuals show that the model predicts better at later time points than at earlier time points.

Residuals for veterinary medicine as well as industry, sulfamethazine residue violations have continued to occur.

From this study, we recommend that procedures be put in place to protect unvaccinated animals from contamination exposure. Techniques need to be developed to ensure contamination is removed from the environment. Plasma levels of approximately 1 μg/mL from contamination are significant when compared to feed-additive studies, with plasma sulfamethazine levels of 9.8 to 16 μg/mL. The PBPK model suggests that 0.039 mg/kg is capable of causing plasma drug levels to exceed tolerance. These blood levels are consistent with an 11-kg pig receiving less than 4 mg of sulfamethazine orally per day. With such low sulfamethazine levels contaminating pig carcasses, residue violations may continue to occur despite producer and veterinary compliance. Animal housing and husbandry throughout the entire production process remains important. Better approaches to model antibiotic use in populations of animals are needed in addition to research on the efficacy of water medications.

ACKNOWLEDGMENTS

The authors thank Patty Benack for assistance with pig handling, study logistics, and supplies. Brett Ruelow was key in troubleshooting the high-performance liquid chromatography techniques. This research was made possible by the North Carolina State University College of Veterinary Medicine Animal Health Formulation Fund.
Pharmacology of tetracycline water medication in swine

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Pharmacology of tetracycline water medication in swine¹

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ABSTRACT: Medicating drinking water with tetracycline is commonly used in swine production systems to treat and prevent disease outbreaks. However, little information is known of the pharmacokinetics of this medication in water formulations. Twenty-four barrows, divided into 1 control group (of untreated animals) and 3 equally sized treatments groups (n = 6/group), were treated with tetracycline water medication for 5 d at 125, 250, and 500 mg/L. Blood samples were collected at 0 (prestudy), 1, 4, 8, 12, 21, 32, 48, 56, 72, 86, 96, and 164 h after exposure. Data analysis consisted of noncompartmental pharmacokinetic analysis and statistical analysis of steady state concentrations with repeated measures ANOVA and multiple-comparison testing to determine whether plasma concentrations differed among groups. Derived pharmacokinetic parameters were consistent with previously published feed and intravenous data. Plasma tetracycline concentrations at steady state were 0.033, 0.47, and 0.77 µg/mL for 0-, 125-, 250-, and 500-mg/L, respectively. Treatment group steady-state plasma concentrations were significantly different from plasma concentrations in control animals (P < 0.0001); however, whereas the 125- and 250-mg/L groups were significantly different from the 500-mg/L group (P < 0.0001), their mean plasma tetracycline concentrations did not differ from one another. Furthermore, the study showed that tetracycline oral bioavailability is very small. The dose response curve also shows that concentrations of plasma tetracycline increase linearly, yet not in a 1 to 1 ratio, to the direct increase in water medication dose.

Key words: pharmacokinetics, plasma concentration, swine, tetracycline, water medication

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INTRODUCTION

In commercial swine production, antimicrobial medication often is used after weaning, transport, or during disease outbreaks. Concern has grown over the last decade between increased antimicrobial resistance in human pathogens and food animal antimicrobial use (FDA, 2003). In Europe, this policy led to the ban of feed additives (EU, 2003) and a concurrent increase in the use of water medications (personal observation in European countries). Currently, there is only limited published pharmacokinetic (PK) data on water medication formulations, including amoxicillin (Agerson et al., 1998), sulfamethazine (Mason et al., 2008), and tetracycline (Nieborn and Gyrd-Hansen, 1996).

Although a ban on feed additives is not in effect in the United States, PK studies to evaluate antibiotic treatment and its effects should be conducted before adopting blanket policies prohibiting drug use. Pharmacokinetic studies would characterize therapeutic potential and PK parameters unique to water antimicrobials. In addition to traditional PK parameters, PK-pharmacodynamic studies can potentially relate the impact of antimicrobial use on the food supply and public health (CDC, 2006).

Tetracycline, an antibiotic labeled for the control and treatment of salmonellosis, some enteric bacteria, and...
susceptible respiratory diseases in pigs, was selected as a test compound because of its common use (Aar
estrup, 2005) and lack of PK data from observation-
al studies (Luthman et al., 1989; Pipers et al., 1989;
Reeve-Johnson, 1998). An experiment was designed to
measure tetracycline concentrations in swine plasma.
This study design allowed for group comparisons of 4
doses, including control, and to determine if the dose
response of tetracycline is linear. These studies also as-
essed whether tetracycline concentrations in plasma
consistently reached minimum inhibitory concentra-
tions of quality control (sensitive) bacteria (Isenberg,
2004; CLSI, 2007; Qaiyum, 2007).

MATERIALS AND METHODS

All animals used in this study were housed and
treated in accordance with the North Carolina State
University Internal Animal Care and Use Committee
standards.

Animals, Treatment, and Facility

Twenty-four Yorkshire-Landrace cross barrows were
housed at North Carolina State University’s Swine
Unit Facility in individual pens on concrete. Barrows,
approximately 8 wk of age and weighing 16 to 18 kg at
the initiation of the study, were assigned to 1 of 4 treat-
ment groups (n = 6/group). One group received 0.5
times the label dose (125 mg/L) of tetracycline hydro-
chloride; the second, a label dose (250 mg/L); the third,
2 times the label dose (500 mg/L); and the control
group received water without tetracycline. Treatments
of 125, 250, and 500 mg/L were achieved by dissolv-
ing a preweighed amount of an approved tetracycline
water medication (AmTech, IVX Animal Health Inc.,
Saint Joseph, MO) into 19.2 L of water in individual
barrows for each animal. All animals were given 5 d
to acclimate to the facility and adapt to the barrow
drinking system before the start of the trial. The Nal-
gene barrows (Thermo Fisher Scientific, Waltham, MA)
were suspended from the ceiling and attached to the
drain plumbing via 0.9 to 1.4 m of 1.27-cm plastic tub-
ing and plumbers fittings. Arato80 drinkers (Aratowerk
GmbH & Co., Kln, Germany) were mounted in the
individual pens to minimize waste and spillage of wa-
ter used. Water flow rates varied between 500 and 800
mL/min, which is consistent with flow rates needed to
prevent dehydration in adult pigs (Leibbrandt et al.,
2001). Daily maximum and minimum temperatures
within the barn were collected with a thermometer
and recorded daily.

Feed and Water Composition

Water was assessed the month before the trial by the
North Carolina Agronomy department to determine
water quality on the farm. A 17% protein antibiotic-
free feed, ground on site, was fed to all animals ad
libitum. Animals did not receive any antimicrobials or
growth promotants at least 10 d before the start of the
study. Carbadox, which did not interfere with HPLC,
was the only medication these pigs received in the nur-
sey. Water was changed and tetracycline was mixed
every other day (i.e., d 1, 3, and 5) except for 2 pigs.
One pig drank excessively, using most of 1 carbony every
day; therefore, the water was replaced daily. One other
barrow fell on d 2, and the water was replaced. Water
use by each pig was measured with a graduated cylin-
der (to within 10 mL) on 2 separate days, the day be-
fore the study began, and on d 2, which was consistent
with daily water use. Water samples, randomly chosen,
were collected daily except for on d 2 and 5 when all
carbos were collected.

Other Measurements

Free catch urine samples were attempted daily (AM
only). Urine specific gravities were determined using a
refractometer, and tetracycline concentrations were de-
termined. All animals were weighed at the start of the
acclimation period and on the final day of the study.

Plasma Sample Preparations
and Chemistries

Blood was collected from each pig via venipuncture
of the anterior vena cava into 10-mL sodium heparin
vials before administration of the tetracycline water
medication (time 0, 800 h) and then at 4, 8, 12, 24, 32,
48, 56, 72, 80, 96, and 104 h after dosing. Whole blood
(before centrifugation) was assayed for hematocrit with
microcapillary tubes and total protein (via specific
gravity) on d 1. Antech Diagnostic Labs (Ithaca, NY)
performed urine creatinine (daily as collected) and se-
rum creatinine on d 1 and 5 for all pigs. Plasma was
harvested from all blood samples after centrifugation
(1,110 × g, 15 min; 4°C) within 1 h of collection and
split into 2 samples, for processing that week and for
storage at -80°C.

HPLC Analysis

A Waters Alliance 2695 HPLC with vacuum pump
and autosampler (Milford, MA) was used for tetry-
cycline quantification. The assay was a modified version
of Cheng et al. (1997) and Santos et al. (1996). Two
hundred microliters of plasma were added to an equivalent
volume of releasing agent (78% water, 20% acetonitrile,
2% o-phosphoric acid) in a YM 10,000 Ultradev kit (Mil-
lipore Corporation, Milford, MA). The Ultradev appar-
atus was placed in an Eppendorf tube and centrifuged at
7,840 × g for 30 min at 22°C. Tetracycline was quanti-
ified using a Photodiode Array at 354.4-nm wavelength,
with peak integration by Empower software (Waters
Corporation). Samples were held away from light at
4°C for up to 7 d before a 4-min run on a Waters Atlantis
4.6- × 150-mm C18 column in 28% acetonitrile, 72%
0.05 M oxalic acid buffer at 37°C. Limit of quantification, the least concentration that can be reproducibly quantified, for plasma and distilled water was 0.1 μg/mL, and the limit of detection was consistently at or below 0.05 μg/mL. A linear curve for well water from the farm spiked with tetracycline from 80 to 800 ng/mL was adequate for our water samples analyzed by HPLC. All values were within the accepted CV of 20%, based on daily standard curves.

