ABSTRACT

MZyk, Danielle Alethea. Influence of Age and Disease on the Pharmacokinetics and Disposition of Danofloxacin and Tulathromycin in Dairy Calves. (Under the direction of Dr. Geof W. Smith and Dr. Ronald E. Baynes.)

Calfhood diseases have a major economic impact on beef and dairy operations, due to the costs associated with mortality, treatment and the long-term effects on growth and performance. A very limited number of drugs are approved for use in calves and heifers, and questions remain about the relationship between plasma and tissue concentrations in young calves. In addition, a majority of published pharmacokinetic studies are performed on healthy animals, which may not reflect physiological differences of calves with respiratory disease. Therefore, the purpose of this research was to determine the impact of age and disease on the distribution of two classes of antimicrobial drugs in dairy calves.

In order to examine this, pharmacokinetic studies were performed that measured concentrations of a fluoroquinolone (danofloxacin) and a macrolide (tulathromycin) in plasma, interstitial fluid and pulmonary epithelial lining fluid. Interstitial fluid was measured to represent the free drug concentrations into tissues and sites of action. Studies were completed in dairy calves at 3-weeks and 6-months of age. A respiratory disease model was also used to simulate disease in calves in the different age groups. As predicted, these studies confirmed our hypothesis that age and disease had a significant effect on distribution of both danofloxacin and tulathromycin into interstitial fluid.

In vitro experiments were also performed to determine plasma protein binding of commonly used drugs in calves as they age. Of the four drugs evaluated (flunixin, danofloxacin, tulathromycin, and florfenicol) plasma protein binding did not change significantly as calves...
matured, although the levels of alpha1-acid glycoprotein, a major drug binding protein, decreased significantly from birth to 21 days of age.

In summary, these studies demonstrated that age and disease impact the distribution and pharmacokinetics of tulathromycin and danofloxacin in dairy calves. The results of these studies have important implications for practitioners, producers and regulatory agencies. By identifying the potential impacts of a patient’s age and physiological status, dosing regimens can be tailored to better meet the needs of the individual patient. Regulatory agencies can use this information to guide drug approvals in these young calves and understand the effects of age and disease on drug pharmacokinetics and distribution.
Influence of Age and Disease on the Pharmacokinetics and Disposition of Danofloxacin and Tulathromycin in Dairy Calves

by
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DEDICATION

To my husband, Philip, for his love, advice, patience and faith. Also, to my beloved animal companions, Tigger and Finley, who taught me the true meaning of dedication, selflessness and, above all else, unwavering loyalty.
BIOGRAPHY

Danielle Alethea Mzyk was born in Santa Clara, California on January 9th, 1990. She received her Bachelor of Science degree in Zoology from North Carolina State University. She then enrolled in the dual degree program at North Carolina State University College of Veterinary Medicine, where she is completing her Doctorate in Veterinary Medicine after completion of her Doctorate of Philosophy Degree in Comparative Biomedical Sciences.
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CHAPTER 1

Introduction

Calfhood diseases have major negative economic consequences on beef and dairy operations owing to costs associated with treatment, long-term effects on growth and performance, and death of affected calves. With very limited approved drugs for use in “pre-ruminant” calves and heifers, questions remain about the effectiveness of these drugs in young animals. It is important to promote judicious use of antibiotics in calves in an effort to limit the development of resistant bacteria, and violative residues. It is essential that we account for the developmental changes that occur in neonatal calves that can affect the pharmacokinetics and/or efficacy of selected drug therapies.

Pediatric patients are distinctly different from their adult counterparts, especially in regard to pharmacological therapy, due to the many physiological changes that take place as they age. In the field of veterinary medicine, where these age groups mature much more rapidly than human pediatric patients, these physiological changes can be profound. Ruminants, including cattle, goats and sheep, are unique due to the fact that their gastrointestinal tract matures from a pre-ruminant/monogastric digestion to a fully functioning rumen. In addition, as in other species, their maturation is accompanied by changes in four key pharmacokinetic (PK) processes: absorption, distribution, metabolism and elimination (ADME). These differences between neonatal calf vs adults ADME processes can impact the PK profile of a drug. An understanding of these differences and likely outcome is important to ensure effective antimicrobial therapy.

In addition to age, disease status can also have a significant impact on drug distribution and clearance. Examples of diseases in cattle that could potentially impact these PK processes include respiratory disease (DeDonder et al. 2016), mastitis (Kissell et al, 2015), and sepsis
Additional considerations of pharmacodynamics (PD) and disease progression need to be factored into dose evaluation for neonatal calves. Since the immune system of these calves are not fully developed, it is preferable to use drugs with bactericidal activity. However, the precise PK/PD target necessary to achieve the desired clinical outcome may not be identical for ruminating calves versus preruminant calves. Potential reasons for such age-related differences include a dissimilarity in the partitioning of drug from the blood to the infection site or the possible need to achieve a greater microbiocidal activity in the presence of the yet immature immune system (i.e., greater reliance on the drug versus the host response). By characterizing PK differences in healthy preruminant and ruminating cattle, we can more accurately predict infection site exposure.

A majority of respiratory infections occur in the extracellular (or interstitial) fluid sites in the lungs. Only free, unbound drug at this site of action will be available to fight bacteria. By studying the concentrations of free drug in the interstitial fluid in both healthy and sick calves, we can more accurately predict which drugs will reach clinically effective concentrations.

The purpose of this research was therefore twofold. First, the blood and tissue concentration of free drug was assessed in order to determine PK differences of two different drugs in healthy calves of two different ages. Second, physiological factors were further investigated to determine the age-associated impact of clinical disease on drug penetration and distribution into the pulmonary epithelial lining fluid as well as interstitial fluid. Chapter 2 provides a review of the literature regarding the impact of age and disease on the PK and tissue distribution of drugs in the cattle to lay the scientific foundation for these studies. Chapters 3, 4, 5, and 6 details the PK studies performed to begin to assess the impact of age, disease and protein binding in calves. Chapter 7 contains a summary of our findings and details plans for
future research to help optimize the safe and effective use of antimicrobial therapies for neonatal calves.
References


Literature Review: Developmental factors affecting drug pharmacokinetics in calves

Absorption

Neonatal calves differ in terms of physical size, body composition, physiology, and biochemistry when compared to older calves and adult cattle. Growth and development occur particularly rapidly during the first 6 months of life. Body weight ideally doubles by 60 days of age and proportions of body water, fat, and protein continuously change as calves mature and develop (Dairy Calf and Heifer Association 2017). Major organ systems mature in size as well as function during birth. Additionally, the pathophysiology of some diseases may change during the neonatal period and differ from adults. Understanding these age effects provide better insights that help identify potentially clinically important differences in pediatric populations of cattle.

A variety of methods are used to administer drugs to cattle, the most common of which involve extravascular routes, including intramuscular, subcutaneous and per os. A therapeutic agent must overcome chemical, physical, and biologic barriers prior to being absorbed into the systemic circulation. The process of absorption depends on drug solubility, permeation (active and passive) and metabolism. Absorption characteristics are often described of in terms of the rate and the extent. Developmental changes at the absorption sites, including the gastrointestinal tract, skin, and lungs, can influence the rate and extent of the bioavailability of a drug.

Gastrointestinal Development in Calves

In a newborn calf’s gastrointestinal tract, several age-related anatomic and physiological changes have the potential to affect drug absorption. At birth, the abomasum is the largest component of the gastrointestinal tract. Young calves’ reticular groove serves as a direct line for milk to bypass the rumen and empty into the omasal canal and abomasum (Guilloteau, Zabielski,
Rumen development is a dynamic process that occurs over a period of time; therefore, determining the exact moment that a young calf transitions from a preruminant to ruminant animal is difficult. The rates of rumen development and maturation vary among calves and are dependent on nutrition and diet (Stobo, Roy, and Gaston 1966). At birth, the rumen is small, and the development and maturation of rumen functions are delayed in calves fed exclusively milk diets as compared to calves that are not fed diets containing exclusively milk. When calves are fed starter, or grain, the microbial population in the rumen begins to ferment carbohydrates into volatile fatty acids (butyric, propionic, and acetic acids). The production of butyric acid, and to a lesser extent propionic acid, is primarily responsible for rumen maturation and the development of functional rumen papillae (Drackley 2008). In cattle, rumen function has a substantial effect on the pharmacokinetics of drugs, and age-associated changes in the rumen pH, extracellular fluid composition, and gastrointestinal transit time affect the solubility and absorption of orally administered drugs. In the rumen, microflora can inactivate orally administered drugs, thereby decreasing drug bioavailability and absorption (P.-L. Toutain, Ferran, and Bousquet-Mélou 2010). Drugs that are extensively inactivated by rumen microorganisms or undergo extensive metabolism in the liver would be expected to have a higher rate and systemic bioavailability (extent of absorption) in preruminant calves. For oral bioavailability studies performed in humans, the rate at which most drugs are absorbed is slower in neonates than in older children, prolonging the time it takes to achieve maximal plasma levels of orally administered drugs (Kearns et al., 2003).

Changes in the intraluminal pH across different segments of the gastrointestinal tract can directly affect both the stability and the degree of ionization of a drug, thus influencing the relative amount of drug available for absorption. In young calves, the pH of the whole intestinal
content ranges from 5.5 to 6.5, whereas in older calves such values are only found in the proximal duodenum, and the ileum and cecum were > 7.0 (Miyashige and Yahata 1980). The pH of the gastrointestinal lumen contents can affect both the extent and rate of drug solubility by modifying the proportion of ionized to unionized drug. Age-associated differences in pH may affect oral drug absorption. For example, the systemic bioavailability of orally administered procaine penicillin G, a weak acid, to calves demonstrated a 46% lower area under the curve (AUC), along with a shorter time to maximum concentration (T_max), and lower maximum concentrations (C_max) in the 5-week-old calves as compared to 1-week old calves (Musser and Anderson 2001).

Gastric emptying and intestinal motility are important determinants for the rate of drug absorption in the small intestine, the major site of drug absorption. Unfortunately, few studies have evaluated the effect of these developmental changes on drug absorption in young calves. The few bioavailability studies published in cattle demonstrated rapid absorption into the systemic circulation after oral administration of cephlosporin antibiotics and meloxicam to preruminating calves and generally lower serum/plasma concentrations in ruminant calves (Soback et al. 1987; Mosher et al. 2012).

Oral absorption of compounds can be altered by food consumption through several mechanisms including changes in pH or motility/emptying time as well as binding of drugs to feed content. Differences in the physical and chemical characteristics of the feed for young and older cattle can impact the flow of ingesta through the gastrointestinal tract. Factors including volume of feed, osmolarity, and energy content has been shown to decrease abomasal emptying in calves, while the capacity of the abomasum is relatively constant in adult ruminants due to a continuous flow of ingesta from the rumen (MacPherson et al. 2016; Burgstaller, Wittek, and
Smith 2017; Nouri and Constable 2006). Changes in transit time and pH can potentially enhance or hinder drug solubility, based on the physicochemical properties specific to each drug.

**Transporters**

Data on the developmental differences in the activity of intestinal drug-metabolizing enzymes and efflux transporters that can markedly alter the bioavailability of drugs are quite limited in cattle. Transporters play an important role in determining the pharmacokinetics and pharmacodynamics of drugs. There are many transporters expressed on the intestine. The two main families include efflux transporters (ATP-binding cassette (ABC) family) and uptake (SLC family) transporters. These two families can transport endogenous compounds or xenobiotics, including some drugs and their metabolites. In the intestines, one of the main efflux transporters includes the P-glycoprotein (P-gp) transporter. P-gp belongs to the ABC transporter family and involved in the pharmacokinetics of a wide range of drugs and xenobiotics and expressed in a number of tissues, including liver, blood–brain barrier, and intestine, where it functions as the efflux pump for xenobiotics before they can access the central circulation (Liu and Liu 2013; J. H. Lin 2003). Age related changes on P-gp expression may impact the drug bioavailability in young vs older animals. In rats, the timing of expression of P-gp has been shown to be tissue-specific with lymphocytic and hepatic P-gp expression increasing with age, while renal P-gp content was lower in the kidneys of older rats (Warrington, Greenblatt, and von Moltke 2004). Although age-related differences in bioavailability may reflect changes in liver size, blood flow and metabolic enzyme expression (Koch-Weser et al. 1982), this data has not been determined in ruminant species.

SLC transporters are responsible for intracellular movement of a wide variety of substrates across biological membranes (L. Lin et al. 2015). These transporters have important
roles in many physiological processes ranging from the cellular uptake of nutrients to the absorption of drugs and other xenobiotics. The tissue distribution of several SLC and ABC transporters in cattle showed high levels of expression in the liver and extra-hepatic tissues, including nasal mucosa, lungs and skeletal muscle (Zancanella et al. 2013; Kandimalla and Donovan 2005; Kadarmideen et al. 2011). Unfortunately, the ontogeny of drug transporters in any ruminant species is not described, and thus, any influence transporter ontogeny has on drug absorption and pharmacokinetics is a gap in current literature.

**Parenteral Administration**

Absorption of drugs from parenteral routes of administration, including intramuscular and subcutaneous injections, may be altered in young calves due to differences in muscle mass and/or muscular perfusion. Additionally, absorption can vary as a function of a drug’s physiochemical properties, such as molecular weight, lipid and water solubility and pKa. Water soluble drugs tend to have greater intramuscular absorption in neonatal humans than children or adults due to higher muscular water content and increased density of skeletal muscle capillaries (Kearns et al. 2003). Similarly, decreased muscle perfusion in young calves may result in slower rates of intramuscular drug absorption and lower peak serum concentrations. This was demonstrated in calves administered intramuscular amoxicillin trihydrate. Significantly higher plasma peak drug concentrations and shorter biological half-lives were noted in preruminant calves than in 5-month-old ruminant calves and dairy cows. Although differences in volume of distribution and clearance cannot be ruled out, absorption from IM injections of amoxicillin trihydrate may be also influenced by age-related differences in drug absorption capability at the injection site (Nouws et al. 1986). Depending on drug formulation and absorption capability at
the site of injection, s.c injections in preruminant calves may differences in blood flow to skeletal muscles, although generally, the rate of absorption can be unpredictable.

**Distribution**

After gaining access to the systemic circulation through absorption, age-dependent changes in body composition, plasma protein binding and cardiac output/blood flow to key drug metabolism organ systems can influence the space a drug distributes into. A drug's physicochemical properties (fat or water solubility) also contribute to distribution differences noted in neonates vs adults.

**Total Body Water**

Total body water is defined as the water contained within tissues and organs. It can be divided into two physiologically relevant compartments: intracellular fluid (ICF) and extracellular fluid (ECF). ICF is the total amount of fluid contained within all the cells in the body. This fluid is high in potassium and low in sodium and chloride (Khanorkar 2012). ECF is divided into several smaller compartments consisting of interstitial fluid (ISF), plasma and transcellular fluid. ISF is contained within all the body tissues and serves a transport space for oxygen, nutrients, cell waste and chemical messengers, but is typically low in protein concentration (Khanorkar 2012). Plasma, as compared to ISF, contains higher concentrations of protein and whole blood contains red and white blood cells in addition to plasma. The plasma can easily be sampled for drug concentration determination in many animal species, and serves as a “central compartment” when determining the extent of a drug’s volume of distribution. The smallest compartment of ECF is transcellular fluid, which represents fluid produced by cells. Examples of transcellular fluid includes cerebrospinal fluid, gastrointestinal fluids, urine, joint fluid and aqueous humor.
The relatively larger extracellular and total body water spaces in neonatal ruminants (Wrenn et al. 1962) as compared with adults, coupled with lower ratios of body fat, can affect plasma levels of drugs. Age-related changes in amount and distribution of total body water are important because the volume into which an amount of drug distributes can greatly affects the resulting concentration (Constable 2003). Since young animals have a higher percent of extracellular water, highly water-soluble drugs, such as beta-lactams, are greatly influenced by the distribution of total body water, leading to lower concentrations in the central compartment (Brumbaugh 2003; Brown, Chester, and Robb 1996).

When assessing the drug partitioning into different fluid compartments, many studies report volume of distribution differences as animals age. The volume of distribution corresponds to the relationship of an amount (A) of drug in the body at a given time (At), and plasma (blood) concentration at that time given (Pt) by the following equation (P. L. Toutain and Bousquet-Mélou 2004):

$$V_d = \frac{A_t}{P_t}$$

A drug that has a high volume of distribution generally has the ability to bind to tissues, cross cell membranes, and move between the intravascular and extravascular compartments. Body composition and drug characteristics influence the distribution of a drug in individuals. In addition to changes in body composition, tissue perfusion, which is driven by hemodynamic processes such as cardiac output and organ blood flow, contribute to distribution of drugs. During the neonatal period, calves cardiovascular and respiratory systems undergo continuous morpho-functional changes, with dramatic increases in pulmonary blood flow after birth (Piccione et al. 2010; Rudolph 1970).
**Blood Flow Maturation**

Blood flow to the kidney and liver of ruminants has been shown to change significantly with age. The glomerular filtration rate (GFR) is a measurement of kidney function and is dependent on the effective filtration pressure and the area/permeability of the active filtration membrane in the kidneys glomerulus. GFR values in beef cattle weighing less than 300 kg and aged less than 1 year of age had large variability, but the GFR in cattle weighing 300 kg or more and aged 1 year old or more remained relatively stable. The increased variability seen in younger calves may be influenced age-associated increases in renal blood flow, and reabsorption-secretion efficiency of the renal tubules during the postnatal period (Murayama et al., 2014). In sheep, arterial blood flow to the liver per unit weight was low in the fetus, significantly greater in the immediate newborn but declined by 16 weeks of age. Conversely on a body weight basis, portal blood flow to the liver, gut and intestines on a weight basis, changed little with age (Apatu and Barnes 1991).