**Pharmacokinetic Analysis**

Pharmacokinetic analysis was performed on each group with WinNonLin (Pharsight, Mountain View, CA) using a noncompartmental analysis to calculate mean residence time (MRT), area under the moment curve (AUMC), area under the curve (AUC), and the AUC between 32 and 48 h (AUC_{32-48}). Due to animal use limitations and the availability of previously published data, an apparent oral bioavailability (F_{oral}) was estimated using experimental oral data and intravenous data from Kniffin et al. (1989), including the intravenous dose and AUC. The following equation was used to calculate F_{oral} for each animal:

\[
F_{oral} \times \frac{\text{Dose}_{oral}}{\text{AUC}_{oral}} = \frac{\text{Dose}_{iv}}{\text{AUC}_{iv}},
\]

where \( F_{oral} \) is the apparent oral bioavailability of the tetracycline, \( \text{Dose}_{oral} \) is the dose from the water (measured concentrations of the water medication multiplied by the amount of water consumed over 24 h) for the animal (Supplemental Table 1 in [http://jasassword.com/content/vol87/issue11/], AUC_{oral} is the AUC_{32-48} from WinNonLin for that animal, and AUC_{iv} and \( \text{Dose}_{iv} \) are the AUC and corresponding intravenous dose from the Kniffin et al. (1989) study. The calculated \( F_{oral} \) was then used to estimate clearance and volume of distribution at steady state for each group based on the following equations:

\[
Vd \times \frac{\text{Dose}_{oral}}{C_{max}} C_{max}^* = \frac{\text{Dose}_{oral}}{C_{max}} C_{max}^* = 24,
\]

where \( Vd \) is the volume of distribution at steady state, \( C_{max} \) is clearance for the group, \( F_{oral} \) is apparent bioavailability of the group, \( \text{Dose}_{oral} \) is the dose (water consumption multiplied by the concentration of water) over a 24-h period for the group, and \( C_{max} \) is the concentration of the group at steady state. For each group, the individual animal peak plasma concentration (C_{max}) is reported with its corresponding time (T_{max}).

**Statistical Analysis**

A randomized block design was employed to test the hypotheses that tetracycline concentrations in plasma differed among 4 treatment groups and if steady-state concentrations of any group reached 1 μg/mL. Daily BW gain and group BW pre- and post-study were calculated and compared via 1-way ANOVA. Daily maximum and minimum temperatures were used to calculate a mean maximum and minimum temperature during the study. Two-way repeated ANOVA was performed (SAS Inst., Inc., Cary, NC) using PROC MIXED with dose and time as independent variables for comparisons at steady state (from 32 to 194 h). Multiple comparison testing was performed with Scheffe to determine if treatment of tetracycline groups 125, 250, and 500 mg/L were different. The data were then grouped to determine if treatment groups displayed a linear exposure-concentration curve. One-way ANOVA, using dose as the independent variable with PROC MIXED, was also performed on group PK parameters. All errors were calculated as per error analysis methods by Taylor (1982).

The ANOVA compared plasma steady state concentration (32 to 194 h) points across all groups. Steady state is considered the time at which at least 5 half-lives have transpired. Previous data (Kniffin et al., 1989; Nielsen and Crad-Hansen, 1996) and our current analysis indicate that the half-life for tetracycline in swine ranges from 4.5 to 6 h. Therefore, steady state should be reached between 20 and 30 h, and the first time point after steady state was reached, 32 h, was used as a conservative initial time for steady-state PK analysis.

**RESULTS**

All animals remained healthy throughout the study irrespective of treatment group as evidenced by values for BW, hematocrit, serum creatinine, and urine specific gravity (except pig 13 discussed below) typical for pigs of this age (Supplemental Table 1 available online at [http://jasassword.com/content/vol87/issue11/]). Daily peak temperatures were elevated at the beginning of the week, were less in the middle of the week, and then increased to above 32°C by the end of the week (Table 1). Plasma concentrations of tetracycline from each group increased from below the limit of detection to steady-state concentrations within a 24-h period (Figure 1). Steady-state concentrations between groups given 125 and 250 mg/L were not different from each other but were different from control (P < 0.0001) and the 500 mg/L (P < 0.0001) treatment (Table 2). The AUC were also different between the control (P < 0.0001) and closed groups, 125 and 500 mg/L, (P < 0.0001) treatments, 250 and 500 mg/L (P = 0.0025) groups. A linear but indirect exposure-concentration relationship of tetracycline water concentration and plasma concentrations is presented in Figure 2. This is consistent with reports in human literature and resources that indicate decreased absorption of larger doses of tetracycline (Chambers, 2006).

Pharmacokinetic parameters (Table 2) were calculated based on the HPLC data as explained in the Materials and Methods section. A water analysis on the farm
Table 1. Daily maximum and minimum ambient temperatures during the study

<table>
<thead>
<tr>
<th>Day</th>
<th>Daily maximum, °C</th>
<th>Daily minimum, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.5</td>
<td>18.1</td>
</tr>
<tr>
<td>2</td>
<td>20.7</td>
<td>17.7</td>
</tr>
<tr>
<td>3</td>
<td>20.7</td>
<td>17.7</td>
</tr>
<tr>
<td>4</td>
<td>23.3</td>
<td>21.1</td>
</tr>
<tr>
<td>5</td>
<td>23.3</td>
<td>21.1</td>
</tr>
<tr>
<td>6</td>
<td>23.3</td>
<td>21.1</td>
</tr>
</tbody>
</table>

Average: 21.1 ± 0.2

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Table 3) shows that the water quality was appropriate for the study and did not result in elevated levels of tetracycline radio-complexing. Water tetracycline concentrations were measured for each animal on 3 d or more, and freshly mixed tetracycline water concentrations were within the assay variability (20%) of the expected concentration (Table 4), which further supports water quality. Water consumption was not different across all groups of pigs. Based on linear scatter plots, there was no observable relationship between the water consumption and flow rates on tetracycline steady state or average plasma concentrations.

**DISCUSSION**

Pharmacokinetic parameters of MRT, AUC, and Cmax were consistent with previously collected data (Krakoff et al., 1989; Nielsen and Gysel-Hansen, 1996) from pigs given tetracycline orally. The main differences and included test variable among the treatment groups are the concentration at steady state (Ct). This is significant because the other parameters of half-life, MRT, F, and Cmax, are all similar to those previously reported. Based on statistical analysis, parameters with at least one difference were plasma concentrations at steady state, as discussed above, and the corresponding AUC. Calculated clearances are similar to previously reported values but should be interpreted carefully based on the age of the pigs in our study and our calculation of apparent bioavailability. An apparent bioavailability, or more correctly the ratio between the amount of tetracycline in water that was used by the pigs compared with the amount of an intravenous dose, was calculated at approximately 6% across all treatment groups. This ratio may be used in the future to help determine how much tetracycline in water would be needed to reach known concentrations in the blood. Essentially, this ratio theoretically means that a dose of tetracycline in water would have to be approximately 20 times the dose given intravenously to approach the AUC of the intravenous dose. This is based only on this study and may not hold for other scenarios; however, it provides an estimate of the magnitude of the difference between an oral dose in a production setting and an intravenous dose. This may not be a true bioavailability as is observed by the potentiometric discrepancy in the clearances. Clearance rates were similar among the groups but slightly less (0.10 µg × mL⁻¹ × h⁻¹) than those previously reported between 0.16 and 0.22 µg × mL⁻¹ × h⁻¹ (Krakoff et al., 1989; Nielsen and Gysel-Hansen, 1996), which adds credence to the apparent bioavailability not being a true bioavailability. Based on this Foral value, volumes of distribution at steady state (Vdss) also were calculated but cannot be directly compared with previous PK studies values of Vdss or Vdtotal because they may not be equivalent (Williams, 1999).

It is interesting to note that a few of the animals appeared to overshoot steady state at 12 h, as seen by the plasma concentrations, but steady state is not reached for another day. Furthermore, the peak concentration for the 500 mg/l group is at 12 h. However, for some of these animals, the peak at 12 h was subsequently followed by a drop to steady-state concentrations by 32 h. This may be explained by the fact that some animals drank very little overnight and that the first day of the study was warm.

Furthermore, 2 animals on the dose response curve had greater than anticipated concentrations (greater than 1.5 SD from the average, with each included in the average). The first outlier, within the 125 mg/L group, used approximately 12 L of water within 1 d. The second outlier did not exhibit any measurable difference in BW, water consumption, or other factors that explain the high plasma concentrations. Exclusion of both animals increases the coefficient of determination for mean concentrations at steady-state from 0.75 to 0.91 for the dose-response relationship.