**Plasma Protein Binding**

Drug plasma protein binding is a critical parameter in drug distribution as well as pharmacological activity. It is the unbound drug is pharmacologically active and free to move to and from the vasculature and site of action. Most studies report PK parameters derived from the total plasma concentrations over time, but neglect to report the more clinically relevant unbound drug concentrations (Greenblatt, Sellers, and Koch-Weser 1982). This may lead to a misinterpretation of clinical implications of the published PK data and consequently, to errors in drug dosing. This is particularly problematic in pediatric populations where as compared to adults, there can be significant differences in drug protein binding and drug metabolism. The binding of drugs to plasma proteins tends to be lower in pediatric patients (McNamara and
Decreased plasma proteins, and high concentrations of endogenous compounds that interact with these binding sites contribute to decreases in plasma protein binding. An increase in unbound fraction of drugs increases the distribution to the rest of the body. Plasma protein binding is typically calculated as follows:

\[ \%\text{PPB} = \left( \frac{C_{\text{total}} - C_{\text{free}}}{C_{\text{total}}} \right) \times 100 \]

Where \( \%\text{PPB} \) is the percentage of binding to plasma proteins, \( C_{\text{free}} \) is the concentration of the free drug and \( C_{\text{total}} \) is the initial total (bound and unbound) concentration of drug in plasma (Zhang and Surapaneni 2012). For some drugs, saturable protein binding may result in differences in \( \%\text{PPB} \) as a function of the total drug concentration.

**Plasma Proteins**

Acidic drugs bind largely to albumin, while basic drugs can associate with a number of plasma components including \( \alpha \)-acid glycoprotein (AGP) and albumin (Piafsky 1980). Albumin and AGP concentrations can show large fluctuations due both to physiological and pathological conditions (Tóthová, Nagy, and Kovác 2013). Changes in the amount of circulating plasma proteins, can alter the distribution of drugs. In Holstein dairy calves, albumin levels have been shown to increase after birth to 80 days of age, although some reports showed a decrease in the total protein from blood samples until day 14 and increases after 84 days (Knowles et al. 2000; Mohri, Sharifi, and Eidi 2007). In newborn humans, AGP concentration is half that of the adult concentration but was reported to be higher in neonatal pigs as compared to adults (Tagawa et al. 1994).

It is assumed that the unbound drug concentrations in plasma reflect unbound drug concentrations at the site of action under equilibrium conditions. Plasma protein binding of drugs tends to be reduced in neonates, although this has not been examined in cattle (McNamara and
The measurement of free drug concentrations has been complicated by sampling techniques that may have led to misinterpretation of drug concentrations at the site of action. High total tissue concentrations based on tissue biopsies may accurately reflect pharmacologically active drug since they may represent both unbound and bound drug. Only the unbound extracellular concentrations are free to bind to its intended target. Therefore, the sampling of unbound drug in the ISF may give insight into drug distribution and activity at effector sites.

**Interstitial Fluid**

Interstitial fluid is composed of ions, small molecular weight proteins/lipids and water (Fogh-Andersen et al. 1995). ISF is a major component of extracellular fluid and therefore, using an ultrafiltration method, unbound/free drug concentrations can be measured. The ultrafiltration membrane excludes proteins and protein bound drugs, and allows free drug to diffuse across the membrane into the ISF. Drug concentrations measured in this fluid reflect free/unbound drug concentrations. For many diseases, particularly respiratory disease in cattle, the site of infection is the extracellular fluid in the respiratory tract. In order for a drug to reach this site of infection, it must be unbound, unionized and free to cross biological membranes. Unlike ISF, tissue biopsies and homogenization measure the total (bound and unbound) concentration, which can overestimate the concentrations at a particular site. To combat this, techniques have been developed to measure unbound drug concentrations including ultrafiltration, tissue microdialysis and tissue cages (Zhang and Surapaneni 2012).

Ultrafiltration probes have been used successfully to collect protein-free interstitial fluids of many different species including dogs, horses, calves and sheep (Bidgood and Papich 2003; Jennifer L. Davis, Salmon, and Papich 2006; Messenger, Papich, and Blikslager 2011; J. L. Davis, Foster, and Papich 2007; Underwood et al. 2014; Sojka et al. 2000). These probes have
typically been placed in the subcutaneous space, but other locations,

![Subcutaneous ultrafiltration probe for collection of interstitial fluid in a calf](image)

**Figure 2.1. Subcutaneous ultrafiltration probe for collection of interstitial fluid in a calf**

including pleural space, bone, muscle, gastrointestinal tract and lamellar tissue have also been sampled (Underwood et al. 2014; J. L. Davis, Foster, and Papich 2007; Warren et al. 2014). The ultrafiltration probe contains three semi-permeable loops connected to a tube extending to the exterior of the animal and uses negative pressure for fluid collection. Through small pores in the loop, water, electrolytes, and low molecular weight molecules (less than 30,000 Da) are allowed to pass and larger molecules, including proteins, protein-bound drugs and fats are excluded (Garrison et al. 2002).

Similar to ultrafiltration, ISF can be collected through microdialysis. This technique involves the collection of unbound drugs in perfusate of different tissues that is controlled by diffusion of analytes through a membrane. Drawbacks to this sampling technique include dilution of the analyte, requiring very sensitive analytical techniques to quantify the
concentration as well as the need for restraint during sampling. Although microdialysis is minimally invasive and an accurate measure of ISF concentrations, each probe must be calibrated to account for dilution (Garrison et al. 2002; Musteata 2009). Microdialysis probes must also be connected to a perfusion system, making it difficult to use in veterinary species. However, unlike ultrafiltration, microdialysis provides an opportunity for frequent samples, avoiding time delays, and subsequent averaging over time that is characteristic of ultrafiltration systems.

In addition to protein binding, drug distribution processes are dependent on the lipid-water partition coefficient, degree of ionization as well as a drug’s ability to overcome physiological barriers. For many drugs, physiological barriers limit drug distribution into specific sites. For example, the permeability of drugs through the blood-brain barrier, when intact, has been shown to be similar in both adults and pediatric patients (>4 months of age) (Jacobs et al. 1986). When this site is invaded by bacteria or viral infections, the protective effect is lost with a resulting increase in drug penetration and accumulation (Chowdhury and Tunkel 2000).

Metabolism

Hepatic Development

The liver is comprised of a functional unit known as an acinus which includes a central hepatic vein connected to multiple portal triads. Within the three different zones of the acinus, each group performs specific physiological functions. Neonatal patients undergo dramatic developmental changes in the physiological and biochemical processes in these cells that determine drug metabolism in the liver over the first few months of life. The liver is also responsible for several critical functions including the regulation of blood glucose levels,
synthesis of multiple serum proteins and bile, as well as the biotransformation of xenobiotics. These functions have been shown to be functionally immature in different ages of calves (Donkin and Armentano 1995; Donkin, Bertics, and Armentano 1997; Gray et al. 1995). In addition, immature drug metabolizing pathways and transport mechanisms probably have the greatest impact on hepatic drug elimination in the developing neonate.

The liver plays a critical role in modulating the systemically available fraction of an orally available compound, as well as the biliary excretion, and metabolism of xenobiotics and endogenous by-products of metabolism. Ontological changes in the metabolism and clearance pathways of the liver are a critical source of pharmacokinetic variation in neonates. As neonates age, physiological changes occur in the blood flow and metabolic capacity of the liver. Studies in lambs suggest high fetal hepatic blood flow, constituting 30% of total cardiac output (Edelstone, Rudolph, and Heymann 1978; Botti et al. 1982). An organ’s ability to clear drugs from the systemic circulation is called the extraction ratio, which can be defined as the ratio of the rate of elimination to the rate of presentation (Benet and Zia-Amirhosseini 1995). Age related changes in hepatic blood flow govern the hepatic clearance of drugs that exhibit high extraction ratios, while the unbound fraction is the primary determinant for hepatic clearance for low extraction ratio drugs.

Variations in species metabolism of certain drugs has been well characterized (Graham, 2004). Metabolism, predominantly occurring in the liver, can be divided into two phases. Phase I reactions involve modifying the compound of interest through chemical reactions including hydroxylation, oxidation, hydrolysis and reduction which renders the compound either active or inactive. Among many enzyme systems involved in Phase I metabolism, the one responsible for the metabolism of many foreign substances is the Cytochrome P450 family of enzymes (CYPs).
Each CYP has a specificity for certain substrates and is the site of many differences in overall metabolism of veterinary pharmaceuticals. In contrast to Phase I, Phase II reactions typically involve conjugation by adding functional groups that increase molecular weight and polarity and generally (although not always) result in bioinactivation. The increase in weight and polarity make the compound more easily excreted in either the urine or bile. Depending on the animal species, gender, age and breed, these Phase I and Phase II metabolic pathways are key components of determining differences that could exist between individuals and patients.

In calves, cytochrome P450 enzyme activity increases 2-fold during the first week after birth. Enzyme activities in 1-day-old calves have been shown to be only 17% to 50% of those in 42-day-old calves (Shoaf et al. 1987). Selective changes in cytochrome P450 expression during the neonatal period can result in decreased half-lives and increased clearance of certain drugs as neonates mature (Gilman 1990). CYP enzymes in calves with functioning rumens showed an increase of over 50% of enzymes expression when compared to veal (immature rumen function) calves (Kawalek and el Said 1994). Similar age-associated changes in CYP enzymes have been noted in other species including humans, sheep, and dogs (Kawalek and el Said 1990b; Kawalek and el Said, 1990a; Hines 2002).

Phase II reactions are synthetic conjugation reactions and include glucuronidation, sulfation, glutathione conjugation, and acetylation. The activity of Phase II enzymes, including glutathione-S-transferase, are crucial to detoxification pathways found in the liver. In young veal calves, the activities of glutathione-S-transferase enzyme were found to be lower than those found in older calves with a functioning rumen. Age-associated maturational differences in enzyme function lead to high variability for many biotransformation and elimination pathways, which can influence PK parameters in pediatric populations.
**Excretion**

While there are numerous routes of excretion, including bile, exhalation, skin, intestinal efflux and kidneys, the focus of this discussion will be on the kidney, due to the marked importance of maturation associated differences of elimination processes for many drugs and their metabolites.

The kidney is responsible for a wide variety of functions in the body. The kidneys filter the blood to excrete metabolic wastes and retain water and electrolytes. The nephron is the unit of the kidney responsible for these functions. The nephron is composed of the glomerulus, where the blood is filtered, and various distinct segments of the renal tubule, where filtered substances are absorbed and plasma components are secreted into tubular fluid. The maturation of these functional segments continues throughout infancy, leading to pharmacological and physiological changes in drug clearance. Developmental changes in the kidney include changes in the number of functional nephrons, glomerular filtration rate (GFR) and tubular secretion and absorption. The kidneys mass relative to age (which correlates with body mass) is generally greater in neonates than adults (Rubin et al. 1949). At birth, the kidneys are anatomically and functionally immature, which hinders renal function. During post-natal development, maturational changes in nephron function and perfusion have been shown in rats, sheep and dogs (Aperia, Broberger, and Herin 1974; Evan et al. 1979; Solomon 1977). The GFR increases due to an increases in cardiac output and decreases in renal vascular resistance (Arant 1987). Interestingly, the neonatal calf kidney has been demonstrated to be functionally mature within a few days of birth with respect to glomerular filtration rate and renal plasma flow (Dalton 1968; Dalton 1968). Development of glomerular filtration in cattle reaches adult levels by one to three days after birth and secretion processes may require up to one or two weeks to be completely developed (De Backer 1986).
This is important for drugs, such as sulfonamide antibiotics that are eliminated by the kidney. These compounds show lower clearance values in one and three week old calves than in seven and fifteen week old calves (Baroni et al. 2008). Changes in clearance by the kidney can also be influenced by urine pH. Ruminanting calves tend to have a more alkaline urine than preruminant calves (Watson et al. 1987).

The ontogeny of transporters in the kidney are important when determining renal elimination changes as neonates mature. The function of organic anion/cation transporters in renal tissue is low at birth in humans and other mammalians, increasing over the first few weeks of neonatal life, and subsequently declining to adult levels (Sweet, Bush, and Nigam 2001; Dutt et al. 1992). This increase demonstrates the simultaneous importance of the maturation of transporter systems in addition to the growth of the kidney itself.

Table 2.1 Plasma elimination half lives in different ages of calves

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Animal Age (months)</th>
<th>T1/2 (h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin trihydrate</td>
<td>10</td>
<td>IM</td>
<td>3-4 weeks</td>
<td>5.2</td>
<td>Nouws et al. 1996</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>IM</td>
<td>5 months</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>IM</td>
<td>4-6 years</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Sulfamethazine</td>
<td>60</td>
<td>IV</td>
<td>1 week</td>
<td>9.46</td>
<td>Baroni et al. 2008</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>IV</td>
<td>3 weeks</td>
<td>9.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>IV</td>
<td>7 weeks</td>
<td>8.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>IV</td>
<td>15 weeks</td>
<td>7.45</td>
<td></td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>7.54</td>
<td>IV</td>
<td>3 weeks</td>
<td>13.5</td>
<td>Nouws et al. 1983</td>
</tr>
<tr>
<td></td>
<td>6.88</td>
<td>IV</td>
<td>12 weeks</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>IV</td>
<td>14 weeks</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>IV</td>
<td>1 day</td>
<td>11.7</td>
<td>Reiche et al. 1980</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>IV</td>
<td>1 week</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>IV</td>
<td>10-12 weeks</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>IV</td>
<td>Adult</td>
<td>4.4</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.1 Continued

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Route</th>
<th>Time</th>
<th>AUC (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftiofur</td>
<td>2.2</td>
<td>IV</td>
<td>1 week</td>
<td>16.1</td>
<td>Brown et al. 1996</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>IV</td>
<td>1 month</td>
<td>17.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>IV</td>
<td>3 months</td>
<td>8.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>IV</td>
<td>6 months</td>
<td>5.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>IV</td>
<td>9 months</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>22</td>
<td>IV</td>
<td>1 month</td>
<td>93.9</td>
<td>Volner et al. 1990</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>IV</td>
<td>3 months</td>
<td>44.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>IV</td>
<td>6 months</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Antipyrine</td>
<td>15</td>
<td>IV</td>
<td>1 weeks</td>
<td>12</td>
<td>Janus et al. 1996</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>IV</td>
<td>two weeks</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>IV</td>
<td>4 weeks</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>IV</td>
<td>6 weeks</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>IV</td>
<td>8 weeks</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>IV</td>
<td>12 weeks</td>
<td>6.1</td>
<td></td>
</tr>
</tbody>
</table>

**Disease induced alterations in pharmacokinetics**

The pathophysiologic changes occurring in disease can profoundly alter the pharmacokinetic behavior of a drug, particularly drugs that are cleared by hepatic or renal pathways (Rodighiero 1999, 1989; Levy 1977). Processes such as inflammation, endotoxemia and stress can also significantly alter a drug’s absorption, distribution, metabolism, and elimination (van Miert 1990). The severity of these different responses to disease may vary depending upon the pathophysiological changes and nature of the physiological stressor. In disease, fever and changes in blood flow to various organs can have a pronounced influence on distribution of drugs due to changes in heart rate, renal/hepatic blood flow, and enzyme function (Kasting, Veale, and Cooper 1982). Changes in blood flow and enzyme function can greatly alter the disposition and clearance of drugs. For goats with experimentally induced fevers, plasma drug concentrations, AUC and volume of distribution of amikacin were significantly
higher, while clearance values were significantly lower for most time points compared to healthy goats (Agrawal, Singh, and Jayachandran 2001).

In ruminants, many of the previously published pharmacokinetic studies in diseased animals have focused on the plasma concentrations of various antimicrobials. However, since most infections occur in tissues other than the plasma, an understanding of drug distribution and efficacy to the site of infection is critical. The presence of inflammation can potentially increase penetration of drugs across the blood-alveolar barrier due to increases in membrane permeability (Baldwin, Honeybourne, and Wise 1992). Some drugs, including macrolides, have been hypothesized to accumulate in inflammatory cells at the site of infection and increase drug concentrations in extracellular fluid (Baldwin, Honeybourne, and Wise 1992; Modric, Webb, and Davidson 1999). In one study, concentrations of florfenicol in tracheobronchial secretions in calves infected with Pasteurella multocida were significantly higher than those in healthy calves (Ramadan and Abd El-Aty 2011). In calves with significant areas of pulmonary consolidation due to bronchopneumonia, danofloxacin concentrations in consolidated lung tissue were reduced by 41% and blood flow decreased in consolidated lung tissue (Apley and Upson 1993). Pathological changes associated with mastitis, are responsible for alterations in the udder tissue and milk. Permeability of blood vessels and other inflammatory responses affect the functional integrity of the udder (Mestorino and Jorge 2012). Such changes may alter the PK of antimicrobial drugs and can also affect drug excretion to milk (Kissell et al. 2015; Cagnardi et al. 2010). Other factors, including changes in composition and pH of milk may also impact pharmacokinetic parameters of drugs.

In respiratory disease of cattle, the initial site of infection is the airways of the lower respiratory tract. Antimicrobial concentration at these sites of infection is a critical piece of
information to predict antimicrobial efficacy. Sampling these sites has been attempted to determine the concentrations in the airways (DeDonder et al. 2015; Foster, Sylvester, and Papich 2017). However, modeling based on plasma, lung homogenate and tissue cages can both under and over-estimate drug concentrations in the airways (Winther 2012; Kiem and Schentag 2008).

Measurement of drug concentrations in pulmonary epithelial lining fluid (PELF) may reflect concentrations of active drugs at the site of action. Bronchoalveolar lavage (BAL) has been widely used in veterinary and human medicine to estimate antimicrobial concentrations in the lower respiratory tract (Drusano 2005). Although widely used, the use of BAL to determine concentrations has its limitations (Drusano 2005; Rennard et al. 1986). In order to overcome the variability in the recovery volumes of PELF, urea is measured in both BAL fluid and plasma to estimate the quantity of drugs in the PELF. Urea, an endogenous substance, is considered to be in equilibrium between the blood and tissues in healthy individuals. Measuring the ratio of urea in plasma and BAL fluid is used as a dilution factor to calculate concentrations of substances in PELF. Overestimation of urea concentrations in BAL fluid due to contamination with blood or increased dwell time can falsely increase the estimated volume of PELF, which would result in an underestimation of drug concentrations in bronchial secretions (Marcy et al. 1987).

**Summary**

Clinical therapeutic success is largely impacted by the age-specific changes in the host. In neonatal calves, immature pharmacokinetic and pharmacodynamic functions and patient parameters of age and disease state are important considerations when selecting an efficacious therapeutic. Absorption can be affected by the differences in gastric physiology as the rumen develops. Low plasma protein concentrations and a higher body water composition can change
drug distribution. Metabolic processes in the liver are often immature at birth, which can lead to a reduced clearance and a prolonged half-life for many drugs. Renal excretion is also lower in neonates due to immature glomerular filtration, tubular secretion, and reabsorption.

Understanding these age effects in developing calves, as well is identifying key impacts of disease on key pharmacokinetic parameters provides a mechanistic way to identify safe and effective therapies in these animals.

References


Chapter 3
Pharmacokinetics and distribution in interstitial fluid and pulmonary epithelial lining fluid of danofloxacin in ruminant and preruminant calves

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2017
Abstract

The objective of this study was to compare active drug concentrations in the plasma versus different effector compartments including interstitial fluid (ISF) and pulmonary epithelial lining fluid (PELF) of healthy preruminating (3-week old) and ruminating (6-month old) calves. Eight calves in each age group were given a single subcutaneous (s.c.) dose (8 mg/kg) of danofloxacin. Plasma, ISF and bronchoalveolar lavage (BAL) fluid were collected over 96 h and analyzed by high pressure liquid chromatography. PELF concentrations were calculated by a urea dilution assay of the BAL fluids. Plasma protein binding was measured using a microcentrifugation system. For most preruminant and ruminant calves, the concentration-time profile of the central compartment was best described by a 2-compartment open body model. For some calves, a third compartment, was also observed. The time to maximum concentration in the plasma was longer in preruminating calves (3.1 h) versus ruminating calves (1.4 h). Clearance (CL/F) was 385.15 and 535.11 mL/h/kg in preruminant and ruminant calves respectively. Ruminant calves maintained higher ISF/plasma concentration ratios throughout the study period compared to that observed in preruminant calves. Potential reasons for age-related differences in plasma concentration-time profiles and partitioning of the drug to lungs and ISF as a function of age are explored.
**Introduction**

Danofloxacin is a synthetic fluoroquinolone antibiotic licensed for use in cattle in the United States for the treatment of bovine respiratory disease (BRD) associated with *Mannheimia haemolytica* and *Pasteurella multocida*. It exerts efficacy against Gram-negative and Gram-positive bacteria by inhibiting bacterial DNA gyrase and topoisomerase, thereby blocking DNA replication and effectively killing the bacteria (Drlica, 1999).

Extra-label drug use of antibiotics in food producing species has led to concerns over an increase in the selection of resistant microbial strains, with the potential for horizontal gene transfer and for the spread of resistant strains to other animal and human pathogens (Martinez et al. 2014). In situations when extra label use is a necessity, it is essential that the prescribing practitioner select a drug that is appropriate for the targeted pathogen, the administered dose is safe, and the selected dosing regimen is consistent with achieving a systemic drug exposure profile that is consistent with efficacy. Integral to defining an appropriate dosing regimen is an understanding of the pharmacokinetics (PK) of that drug in the targeted patient population and tissue compartments.

Danofloxacin is approved for use in ruminating calves but does not currently have an approval for veal or preruminating calves. While one may consider defining the dose in younger calves simply by adjustments based on body weight, such dose adjustments fail to recognize potential age-associated differences in drug PK. Therefore, the PK of danofloxacin in preruminant and ruminating calves needed to be examined.

Several reports have described the age-associated bovine PK for a variety of drugs. Considering the findings associated with sulfamethazine (Nouws et al., 1986), phenylbutazone (Volner et al., 1990), ceftiofur sodium (Brown et al., 1996) and meloxicam (Mosher et al., 2011),
there appears to be a trend towards a lower total systemic clearance (CL) and larger volumes of
distribution at steady state (Vss) in neonatal calves as compared to adults (approximately 6
months of age). However, these relationships may not be universal as the Vss of the
fluoroquinolone enrofloxacin was suggested to be smaller in one day old calves as compared to
one week old calves (Kaartinen et al., 1997).

Additional considerations of pharmacodynamics (PD) and disease progression need to be
factored into dose evaluation for neonatal calves. Since the immune system of these calves are
not fully developed, it is preferable to use drugs with bactericidal activity. This is one of the
reasons why fluoroquinolones such as danofloxacin are particularly attractive since they are
known to exert concentration-dependent action (Lees and Shojaee Aliabadi, 2002). Typically,
fluoroquinolone efficacy has been linked to the extent of exposure over a 24-hr period (Area
Under the Curve, AUC\textsubscript{0-24h}) at steady state relative the minimum inhibitory concentration (MIC)
of the targeted pathogen (AUC/MIC) and to the ratio of peak plasma drug concentrations (C\text{max})
to the minimum inhibitory concentration (C\text{max}/MIC) (Andes & Craig, 2002). Consistent with
this pharmacokinetic/pharmacodynamic (PK/PD) relationship, danofloxacin has been shown to
achieve high concentrations in lung tissues, with rapid penetration into the pulmonary fluids of
calves with respiratory disease (Terhune et al., 2005). However, the precise PK/PD target
necessary to achieve the desired clinical outcome may not be identical for ruminating calves
versus preruminant calves. Potential reasons for such age-related differences include a
dissimilarity in the partitioning of drug from the blood to the infection site or the possible need to
achieve a greater microbiocidal activity in the presence of the yet immature immune system (i.e.,
greater reliance on the drug versus the host response).
The objective of this study was to compare the drug concentrations (free and total) in the plasma versus concentrations in the ISF and PELF of healthy preruminating (3-week old) and ruminating (6-month old) calves. It is important to acknowledge that the extra label use of fluoroquinolones in major food producing species is prohibited by law. However, it was selected for use in this study because it is approved for use in ruminating calves, has well-defined pharmacokinetic/pharmacodynamic (PK/PD) characteristics and the importance of the use of peak concentration and extent of drug exposure for determination of its antimicrobial effects needed to be examined. By providing more data on the effect of age on PK parameters in preruminant calves, these results could provide the scientific foundation upon which to base assessments of potential future applications for the use of antimicrobial compounds for use in neonatal calves.

**Materials and Methods**

**Animals**

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee. Weaned and unweaned male Holstein calves were bought from the North Carolina State University Dairy herd. Eight unweaned Holstein calves, 2-3 weeks of age, weighing between 41-53 kg were classified as preruminants. These calves were housed in individual hutches at the University dairy, were fed commercial milk replacer twice a day, and had free access to water and calf starter ration ad libitum throughout the study. Eight weaned calves, 6 months of age and weighing between 151-214 kg at time of study, were classified as ruminating calves. The calves were group housed indoors on a concrete floor bedded with wood shavings. Ruminant calves were maintained on grass hay and water *ad libitum* and were supplemented with grain. None of the calves had any previous history of disease or antibiotic
administration and had normal physical examinations prior to start of the study. The extra
label use of fluoroquinolones is expressly prohibited in food producing animals in the United
States and these calves were not allowed to enter to human food supply at the completion of the
study.

**Drug Administration and Blood Collection**

All calves were weighed on a digital scale on the morning of the study commencement
for determination of the administered dose. Approximately 24 h prior to start of the study, calves
were restrained for intravenous catheter placement. The area where the catheter was to be placed
was clipped and cleaned with alternating scrubs of chlorhexidine and isopropyl alcohol. Using
sterile technique, a 14 G x 3.25 mm catheter (AngiocathTM, BD, Franklin Lakes, NJ, USA) was
inserted into the right jugular vein with an extension set and sutured to the skin using a 2-0
monofilament suture. Catheters were flushed three times a day using 6 mls of 10 units/ml of
heparin saline. A single s.c. injection of danofloxacin (8 mg/kg) (AdvocinTM; Zoetis, Florham
Park, NJ, USA) was administered to each calf in the neck per label instructions. Blood samples
were taken from the jugular vein and were transferred to lithium heparinized tubes at 0
(pretreatment), 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 and 96 hours post administration of
danofloxacin. These samples were stored on ice until centrifugation at approximately 3500 g for
20 min to collect plasma. The plasma samples were stored -80°C until analysis.

**Interstitial Fluid Collection**

All calves were implanted with s.c. ultrafiltration interstitial fluid (ISF) probes on the side
of the neck opposite site of s.c. injection (BASI Inc, West Lafayette, IN). Each probe contained
three semi-permeable loops connected to a non-permeable tube that extended outside the animal
and attached to a 3 ml no additive plastic vacutainer tube. This tube provided negative pressure
for fluid collection through small pores in the probe membranes. These pores allowed for the movement of water, electrolytes and low molecular weight molecules (<30,000 Da) to pass into collection tube. This pore size excludes large molecules such as proteins, protein bound drugs, and cells. Probes were placed with calves under sedation with xylazine [Rompun® Injectable (20mg/ml); Bayer Animal Health Division] at a dose of 0.05-0.1 mg/kg in the cervical neck muscles. Probes were placed twenty four hours before the start of the trial to allow them adequate time to equilibrate. One probe was inserted into each calf subcutaneously in the area cranial to the scapula. All probes were placed through a small stab incision using an introducer needle provided by the company. The ISF was collected at 0 (pretreatment), 2, 4, 8, 10, 12, 24, 48, 72 and 96 h after s.c. administration of danofloxacin. Since each ISF sample represents fluid collection over a certain amount of time (i.e. not instantaneous sample), a lag time was calculated based on the length of the tube and fluid collected over time for each sample. The fluid collected was frozen at -80°C until analysis.

**Lung Fluid Collection**

In order to determine drug concentrations in PELF, a bronchoalveolar lavage (BAL) was performed using a method described previously (Poulsen and McGuirk, 2009). Briefly, BALs were performed in all preweaned calves using a sterilized, flexible 10 French X 36 inch catheter with a 3-cc balloon cuff and in ruminating calves a 24 French X 59 inch catheter was used (Mila International, Inc. Medical Instrumentation for Animals, Florence KY). At each time point, the calf was restrained and the head and neck of the calf were extended to facilitate passage of the sterile BAL catheter. The BAL catheter was introduced into the ventral meatus of the nose through which it was advanced down the trachea until it was wedged in a terminal bronchus. Repeated coughing was used as an indicator of appropriate placement. In the wedged position,
the balloon cuff was inflated to create a seal and the catheter was held firmly in place while the
guide-wire was removed. At each time point, 100 ml of sterile saline were infused into the lungs.
Immediately after the infusion, negative pressure was applied to aspirate fluid. The volume of
fluid that was retrieved ranged from 0 to 55 ml of clear to mildly turbid foamy fluid. The fluid
sample was placed into a sterile collection tube, the total amount recorded and placed on ice until
centrifugation. The BAL samples were centrifuged at 300 g for 10 min and supernatant fluid was
separated from cell pellet and frozen at -80°C until analysis.

Drug Analysis

Plasma and BAL fluid were analyzed by high-performance liquid chromatography
(HPLC) with fluorescence detection following solid phase extraction (SPE). The ISF was
injected directly onto the HPLC. Danofloxacin was extracted from plasma and BAL supernatant
using a method described previously by Davis et al (2007). Briefly, a 3 ml/60mg HLB cartridge
(Waters Corporation, MA) was conditioned and equilibrated with 2 ml of methanol followed by
2 ml of water. A 500 µl of plasma sample was added to cartridge followed by a wash step using
2 ml of 95:5 water:methanol. The drug was then eluted into glass tubes with 1 ml of methanol
followed by another 1 ml of methanol. The eluted sample was evaporated to dryness under
nitrogen at 40°C and reconstituted in 500 µl of mobile phase [water:methanol with 0.1% tri-
fluoroacetic acid (85:15, v:v)] for plasma and 250 µl for BAL samples for HPLC analysis.
The extracted plasma and BAL samples were analyzed using a Waters XBridge C18 3.5µm (4.6
x 100mm) HPLC column (Waters Corporation, MA) at 800 µl/min with an injection volume of
25 µl. Danofloxacin was detected using fluorescence (W2475) at an excitation wave length of
280 nm and an emission wave length of 400 nm. Validation standards were prepared over a
linear range for each matrix (plasma, ISF, sodium chloride 0.9%, mobile phase) and were used to
construct calibration curves. These standards were validated over the range 0.001-5.0 µg/mL in fortified (spiked) blank plasma, BAL and ISF with danofloxacin (reference drug standard was provided by Zoetis) to validate the HPLC analysis. Over the validated range, the percent coefficient of variation (%CV) for inter and intra-day averaged 10.7% with an average recovery of 95.9%. The limit of quantification (LOQ) was 5 and 10 ng/ml and the limit of detection (LOD) was 1 and 5 ng/ml for plasma and BAL, and ISF respectively.

Danofloxacin Concentrations in PELF

Estimation of the amount of PELF sampled by BAL fluid was performed using the urea dilution method as described previously in cattle (Giguère, S. et al. 2011). Urea nitrogen concentrations in serum (UreaSERUM) and in BAL fluid (UreaBAL) were determined by use of a urea test kit (Urea test kit; Sigma Chemical, St Louis, MO, USA) and the absorbance values measured by use of a spectrophotometer. The volume of PELF (VPELF) in BAL fluid was derived from the following equation:

\[ V_{PELF} = V_{BAL} \times \left( \frac{Urea_{BAL}}{Urea_{SERUM}} \right) \]

in which \( V_{BAL} \) is the volume of recovered BAL fluid. The concentration of danofloxacin in PELF (DANOPELF) was derived from the following relationship:

\[ DANO_{PELF} = DANO_{BAL} \times \left( \frac{V_{BAL}}{V_{PELF}} \right) \]

in which \( DANO_{BAL} \) represented the measured concentration of danofloxacin in BAL fluid.

Plasma Protein Binding

Pooled plasma was collected from 5 healthy calves that have not received danofloxacin. Plasma aliquots of each age of calf was spiked with danofloxacin, generating samples in triplicate with concentrations of 0.01, 0.1 and 1.0 µg/mL, 10.0 µg/mL (Davis et al., 2007). All samples were allowed to stand at room temperature for 30 minutes. A 1 ml sample of each
standard was added to the microcentrifugation device and was centrifuged at 4000 g for 10 minutes. The ultrafiltrate was analyzed using HPLC without extraction to determine unbound concentration and protein binding of danofloxacin was then determined. Non-specific binding of danofloxacin was determined to be minimal in the microcentrifugation device and filter.

**Pharmacokinetic Analysis**

Unless otherwise indicated, all PK parameter values are expressed relative to the unbound danofloxacin concentrations taking into account the nonlinearity in protein binding as a function of drug concentrations.