The high temperatures ranged from 28.4 to 33.2°C for the whole week. According to previous studies, the maximal estimated water consumption of 1 L of water per 10 kg of BW is expected each day for pigs up to 50 kg of age (Harvey, 1994). Based on this estimated water consumption for pig BW of 15 to 20 kg, the expected water consumption for the pigs in our study was 1.5 to 2 L of water daily. However, the pigs in our study consumed a larger amount of water relative to their size. The increased water consumption for the animal size could be explained by increased ambient temperatures as compared with other studies. On average, the water consumption was 3 to 1.1 L per pig per day (Supplemental Table 1 available online at http://jsw.sagepub.com/content/vol87/issue1/). Due to the warm ambient temperatures, it is anticipated that plasma concentrations of these animals are close to peak attainable concentrations based on the amount of water consumed (Harvey, 1998, 1994).

Only 1 animal appeared to use water excessively, up to 12 L, over one 24-h period. This may be due to either psychogenic water drinking, which can occur in a small proportion of animals from multiple species or from the pig playing with the drinker. The urine-specific grav-
Figure 1. Individual animal plasma concentrations over time by treatment group. A) Plasma concentrations from pigs (n = 6) exposed to 125 mg/L tylosin in water. B) Plasma concentrations from pigs (n = 6) exposed to 250 mg/L of tylosin in water. C) Plasma concentrations from pigs (n = 6) exposed to 500 mg/L. Note that for many animals, there was a slight overshoot of steady-state concentrations at 12 h on day 1 and a decline in plasma concentrations when the temperatures were less in the middle of the week. Then, at the end of the week, as water consumption likely increased with increased ambient temperature, the plasma concentrations rose again. All units are the same units for easier comparison.
of that pig on that same day was 1.003, which is consistent with moderate washout and excessive consumption of water. Regardless of this particular animal, increased water consumption is the only variable that appears correlated to increased temperatures.

It was also noted that the tetracycline from the 250-mg/L group began darkening in color by the late afternoon of day 2. This color change was also seen in the 125- and 500-mg/L-treated carboys at later times. The control water never changed color. This color change was presumed to be related to degradation from tetracycline exposure to light; however, a study by Wu and Fussell (2005) suggested that increased temperatures and increased humidity cause degradation of tetracycline and not light exposure. The HPLC assay continued to detect the tetracycline present in the water even after the color change began. Follow-up studies are being performed to better characterize the antimicrobial

Figure 2. Exposure-response relationship between tetracycline water medication concentration and individual animal steady-state plasma concentrations of tetracycline. The line represents the relationship, based on least squares best fit, between the average individual animal steady-state concentrations and the treatments exposure concentrations. Removal of 3 data points because each steady-state concentration was greater than 1.5 SD from the calculated means, using all data points increases R² to 0.91.
Table 3. Solution analysis report from the North Carolina Agronomy Department. (Rochelle)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.15</td>
</tr>
<tr>
<td>Electrical conductivity, uS/cm</td>
<td>186</td>
</tr>
<tr>
<td>Sodium adsorption ratio</td>
<td>0.50</td>
</tr>
<tr>
<td>Total alkalinity (CaCO₃), mg/L</td>
<td>50</td>
</tr>
<tr>
<td>Hardness, mg/L</td>
<td>63</td>
</tr>
<tr>
<td>Mg, mg/L</td>
<td>0.82</td>
</tr>
<tr>
<td>Ca, mg/L</td>
<td>17.59</td>
</tr>
<tr>
<td>K, mg/L</td>
<td>1.52</td>
</tr>
<tr>
<td>Mg, mg/L</td>
<td>0.62</td>
</tr>
<tr>
<td>Mn, mg/L</td>
<td>0.99</td>
</tr>
<tr>
<td>Sr, mg/L</td>
<td>0.72</td>
</tr>
<tr>
<td>F, mg/L</td>
<td>0.18</td>
</tr>
<tr>
<td>Cu, mg/L</td>
<td>8.09</td>
</tr>
<tr>
<td>Na, mg/L</td>
<td>10.60</td>
</tr>
<tr>
<td>Cl, mg/L</td>
<td>1.24</td>
</tr>
<tr>
<td>Zn, mg/L</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Water from the farm well was assessed before the study. This assay tested for the suitability of the water for its intended purpose. Physical properties and concentrations of divalent and trivalent cations which could bind tetracycline were also reported. Recommendations from the report: “The present water quality... from this farm well looks good. Mineral content and soluble salt levels are low and alkalinity is moderate.”

The pH is defined as the log₁₀ of the hydrogen ion activity.

When in meters, which is the SI unit of resistance; 1 ohm is equal to 0.001 M ohm.

Table 4. Tetracycline HPLC assay quantitation compared with the expected amount of tetracycline mixed into carbay water on d 3 of the study

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>True dose/expected dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>1.11</td>
</tr>
<tr>
<td>135</td>
<td>1.09</td>
</tr>
<tr>
<td>145</td>
<td>1.15</td>
</tr>
<tr>
<td>250</td>
<td>0.99</td>
</tr>
<tr>
<td>350</td>
<td>0.99</td>
</tr>
<tr>
<td>500</td>
<td>0.88</td>
</tr>
<tr>
<td>1000</td>
<td>0.88</td>
</tr>
<tr>
<td>1500</td>
<td>1.11</td>
</tr>
<tr>
<td>2000</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Average proportion of water dose to expected value: 0.99 ± 0.01

The pH value is reported as 6.15, the electrical conductivity as 186 uS/cm, and the sodium adsorption ratio as 0.50. The total alkalinity (CaCO₃) is 50 mg/L. The hardness is 63 mg/L, and the Mg concentration is 0.82 mg/L. The Ca concentration is 17.59 mg/L, and the K concentration is 1.52 mg/L. The Mg concentration is 0.62 mg/L, and the Mn concentration is 0.99 mg/L. The Sr concentration is 0.72 mg/L, and the F concentration is 0.18 mg/L. The Cu concentration is 8.09 mg/L, and the Na concentration is 10.60 mg/L. The Cl concentration is 1.24 mg/L, and the Zn concentration is 0.82 mg/L.

Tetracycline water medication is used in the study. According to the North Carolina Agronomy Department report, the water was adequate and appropriate for farm animals. The concentrations of Ca, Mg, Mn, Zn, Fe, and Cu when added together are less than 26 mg/L. This implies that cation-complexing effects from water are relatively small, especially in respect to feed sources. The water analysis also reported that the water was slightly acidic, pH 6.15, which would tend to stabilize the tetracycline (Harvey, 1998; Wu and Fasbender, 2005). The stability is supported by HPLC analysis on water samples taken at the start of the study and those measured right after mixing. Based on previous work, the medication should be stable for 48 h (Harvey, 1998).

After addressing the typical concerns (cation-complexing, water concentrations, and differences among groups) and new problems noted (water color change and indirect linear exposure-concentration), tetracycline water medication concentrations as dose were found to be consistent with tetracycline feed additive concentrations. In one feed additive study, Neff et al. (1989) dose 1 g/t with 0.55 g of tetracycline per kilogram of BW. Tetracycline concentrations reached 0.1 to 0.66 µg/mL (peak). Another study administered a single water dose of tetracycline in fasted and fed pigs (Niksen and Gyrd-Hansen, 1996). Animals received a dose of 45 mg/kg of tetracycline, and peak tetracycline concentrations of 4 µg/mL were seen in fasted pigs; however, when fed pigs were given this single oral dose, peak tetracycline concentrations only reached 0.8 µg/mL. These data are consistent with the data reported by Lauthman et al. (1989) on oral tetracycline use. According to our study, if pigs consumed only 2 L of treated water per day, then they would have received a 25 or 50 mg/kg dose of tetracycline for the 250- and 500-mg/L, treatment groups, respectively. Because it is impractical to fast pigs in a production facility, this information indicates that water medication values are in line with feed additive concentrations and will likely have similar efficacy. Furthermore, based on the Clinical and Laboratory Standards Institute guidelines, sensitive control bacteria used in antibiotic susceptibility assays need at least 0.1 µg/mL for growth inhibition (CLSI, 2007). Only in a few pigs is 1 µg/mL reached or exceeded, and even this level is not consistently met by any animal or group. Based on treatment group PK analyses, it is evident that all steady state plasma concentrations are inadequate for treatment of highly susceptible bacteria. Furthermore, tetracycline has been used more than any other medication in actual farms in Europe and the United States (Aarestrup, 2005). Burner et al. (2007) showed that Escherichia coli are highly resistant to tetracycline with minimum inhibitory concentrations above 16 µg/mL, which is often considered the breakpoint of tetracycline.

As a reference for in vivo tetracycline concentrations, the Food and Drug Administration has set a tolerance of tetracycline at 2 µg/mL for muscle, 12 µg/mL for kidney and fat, and 5 µg/mL in liver (FDA, 2005).
Concentrations within the plasma from our study are not even 50% of the least muscle tolerance concentration for any time points. At this time no published tetracycline residue studies are available; therefore, the true tissue concentrations of tetracycline in vivo are unknown. However, based on oxytetracycline tissue concentrations in pigs and ruminants, the concentrations of tetracycline will likely be a maximum of 6 to 7 times the plasma concentrations in the kidney (Mercer et al., 1978). The lung, which is often the target of tetracycline treatment, only reaches or slightly exceeds the concentration in the plasma. Other tissues such as fat reach less than one-half of peak plasma concentrations (Mercer et al., 1978; Craighill, 2003).