Plasma concentrations of danofloxacin were analyzed using a computer software program (Phoenix® WinNonlin/NLME, Version 1.3 Certara, U.S.A. Inc. Princeton, NJ). Non-linear mixed effects modeling of danofloxacin concentrations was performed. The selected model was parameterized by clearance and estimated volume of distribution and absorption rate constant. Model selection was based on goodness of fit plots, statistical significance between models using lowest Akaike information criteria (AIC) values obtained in Phoenix® software and coefficient of variation (CV%) of the parameter estimates. An examination of covariates was performed to determine if there were any factors that may explain the variability in Cl/F, V/F and/or Ka. Covariates investigated included age, weight and physiological status (preruminant vs ruminant). No covariates were found to significantly improve model predictions for any parameter for danofloxacin. A student’s t-test from compartmental values for age differences in the two groups of calves was performed to confirm the results obtained from NLME.

Compartmental modelling was performed to determine PK parameters in each age group. In order to select between a one-, two- or three-compartment open body model, the AIC and the highest $R^2$ values, were evaluated. A 2-compartment model was determined to provide the best
fit and therefore was the model used to describe the danofloxacin concentration-time curve for each calf. Parameter estimates were obtained by minimizing the sums of the weighted deviations using a weighting factor of $1/Y_i^2$, where $Y_i$ is the observed plasma concentration at time $i$. For the selected model, $R^2$ values ranged from 0.81-0.99 for preruminant calves and from 0.87-0.98 for ruminant calves.

Noncompartmental analysis (NCA) was also performed to generate estimates of the area under the curve (AUC), maximum plasma concentrations ($C_{\text{max}}$), time to $C_{\text{max}}$ ($T_{\text{max}}$) (plasma and tissue fluid) and the terminal elimination half-life ($t_{1/2}$) (plasma). The AUC was estimated from time 0 to the last measured concentration (as defined by the LOQ) using the log-linear trapezoidal method. Values of $t_{1/2}$ were generated using $0.693/\lambda z$ where $\lambda z$ is the slope of the log-linear portion of the terminal depletion phase (terminal portion of the curve out to 96 hours for preruminant calves and 72 hours for rumiannt calves. Slopes were also estimated from $T_{\text{max}}$ to hr 24 postdose to explore the potential difference for bias in the half-life estimate due to the slower input rate preruminating versus ruminating calves.

All PK parameters were reported as a mean ± SD. Since the $t_{1/2}$ was estimated as $0.693/\text{mean } \lambda z$, the $t_{1/2}$ values are reported as harmonic mean ± standard deviation (SD). The SD was estimated using the Delta method, where:

$$SD = HM^2 \times \left[ \frac{\sum_{i=1}^{n}(Hi - MH)^2}{n - 1} \right]$$

where MH is the mean of the harmonic values (MH) for

$$\frac{\sum_{i=1}^{n} \frac{1}{X_i}}{n}$$
Hi is the harmonic value (λz) and HM is the harmonic mean being reported (t₁/₂).

ISF/Plasma concentration ratios at each time point were determined for each calf using the concentration of ISF (unbound drug) to the total (bound and unbound) and unbound danofloxacin plasma concentration. Statistical analyses were performed on the mean PK parameters of preruminant vs ruminant calves using Sigma Plot (Systat Software, Inc, San Jose, CA, USA); P-values of ≤0.05, were considered statistically significant. The normality assumption was tested for each variable set with the Shapiro–Wilk W test, which is the preferred method for testing the normality of data when the sample size is small (Ghasemi and Zahediasl., 2012). NLME modeling of the PELF data was unsuccessful as the data was sparse.

Due to missing values and the sparse nature of the dataset, PELF was evaluated using a non-compartmental analysis from the perspective of concentrations as a function of sampling times and the corresponding averages of those concentrations at each time point for the ruminant and preruminant calves. NLME modeling of the PELF data was unsuccessful as the data was sparse.

Results

There were no adverse reactions noted from placement of ultrafiltration devices, catheters, procedures or from the danofloxacin administration. Plasma was obtained at each time point. The 96 hour sample for ruminant calves was mislabeled and could not be matched with an individual calf and was excluded from analysis. Therefore, ruminant calves profiles were truncated to hr 72 postdose. Over some sampling intervals, fluid was not collected into the ISF probe or the probes were pulled out by the calves. Therefore, ISF data are missing at these time points. There was no significant binding of danofloxacin to the ultrafiltration probe.

1Feiveson AH (2005). What is the delta method and how is it used to estimate the standard error of a transformed parameter?: Explanation of the delta method, http://www.stata.com/support/faqs/statistics/delta-method/
Several challenges were encountered during efforts to capture the BAL fluid. Irrespective of age, BAL fluid volumes collected ranged from 0 to 55 ml. At the 2-hr sample for preruminant calves, the lavage yielded no lung fluid for 3 of the calves. Greater success was achieved at the 12 and 24-hr time points where fluid could not be aspirated from only one preruminant calf each of these timepoints. The variability of fluid return with the BAL procedure in the preruminant calves could be attributed to anatomical differences and the corresponding difficulties in achieving proper placement of the catheter in the terminal bronchus.

*Plasma*

Although drug samples at the 96 hr could not be included in the analysis for the ruminating calves, the use of the compartmental analysis enabled an analysis of values to time infinity in all subjects. To insure that assessments of CL/F and Vd/F are not biased by the loss of the area defined by the 72 to 96 hr trapezoid, these estimates could not be reliably compared on the basis of data generated by the NCA, but were extrapolated out to 96 hours for both groups of calves in the compartmental analysis.

Age-associated differences were observed in several PK parameters. The AUC and $T_{\text{max}}$ values tended to be greater in the preruminant as compared to ruminating calves (Table 1). Using the compartmental analysis, $K_{01}$ (absorption rate constant) values tended to be lower in preruminants versus ruminants (Tables 1 and 2).

Despite the observed differences in mean values as a function of age, the $\text{AUC}_{0-96}$ was not statistically significant between the two groups, which likely reflects a high degree of variability and the limited number of study subjects per age group. The elimination rate constant ($K_{10}$) tended to be smaller in the preruminants (slower elimination). The rate of drug distribution from the peripheral compartment back into plasma ($K_{21}$) values tended to be smaller (slower
partitioning back into the plasma) in the ruminating versus preruminating calves. As discussed later, these findings may be useful in explaining the observed relationship between ISF/plasma concentrations in ruminants versus preruminants. Compartmental parameter rate estimates in the individual ruminant and preruminant calves are depicted in Appendix Figure 2.

After s.c. administration, the maximum unbound danofloxacin concentration in plasma $C_{\text{max}}$ values, as generated via our compartmental analysis, achieved a value of 1.79 µg/mL for preruminant and 2.12 µg/mL in ruminant calves. The corresponding mean $T_{\text{max}}$ values were 3.125 and 1.44 hr, respectively. The mean terminal elimination $t_{1/2}$ was estimated as 20.36 hours for preruminant calves and 15.31 hours for ruminant calves (Table 1). These differences were not statistically significant. To ascertain if this difference may have been biased by drug input rates, slopes were also estimated from $T_{\text{max}}$ to 24 hr. In this case, the profile depletion rates were similar in pre-ruminating and ruminating calves, with corresponding rather than the terminal elimination phase, the $t_{1/2}$ estimates ranging from 4.23 – 10.16 hrs in the preruminant calves and 4.0 to 5.44 hrs in the ruminant calves (Table 3). These values were substantially less than the slopes observed in the terminal depletion phase.

A non-linear plasma protein binding was noted, with protein binding decreasing as concentration increased, ranging from 67 to 17% bound as danofloxacin concentrations varied from 0.01 to 10 µg/mL (Table 4). Importantly, at 10 µg/mL, the preruminant binding was substantially lower than that of the ruminant calves. $K_d$ and $B_{\text{max}}$ values were estimated using the plasma protein binding data in preruminant and ruminant calves using a graphic software (GraphPad Software, Inc, La Jolla, CA). The relationship between bound and free concentrations in plasma was best described by a saturable binding model using the following equation:
\[ C_{\text{bound}} = B_{\text{max}} \cdot \frac{C_{\text{free}}}{(C_{\text{free}} + K_D)} \]

Where \( B_{\text{max}} \) is the maximum amount of drug that is bound to in the plasma (maximum binding capacity) and \( K_D \) is the equilibrium dissociation constant which reflects the equilibrium concentration at which the drug meets half maximal binding capacity. \( B_{\text{max}} \) and \( K_D \) values showed differences in preruminant and ruminant calves. \( B_{\text{max}} \) value was estimated to be 6.09 µM (2.176 µg/ml) and 21.66 µM (7.740 µg/ml) and \( K_D \) values of 5.39 µM (1.925 µg/mL) and 24.88 µM (8.892 µg/mL) in preruminant and ruminant calves respectively. The unbound fraction was calculated using the free concentration, \( B_{\text{max}} \) and \( K_D \) values using the formula previously described (Toutain & Bousquet-Melou, 2002). The unbound fraction was not significantly different between the two groups (Table 4).

**Interstitial Fluid Data**

Using an in vivo ultrafiltration technique to collect ISF in repeated samples allowed for the monitoring of unbound drug disposition over time and was less invasive than tissue biopsies. Although some samples were missed due to occlusion of the probe or the calf movement around the hutches, these devices were well tolerated and collected between 0.05 µl to >2 ml of fluid for drug analysis.

The mean (±SD) plasma and ISF samples from both groups of calves are shown in Fig. 1 (n = 8 calves in each group). A comparison of ISF and plasma (bound and unbound) concentrations are shown in Fig. 2 for each group of calves. The \( T_{\text{max}} \) for ISF fluid occurred later than in plasma irrespective of calf age. Whether considering the ratios of ISF to total or free plasma danofloxacin concentrations, the ISF/Plasma concentration ratios tended to be higher in ruminant calves as compared to that seen in the preruminant calves (Fig. 3).
Pulmonary Epithelial Lining Fluid

Average PELF concentrations from the BAL samples were reported in Table 5 and are seen graphically in Figure 4. The maximum concentration in PELF was noted to occur at 2 hours post administration in both preruminant and ruminant calves, with estimated PELF concentrations far exceeding the concentrations seen in plasma and ISF. Greater variability in drug concentrations in PELF was seen in the preruminant calves as compared to ruminating calves.

Discussion

Danofloxacin is approved in the United States for the treatment of bovine respiratory disease in calves. It is not approved for use in cattle intended for dairy production or for calves to be processed for veal. In the United States, extralabel use of fluoroquinolone antibiotics is prohibited by law (Davis et al., 2009). Although the calves used in this study were Holsteins, these steers were not meant for veal production. They were all ultimately shipped to a feedlot to be finished as beef. Danofloxacin was chosen in this study as part of a larger project to compare the PK of different antimicrobials in both healthy and diseased calves of varying ages to examine what differences may be present related to age and physiologic development.

Half-Life comparisons

Danofloxacin is labeled to be given once s.c. at 8 mg/kg for the treatment of bovine respiratory disease. Past studies on the PK parameters of danofloxacin in 2-8 month old calves have reported $t_{1/2}$ values ranging from 2.65 – 7.4 hours when administered at 1.25 mg/kg intravenous (Friis, 1993., Giles et al., 1991, McKellar, Gibson et al., 1999, Shojaei Aliabadi and Lees, 2003). The reported mean plasma $t_{1/2}$ in this study was considerably longer than these other reported values. Using NCA, the $t_{1/2}$, for the first distribution phase (from 4 to 24 hours for
preruminant calves and 1-24 hr for ruminant calves) was very similar to those values reported in previous studies, indicating that the observed difference between our results and those reported by other investigators primarily reflected our ability to capture a second depletion phase. Most of previous studies reported total (unbound + bound) concentration, which may miss significant changes as a function of age in clearance, volume of distribution and other parameters. Since only unbound drug is free to move across barriers and is active to fight infections, reporting unbound concentrations is a more clinically significant when determining drug concentrations at the active sites.

All calves used in this study remained clinically normal throughout the study, but it has been noted previously that disease status may also affect the $t_{1/2}$ of danofloxacin. Apley et al. (1993) reported that crossbred steer calves with acute pneumonia had a $t_{1/2}$ of approximately 6.3 hours (Apley et al., 1993). Estimates of $t_{1/2}$ can be influenced by several physiological and experimental factors, including the experimental design (such as sampling time) and the composite effects of drug distribution and free drug clearance (Toutain et al., 2004). The ramifications of such differences as it pertains to unbound drug concentrations at the site of infection need to be considered from the perspective of the etiology of any age- or disease-associated change in drug PK.

Absorption Comparisons

While there were no statistically significant differences in the observed preruminant vs ruminant danofloxacin $C_{\text{max}}$ values, there was a significant difference ($P < 0.006$) in the time to maximum concentration ($T_{\text{max}}$). Preruminant calf peak concentrations were observed between hrs 2-4 in all but one calf. In contrast, the corresponding ruminant $T_{\text{max}}$ values ranged between 0.5 to 1 hr in all but 1 ruminating calf. The significant difference noted between these two age
groups of calves following s.c. injection was reflected in the fitted absorption rate constants (K01). From the NLME model, the reported K01 values was around 2.18 h⁻¹. Considering some of the physiological changes that occur during the maturation of neonatal humans (Batchelor and Marriott, 2015), the larger K01 values observed in the older calves could be a function of such maturation-induced differences as higher body temperature, body composition (lower tissue fluid volumes) and a greater local tissue blood flow as compared to that of neonates. With respect to the latter point, pediatric human patients have been shown to have relatively lower density of skeletal muscle capillaries than that in adults. This may have also contributed to the lower bioavailability of drugs after intramuscular and s.c. routes of administration in neonates (Carry et al., 1986). An increase in vascular perfusion to certain muscle groups increase with age and may impact drug absorption rates (Greenblat, 1976).

Plasma Protein Binding

Initially, protein binding was thought to potentially contribute to some of the kinetic differences observed between preruminant vs ruminant calves. Since only the unbound fraction can reach the site of action, the distribution and availability are greatly influenced by the degree of protein binding of certain drugs. Frequently, the unbound fraction of many drugs is higher in human neonates and infants as compared to that of adults because of their lower concentration of binding proteins (Kearns et al. 2003). Coupling this difference in protein with a large extracellular fluid volume may account for the larger drug volume of distribution occasionally observed in neonates.

In the current study, within the range of concentrations observed in the plasma (0.01 to 1.0 µg/mL), only small differences in plasma protein binding were observed as a function of age. Therefore, plasma protein binding is unlikely to have had a significant impact on the age-
associated PK differences observed in this study. This conclusion is supported by examining the similarity in the relationship between total vs free drug concentration/time profiles in preruminant versus ruminating calves (Figure 1). Similar estimates of danofloxacin protein binding were reported in buffalo calves (36% at concentrations ranging from 0.0125 μg/ml to 1 μg/ml). (Sappal et al., 2009). A strong non-linear binding has already been reported by Friis et al (Friis, 1993) not only in plasma, but also in bronchial and nasal secretions.

Although average protein binding was similar in the linear range of 0.01 to 1 μg/mL, percent protein bound tended to be higher in ruminant calves at 10 μg/mL. The origin of these differences were not investigated, however, in human pediatric patients, a decrease in gestational age was directly proportional with a decrease in binding capacity of albumin, but did not affect the binding affinity to albumin (Bender et al., 2007). There was a lack of fit of the algorithm used to estimate $B_{\text{max}}$ and $K_D$ at lower concentrations which was noticed in the predicted and observed values for both groups (Table 4). This could reflect that the danofloxacin binding may be better defined by a different equation than was used. Differences were noted between the two age groups suggests a binding capacity and efficiency changes as a function of age- but the validity of such assumptions is the subject of ongoing research. Although plasma protein binding of fluoroquinolones is usually associated with albumin, there is some evidence that this group of drugs may bind to other binding proteins, including α1-acid glycoprotein (Barbato et al., 2007). In calves, increase in α1-acid glycoprotein concentrations are seen over the first 30 days of life which could explain differences noted in $B_{\text{max}}$ and $K_D$ values in these two age groups (Tothova et al., 2015). When unbound concentrations tend towards the number of available binding sites, protein binding becomes concentration dependent (i.e. saturable binding). Clinical differences in PK were not observed in this study. In future studies, alternative algorithms for describing the
relationship between drug concentration and free fraction should be explored.

Based upon the PR and R plasma concentrations noted in this study, assessments of the potential impact of protein binding on danofloxacin PK should be limited to a range of 0.01 – 1.0 µg/mL. Based upon the in vitro data generated within this concentration range, there were no differences in the free fraction as a function of age. For this reason, so long as the danofloxacin blood levels remain within this concentration range, age-associated differences in protein binding should have negligible influence on danofloxacin clearance of distribution kinetics.

**Clearance and volume of distribution comparisons**

The CL/F values of danofloxacin did not differ significantly between ruminant and preruminant calves. Assuming bioavailability (F) was very similar, clearance values (578 mL/kg/hr) determined in 11-13 week old calves were similar to those reported in the current study (Sarasola et al., 2002). At this concentration and dose range, the clearance does not appear to change, implying that a change in dose would not change PK parameters. Although significance was not seen when comparing 3 week old vs 6-month old calves, maturation differences of clearance values in younger calves (less than three weeks of age) may impact the intrinsic clearance and elimination of danofloxacin.

In urine, unchanged danofloxacin accounted for 88-94% of total drug, while the desmethyl metabolite accounted for the remainder (Pfizer Inc, 1989a). At birth, both phase I (primarily oxidation) and phase II (conjugation) metabolic enzymes in the liver may be immature, as well as lack of functional tubular transporters in the kidneys. Maturation of different elimination pathways (including liver and kidney) may impact drug metabolism and clearance and may explain why the impact of age on the reported mean half-lives vary depending on the class of drugs studied (Reiche et al. 1980; Nouws et al. 1983). The increase in glomerular
filtration rate (GFR) in neonates during the first few weeks of life is mainly due to an increase in renal blood flow, which would increase the clearance values of drugs eliminated by renal pathways as calves mature (Baroni et al. 2008).

Statistical significance was not achieved in the age comparison of CL/F, Vd/F or of t1/2 values. It is likely that the lack of statistical significance was largely attributable to study size. If the study was powered differently (contained a larger number of study subjects), statistically significant differences in these parameters may have been detected.

**Interstitial Fluid/Plasma Ratio Comparisons**

The observed PK differences in neonatal versus mature calves led to a question on how these age-related differences may influence the relative concentration vs time profiles (free or total) in the ISF or PELF vs plasma. With respect to the ISF, this relationship was described both by the total or free danofloxacin concentration versus time profiles in plasma. ISF/plasma ratios were examined before and after correcting the plasma concentrations for protein binding, taking into account the non-linearity of plasma protein binding reported in both groups of calves (Figure 3). Since the plasma total drug concentrations exceed the free drug concentrations, in plasma the ISF/plasma ratios are greater when considering the ISF concentrations (which are free values) to the free versus to the total plasma drug concentrations (Figure 3). Although the depletion phase ISF concentrations were consistently higher than the plasma concentrations for both age groups, after an initial lag time, the ISF concentrations tended to more closely follow the total plasma concentration vs time profile in preruminants than it did in ruminants (Figure 2).

The observation that the best fit was a 2-compartment model is inconsistent with the suggested presence of a deep compartment in some ruminant calves. Upon examining the data,
although there is a suggestion of a change in slope (which would be consistent with a third, or deep, compartment), there were an insufficient number of time points to adequately characterize that final depletion component. However, based upon ISF data and visual examination of the profiles, the authors believe that a deep compartment was in fact present.

Differences were noted in the shapes of the ratios of ISF/plasma in preruminants versus ruminants as a function of time (Figure 3). For the preruminants, the ratios peaked at 24 hours but then tended to remain relatively consistent through the remaining sampling times as compared to ruminant calves. In contrast, the ratios continued to decline over the duration of sampling period in ruminant calves. There are several factors should be considered when trying to understand the observed ISF/plasma relationships:

1) The method used for capturing the ISF reflects an averaging of concentrations over a collection period. Accordingly, when estimated at the time of fluid sampling, the measured concentration will exceed the actual concentration of drug in the ISF at any given moment in time. Thus the use of ultrafiltration tends to over-estimate ISF concentrations at a point in time. However, with the longer interval over which the ISF samples were collected toward the terminal portion of the curve, the “averaging” effect would be expected to increase and not decrease the ISF/plasma ratios over time (i.e., a magnification of the experimental error). It is unlikely that this confounding factor was responsible for the observed decrease in the ISF/plasma ratios as a function of time in ruminants.

2) The concept of rate-limiting factors needs to be considered. In this regard, the trend towards slower Cl/F (and K10) and faster K21 values in preruminant as compared to that in ruminants can lead to a greater similarity in ISF vs plasma concentrations in preruminant versus ruminating calves. More explanation on calculating transfer rate differences are
discussed further in the supplemental appendix.

3) A potential physiological basis for this difference in shapes of the ruminant versus preruminant ISF/plasma ratio profiles may be related to the higher amount of body fat in the older animals. Since danofloxacin is a lipophilic drug, it could more easily distribute into the body fat of older animals. The body fat could then serve as a “deep compartment” whose effects would be evident primarily at the very low drug concentrations. Adipose tissue in cattle has been shown to express α1-glycoprotein, which may bind to danofloxacin in the tissues (Rahman et al., 2015). This is consistent with the smaller K21 values seen in the ruminating vs pre-ruminating calves. When this deep compartment becomes the rate limiting factor in the elimination of drug from the central compartment, the depletion of drug from the plasma (CP) becomes slower than was observed at earlier timepoints. Simultaneously, with regard to the ISF, with the K12 being similar to that of the K21 in ruminating calves, the ISF concentrations temporarily declines at a rate slightly faster than that of the blood (as was seen on the average values plotted in Figure 1). In view of this proposed scenario, the decrease in ISF/plasma would not be as evident in the preruminants both because of slower K10 values relative to other intercompartmental rate constants and because of the lower amount of body fat would limit the influence of this potential “deep compartment”. With these similar K12 values but smaller K21 values in ruminating versus preruminating calves, one might expect that the danofloxacin Vd/F values would have been larger in the older calves. However, similar values were observed as a function of age (with mean values of preruminants exceeding that of the ruminating calves). It is likely that this apparent inconsistency is attributable to the magnitude of variability observed across individual calves.
**Potential PK/PD Targets**

Ultimately, one of the goals of this work is to facilitate the use of plasma concentration vs time profiles as a mechanism for generating age-associated dose adjustments. However, this assumes that preruminant and ruminating calves have identical PK/PD targets. It is now recognized that the use of targeted plasma concentrations as the PK/PD metric for dose evaluation is best when it is linked to the outcome of clinical trials (Ambrose et al., 2001). However, within veterinary medicine, practitioners often rely upon prior literature-based targets and an assumption that the plasma concentrations in normal healthy animals reflect the free (active) drug concentrations at the site of infection. Therefore, as part of this study, sampling of the PELF should relate to the concentration of free (active) drug in lung fluids. Past methods for determining lung concentrations in many veterinary species include biopsies, bronchial microsampling probes and bronchoalveolar lavages (BAL) and tissue homogenate (Winther, 2012., Giguère et al., 2011, Menge et al. 2011, Bidgood and Papich, 2005.; Davis et al., 2006, Davis et al., 2007, Messenger et al., 2012, Warren et al., 2014). Tissue homogenate represents a composite of cells, plasma, interstitial, pulmonary epithelial lining fluids as well as tissue concentrations, which may falsely increase the estimates of drug concentration at the specific sites of infection and lead to incorrect clinical drug distribution conclusions (Mouton et al. 2008). Lung homogenate does not allow for the differentiation between intracellular vs. extracellular drug concentrations, or the degree of binding that may have occurred between drug and pulmonary tissues (Gonzalez et al., 2013). However, for many veterinary species, cost and availability for many of these techniques limits their use in PK studies.

The PELF is secreted extracellularly in the respiratory tract and is a potential site for bacterial colonization, making it an ideal target for evaluating the relationship between plasma...
PK/PD versus target site exposure in pneumonia in calves and for designing dosage regimens for therapeutic purposes in calves. To obtain these data, a BAL technique was used to harvest PELF. The observed higher concentrations in PELF as compared to plasma and ISF most probably reflects methodological associated biases in PELF danofloxacin concentrations. In this regard, several limitations with this method have been described in literature, including under- and/or overestimation of drug concentrations in this matrix. The volume of PELF harvested from the BAL fluid is usually estimated using a dilution marker such as urea. When urea is used as a dilution marker, the dwell time of fluid infused in the airways during the BAL procedure may represent an important source of error and experimental variability (Baldwin et al., 1992; Dargaville et al., 1999). Several other limitations include overestimating drug concentrations in PELF due to potential ‘contamination’ of the fluid from cells, the dilution factor caused by repeated infusion of large amounts of fluid in the airway, and the problems with using the “urea correction method to adjust for this dilution. Other studies performed in larger calves showed PELF concentration measurements are inherently highly variable and affected by the method of collection.

Lung microdialysis method may offer a better overall determining free drug concentration in the PELF (Kim and Schentag, 2008). Previous studies using microdialysis have correlated free antibiotic concentrations in lungs with unbound concentration in muscle tissue (Marchand, Dahyot et al., 2005). Moreover, recent evaluation of PELF concentrations of ciprofloxacin and enrofloxacin in mature calves showed a PELF/plasma ratio of about 40% based upon PELF values harvested via bronchial absorptive swabs (Foster et al., 2016). Nevertheless, since any error-associated bias attributable to our method of PELF sampling would be expected to equally influence the data generated in all of the study animals, conclusions derived from the comparison
of danofloxacin lung concentrations of age are expected to be valid.

When comparing the mean PELF concentrations in ruminants versus preruminants, it would appear that the concentrations tended to be markedly higher in the neonatal calves. However, when examining the individual subject data (Figure 4), it is evident that such conclusions may be incorrect. The range of values observed in the preruminating and ruminating calves was very large, and the reliability of the means was further compromised by missing datapoints, particularly in the preruminant treatment group. Therefore, the PELF values should be considered as only a rough approximation of pulmonary drug concentrations. Particularly in the preruminating calves where substantial technical difficulties were encountered, it may be necessary to identify some alternative procedure for estimating active drug concentrations in the lung.

The measurement of the unbound danofloxacin in ISF and PELF is ideal since most respiratory pathogens seen in calves are extracellular bacteria. By measuring the drug concentration in the active sites of infection, a more accurate conclusion of clinical efficacy can be determined. Many papers report the use of specific target ratios using AUC and $C_{\text{max}}$ values to determine clinical efficacy (Tessman et al. 2011). The problem with extrapolating these ratios from ruminating calves to preruminant calves is that targets to achieve efficacy may vary between the two age groups. The host immune competency as well as bioburden at the site of action may differ between these two groups and more research is needed to determine age effects on clinical efficacy, especially considering younger calves may not have a fully mature immune system.

A limitation in the current study is relying on the use of the statistical t-test to determine significance. Since preruminant calves have a larger variability, there is the possibility that there
was an underlying violation of homogeneity of variances across age groups for the parameters undergoing evaluation. Furthermore, given the large variability, the power to detect statistical differences was low. In this regard, a larger sample size for each group would have helped to achieve a better study power.

Conclusions

Trends towards differences in CL/F, rate of drug absorption, Vd/F and t½ were observed as a function of calf age. However, the lack of study power prohibited the detection of statistically significant differences. A deep or third compartment, similar to previous studies, was detected and was most evident in the ruminating calves. This contributed to age-associated differences between the relationships of ISF/plasma over time.

The ability to quantify drug concentrations out to 96-hrs postdose and the successful utilization of in vivo ultrafiltration and BAL techniques provided the opportunity to obtain unique insights into potential factors responsible for age-associated differences in danofloxacin PK in calves. Although efforts to explain the source of these differences remain speculative, it has identified additional questions that need to be resolved. Specifically, if an objective is to use data derived from ruminating calves to support approvals in preruminating animals, it is evident that further examination is needed to determine if there are differences in the impact of disease on drug PK and the relationship between drug exposure versus response. In addition to clinical reasons for resolving these issues, the alteration of drug PK as a function of age and disease could impact withdrawal time considerations.

There are limited data in veterinary species addressing pediatric specific PK data and these results raise several questions about the physiological changes that may impact PK in animals of various age, as well as disease state. Further studies are warranted to compare concentrations in
PELF and ISF of diseased vs healthy calves, and the impact of these relationships on drugs presenting with distributional characteristics that differ from those associated with the fluoroquinolones.

Acknowledgments

The authors would like to thank the staff at the Lake Wheeler Dairy at NC State University for their help with this project, Dr. Jennifer Halleran and Ginger Hobgood with sample collection, Dr. Jennifer Davis for help in PK data analysis and Jenna Schrimer for her help with assay development.
Table 3.1 Danofloxacin Plasma pharmacokinetic parameters in preruminating vs ruminating calves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Preruminating Calves</th>
<th>Ruminating Calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0-96}$ (h*ug/mL)</td>
<td>23.23 10.07</td>
<td>15.17 1.83</td>
</tr>
<tr>
<td>Cl/F (mL/h/kg)</td>
<td>385.15 132.15</td>
<td>535.11 66.83</td>
</tr>
<tr>
<td>C$_{max}$ (ug/mL)</td>
<td>1.79 0.43</td>
<td>2.12 0.53</td>
</tr>
<tr>
<td>t½ (h)</td>
<td>20.36 2.46</td>
<td>15.31 4.97</td>
</tr>
<tr>
<td>T$_{max}$ (h)</td>
<td>3.13* 1.25</td>
<td>1.44* 1.11</td>
</tr>
<tr>
<td>Vz/F (mL/kg)</td>
<td>4125.01 3842.5</td>
<td>2693.8 1709.3</td>
</tr>
</tbody>
</table>

Table 1. Mean pharmacokinetic parameters from 2-compartment model after single S.C. injection of 8 mg/kg danofloxacin in 3 week old vs 6 month old calves. Half life parameter is reported as harmonic mean. *Indicates significantly different by t-test (P <0.05)
Table 3.2 Danofloxacin K01 (absorption rate constant) for preruminant and ruminant calves from compartmental analysis

<table>
<thead>
<tr>
<th>Calf #</th>
<th>Preruminant (1/hr)</th>
<th>Ruminant (1/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.73</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td>0.52</td>
<td>19.5</td>
</tr>
<tr>
<td>3</td>
<td>0.90</td>
<td>2.8</td>
</tr>
<tr>
<td>4</td>
<td>0.91</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>2.2</td>
<td>24.3</td>
</tr>
<tr>
<td>6</td>
<td>0.81</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>0.69</td>
<td>24.5</td>
</tr>
<tr>
<td>8</td>
<td>0.89</td>
<td>10.8</td>
</tr>
</tbody>
</table>
Table 3.3 Non-compartmental analysis to determine terminal half life after injection of 8 mg/kg danofloxacin in 3 week old vs 6 month old calves

<table>
<thead>
<tr>
<th>Calf #</th>
<th>Preruminant (4-24 hours)</th>
<th>Ruminant (1-24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.43</td>
<td>5.44</td>
</tr>
<tr>
<td>2</td>
<td>10.16</td>
<td>4.62</td>
</tr>
<tr>
<td>3</td>
<td>7.07</td>
<td>4.75</td>
</tr>
<tr>
<td>4</td>
<td>6.02</td>
<td>4.64</td>
</tr>
<tr>
<td>5</td>
<td>5.29</td>
<td>4.00</td>
</tr>
<tr>
<td>6</td>
<td>4.23</td>
<td>4.45</td>
</tr>
<tr>
<td>7</td>
<td>5.66</td>
<td>4.08</td>
</tr>
<tr>
<td>8</td>
<td>4.64</td>
<td>4.38</td>
</tr>
</tbody>
</table>

Reported half life was calculated using the equation $0.693/\lambda_z$ value for the first distribution phase after $C_{\text{max}}$ is reached for each group of calves.
Table 3.4 Average % Plasma protein binding of Danofloxacin using microcentrifugation

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Preruminant Mean</th>
<th>Ruminant Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>68.3</td>
<td>65.6</td>
</tr>
<tr>
<td>0.1</td>
<td>67.0</td>
<td>63.4</td>
</tr>
<tr>
<td>1.0</td>
<td>46.8</td>
<td>44.9</td>
</tr>
<tr>
<td>10.0</td>
<td>17.64</td>
<td>33.2</td>
</tr>
</tbody>
</table>

Percent plasma protein binding of danofloxacin spiked plasma from preruminant (3 week old) and ruminant (6-month-old) calves. Samples were spiked in triplicate at four concentration levels (0.01, 0.1, 1.0 and 10 µg/mL) and averaged.
Table 3.5 Pulmonary epithelial lining fluid danofloxacin concentrations in preruminant vs ruminant calves

<table>
<thead>
<tr>
<th>Sample Time (Post injection)</th>
<th>Preruminating Calves</th>
<th>Ruminating Calves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>2 hours</td>
<td>5.4</td>
<td>6.3</td>
</tr>
<tr>
<td>12 hours</td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.62</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Mean ± SD in PELF determined from bronchoalveolar lavage after single S.C. injection of 8 mg/kg danofloxacin in ruminant and preruminant calves
Figure 3.1 Average danofloxacin concentrations in ruminant calves PELF

Average danofloxacin concentrations in ruminant calves PELF. Dashed line represents the average PELF concentrations from ruminant calves. Two data points were omitted from analysis from calf 7.
Figure 3.2 Mean plasma (bound and unbound) ± SD and ISF concentrations after single S.C. injection of 8 mg/kg danofloxacin in calves. Preruminant (A) and ruminant calves (B) and average Interstitial fluid danofloxacin concentrations ± SD (C).
Figure 3.3 Mean total plasma (a) and unbound (b) and ISF concentration after single S.C. injection of 8 mg/kg danofloxacin in preruminant and ruminant calves.
Figure 3.4 ISF/Unbound plasma danofloxacin concentration ratios in ruminant and preruminant calves. Each point is a calculated geometric mean at each time point.
References


Chapter 4

The effect of age on the pharmacokinetics and distribution of tulathromycin in interstitial and pulmonary epithelial lining fluid in calves

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2018
Abstract

The aim of this study was to determine the influence of age on the pharmacokinetics (PK) of tulathromycin and its distribution into the interstitial fluid (ISF) and pulmonary epithelial lining fluid (PELF) of healthy Holstein calves following a single subcutaneous (SC) administration of a 2.5 mg/kg dose of tulathromycin Draxxin™. Plasma tulathromycin concentrations were determined using liquid chromatography-tandem mass spectrometry. The PK parameters associated with the plasma concentration versus time profiles were analyzed via a non-linear mixed effect model. PELF concentrations were calculated by a urea dilution assay of the bronchoalveolar lavage fluids. Plasma protein binding was measured using a microcentrifugation system.