Injectable antibiotics are still used in livestock production; however, due to labor constraints, the possibility of needle breakage, and the expense of injectable medications, other methods of medicating animals are used first. Food producers need to be aware of the medications available and what they actually treat. Based on this study and correlation with previous work on tetracycline oral medications in pigs, tetracyclines should not be used enterally to treat salmonellosis and respiratory disease. There may still be possible gastrointestinal effects from using oral tetracyclines for prevention of scours and other enteric disease, but this needs to be assessed further.

LITERATURE CITED


Stability and Bioactivity of Tetracycline Water Medication in Swine Production Units

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Summary

Tetracycline hydrochloride-treated water on commercial farms may change color over time but its stability is unknown. Water samples containing tetracycline, darkened to yellow or brown, were assayed by HPLC and MIC micro-broth dilution for biological activity. Water samples were biologically active in the expected bioactivity range, but safety could not be determined.
Tetracycline hydrochloride (TcHCl) added to water is indicated for use in swine for the therapeutic treatment for *Escherichia coli* scours and susceptible respiratory pathogens. It was suggested that medications be mixed daily for use in swine facilities; however, mixing of water medications is often only performed when the batch of medication has been used. However, tetracycline hydrochloride medications may change color in water after mixing. During two recent studies, it was observed that the stock solution of tetracycline hydrochloride medicated water began to change color between 24 and 48 hours after mixing of the medication. At least two reports state that tetracycline is deactivated by a combination of high humidity and temperatures over 100°F. Tetracycline has also been shown to degrade after exposure to ultraviolet and fluorescent light.

Mason et al took water samples from carboys from which 24 individual pigs were exposed to TcHCl medicated water at three different concentrations (the label dose (250 ug/mL or ppm, twice the label dose (500ppm) and one-half the label dose (125ppm). The medicated water sample concentrations of tetracycline were measured via HPLC. Those HPLC results are compared to MIC microdilution assays used to test the bioactivity of those same medicated water samples. The bacterial standard (*E. coli* ATCC25922) for the Clinical Laboratory Standards Institute (CLSI) was compared to both standard TcHCl MIC assays and the reported MIC. This study was performed because of changes in color of water medicated with tetracycline hydrochloride and the lack of knowledge of its stability and bioactivity once the color change has occurred. Therefore, we proposed that TcHCl in water is adequately stable and biologically active despite its color change.
Materials and Methods. HPLC analysis was performed on a Waters Alliance 2695 High Performance Liquid Chromatograph with autosampler (Waters Corporation, Milford, MA) coupled to a Photodiode Array. Peak integration at 354.4-nm wavelength by Empower software (Waters Corporation, Milford, MA) was used for tetracycline quantification for each run on a Waters Atlantis 4.6- × 150-mm C18 column held at 35°C during a 4 min run time in 28% acetonitrile, 72% 0.05 M oxalic acid buffer mobile phase. All samples were kept in darkness at 4°C and were initially run within 14 d of collection. Two hundred microliters of plasma were added to an equal volume of releasing agent (78% water, 20% acetonitrile, 2% o-phosphoric acid) in a YM 10,000 Ultracel kit (Millipore Corporation, Milford, MA), placed in an Eppendorf tube and centrifuged at 7,840 × g for 30 min at 22°C. The lower limit of quantification, the smallest concentration that can be reproducibly quantified, for all water samples was 0.1 µg/mL, and the limit of detection was consistently at or below 0.05 µg/mL. The standard curve for water sample standards from 80 to 800 µg/mL had an R² of 0.99 and intraday CV less than 15%. The clean up assay was based on Dorr et al.¹ and the HPLC method was a modified version of Cheng et al.⁵ and Santosa et al.⁶. This technique was identical to the procedure used by Mason et al.².

Mueller Hinton broth (Sigma-Aldrich, St. Louis, MO) was made and cation adjusted to CLSI specifications ⁷,⁸ with CaCl₂ and MgCl₂ and then steam autoclaved. Two stock solutions of 250 µg/mL of tetracycline hydrochloride water medication (AmTech, IA) and tetracycline hydrochloride standard (Sigma, Thermo Fischer, Milford, MA) were made and filter sterilized (0.45 µm) under vacuum. These two standards were run on HPLC to confirm concentrations.
CLSI clinical isolate standards of *E. coli* ATCC 25922 were cultured at 37°C on MacConkey agar. CLSI procedures 7-9 were followed for media preparation and for MIC testing. Briefly after 24h, a suspension of *E. coli* ATCC25922 was prepared by suspending growth in PBS to a McFarland turbidity standard of 0.5 with a nephelometer. This standard was diluted 1:100 into the above mentioned Mueller Hinton broth to give approximately $10^6$ colony forming units (CFU)/mL. This suspension was used in the bacterial susceptibility testing. Two sets of serial dilutions of $10^{-2}$ to $10^{-6}$ were made in sterile glass vials for confirmation of the concentration of the standard suspension. An initial 1:100 dilution was made and serially diluted at 1:10 into vials to achieve the $10^{-2}$ to $10^{-6}$ concentration of the original standard suspension. Then 10 µL from each of the serial dilutions was plated onto two separate quadrant MacConkey agar plates, dried and then incubated at 37°C. Plate counts were performed approximately 24 h later on quadrants containing 30-300 colonies. Sterile Costar round bottom 96 well plates with lids (Thermo Fischer Scientific, Milford, MA) were obtained for all experiments. Water samples from individual carboys collected from the study (Mason et al3) were thawed. A 0.01 mL aliquot of cation adjusted Mueller Hinton broth was dispensed into each well of the 96 well plates. An aliquot of 0.01 mL of each water sample and control was then dispensed into the first column of wells with an additional 0.01 mL of Mueller Hinton broth. One-hundred microliters from the first well was used to begin a serial dilution across the columns of the plates, as per the CLSI method. Plates were then covered with lids and cultured for 20-24 hours at 37°C before being read for the lowest dilution that demonstrated no visible growth. All samples and controls were performed in duplicate. Any samples that varied more than one level of dilution were repeated.
**Results.** HPLC analyses of both TcHCl standard and water medication solutions were within the expected AUC integration range. These controls exhibited the expected inhibition for the low end of the MIC range of *E. coli* ATCC 25922. HPLC analysis results (Table 1) show that the various treatment group levels from HPLC analysis were very close to the expected tetracycline concentrations. The HPLC assay showed that there is some degradation of tetracycline over time, but the grand mean for all concentrations was 84% of the expected levels based on mixing concentrations (i.e. not including the control samples). Figure 1 shows comparative chromatographs of TcHCl as it ages. The first chromatograph (A) shows the TcHCl standard, while chromatograph B represents the medicated water samples at ½ the concentration strength of chromatograph A. The final chromatograph (C) demonstrates the change in TcHCl after refrigeration for 6 months to exaggerate the changes discussed below. On the 3 chromatographs, the first peak changes markedly from chromatographs A&B to chromatograph C. These differences show that the second and tallest peak represents TcHCl while the smaller initial peak corresponds with an epimerization product. The small peak enlarges as tetracycline ages and becomes very large in the oldest tetracycline standard as seen in chromatograph C.

The MIC assay results (Table 1) show that TcHCl concentrations were within one microdilution of the expected value based on HPLC. All control samples (i.e. tetracycline stock solutions of both the water medication product and a chemical standard) inhibited bacterial growth at between 0.5 and 1 µg/mL, while bacteria grew in all of the untreated water samples. These results and those of the medicated water samples were consistent with the HPLC analysis run shortly after collection of water samples.
Discussion. The HPLC analysis shows that tetracycline, despite its change in color, was still biologically active against *E. coli* ATCC25922 at the CLSI reported susceptible concentrations. Tetracycline hydrochloride (TcHCl) inhibited bacteria at concentrations consistent with HPLC analysis despite freezing and thawing. There may be increased variability in the MIC dilution assay due to the delay in processing of samples and the thawing process. The control runs for TcHCl for both the chemical standard and water medication standard solutions were consistent across plates and days. The TcHCl standards results were consistent with the MIC values for *E. coli* ATCC25922 and thus should be able to inhibit the growth of susceptible bacteria. Table 1 shows that as tetracycline ages, there is no significant difference in the microbial inhibition for the first 24 to 48 hours. Based on this information, the stability of the drug is adequate for at least the first 24 hours after mixing in water. However, there is a slightly greater drop of the stability of the 500 ppm medicated water samples, compared to either the label dose or one-half label dose medicated solutions. This drop in stability may be consistent with the decreased buffering capacity of the water when a large amount of drug is present. It has been reported that tetracycline hydrochloride is more stable in acidic conditions than normal to alkaline pH. This may be due to the ability of the increased number of hydrogen atoms to prevent epimerization of the drug.