For most 3-week and 6-month old calves, the plasma concentration–time profiles were best described by a two-compartment open body model. The maximum total (free plus protein bound) concentrations in the plasma ($C_{\text{max}}$) were greater in 3-week old calves (1.34 ± 0.45 μg/mL) as compared to that of the 6-month old calves (0.82±0.45 μg/mL). Clearance values (CL/F) were significantly lower in 3-week old calves but the volume of distribution (Vd/F) significantly higher in 6-month old calves. A downward trend in the plasma protein binding of tulathromycin was observed in plasma derived from 6-month old calves (63% to 39% for tulathromycin concentrations ranging from 0.1 to 1.0 μg/mL) but a lower and relatively constant fraction bound was observed in 3-week old calves (22% - 24% bound at tulathromycin concentrations ranging from 0.1 to 1.0 μg/mL). The older calves maintained higher ISF concentrations throughout the study period compared to those seen in younger calves. PELF concentrations of tulathromycin tended to be higher in 3-week old calves and reached higher maximum concentrations than seen in plasma in both groups. An age-associated difference in
plasma and ISF concentration time curves is consistent with maturational changes in calf physiology, resulting in altered tulathromycin exposure characteristics in the plasma, lungs and ISF.
Introduction

Bovine respiratory disease (BRD) continues to be a significant cause of morbidity and mortality in young dairy and veal calves (Brscic et al., 2012; Ames, 1997). Pneumonia in dairy calves is multifactorial, and its occurrence and severity is impacted by herd management, animal age, calf immunity, and the environment (Jennings and Glover, 1952). Tulathromycin, a semi-synthetic macrolide antibiotic of the subclass triamilide, has been shown to be safe and effective for the treatment of BRD associated with *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, and *Mycoplasma bovis*, and for the control of respiratory disease in cattle at high risk of developing BRD associated with *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, and *Mycoplasma bovis*. Tulathromycin has excellent bacteriostatic and some bactericidal activity against many of these pathogens. (Godinho, 2008). Currently, it is approved for use in multiple ages of calves, including those to be processed for veal, with an extended meat withdrawal time in veal calves.

Similar to other macrolide antibiotics, pharmacokinetic (PK) studies of tulathromycin in cattle, swine, deer, bison and foals demonstrate rapid absorption following subcutaneous (SC) and intramuscular (IM) injection, extensive accumulation in lung tissue, and prolonged elimination half lives in lung homogenate and pulmonary epithelial lining fluid (PELF) (Benchaoui et al., 2004; Scheuch et al., 2007; Villarino et al., 2013; Bachtold et al., 2015a; Bachtold et al., 2015b; Foster et al., 2016). In previous PK studies, tulathromycin was measured in plasma, PELF, and the interstitial fluids (ISF) of mature, clinically healthy calves (Foster et al., 2016). Although a plasma PK characterization in pre-ruminating calves has been previously reported (EMA, 2003), similar assessments comparing blood, ISF and PELF have not as yet been reported for pre-ruminating calves. Maturation can affect drug metabolism (Shoaf et al., 1987;
Alcorn and McNamara, 2002), transporter function (Pácha, 2000), body composition [including body fat, muscle and water content (Wrenn et al., 1962)], blood flow (Varga and Csáky, 1976), and blood composition in terms of plasma proteins and cellular constituents (Nagy et al., 2014).

With regard to the latter, bovine neutrophils exhibit a high-affinity for tulathromycin (Evans, 2005). The number of circulating neutrophils decrease with age from birth to 30 days of age in Holstein heifer calves, with maximum values occurring between birth and 8 hours of life (Benesi et al., 2012). In cattle, endotoxins induced a higher rate of neutrophil migration in neonatal calves as compared to that of adults (Zwahlen and Roth, 1990). In rats, previous studies have correlated an immature neutrophil function with a decrease in lung injury (Calkins et al., 2002), indicating that age impacts not only the neutrophil function but also the pathogenesis and progression of disease.

Ultimately, a determination of effective dosing strategies should relate the concentrations at the site of action to the in vitro susceptibility of the target bacteria. For some drugs, free drug concentrations in the blood can be used to approximate concentrations available to treat extracellular infections. This is not the case for macrolides where drug concentrations in the lung, ISF and blood can be markedly different. Accordingly, appreciating the distribution patterns of antimicrobials into the ISF and PELF in different subpopulations may improve the pharmacokinetic-pharmacodynamic (PK-PD) correlations for many bacterial pathogens. However, the extent to which age influences drug exposure (clearance), and plasma versus tissue (lung and ISF) tulathromycin concentrations have yet to be evaluated relative to that seen in ruminating (mature) calves.

With these points in mind, the objective of this study was to characterize tulathromycin drug penetration into PELF and ISF of different age calves after administration of a single SC
dose of 2.5 mg/kg body weight. The results of this study will provide insights into the ways that drug PK in cattle may differ as a function of age.

**Materials and methods**

**Animals**

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee. Holstein steer calves were bought from the North Carolina State University Dairy herd. Eight unweaned Holstein calves, two to three weeks of age, weighing between 41-53 kg were housed at the University Dairy and fed a commercial non-medicated milk replacer twice a day, and had free access to water and calf starter ration *ad libitum* throughout the study. Eight weaned calves, 6 months of age and weighing between 151-214 kg at time of study, were also enrolled. These older calves were group housed indoors at the University lab animal facility. The 6-month old calves provided *ad libitum* access to grass hay and water and their diets were supplemented with grain. All calves were confirmed healthy via physical exams conducted prior to the start of the study and none of the calves had a previous history of disease or antibiotic administration.

**Drug Administration and Blood Collection**

All calves were weighed on a digital scale on the morning of the study commencement for determination of the administered dose. Approximately 24 h prior to start of the study, calves were restrained for intravenous catheter placement. The area where the catheter was to be placed was clipped and cleaned with alternating scrubs of chlorhexidine and isopropyl alcohol. A 14 G x 3.25 mm catheter (Angiocath, Becton Dickinson) was inserted into the right jugular vein with an extension set. The catheter was sutured to the skin using a 2-0 monofilament suture and were flushed four times a day using 6 mLs of 10 units/mL of heparin saline. A single SC injection of
tulathromycin (2.5 mg/kg) (Draxxin, Zoetis) was administered to each calf in the neck per label instructions. Blood samples were taken from the jugular vein 0 (pretreatment), 0.25, 0.5, 1, 2, 3, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, and 312 hours post administration of tulathromycin and the samples were transferred to lithium heparinized tubes. These samples were stored on ice until centrifugation at approximately 3500 g for 10 min to collect plasma. The plasma samples were stored -80°C until analysis.

**Interstitial Fluid Collection**

All calves were implanted with SC ultrafiltration interstitial fluid (ISF) probes (BASI Inc.) on the side of the neck opposite site of the SC tulathromycin injection. Each probe contained three semi-permeable loops connected to a non-permeable tube that extended outside the animal and attached to a 3 mL plastic vacutainer tube without clotting agents. This tube provided negative pressure for fluid collection through small pores in the probe membranes. These pores allowed for the movement of water, electrolytes and low molecular weight molecules (<30,000 Da) to pass into collection tube while excluding large molecules such as proteins, protein bound drugs, and cells. Probes were placed while the calves were sedated with xylazine [Rompun Injectable (20 mg/mL), Bayer Animal Health Division] at a dose of 0.05-0.1 mg/kg in the cervical neck muscles. Probes were placed twenty four hours before the start of the trial to provide the time necessary to allow for equilibration with the surrounding ISF. One probe was inserted into each calf subcutaneously in the area cranial to the scapula. The ISF was collected at 0 (pretreatment), 2, 3, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, and 312 hours following SC tulathromycin administration. Since each ISF sample
represents fluid collection over a certain amount of time (i.e. not an instantaneous sample), a lag time was calculated based on the length of the tube and the fluid volume collected over time for each sample. The fluid collected was frozen at -80°C until analysis.

Lung Fluid Collection

To determine drug concentrations in the PELF, a bronchoalveolar lavage (BAL) was performed using a method previously described (Poulsen and McGuirk, 2009). Briefly, BALs were performed in all calves using either a sterilized, flexible 10 French X 36 inch catheter with a 3-cc balloon cuff (3-week old calves) or a 24 French X 59 inch catheter (6-month old calves) (Mila International, Inc.). At each time point, the calf was restrained and the head and neck of the calf were extended to facilitate passage of the sterile catheter. The BAL catheter was introduced into the ventral meatus of the nose and advanced down the trachea until it was wedged in a terminal bronchus. The balloon cuff was inflated to create a seal and the catheter was held firmly in place while the guide-wire was removed. At each time point, 100 mL of sterile saline were infused into the lungs and the fluid was immediately aspirated, limiting the dwell time in the lungs. The volume of fluid retrieved ranged from 0 to 42.5 mLs of clear to mildly turbid foamy fluid. The sample was placed into a sterile collection tube, the total volume recorded, and sample subsequently placed on ice until centrifugation. The BAL samples were centrifuged at 300 g for 10 min, and the supernatant fluid separated from cell pellet and the supernatant stored frozen at -80°C until analysis. Prior to the start of the study, all 8 calves were
randomly allocated to one of two groups. Group 1 calves were sampled at 3, 12, 24 and 72 hours postdose and Group 2 was sampled at 3, 12, 48 and 96 hours postdose.

**Drug Analysis**

Quantitative analysis of tulathromycin concentrations in plasma, ISF and PELF was accomplished using triple quadrupole mass spectrometry (UPLC-MS/MS; Waters, Milford, MA, USA). An analytical method for determination of tulathromycin concentrations in various matrices was developed for this study. Plasma samples for UPLC-MS/MS analysis were prepared using a solid phase extraction cleanup. For each assay matrix, calibration curves were constructed spanning over the calibration range 5–1000 ng/mL. The $R^2$ values for the calibration curves were 0.99. Intraday and interday percent coefficient of variation (%CV) were less than 20%, and the accuracy ranged from 102 to 106%. The lower limit of quantification (LLOQ) was 5 ng/mL with a precision of 8% and an accuracy of 105%.

**Plasma:** 500-μL of plasma was mixed with 500-μL of 4% phosphoric acid, vortexed for 10 seconds to pre-treat samples. The 1 mL pretreated sample was then loaded onto Oasis 3 cc PRiME HLB cartridges (Waters Corporation) with 60 mg sorbent cartridges and pulled through with a vacuum at ~3 psi. Each sample was then washed with 1 mL of 5:95 (v:v) Methanol:water. Samples were then eluted from cartridge using 400-μL of 60:40 (v:v) Acetonitrile:Water with 0.1% formic acid. The collected liquid was then transferred to a vial and 5-μL aliquot was analyzed by the UPLC-MS/MS.

**Interstitial Fluid:** 100-μL of ISF from each sample was loaded into UPLC vials and a 5-μL aliquot was injected onto the UPLC-MS/MS.
PELF/BAL Supernatant: 100-μL of BAL supernatant from each sample was filtered through a 0.2μm filter and loaded into UPLC vials and a 5-μL aliquot was injected onto the UPLC-MS/MS.

Tulathromycin Concentrations in PELF

Estimation of the amount of PELF sampled from BAL fluid was performed using the urea dilution method previously described in cattle (Mzyk et al., 2016). To determine urea nitrogen concentrations in serum (Urea<sub>serum</sub>) and BAL fluid (Urea<sub>BAL</sub>), a urea test kit (Urea test kit; Sigma Chemical) was used and the absorbance values measured by spectrophotometry according to manufacturer guidelines. The volume of PELF (V<sub>PELF</sub>) in BAL fluid was derived from the following equation:

\[ V_{PELF} = V_{BAL} \times \left( \frac{\text{Urea}_{BAL}}{\text{Urea}_{serum}} \right) \]

In which V<sub>BAL</sub> is the volume of recovered BAL fluid. The concentration of tulathromycin in PELF (TUL<sub>PELF</sub>) was derived from the following equation:

\[ TUL_{PELF} = TUL_{BAL} \times \left( \frac{V_{BAL}}{V_{PELF}} \right) \]

In which TUL<sub>BAL</sub> is the measured concentrations of tulathromycin in BAL fluid.

Plasma Protein Binding

Plasma from five healthy calves of both ages (3-weeks and 6-months) were pooled to determine in vitro plasma protein binding. Plasma was spiked at three different concentrations (0.1, 0.5 and 1.0 µg/mL in triplicate. All samples were allowed to stand at room temperature for 30 minutes in the dark to equilibrate. A 1 mL sample of each standard was loaded onto a ultrafiltration device (Centrifree Ultrafiltration Device; Millipore Sigma) and was centrifuged for 2,000 x g for 10 minutes. The ultrafiltrate was analyzed using UPLC-MS-MS to determine
unbound concentration and plasma protein binding. Non-specific binding of tulathromycin was determined to be <1% in the device and filter.

*Pharmacokinetic Analysis*

Unless otherwise indicated, all PK parameter values are expressed relative to the unbound tulathromycin concentrations determined from *in-vitro* protein binding assays performed in pooled 3-week old and 6-month old calf plasma.

The influence of age and weight on primary and secondary PK parameters were determined using a nonlinear mixed-effect model (Phoenix® WinNonlin/NLME, Version 1.3 Certara). The population base model was fitted as a multiplicative two-compartment model parameterized by clearance. Model selection was based upon precision of parameter estimates, and goodness-of-fit plots (e.g., residual plots) and statistical significance between models using lowest log-likelihood ratio (-2LL) values obtained in the software. A preliminary non-compartmental and compartmental analysis was conducted with Phoenix WinNonlin in order to obtain the initial estimates for the parameters of the basic model (i.e. no covariates).

Assessment for age and weight was conducted by the individual addition of each term to the base model and the change in the objective function was noted. A box plot of effect of the covariate (age and weight) on each parameter showed that clearance (CL) expressed as a function of bioavailability (CL/F) was the parameter most likely affected by age. Age was added to the base model as a categorical covariate (where 3-week old calves = 3 and 6 month old calves = 24), and its effect on the PK parameters, the apparent volume of distribution (Vd) expressed as a function of bioavailability (Vd/F) and CL/F was evaluated using a likelihood ratio test. A P-value ≤ 0.05 was considered to be significant. Age was found to significantly improve model predictions for CL/F, but not Vd/F (p < 0.001). After age was determined to improve the model,
this covariate remained in the final model. All PK parameters, except for elimination half life (T½), were reported as a geometric mean. The T½ was estimated for individual calves from the NLME according to the formula:

\[ T_{1/2} = 0.693 \frac{V_{d}/F}{C_l/F} \]

The T½ values are reported as harmonic mean ± standard deviation (SD). The SD was estimated using the Delta method as previously described (Lam et al., 1985; Feiveson, 2005) where:

\[ SD = HM^2 \times \sqrt{\frac{n}{n-1}} \sum_{i=1}^{n} \left( H_i - MH \right)^2 \]

And where MH is the mean of the harmonic values (MH) for

\[ \sum_{i=1}^{n} \frac{X_i}{X_i} \]

H_i is the harmonic value (λz) and HM is the harmonic mean being reported (T_{1/2}).

ISF/plasma concentration ratios at each time point were determined for each calf using the concentration of ISF (unbound drug) to the total (bound and unbound) and unbound tulathromycin plasma concentration. Statistical analyses were performed on the ISF concentrations of both groups of calves using SigmaPlot (Systat Software, Inc.); Levels of significance were set at P-values of ≤0.05 using a two-tailed test. The normality assumption was tested for each variable using the Shapiro–Wilk W-test, which is the preferred method for testing normality of data when the sample size is small. (Ghasemi and Zahediasl, 2012). Efforts to employ NLME modeling of the PELF was unsuccessful due to the apparent random fluctuations of values within an individual. Therefore, the evaluation of the data generated from the PELF
was limited to a simple numerical comparison of the average concentrations at each time for each group of calves.