In relation to this stability concern, it was noted on HPLC analysis that the chromatographs contained a small peak that increased in size when the samples were run 2 weeks after they had been thawed. This peak placement is consistent with peaks associated with tetracycline epimerization products called epitetracyclines. One tetracycline called quatrimycin displays
an increased UV spectral peak at approximately 270 nm to tetracycline’s greater peak at 250 nm
11. The results of the chromatographic analysis correspond with this non-toxic epitetracycline peak, shown in detail on HPLC analyses by Naidong et al 10 and Stephens et al 11. Although we did not run standards for tetracycline epimers, there are several papers that discuss the metabolism and formation of these products as well as their HPLC characteristics 3,10-12. The epimerization product is not necessarily responsible for the color change of the medicated water but its placement is consistent with reported peaks on HPLC analysis. It is possible that a carrier or other molecule present in the TcHCl formulation is responsible for the color change.

It is well known that expired or aged tetracycline products may result in renal damage, a reversible Fanconi’s syndrome of proximal tubular necrosis with proteinuria in mammals, of which anhydro-4-epitetracycline is the reported cause 13,14. The enlarged initial chromatographic peak (Figure 1A) is not consistent with the chromatographic elution of peaks reported for epimers that result in renal damage. However, toxicology is beyond the scope of this study, but further evaluation of the safety of TcHCl should be performed in the future.

**Implications.** Our study showed that tetracycline water medication is effective in inhibiting the growth of susceptible bacteria, even after a mild to moderate color change. The stability of the product when used on label is within the margin of error of the assay for the first 24-48 hours after mixing for on-label preparations.

**Acknowledgements.** Authors would like to thank Paul Orndorff for the use of lab space; Meghan Faults for the donation of bacterial strain *E. coli* ATCC25922 to complete this study.
The authors would also like to thank Dr. Sid Thakkur for the use of the MacFarland meter and suggestions for the MIC micro-dilution assay. This work was supported by Pfizer Animal Health.
Table 1: Comparison of HPLC Assay and MIC range for bacterial inhibition

<table>
<thead>
<tr>
<th>Treatment Group (µg/mL)</th>
<th>Average Concentration on HPLC (µg/mL)</th>
<th>Expected HPLC to Treatment Group (%)</th>
<th>CV(^a) for HPLC Concentrations (%)</th>
<th>Water Concentration Ranges (µg/mL)(^b)</th>
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<tbody>
<tr>
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<td>0.00(^a)</td>
<td>100</td>
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<td>182.56</td>
<td>73</td>
<td>20.03</td>
<td>250-500</td>
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</table>

\(^a\) CV is Coefficient of variation

\(^b\) Water concentrations were back calculated from the MIC found for the bacteria based on control runs.

\(^c\) All concentrations of tetracycline were below the limit of detection for unmedicated water samples. The rest of the results were from the 6 controls were identical for treatment concentration and Coefficient of Variation

\(^d\) ND is not done. There was only one acceptable sample run and therefore CV was not calculated.
<table>
<thead>
<tr>
<th>Treatment Group (µg/mL)</th>
<th>Average Concentration on HPLC (µg/mL)</th>
<th>Expected HPLC to Treatment Group (%)</th>
<th>CV for HPLC Concentrations (%)</th>
<th>Water Concentration Ranges (µg/mL)</th>
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<td>250-500</td>
</tr>
<tr>
<td>Grand Mean</td>
<td>398.66</td>
<td>80</td>
<td>6.90</td>
<td>250-500</td>
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</table>
Figure 1: Chromatographs of three different aged water samples containing tetracycline. A.) Standard water sample containing 500 ppm tetracycline. B.) Water sample from farm containing 250 ppm of tetracycline. C.) A water sample containing 250 ppm of tetracycline after being stored for 6 months at 4°C. As the tetracycline ages the first visible peak begins to enlarge in relation to the major (tetracycline) peak. This peak appears to correspond to the increase in 4-epitetracycline formation as described in the literature.
References


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Variables Important to the Population Pharmacokinetic Modeling of Tetracycline Water Medication in Swine

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ABSTRACT

Population based mixed effects pharmacokinetic modeling has not been applied to water medications despite their common use in swine production units. We propose a non-linear mixed effects model to simulate and predict factors that may determine plasma concentration levels of tetracycline water medication in swine. The model identified several covariates that may have an effect on the plasma concentration levels in populations of exposed animals. These covariates combined with knowledge of the pharmacokinetic and chemical properties of currently approved water medications may result in more prudent use of water medications in the future.
INTRODUCTION

Water medications have been used extensively in the livestock industries to treat populations of animals. However few pharmacokinetic studies have been conducted on water medications in general (2, 20, 21). This is likely related to concerns over determining the correct dosing levels and adequately modeling the pharmacokinetics of the drug (29). One potential technique that could provide insight and improved pharmacokinetic modeling is population based modeling using non-linear mixed effects (19). It appears that few population pharmacokinetic studies have been performed in veterinary medicine (3, 17, 18, 25, 27), and none on water medications. Only one study by del Castillo et. al.(3) looked at the population pharmacokinetics of doxycycline in a slurry (liquid) feed in swine. However, because this was slurry feed, the factors most important to plasma concentration levels in pigs from water medication treatments has not been investigated. Therefore this topic requires further investigation. Mason et al (20) determined the pharmacokinetics of three tetracycline water medication treatment levels in individually housed swine, while Dorr et al (4) investigated the pharmacoepidemiology of tetracycline water medication in an industrial setting. However, the predictability of plasma concentration levels was extremely variable and unable to be characterized by either of these methods alone.

Thus an understanding of water medication pharmacokinetics and the factors that contribute to in vivo plasma levels is important given the frequency and use of antibiotics in the swine industry (1). Therefore we used data collected by Mason et al (20) to develop a population pharmacokinetic model on tetracycline administered as a water medication to
swine. This model was then externally validated with data independently collected by Dorr et al (4) to validate our model. This model can be used to predict plasma concentration levels in swine and more importantly has helped to identify production factors and pharmacokinetics factors that will affect plasma tetracycline levels in pigs.

MATERIALS AND METHODS

Study Design. A population pharmacokinetic program (Phoenix WinNonMix NLME, Pharsight Corp., Mountain View, CA) was used to conduct all of the population PK analyses. The model was a non-linear mixed effects model using written code (i.e. not a pre-coded model from the program database). Three data sets were input separately in individual spreadsheets as different populations of animals. The training set data were used to develop the initial model, which was written as a one compartment pharmacokinetic model. The tow other datasets, from two separate populations, were compared to the best models for analysis (150 ppm dataset) and validation (80 ppm dataset). Plasma concentrations were set as the dependent variable. The program was run using first order conditional estimate (FOCE) with the Lindstrom-Bates algorithm (11). This algorithm was used for early model selection due to the time to run the FOCE-Extended least squares algorithm (ELS) and independently compared to the FOCE-ELS models to determine the best fit (34). However, in the end, the final model predictions and selection for figures was run with the FOCE-LB due to no obvious model fit benefit from the extended least squares algorithm. A Gaussian curve to the data distributions based on the initial data set allowed for adequate model fit and required much less computational time to run.
A priori parameter estimates were used for model development (20) and to elucidate which potential covariates improved model fit (30, 35). The initial parameter estimates for the model validation datasets were initiated at the training data population average for each parameter. Covariates were included in the model based on statistical analysis (one-way ANOVA in SAS 9.1 Cary, NC) and graphical comparisons for residuals, before inclusion in the model. Some parameters, such as bioavailability, were set as fixed values because little variability existed in the initial parameter estimates and there was statistical and graphical support that they did not contribute to the model fit. By fixing this parameter, parameter degeneration was prevented for the rest of the model and a better fit was achieved for most of the data (31).

**Structural model determination.** Due to the type of information available from the previous study data collected on water medications and tetracycline pharmacokinetics (9, 24), both one and two compartment models were attempted for modeling the data. The three initial PK parameters included in the structural model were: volume (of the plasma compartment), absorption (bioavailability) and elimination (Kel) (16, 32).

**Covariate Selection.** The initial linear equation predicted by Mason et al (20) was a direct correlation between water concentration and plasma concentrations in treated pigs and was set as the default or null covariate model (which included only water concentration). Several parameters including volume of distribution at steady state, body clearance and apparent bioavailability were calculated in the study by Mason et al. The use of covariates (as surrogates) for bioavailability and clearance did not prove useful. Serum creatinine (often a
covariate for clearance) was found to be non-predictive because all values for the population of animals were within 0.1 mmol/dl of each other. The urine specific gravity was also not predictive, due to highly concentrated urine in all animals. Bioavailability (F) was found to be consistent across all animals and when included in the structural model as a variable parameter. Model parameter estimates for F were very close to literature values of 0.06 or 6% (20, 24). Finally, body weight was linked to water use and volume of distribution and was therefore considered a covariate to two different factors, volume of the central compartment and tetracycline dose.

Based on both graphical and ANOVA comparisons, measured water use proved to be a useful parameter as did individual and group body weights. The water use in the training data set was high, compared to expected water use based on other investigators (7, 8). The Dorr data also fell significantly below the predicted linear relationship predicted by the null model (Figure 1). Based on this information and previous studies, water use was positively correlated with increased temperatures (23). Thus, temperature was included in the model covariate selection. Of all of variability in the proposed PK models, the most significant variable to data fit was water use rate for each animal. Therefore the various PK models included a variable drinking rate (called drate in the model code) that is the only difference represented by 5 different equations (Table 1) that included the above identified covariates and model structure.

**Model Selection and Validation.** These five models were run individually and compared to each other and the linear model based on graphical fit, Akaike’s Information Criteria (AIC),
Bayesian Information Criteria (BIC) and the loglikelihood ratios (LLV) (10, 12, 13, 15). The coefficients of variation, standard deviations of the observed values, and comparison of each model’s parameter estimates to literature parameter values for animals of similar ages (9, 14, 20, 22, 24) were also taken into consideration of the model fits.

RESULTS

Initial model testing was used to select between a one and two compartment model. Although the two compartment model was able to fit the data, the coefficients of variation for all parameter estimates were high in relation to the coefficients of variation for the one-compartment model. Therefore, the two compartment model was eliminated.

The five model covariate relationships were then run under the structure of the one-compartment model. By comparing the five models using the training data, two models, Model 3 and 4, were found to fit the data poorly (Table 1). The other three models showed a similar fit with Model 1 exhibiting the lowest scores for each of the AIC, BIC and LLV tests (Table 1). The graphical fits of Models 1 and 2 appeared to mimic an asymptotic fit, while Model 5 exhibited an almost sine wave pattern, better following peak and trough levels, based on temperature variations. Using LLV, BIC and AIC, Model 5 exhibited a slightly lower fit than either Models 1 or 2. However, this model was included in subsequent comparisons due to the graphical fit and consideration of covariate selection.

The final model selection was then based on the effect of the Dorr data for 150 ppm exposure to tetracycline. This data set was run on the above three covariate models since this was an independently collected data set. Covariates are listed under each model with model-
specific based parameter estimates (Tables 2 and 3). The performance of the models showed that Model 1 exhibited poor parameter estimates of drinking rate, due to high variability, and volume of distribution outside of physiologic ranges for tetracycline. The variability in estimates for Model 1 can be compared to Models 2 and 5 which also exhibit goodness of fit based on AIC, BIC and LV (Table 1).

The two more accurate models were compared using simulations compared to the Dorr 80 ppm validation dataset. Individual graphic results for the two final model predictions of Mason data are in Figure 2. The conditional weight residuals for each model (2 and 5) are shown graphically for both the 150 ppm and 80 ppm population values (Figure 3). The weighted residuals for the population etas represented an approximate Gaussian distribution for Model 5 data better than for Model 2 data (data not shown). Model 5 predictions for individual pigs from Dorr’s 150 ppm dataset are in Figure 4. The predictive check data exhibits some values that fall below the expected distribution, but overall the data are encompassed by the model prediction of a 5% to 95% confidence interval (26). Finally, simulations using both models were run for the Dorr 80 ppm data to compare models (Figure 5). When the parameters are set to the model predictions from the initial training set predicted values (Table 2), the simulations exhibit a robust fit to the data for the 90% confidence interval.

**DISCUSSION**

Based on the tetracycline water medication study by Mason et al, there is an initial linear relationship between plasma concentration and the concentration of tetracycline in the
water of pigs dosed in commercial settings (Figure 1). This relationship is predicted by the following equation:

\[
\text{Plasma Concentration} = 0.0019 \times \text{Water concentration} + 0.0435 \quad (1)
\]

This relationship appears to hold for the animals in the Mason study with a \( R^2 \) for approximately 0.79. The equation applied to a certain extent in the Dorr study but the average concentrations are less than those for the Mason et al data. This linear relationship does not address the variability in animals that is seen from the perspective of the population.

The major differences in animal plasma levels from the same treatment group (i.e. similar water concentration levels of tetracycline), when using the previously calculated population parameters, is not the apparent bioavailability or the values of the clearances. The obvious variability appears to arise mostly from water use in this modeling scheme. The water consumption is what determines the intake dose of the water medication, when compared to the base water concentration alone. With water medications, the typical pharmacokinetic programs cannot be easily used because they require a known dosing schedule. However, truly knowing how much an animal is dosed is not easily extrapolated (23, 33); therefore, by using an approximation based on calculated PK parameters and known water use, a theoretically determined dose of tetracycline that would be consumed can be related to a population of animals. This is what has been done with the model and the simulations with the ability to determine the factors that appear most important for the plasma levels in the study.
The model results show that in order to adequately model the dose of tetracycline with a true physiologic variability, not a random error from an average, we need to know several covariates. These covariates and fixed effects include daily water use (for each animal or the group); average body weights with a range or variance; temperature when available, and finally the anticipated or known concentration of the water medication. Based on the data and modeling, both Model 2 and Model 5 can adequately model the data. However, when more data is available, i.e. daily high and low ambient temperatures, it appears that Model 5 could potentially be more useful. Model 5 also exhibited less variability in the drinking rate than Model 2, which is most likely due to the temperature effect being removed from the primary drinking rate. This relationship is further supported by Figure 1 which shows that water concentration is not the only factor contributing to the water medication levels.

When the two models are compared, with and without the temperature variable, the validation dataset etas appear more skewed and random in Model 2 than when temperature is controlled in Model 5. Unfortunately the validation datasets did not contain temperature data and, therefore, Model 5 temperatures for validation datasets were based on a likely range. However, due to this artifact, the temperature accuracy is unknown, which prevents Model 2 and 5 from being accurately compared. This inaccuracy is due to unknown variability in temperature, which may mask its true effect on dosing in the three populations.

Unfortunately, we also do not have any information on water consumption in the barn from the comparison and validation datasets; therefore, the similarity in dosing rates between
the training and validation data sets is likely due to the model converging on the initial parameter estimates. Furthermore, there is no published relationship between the temperature and water use for grower-finisher pigs in the literature. The only published relationship we found is based on the equation from Vandenheede and Nicks for 150 kg sows (23).

\[ I = 0.92T - 1.52 \]  

(1),

where \( I \) is water intake, \( T \) is temperature and 1.52 is the y-intercept for baseline water consumption. When applied on a milligram per kilogram basis, water use is influenced by temperature. Based on the average drinking rate from our groups per day compared to the high temperature, drinking rate can be estimated by the following equation:

\[ \text{Water use} = ((\text{Temperature}-20)+\text{Constant})\times\text{Wt} \]  

(2),

where, temperature is in degrees Celcius, \( \text{Wt} \) is the body weight (kg) and \( \text{Constant} \) is a drink rate per hour within the thermoneutral zone for that age pig. This equation appears to hold pretty well for the relationship to water consumption and it is predictive in Model 5. We see similar values can be achieved for sows (150kg body weight) for temperature ranges over which the above equation is valid, however the low end of the thermoneutral zone temperature for sows would be considered 10ºC. One other major difference about these animals is that they are growing. Thus, their water use may be much higher (mg/kg) than a sow due to the fact that they have a higher concentration of body water (23).

Body weight fits into this model well because it directly affects the apparent volume of distribution and the dose (mg) of tetracycline. A large variability is present in the model
volumes (of distribution) which can be assumed to be due to age variability or due to model misspecification. However, it is difficult to determine which. Data from the literature shows a wide range of volume values from less than 1 L/kg to 4.5 L/kg for tetracycline (9, 24). These differences may be affected by the plasma volume or total body water for various animals which is linked with age. In young animals, due to body water levels being very high, volumes of distribution may be higher than in older animals because as animals age, body fat percentage increases.

In addition to wide changes in Vd, total body clearance (Ke) is the other important factor to consider. This factor showed values of between 0.08 and 0.27 across the model. It is best to compare Ke across animals of the same age group, not across individual animals. The literature values reported by Kniffen et al (9) and Nielson and Gyrd (24) were based on 12 week and older aged pigs whereas in Mason et al (20) and Mevius et al (22), pigs were only 8-9 weeks old at the start of the study. Although this is only a four week difference at the start of the study, the clearance of young animals is often “impaired” up to 16 weeks of age in many of our domestic species that reach puberty at approximately 6 months of age (6). Therefore, the clearance for younger animals may in fact, as the model predicts, be much lower than for the validation set of 12 week old animals, which match the literature values of 0.16 to 0.25 ml/kg/h clearance rates.

This age related change has been documented for gentamicin in very young pigs (newborn) which have a lower renal clearance and a larger volume of distribution than 42 day old pigs (5). This phenomon is also seen for doxycycline in which pigs at 18 kg have
a substantially lower clearance (28) than pigs weighing 25 kg (3). However, for tetracycline this is not explicitly documented in the literature, but the time line of development to reach adult renal and hepatic clearance for pigs has not been fully elucidated.

This potential immaturity in body clearance is also supported by the rapid drop in percent body water from approximately 80% to 50% between 28 and 180 days of age, with a plateau between 28 and 89 days at 65% (23). This drop in percent body water also appears to correspond to an inverse rate (increase) of renal clearance over time for young animals. Therefore, the clearance values reported by Mason et al appear to be potentially correct both physiologically and by the model in light of this interpretation. Unfortunately, there does not appear to be a lot of research conducted on the clearance of juvenile pigs, which is the age-range most dosed with antibiotics.