**Results**

No adverse reactions were observed following placement of the ISF probes, jugular catheters, or tulathromycin injection. However, due to malfunctions in the ISF probes in several calves, fluid was not collected over some sampling intervals. Therefore, only incomplete ISF concentration versus time profiles were available in some calves from the two ages groups.

During BAL collection, sample volumes ranged from 0 to 42.5 mLs. At the 72 h sample for 6-month old calves, the lavage yielded no lung fluid for two of the four of the calves. The variability of fluid retrieved with the BAL procedure in the 3-week old calves could be attributed to difficulties in achieving proper placement of the catheter in their terminal bronchus.

**Plasma**

Based upon free plus protein bound (total) plasma tulathromycin concentrations, age associated differences were observed in several of the primary and derived PK parameters estimated via the NLME model (Table 2). The individual plasma-concentration time curves showed high variability and oscillations in the concentrations determined at the later time points in both groups of calves (Fig. 1). The $T_{\text{max}}$ values tended to occur earlier in 3-week versus 6-month old calves. $\text{CL/F}$ and $\text{Vd/F}$ determined from total plasma concentrations were significantly higher in 6-month old calves. The harmonic mean terminal elimination half life ($T_{1/2}$), was estimated as 67.6 and 44.4 h for 3-week and 6-month old calves respectively. Based upon the fitted estimates of the rate of drug distribution from the peripheral back to central compartments in plasma ($K_{21}$), values tended to be smaller (slower partitioning back into the plasma) in the 6-month vs. 3-week old calves, which is consistent with the larger $\text{Vd/F}$ values.
estimated in the 6-month old calves. Compartmental parameter rate constant estimates in the individual calves are depicted in Appendix (Table. S1).

After SC injection, the tulathromycin plasma $C_{\text{max}}$ values, estimated directly from individual calf data, achieved 1.34 µg/mL in 3-week old calves and 0.82 µg/mL in 6-month old calves. The unbound fraction of drug in plasma varied from 0.36 to 0.61 in 6-month old calves and 0.75-0.82 in 3-week old calves as tulathromycin concentrations varied from 0.1 to 1.0 µg/mL (Table 3). Although a shift from proportionality in protein binding was seen in the older calves, the majority of the profile was constrained within a range where the protein binding was relatively stable. A significant difference was found in the unbound fraction at all tested concentrations in plasma pooled from healthy 3-week and 6-month old calves.

**Interstitial Fluid data**

The collection of ISF using ultrafiltration probes allowed for the monitoring of unbound drug concentration in repeated samples. These devices were well tolerated and were able to collect between 0.05 mL to >2 mL of fluid in most samples. 6-month old calves showed detectable concentrations in ISF at 12 h post dosing. In contrast, tulathromycin was not detected in the ISF of 3-week old calves until 51 h. In both age groups, maximum ISF tulathromycin concentrations were obtained later than that of the plasma. Regardless of whether expressed relative to total or unbound plasma drug concentrations, the ISF/Plasma concentration ratios were significantly higher in the 6-month versus 3-month old calves (Fig. 2).

**Pulmonary Epithelial Lining Fluid**

Average PELF concentrations determined from BAL samples are reported in Table 4 and Figure 3. The average maximum concentrations in PELF occurred at 96 hours. Large variability in drug concentrations in PELF was seen across both groups at all time points.
Concentrations in PELF exceeded blood and ISF concentrations for both ages of calves.

**Discussion**

Developmental changes in body composition, organ functions, ontogeny of drug biotransformation pathways and elimination pathways can impact drug PK in calves (Table 1). This is the first study to evaluate the effect of age on tulathromycin concentrations in plasma, PELF and ISF and plasma protein binding in calves. The use of ultrafiltration probes and the collection of PELF allows for a continuous assessment of active drug in sites of action. Accordingly, the results of this investigation provide an important step toward our understanding of how drug PK, and therefore the targeted dose needed to achieve some desired level of tissue exposure, can be influenced by a calf’s age.

To determine active drug concentrations at infection sites, the accurate measurement of antimicrobial concentrations is crucial for the prediction of drug antimicrobial efficacy. Typical methods for obtaining this information includes the quantification of drug concentrations in plasma, tissue cages, and homogenized lung tissue. The latter is a poor predictor of drug concentrations in the PELF, which is where many lung bacterial infections are localized (Marcy et al., 1987; Nowakowski et al., 2004; Winther, 2012). Alternatively, the ISF has been considered a potential surrogate for drug lung concentrations since it reflects the unbound drug concentration and the partitioning of drug into a tissue compartment. However, such extrapolations are inappropriate for macrolides. For example, tulathromycin, PELF exposure has been shown to reach over 9x higher than plasma and ISF concentrations in ruminating calves (Foster et al., 2016). Lung concentrations of tulathromycin have been shown to be different in bovine pneumonic vs healthy lung homogenates but whole tissue homogenate do not allow for the evaluation of free versus bound drug concentrations in the pulmonary tissues (Pfizer, 2005).
In contrast to our study results, it has been previously reported that there were no statistically significant differences in tulathromycin plasma PK parameters between preruminant calves (4-7 weeks of age) and adult cattle (EMA, 2003). What is not clear is whether or not this discrepancy may be due to PK differences between 3-week old versus 4-7 week old calves (even though both age groups are classified as pre-ruminants). We note that the PK parameter values for tulathromycin in 6-month old calves in our investigation are within the same range as reported in previous studies (Villarino et al., 2014; Foster et al., 2016).

Irrespective of age, plasma tulathromycin concentrations were relatively low. SC administration was associated with rapid absorption and a subsequent slow decline (Fig. 1). The younger calves had statistically significantly lower CL/F and Vd/F values as compared to those of the 6-month old calves. Across age groups, the T1/2 was similar, although was an observed trend for a longer T1/2 in the 3-week old (67.6 hours) versus 6-month old (44.4 hours) calves. Since disposition of drugs refers to the simultaneous effects of elimination and distribution, the observed age related changes in these two processes could account for similarity in T1/2 values.

Assuming similar bioavailability following SC injection, the CL/F values in 6-month old calves (0.33 L/hr/kg) were were higher than those reported by Nowakowski et al., (2004). Although calves enrolled in that study were beef calves with body weights similar to those in current study (181-246kg), the age of the calves used were not reported. We also cannot discount the possibility that the observed differences CL/F values observed in the Nowakowski versus our study was a function of breed.

The lower CL/F values observed in the 3-week old calves in the current investigation could potentially be attributed to age-associated differences in kidney and liver function. Regarding liver function, hepatic clearance depends on several factors including blood flow,
hepatic enzyme activities, transport systems and plasma protein binding (Fernandez et al., 2011). Hepatic blood flow has been shown to be lower in 3-month old calves compared to adults and can impact the metabolism of high extraction ratio drugs (Baird et al., 1975; Araya and Ford, 1982). Since tulathromycin is a low extraction ratio drug, it is unlikely that hepatic blood flow will be a contributing factor to the lower CL/F in young calves (FDA, 2004). Moreover, most the administered drug is eliminated unchanged and only about 10% is metabolized in cattle. Less than 10% each of the metabolites in excreta and tissues were formed by N-demethylation or N-oxidation of the desosamine portion of the molecule, cleavage of the modified cladinose moiety, N-depropylation of the cladinose moiety and ester hydrolysis of the macrocyclic ring. (EMA, 2015). Therefore, it is highly unlikely that age-associated differences in CL/F were due to differences in drug hepatic metabolism.

Alternatively, tulathromycin is predominantly excreted unchanged in the feces. Therefore, maturation differences in elimination pathways in biliary excretion (i.e., via transporter mechanisms) may be responsible for differences in drug elimination. To date, little is known with regard to the maturation of liver efflux transporters in humans or veterinary species. However, ontological changes in glomerular filtration, renal tubular secretion and tubular reabsorption have been well characterized in pediatric patients and could potentially have a profound impact on drug PK profiles (Fernandez et al., 2011). Therefore, it would not be surprising to find similar maturation-associated changes in the hepatic efflux transporters.

The Vd/F is affected by plasma protein binding, tissue binding and the lipid solubility of drugs. Lipid soluble drugs like tulathromycin have very high apparent volumes of distribution, as seen in this study with Vd/F values 3.5 L/kg and 10.5 L/kg in 3-week old and 6-month old calves respectively (Table 2). As animals mature, the body fat:water ratio increases, leading to a greater
sequestration of lipid soluble drugs in the adipose tissue. Volume of distribution also takes into 
account drug distribution into immune cells. Macrolides (which are weak bases) tend to ionize in 
an acidic environment and therefore can accumulate in cells and tissues, particularly 
polymorphonuclear cells due to lysosomal trapping. This lysosomal trapping has been suggested 
to serve as a vehicle that transports drug to an infection site (Frank et al., 1992). While some 
argue that this may be a mechanism for high drug concentrations at the site of infection 
(Scorneaux and Shryock, 1999; Matzneller et al., 2013), others have refuted this assumption 
(Toutain et al., 2016). *In vitro*, macrolides have demonstrated high accumulation in neutrophils 
and macrophages. Irrespective of whether or not these do in fact act a drug delivery mechanism, 
differences in development of the immune system may also have contribute to the larger volume 
of distribution in older calves. Neutrophil counts were demonstrated to be higher than adult 
reference values at 1 day of age but returned to adult levels by 28 days of age (Mohri et al., 
2007).

The changes noted in the plasma kinetics of tulathromycin could be attributed to 
differences in plasma protein binding. Most previous studies determined plasma PK parameters 
using total (unbound + bound) concentrations. Such measures may miss significant changes as a 
function of age in intrinsic clearance or unbound systemic drug concentrations. As only unbound 
drug is free to move across biological barriers and to fight infections, unbound concentrations are 
the more clinically relevant measurement. Reported bovine plasma tulathromycin protein 
binding is approximately 40% [i.e., fraction unbound \((fu)\)= 0.53-0.68 in 6-month old calves 
(Nowakowski et al., 2004; Foster et al., 2016). The *in vitro* plasma protein binding estimated in 
the current study was similar for 6-month old calves (Table 3). In contrast, we estimated the 
protein binding in 3-week old calves to be approximately 17-24%. Similar to our study in calves,
age has been found to influence the plasma protein binding of a variety of drugs in humans (McNamara and Alcorn, 2002).

In calves, concentrations of major drug binding proteins such as albumin, $\alpha_1$-acid glycoprotein (AGP), showed significant changes during the first three months of life (Tóthová et al., 2015; Tóthová et al., 2016). The higher unbound fraction of basic drugs, like tulathromycin, in 3-week old calves may be due to the drug binding properties of AGP (Routledge, 1986). Since the plasma concentration of AGP is relatively low in neonates and since there is only one drug-binding site in each AGP molecule, drug binding to AGP is typically saturable and is readily displaceable (Huang and Ung, 2013).

For macrolides, the use of blood levels for the assessment of dose when treating BRD does not directly mirror drug concentrations at the site of action. Although not the targeted infection site, measuring drug concentrations within the ISF was considered to provide an additional layer of information regarding the way tulathromycin moves through the various tissue compartments and how the observed plasma PK differences in 3-week old and 6-month old calves may have influenced the distribution in ISF vs plasma. Drug concentrations in the ISF have been evaluated for several drug classes, including cephalosporins, fluoroquinolones, and macrolides (Foster et al., 2016; Mzyk et al., 2016). Overall, many macrolides have limited penetration into ISF compartments, as shown by consistently lower drug concentrations in the ISF compared to free drug concentrations in the plasma. These results are consistent with the observed difficulty in achieving therapeutic drug concentrations in human ISF for a wide range of macrolides (Kiang et al., 2014). Therefore, it may be difficult to achieve the macrolide concentrations necessary to successfully treat soft tissue infections.

Tulathromycin concentrations in the ISF were significantly higher in 6-month old calves
as compared to 3-week old calves. This could reflect age-associated differences in body composition and immune system constituents (Fig. 2A). Tulathromycin was below the limit of detection (0.005 μg/mL) in ISF until 48 h after dosing for the young calves, but was detectable in 6-month old calves by 8 h post dose. In addition, ISF/plasma ratios were examined before and after correcting the plasma concentrations for protein binding (Fig. 2B, 2C). Differences were noted in ISF concentrations and ISF/Plasma\textsubscript{unbound} ratios as a function of time in both groups. For the 3-week old calves, the ISF/plasma (total and unbound) ratios peaked at 168 hours after dosing while that of the 6-month old calves reached peak concentrations much earlier at around 48 hours (Fig. 2C). These differences in both concentration and kinetics of ISF drug accumulation may reflect the change in adipose and muscle growth that occurs as animals mature. The older calves tend to have a smaller proportion of total extracellular water as compared to that of veal calves. In younger animals, larger proportion of total body water (especially in adipose tissue, which is where the SC probes were placed) could lead to a dilution effect. The latter would result in the lower drug concentrations found in the ISF. Understanding the partitioning of unbound antimicrobial drug into the ISF and plasma may not be the best modality in determining appropriate therapy in different ages of calves.

Regarding lung concentrations, previous studies have estimated these by such methods as tissue homogenates, lung biopsies, bronchial microsampling and bronchoalveolar lavage (Giguère et al., 2011; Winther, 2012; Mzyk et al., 2016). Comparing tilmicosin concentrations (another macrolide) estimated using direct sampling (bronchial swabs) vs bronchoalveolar lavage, it was concluded that the concentrations estimated using either of these two techniques were not significantly different (Foster et al., 2017). In contrast, the use of lung/tissue homogenates does not allow for a differentiation between intracellular vs. extracellular drug
concentrations or for the binding of drug to the tissues. Therefore, lung homogenates typically overestimate the active concentration of macrolides at the site of infection (Mouton et al., 2008).

Alternatively, PELF is secreted extracellularly in the respiratory tract and is a potential site of respiratory bacterial infections in cattle. Foster et al. (2016) determined that some antimicrobials exhibit higher penetration into the PELF than others and therefore are more effective in the control of BRD. Accordingly, we sought to measure tulathromycin PELF concentrations as a function of calf age. Determining drug concentration in the PELF allows for the evaluation of plasma/ISF/PELF PK-PD exposure relationships, thereby facilitating an assessment of potential therapeutic dosing strategies as a function of the calf age. PELF concentrations of tulathromycin tended to be higher in 3-week old calves (Fig. 3). Interestingly, this contrasts with the relationship observed in the ISF. The higher PELF concentrations in 3-week old calves cannot reflect immature physiological barrier function [P-glycoprotein (P-gp)] in the blood-alveolar interface in the lungs. Since P-gp is believed to efflux substances into the alveolar sac [serving a protective function (Campbell et al., 2003)], the presence of an immature P-gp activity cannot explain the higher concentration of tulathromycin in the PELF in 3-day old calves. Efflux across the pulmonary membrane would increase tulathromycin concentrations in the PELF. Therefore, the involvement of an immature P-gp is not likely. However, if the pH of the PELF were lower in young versus mature calves, ion trapping may have contributed to these observed differences. The pH of the PELF of young versus mature calves have yet to be measured.

When evaluating the mean concentrations in PELF at each time point, interpretation of age related differences is hindered by the high variability seen across both groups (Table 4). Although concentrations in the PELF were higher than that in the ISF and plasma irrespective of
calf age, the variability in PELF concentrations may have been biased by the use of urea to
determine volume of PELF. When urea is used as a dilution marker, the dwell time of fluid
infused in the airways during the BAL procedure may represent an important source of
experimental variability (Dargaville et al., 1999). Since tulathromycin concentrates at high levels
in neutrophils, contamination from ruptured cells during BAL fluid centrifugation process, as
well as irritation from repeated BAL sampling, may over-estimate drug concentration in the
PELF. Although contamination from lysed cells is possible, the ruptured neutrophils alone
cannot explain the high concentrations in the extracellular matrix (Toutain et al. 2016). Given
these limitations and uncertainties, PELF values should be considered as a rough approximation
of pulmonary drug concentrations.

Determining the right dose for drugs used to treat diseases in neonates is critically
important. Physiological differences affecting drug absorption, distribution, metabolism, and
elimination render the extrapolating of doses from mature to pre-ruminating calves unreliable.
Movement of tulathromycin from plasma to the ISF and PELF has been shown to be
significantly different in young vs older calves, which in turn may indicate functional changes in
body composition. The therapeutic success of tulathromycin for the treatment of BRD relies not
only on the drug reaching efficacious concentrations at the site of infection, but also on the
functionality of a calf’s immune system. Although there are greater numbers of phagocytic cells
in the neonatal calf, the function of these cells is decreased until around 4 months of age (Hauser
et al., 1986). Each of these components need to be considered when assessing he magnitude of
dose adjustment necessary when treating disease in young calves.