In conclusion, our non-linear mixed effects model relates plasma tetracycline levels in three groups of pigs to tetracycline concentrations in water and their water use. It appears based on the two best models that water concentration levels of medication and water use are the two biggest factors in determining the plasma concentrations of swine exposed to tetracycline dosed in water. These factors appear to effect plasma concentrations most as clearance and bioavailability exhibit tight estimates when age is considered. These two models were able to predict plasma concentrations for approximately 90% of the population. While the ability to predict plasma levels is necessary, the fact still remains that plasma concentration levels are very low for tetracycline as compared to bacterial MIC levels (14, 20).
The two factors may also apply to all other medications administered via water. Once bioavailability is known, water concentration levels and water use are the major factors that likely contribute to plasma concentration levels. Therefore, in selecting antibiotics for use in water, ideal candidates should have high bioavailability in the treated species and be readily dissolvable in water. If these two criteria are not met, then it appears, as with tetracycline, no matter what the water use and water concentrations of the medication, plasma levels will remain low. As always, prudent discrimination in the initial antibiotic selection should be performed prior to the criteria suggested above.
### Table 1: Model types and Associated Model Selection Criteria for training data (Mason et al)

<table>
<thead>
<tr>
<th>No.</th>
<th>Model Specificationa</th>
<th>LLV</th>
<th>AIC</th>
<th>BIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$\text{drate} = \text{average drate} \times \exp(\text{ndrate})$</td>
<td>-286</td>
<td>588</td>
<td>616</td>
</tr>
<tr>
<td>2</td>
<td>$\text{drate} = \text{average drate} \times Wt \times \exp(\text{ndrate})$</td>
<td>-284</td>
<td>584</td>
<td>612</td>
</tr>
<tr>
<td>3</td>
<td>$\text{drate} = \text{average drate} \times (\text{Temp - 20}) \times \exp(\text{ndrate})$</td>
<td>-310</td>
<td>635</td>
<td>664</td>
</tr>
<tr>
<td>4</td>
<td>$\text{drate} = \text{average drate} \times Wt \times (\text{Temp - 20}) \times \exp(\text{ndrate})$</td>
<td>-489</td>
<td>993</td>
<td>1022</td>
</tr>
<tr>
<td>5</td>
<td>$\text{drate} = (\text{teffect} + \text{average drate}) \times Wt \times \exp(\text{ndrate})$,</td>
<td>-293</td>
<td>602</td>
<td>631</td>
</tr>
</tbody>
</table>

*aModel specifications and their associated selection criteria. LLV is the loglikelihood value, AIC is the Akaike’s Information Criteria, BIC is the Bayesian Information Criteria. Variables in the model include drate, the average population water use rate, calculated from known drinking rates; ndrate, the interindividual variability of the calculated drate; Wt the body weight of the animal; Temp, the ambient temperature in degrees Celcius, where 20°C represents the low end of the thermoneutral zone of early grower pigs; teffect = Temp - 20 in order to allow an additive drinking rate to the model instead of a multiplicative rate.
Table 2: Parameter Estimation for Models 1, 2 and 5 with CVs for Training Data (Mason et al)

<table>
<thead>
<tr>
<th>Population Parameter†</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>CV%</td>
<td>Mean</td>
</tr>
<tr>
<td>Volume (ml/kg)</td>
<td>963</td>
<td>18.9</td>
<td>792</td>
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<tr>
<td>Kel (1/h)</td>
<td>0.253</td>
<td>18.8</td>
<td>0.298</td>
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<tr>
<td>Drate (var)*</td>
<td>203</td>
<td>9.8</td>
<td>8.32</td>
</tr>
<tr>
<td>Concentration st dev. (µg/ml)</td>
<td>0.192</td>
<td>5.0</td>
<td>0.189</td>
</tr>
<tr>
<td>Water use st dev. (ml)</td>
<td>1512</td>
<td>14.0</td>
<td>1604</td>
</tr>
</tbody>
</table>

† CV% is the coefficient of variation of the parameter estimate.

* Units for Drate are ml/h for Model 1, ml/(h*kg) for Models 2 and 5
Table 3: Parameter Estimation of Dorr Data for Models 1,2 and 5 for Second Training dataset (Dorr et al)

<table>
<thead>
<tr>
<th>Population Parameter</th>
<th>Model 1 150ppm</th>
<th>Model 2 150ppm</th>
<th>Model 5 150ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml/kg)</td>
<td>160</td>
<td>910</td>
<td>973</td>
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<tr>
<td>Kel (1/h)</td>
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<td>0.278</td>
<td>0.279</td>
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<tr>
<td>Drate (var)*</td>
<td>41.73</td>
<td>8.05</td>
<td>6.54</td>
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<tr>
<td>Concentration St dev. (µg/ml)</td>
<td>0.121</td>
<td>0.121</td>
<td>0.121</td>
</tr>
</tbody>
</table>

- Units for Drate are ml/h for Model 1, ml/(h*kg) for Models 2 and 5
- † Water use data was not included because no water use was directly measured.
Figure 1. Relationship between water concentration and the plasma steady state concentrations of swine treated with tetracycline water medication. Diamonds represent plasma concentrations from Mason et al. Squares represent pigs exposed to approximately 150 ppm of tetracycline during the winter (Dorr et al); Triangles represent pigs exposed to 80 ppm tetracycline from an independent farm (Dorr et al). The trendline is for data from Mason et al. Notice that the data from Dorr’s data is on average below the trendline for both farms. This is likely due to higher temperatures during the collection period for Mason et al.
Figure 2. Model Estimation of Mason data. The upper panel displays the model estimations for Model 2, which shows little daily variability compared to Model 5 (lower panel). This is because the water use rate varies throughout the day in Model 5 as a function of temperature, while Model 2 uses a constant daily water use rate. Circles are actual water use data (top points) and plasma concentration data (lower points); the complete line is predicted plasma concentrations; dotted line is predicted water use. Each lattice represents an individual animal.
Figure 3: Conditional Weighted Residuals Comparison for Models 2 and 5. Dorr 80 ppm data is modeled by Model 2 and Model 5, respectively in upper A) and B) lower panels. Both models show a bias in the water concentration at 8 hours into treatment; however, Model 2 (A) tends to under-predict later time points, which is likely due to model misspecification. The second set of figures shows residuals for Model 2 (C) and Model 5 (D) for Dorr data at 150 ppm. Both models underestimate later time points and overestimate earlier time points. This may be due to degradation of tetracycline over time during the Dorr experiment.
Figure 4: Model 5 Simulations of Dorr 150 ppm Data using Predictive Check Feature of PK program (n=50). It can be seen that the majority of data falls within a 90% confidence interval as predicted by the model. Circles are actual plasma concentration data; the complete lines show the predicted 5%, 50% and 95% plasma concentrations intervals by the model. Each lattice represents an individual animal.
Figure 5: Simulations of Dorr 80 ppm Data. Lattices in A and C can be compared directly for Models 5 and 2, respectively; while B and D compare the same group of pigs using Model 5 and 2, respectively. There are subtle differences between the outcomes of these two models but both models appear to simulate the data relatively well. Model 5 tends to predict slightly lower concentrations overall than model 2. Circles are actual plasma concentration data; the complete line is predicted plasma concentrations by the model. Each lattice represents an individual animal.


Summary of Results and Future Applications

Sharon E. Mason
For more than 40 years, water medications have been approved to control and treat disease in swine. Over the last two decades their use has increased and they have become a mainstay in the United States and in Europe for the treatment, control and prevention of bacterial diseases. However, during that same time period, few studies have looked at the pharmacokinetic disposition of these medications in swine when dosed via water. Due to the nature of the treatment regimens necessary to administer water medications, traditional pharmacokinetic techniques that can and have been used for individual animals are inadequate to address the dosing of a population. Furthermore, due to the lack of individual dosing and follow up, greater understanding of the consequences on the population as a whole are necessary for the best possible treatment in these herd situations.

In treating any animal with water medication (or other “at-will” dosing regimens), there is a need to know the plasma or tissue concentrations of the antimicrobials or other medications being used in the population. The largest challenge to this understanding is a dosing regimen or determining an amount of drug to which these animals are exposed. Sometimes, in traditional pharmacokinetic studies, this can be circumvented by using AUC ratios. Further research using other non-traditional pharmacokinetic techniques including physiologic based pharmacokinetic modeling (PBPK) and population pharmacokinetic modeling enables understanding of the mechanisms or factors that affect the dose and subsequent \textit{in vivo} levels of water medications used in food animals. Using these pharmacokinetic modeling techniques in conjunction with assays that determine chemical
properties of drugs are necessary to adequately model water medications and to explain the final pharmacokinetic behaviors of the drugs when used in a clinical or farm setting.

Two prominent medications dosed via water in food animals, namely swine, are sulfamethazine and tetracycline. There have been various studies on sulfamethazine in pigs that have described pharmacokinetic profiles with emphasis on its high bioavailability and metabolism. However, more papers have documented contamination of sulfamethazine within farms and abattoirs. In the early to mid-2000s, sulfamethazine had the highest number of residue violations in swine in the United States. Research by Buur in conjunction with FARAD used a PBPK model to show that the approved US label withdrawal time should be extended to prevent future tissue residues. In industry, for the last decade, there has been an industry wide increase to an 80-day withdrawal time of sulfonamide medications in response to residue violations, contamination concerns and exportation restrictions. With this extended withdrawal time, a pig must be younger than 3 months of age when treated with sulfamethazine to still meet the extended 80 day withdrawal time.

The initial study was conducted at the Teaching Animal Unit at North Carolina State University in nursery age pigs to determine the pharmacokinetic parameters in animals this young when orally dosed with sulfamethazine at product approved levels. Eight pigs aged 4 to 5 weeks were treated via oral gavage with an undiluted daily dose of sulfamethazine. Pharmacokinetic parameters for young animals were not directly compared to adult values for this medication, but contamination was seen in the control animals (6 pigs) who received no sulfamethazine in food or water. This study demonstrated contamination in a swine barn
with oral sulfamethazine treatment of a water medication formulation. Secondly, the above mentioned PBPK model was employed to calculate a dose that would mimic the plasma profiles of the control pigs exposed to sulfamethazine via contamination. This PBPK model was validated by the oral data collected on the treated pigs and then showed that a validated PBPK model can be used to calculate a dose of water medication when the plasma profile is known.

The second drug that is used regularly in commercial swine production units is tetracycline. In the case of this drug, much less research has been performed in swine and none on water medication pharmacokinetics at the youngest age at which pigs are exposed. The study design was a two-stage model with rich sampling to determine the common pharmacokinetic parameters in these animals exposed to tetracycline at three different water concentration levels. Twenty-four 8 to 9 week old barrows were housed individually with separate carboy drinking systems. The animals all received tetracycline exclusively in the water and had never been exposed to tetracycline prior to the study. The results showed that bioavailability was extremely low in these pigs which matched reports in previous studies. The pharmacokinetic parameters appeared to be slightly different from those reported for older animals (22-33 kg), with clearance being approximately half that experienced in pigs 12 weeks and older. The plasma concentrations were similar to those observed in other animal studies. Non-compartmental modeling was employed in this situation and it revealed that the pharmacokinetics were slightly different under a repeated dosing regimen of water medications than typical dosing. This study confirmed observations that volume of
distribution often decreases in animals as they age while clearance increases during maturation. The study also helped to show that a dosing rate could not be easily determined from traditional modeling techniques and therefore other pharmacokinetic techniques are necessary to explore water medications further. Namely, there remained a need to determine covariates and other factors that affect the plasma concentrations of tetracycline dosed via water.

During the course of this study, it was noticed that the treated water changed color over time, with the 250 ppm dose changing first, followed by the 500 ppm dose and finally by the 125 ppm dose. The control water did not experience this color change. There remained the question of whether the tetracycline was degrading in the water with this color change and if the medication would be effective against susceptible bacteria. These observations led to the following study that determined the antimicrobial bioactivity of the tetracycline. Initially, all water samples were assayed by HPLC and quantified with ultraviolet spectrum for tetracycline. A couple of control tetracycline compounds were also stored for 6 months and then rerun to determine the tetracycline peak concentrations to document peak changes over time. Samples were compared for bioactivity and HPLC assay quantification to see if they remained effective. Based on HPLC assay, the amount of tetracycline was approximately 77 to 85% of the initial concentration depending on the mixed concentration, while the bacterial assay determined that they were within the expected micro-dilution range for the compound. Therefore, tetracycline, although unstable, was still
effective at close to the expected concentrations and would be expected to treat susceptible bacterial infections, if appropriate levels were acheived in vivo.

The final modeling study used data from the study on tetracycline and data from an independently collected study of three distinct populations to develop a model for plasma concentrations of tetracycline in commercial swine herds exposed to tetracycline in water. The model used the two-stage data to determine which covariates would contribute to the model. Based on the measured data, covariates that were found to be useful were body weight and water drinking rate. The measured effects that contributed to the model were temperature and water concentration of tetracycline. The only fixed effect not directly measured but determined \textit{a priori}, was bioavailability. Based on the model selection process using both Akaike’s Information Criteria and Schwarz Criteria (Bayesian Information Criteria) there were two models that could predict concentrations based on the circumstances. When temperatures were constant or close to the thermo-neutral zone, temperature was not important to the model and therefore, the use of drinking rate as a function of body weight was only necessary. During ambient known temperature fluctuations, values were better predicted using the temperature based model. Overall, population pharmacokinetic modeling was useful in determining factors both farm- and situation-dependent and specific to the pigs to better predict steady-state plasma tetracycline concentrations compared to a general linear model. More importantly, this model helped to elucidate the factors for selection of appropriate medication candidates for dosing in water over other available medications.
In order to validate the model for other water medications, it would be necessary to take a subset of commonly used drugs in swine production and test the above-mentioned model using known PK parameters and using sparse sampling designs to see if the model can be validated for drugs other than tetracycline. If the model were useful for several water medications, swine production could be positively affected and candidate drugs better selected than in the past.

The data from these studies show that various techniques are available and can be used to model drugs for which there is no known specific dose delivered. This is often the case in barn treatments of water medication or in medicating wild animals or pets such as psittacine birds. However, these modeling techniques could be used in phase III clinical trials, where patients take oral chemotherapeutics at home. Instead of treating a group as an intention to treat for PK analysis, the dose could actually be estimated and compared to the rest of the population to better characterize drugs and their efficacy.

Aftermarket surveillance of drugs by FDA is also a major undertaking. The use of population based techniques could predict doses and side-effects better on a per body weight basis than currently used techniques. Determining if doses are inappropriate for patients based on clinical presentations (which may not be overtly visible with pharmaco-epidemiological studies) may be one benefit. This surveillance could be used to educate health care professionals on at-risk populations who need dosing adjustments, based on simulations and population based modeling.
Finally toxicologists are often faced with situations in which there is doubt or no known dose for an exposure. With either PBPK or population based modeling techniques, the use of validated models in humans already exposed or exposed at variable levels, a dose might be determined when other factors are known, or when plasma concentrations in exposed individuals can be simulated with PBPK or population models. Population pharmacokinetic modeling techniques have also been shown to provide useful information not just from the clinical perspective that has been used with human population modeling, but in determining other fixed factors that are not patient-related, but nevertheless can contribute to pharmacokinetic outcomes.

In conclusion, the exploration of modeling techniques in unknown dosing situations is an area of toxicology and pharmacology that heretofore was either not addressed or involved risk factor terms, with dubious merit. In the future, the use of these modeling techniques may continue to provide potentially improved approaches to unknown exposure risks, both intentionally and unintentionally.
APPENDICES
Supplemental Data Published in the Journal of Animal Science
"Pharmacology of tetracycline water medication in swine"

Table 1: Individual pig data divided into treatment groups

<table>
<thead>
<tr>
<th>Parameter/ Pig number</th>
<th>Animal ID ear notch</th>
<th>Hematocrit Day 1, %</th>
<th>Urine Specific Gravity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 1 Serum Creatinine, mg/dL&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day 2 Water Use, mL</th>
<th>Day 2 Serum Creatinine, mg/dL</th>
<th>Weight Gain, lb</th>
<th>Urine Creatinine, mg/dL</th>
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</thead>
<tbody>
<tr>
<td>pig 1</td>
<td>11210</td>
<td>39</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0</td>
<td>3380</td>
<td>10.0</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>pig 2</td>
<td>11304</td>
<td>40</td>
<td>1.028</td>
<td>0.8</td>
<td>4000</td>
<td>14.9</td>
<td>116.6</td>
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<td>pig 3</td>
<td>12208</td>
<td>42</td>
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<td>16.0</td>
<td>Err</td>
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</table>

<sup>a</sup> A few animals had repeated water specific gravity measures, but none were more than 0.006 different from each other in the same animal

<sup>b</sup> Serum creatinine levels were performed on the last day of the study, but no changes were seen with these values

<sup>c</sup> ND no urine was collected on these animals on any day

<sup>d</sup> Err was reported by Antech lab when tried to run test, some urine samples had a very small volume
Code for Model for “Variables Important for Modeling Water Medications”

```r
#Structural Model
test(){
deriv(A1 = drate*Wconc*fa - Ke * A1)  # 1 compartment model
deriv(W = drate)      #mL/hr water use rate
C = A1 / V          # ug/mL units
error(CEps = 1)
error(WEps=1)
observe(CObs = C + CEps)   # observed Conc read in
observe(Water = W + WEps)   # observed water use read in

#Statistical model parameter definitions
stparm(V = (tvV*Wt)*exp(nV) )
stparm(Ke = (tvKe)*exp(nKe))
stparm(teffect = Wt*(Temp-20))
stparm(fa = 0.06)
stparm(drate = (teffect+tvdrate*Wt)*exp(ndrate)) # Model 5
stparm(drate = (tvdrate*Wt)*exp(ndrate))  # Model 2

#Covariates
covariate(Wconc)
covariate(Wt)
covariate(Temp)

#Error Structures
fixef(tvV = c(0, 2600, ))
fixef(tvKe = c(, 0.084, ))
fixef(tvdrate = c(0, 3.856,))
ranef(diag(ndrate,nV,nKe) = c(1,1,1))       # model error structure
sequence{W=0;sleep(56);W=0}       # allows correct water use sampling
}

Additional features of the validation models to account for unmeasured variables
stparm(Wt = (tvWt))
stparm (Temp=(tvTemp))
fixef(tvWt = c( 25, 30, 35))
fixef(tvTemp = c(20, 22,24))
}