Conclusions

In veterinary species, data are limited with respect to the physiological changes
associated with age and its corresponding influence on drug PK. The results of the present study lead us to conclude that age will influence the PK and distribution of tulathromycin when administered as a 2.5 mg/kg SC injection to 3-week old and 6-month old calves. Rapid absorption and extensive distribution to PELF is advantageous when treating bovine respiratory disease, but differences in age clearly impact the disposition of tulathromycin. Lower Vd/F and CL/F estimates resulted in higher plasma levels but lower ISF concentration levels in 3-week vs 6-month old calves. Although concentrations in the PELF were highly variable, they were consistently higher than plasma values at all time points.

Based upon our findings, there is a need for additional studies to compare distribution of tulathromycin in diseased vs healthy calves, as well as the relationship between drug exposure vs. response in different ages of calves.

Conflict of interest statement
Zoetis supplied the standard tulathromycin used for UPLC-MS/MS analysis in this study. Zoetis played no role in the study design nor in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication. None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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### Table 4.1 Developmental Factors Affecting Drug Pharmacokinetics in Neonatal Calves

<table>
<thead>
<tr>
<th>Physiologic Factors</th>
<th>Difference compared to Adults</th>
<th>PK Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absorption</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abomasum pH</td>
<td>↑</td>
<td>↓ Bioavailability (weak acids)</td>
</tr>
<tr>
<td>Abomasum Emptying Time</td>
<td>↑</td>
<td>↑ Absorption time</td>
</tr>
<tr>
<td>Intestinal Drug Transporters</td>
<td>Variable</td>
<td>Unknown in Cattle</td>
</tr>
<tr>
<td>Skeletal Muscle Blood Flow</td>
<td>Variable</td>
<td>Unknown in Cattle</td>
</tr>
<tr>
<td><strong>Distribution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Water:Fat Ratio</td>
<td>↑</td>
<td>↑ Volume of Distribution (hydrophilic drugs)</td>
</tr>
<tr>
<td>Protein Binding</td>
<td>↓</td>
<td>↑ Free fraction of drugs</td>
</tr>
<tr>
<td><strong>Hepatic Metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase I enzyme activity</td>
<td>↓</td>
<td>↓ Hepatic Clearance</td>
</tr>
<tr>
<td>Phase II enzyme activity</td>
<td>↓</td>
<td>↓ Hepatic Clearance</td>
</tr>
<tr>
<td><strong>Renal Excretion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerular Filtration Rate</td>
<td>↓</td>
<td>↓ Renal Clearance</td>
</tr>
<tr>
<td>Tubular Absorption/Secretion</td>
<td>↓</td>
<td>↓ Renal Clearance</td>
</tr>
</tbody>
</table>

↑, changes increased in values; ↓, changes decreased in values
Table 4.2 Population pharmacokinetic parameters from nonlinear mixed effects model (NLME) analysis after subcutaneous administration of 2.5 mg/kg tulathromycin in Holstein calves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Estimate</th>
<th>Stderr</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{MAX}}$</td>
<td>h</td>
<td>0.02</td>
<td>0.81</td>
<td>21.8</td>
</tr>
<tr>
<td>$C_{\text{MAX}}$</td>
<td>$\mu g/mL$</td>
<td>0.67</td>
<td>3.1</td>
<td>18.8</td>
</tr>
<tr>
<td>$\theta V$</td>
<td>L/kg</td>
<td>3.7</td>
<td>0.0045</td>
<td>28.3</td>
</tr>
<tr>
<td>$\theta V2$</td>
<td>L/kg</td>
<td>16.4</td>
<td>4.8</td>
<td>20.2</td>
</tr>
<tr>
<td>$\text{Cl}$</td>
<td>L/kg/h</td>
<td>0.10</td>
<td>0.14</td>
<td>21.5</td>
</tr>
<tr>
<td>$\text{Cl2}$</td>
<td>L/kg/h</td>
<td>0.92</td>
<td>0.02</td>
<td>20.2</td>
</tr>
<tr>
<td>$T_{1/2\ast}$</td>
<td>h</td>
<td>54.3</td>
<td>24.1</td>
<td>17.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Mean</th>
<th>Range</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{MAX}}$</td>
<td>h</td>
<td>0.68</td>
<td>0.25 – 1.0</td>
<td>0.38</td>
<td>0.25 – 0.50</td>
</tr>
<tr>
<td>$C_{\text{MAX}}$</td>
<td>$\mu g/mL$</td>
<td>0.82</td>
<td>0.32 – 1.63</td>
<td>1.34</td>
<td>0.61 – 1.84</td>
</tr>
<tr>
<td>$\theta V/F$</td>
<td>L/kg</td>
<td>10.5</td>
<td>5.2 – 20.4</td>
<td>3.5</td>
<td>2.0 – 7.03</td>
</tr>
<tr>
<td>$\theta V2/F$</td>
<td>L/kg</td>
<td>24.03</td>
<td>16.5 – 37.5</td>
<td>16.47</td>
<td>7.5 – 32.7</td>
</tr>
<tr>
<td>$\text{Cl}/F$</td>
<td>L/kg/h</td>
<td>0.33</td>
<td>0.16 – 0.49</td>
<td>0.14</td>
<td>0.07 – 0.25</td>
</tr>
<tr>
<td>$\text{Cl2}/F$</td>
<td>L/kg/h</td>
<td>1.07</td>
<td>0.37 – 1.42</td>
<td>0.91</td>
<td>0.53 – 1.63</td>
</tr>
<tr>
<td>$T_{1/2\ast}$</td>
<td>h</td>
<td>44.4</td>
<td>30.4 – 123.8</td>
<td>67.6</td>
<td>43.7 – 163.5</td>
</tr>
</tbody>
</table>

CL, clearance per fraction absorbed; $C_{\text{MAX}}$, peak concentration; $T_{1/2\ast}$, elimination half-life; $T_{\text{MAX}}$, time to peak concentration; $V$, apparent volume of distribution per fraction absorbed. The symbol $\theta$ (theta) indicates that this is the typical value for the population. Stderr represents the standard error. CV, Coefficient of variation.

*Terminal half-life was determined using harmonic mean
Table 4.3. Average % Plasma protein binding and calculated % bound of tulathromycin using microcentrifugation

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>6-Month Old</th>
<th>3-Week Old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Bound</td>
<td>Unbound fraction</td>
</tr>
<tr>
<td>0.1</td>
<td>63</td>
<td>0.36</td>
</tr>
<tr>
<td>0.5</td>
<td>49</td>
<td>0.54</td>
</tr>
<tr>
<td>1.0</td>
<td>39</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Percent plasma protein binding of tulathromycin spiked plasma from 3-week-old and 6-month-old calves and calculated % bound using the equation from (Toutain & Bousquet-Melou, 2002). Samples were spiked in triplicate at 3 concentration levels (0.1, 0.5, and 1 µg/mL) and averaged.
<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>6-Month Old µg/mL ± SD</th>
<th>3-Week Old µg/mL ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2.9 ± 2.2</td>
<td>4.9 ± 3.1</td>
</tr>
<tr>
<td>12</td>
<td>2.1 ± 1.9</td>
<td>5.3 ± 4.6</td>
</tr>
<tr>
<td>24</td>
<td>0.5 ± 0.3</td>
<td>4.7 ± 5.1</td>
</tr>
<tr>
<td>48</td>
<td>1.9 ± 0.2</td>
<td>3.6 ± 5.0</td>
</tr>
<tr>
<td>72</td>
<td>1.1 ± 0.5</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>96</td>
<td>4.3 ± 2.0</td>
<td>3.5 ± 2.0</td>
</tr>
</tbody>
</table>

Mean ± SD tulathromycin concentrations in PELF of 3-week and 6-month old calves.
Figure 4.1 Total tulathromycin plasma concentration vs time curve in all calves
Individual Plasma (bound and unbound) concentration time curves after single s.c. injection of 2.5 mg/kg tulathromycin in eight 3-week old (○) and seven 6-month old (▲) calves.
Figure 4.2 Mean interstitial fluid concentrations and ISF/Plasma ratios (total and unbound). Mean ISF concentrations ± SD (a), ISF:Plasma (total) ratios (b) and ISF:Plasma (unbound) ratios (c) of tulathromycin in 3-week old and 6-month old calves. Each point is a calculated geometric mean at each time point.
A)  

B)
C)

**ISF:Plasma\textsubscript{Unbound} Ratios**

- ▲ 6-Month Old Calves
- ○ 3-Week Old Calves

**Concentration (µg/mL)**

**Time (hours)**

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
</tr>
<tr>
<td>0.1</td>
<td>300</td>
</tr>
<tr>
<td>0.01</td>
<td>400</td>
</tr>
</tbody>
</table>
Figure 4.3 Tulathromycin Concentrations in PELF. Individual tulathromycin concentrations in PELF in 3-week old (○) and 6-month old (▲) calves.
References


Chapter 5

Effect of age on plasma protein binding of several veterinary drugs in dairy calves

2018
Abstract

The intent of this study was to determine what influence, if any, increasing age has on the binding of drugs to plasma proteins in cattle. Plasma from each calf (n=20) was taken at 1, 7 and 21 days of age. These were compared to calves at 8 weeks and 6 months of age. The plasma protein binding of danofloxacin, florfenicol, flunixin meglumine and tulathromycin was determined \textit{in vitro} via microcentrifugation using three different drug concentrations spiked into the individual plasma samples derived from each calf. Albumin concentrations were lowest at 1 day and increased until it reached a plateau at 56 days. There were significant decreases in alpha-acid glycoprotein in calves until 21 days of age. However, statistically significant age-effects on plasma protein binding were not observed for any of drugs evaluated in this study. Findings from these calves suggest that age is not an important factor in the binding of these drugs to plasma proteins.
Introduction

Drug plasma protein can influence drug distribution, total drug clearance, and the relationship between total drug concentrations and its pharmacological effects. Failure to recognize the difference between total and unbound drug concentrations can lead to errors in the interpretation of the dose-exposure-response relationships (Benet and Hoener, 2002; Toutain and Bousquet-Melou, 2002; Stern et al., 2016). Nevertheless, most studies report pharmacokinetic parameters derived from the total plasma concentrations over time but neglect to report the more clinically relevant unbound concentrations (Greenblatt, Sellers, and Jan 1982). The lack of distinction between total and free drug concentrations can be particularly problematic when trying to extrapolate drug pharmacokinetic characteristics across populations such as from adults to neonates. Particularly within the human population, it is recognized that there can be a substantial deviation from adult concentrations of binding proteins and metabolizing enzymes. As compared to adults, the binding of drugs to plasma proteins tends to be lower in pediatric human patients (Ignjatovic et al. 2011; McNamara and Alcorn 2002) due not only to lower concentrations of plasma proteins but also to the presence of endogenous compounds that can compete for these binding sites. Furthermore, there is some suggestion that even at comparable concentrations and after considering any potential differences in non-protein substances, the binding properties of the plasma proteins appear to differ in human neonates as compared to that of adults (Kurz, Mauser-Ganshorn, and Stickel 1977). The resulting increases in unbound fraction of drugs could increase the distribution to the rest of the body.

Unfortunately, for many veterinary species, the temporal changes of plasma protein binding have not been well established. In vitro protein binding for a few drugs has been examined in young animals (Abo El Sooud 2003). The influence of developmental changes in
plasma protein binding on pharmacokinetics and drug distribution can make interpretation of total plasma concentrations difficult, especially for drugs that have a narrow therapeutic index. The complex relationship of drug concentration and plasma protein binding is influenced by many factors, including the quantity of binding proteins, total drug concentration and affinity of a drug for each binding protein. Acidic drugs bind largely to albumin, while basic drugs associate with a number of plasma components including α1-acid glycoprotein (AGP) and albumin (Piafsky, 1980). Although changes in these proteins can vary with age and disease, the variation in plasma albumin is relatively low. Conversely, AGP concentrations can show large fluctuations due both to physiological and pathological conditions (Cs Tóthová, Nagy, and Kovác 2013). Changes in AGP concentrations have resulted in changes in distribution and metabolism of basic drugs, complicating a clinical interpretation of the relationship between total drug concentration and drug efficacy in neonatal patients (Routledge 1986).

While danofloxacin, florfenicol and flunixin bind primarily to albumin, there is evidence that some fluoroquinolones (Johnson and Smith, 2006) and macrolides (Bohte et al., 1995) can bind to AGP. The objective of this study was to investigate the effect of age on plasma concentrations of albumin, and AGP and the corresponding effect of these differences on the in vitro plasma protein binding of several commonly used veterinary drugs: danofloxacin, tulathromycin, florfenicol, and flunixin meglumine.

**Materials and Methods**

*Animals and housing*

Twenty healthy, Holstein heifer calves sourced from the North Carolina State University Dairy herd were used in this study. Calves were housed in individual hutches and fed commercial milk replacer twice daily and had free access to water and calf starter ration throughout the study.
Additionally, twenty 8-week old calves and twenty 6-month old calves were simultaneously enrolled. Calves were randomly assigned one of four drugs (danofloxacin, tulathromycin, florfenicol, and flunixin meglumine; n=5 for each group) for determination of in vitro protein binding.

All calves received colostrum at birth, and remained clinically healthy throughout study. Each calf was assessed for adequate passive transfer of immunoglobulins from colostrum, as indicated by a total plasma protein of greater than 5.5 g/dL. No antibiotics or other therapies were administered during the trial.

**Blood Collection**

If a calf showed clinical signs of disease at the time of sampling (cough, nasal discharge, diarrhea) or had a rectal temperature >102.5° F, the sample was excluded from the analysis at that time point. Blood was collected from a jugular vein using a vacutainer needle into sodium heparinized tubes. Samples were collected from each calf at 1, 7, 21 days of age. Plasma was also collected from individual Holstein heifers at 8-weeks (n = 20) and 6-months of age (n=20). Samples were stored on ice until centrifugation at approximately 3500 x g for 20 minutes to collect plasma. Total protein was recorded from each calf at all time points. The plasma samples were stored at -80° C until analysis.

**Drug Analysis**

Flunixin meglumine, florfenicol analytical standards were purchased from Sigma Aldrich (St. Louis, MO). Tulathromycin and danofloxacin analytical standards were donated by Zoetis, Inc. (Kalamazoo, MI). All standards were stored according to manufacturer recommendations. The physicochemical properties of the compounds used in this study are included in Table 1. Calibration curves were constructed for each run spanning over the calibration range 5–1000
ng/mL. The $R^2$ values for the calibration curves were 0.99 for each drug. Intraday and interday percent coefficient of variation were less than 20%, and the accuracy ranged from 94 to 118%. Potency was not determined prior to spiking plasma and drug levels were based upon label concentrations. However, since the same lots were used across all samples and volumes, any difference from the label amount was deemed to not influence the conclusions generated in this study.

**Microcentrifugation**

A microcentrifugation technique was used as previously described (Davis et al., 2007). Plasma was not pooled in order to monitor plasma protein binding changes in individual calves as they aged. Each compound (danofloxacin, tulathromycin, florfenicol, and flunixin meglumine) was spiked for each age of all calves and samples were made in triplicate. For danofloxacin, flunixin meglumine and florfenicol, plasma was spiked with concentrations of 0.05, 0.1, and 1.0 μg/mL. For tulathromycin, plasma was spiked with 0.1, 0.5 and 1.0 μg/mL. All spiked plasma samples were allowed to equilibrate at room temperature for 30 minutes. A 1 ml aliquot of each sample was added to the microcentrifugation device and was centrifuged at 2000 x $g$ for 20 min. The ultrafiltrate was analyzed using HPLC-UV/Fluorescence (florfenicol, danofloxacin) or UPLC-MS-MS (tulathromycin, flunixin meglumine) based upon protocols previously developed in our lab to determine unbound concentration (Kissell et al. 2012; Foster et al. 2016). Non-specific binding was determined to be minimal in the microcentrifugation device and filter for all four drugs.

**Analysis of albumin and alpha1-acid glycoprotein in plasma**

Total protein concentrations of each centrifuged blood sample were determined using a digital refractometer. These values were recorded for each calf. Albumin concentrations were
quantified using a colorimetric-bromcresol green method (Roche Diagnostics). AGP concentrations in plasma were measured with a commercially available sandwich ELISA kit (Bovine Alpha 1-Acid Glycoprotein ELISA Kit, Abcam PLC.). Intra- and inter-assay CV% were <10% and <15% over the range 3.125-100 ng/mL, respectively. Concentrations were interpolated from the standard curve constructed from standards and corrected for sample dilution following the manufacturer protocol.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 11.0 (SAS Inc.). The shapiro-wilk test was performed to assess normality of ordinal/continuous data across calf ages for albumin and AGP. AGP concentrations did not meet the criteria for a normal distribution, so a non-parametric Wilcoxon signed-rank test was used to evaluate changes in concentrations of AGP as well as plasma protein binding as calves aged from 1 day old to 6 months. Albumin concentrations between each group were determined to be normally distributed and evaluated using a one-way ANOVA and each group compared using a student t-test. Significance was set at P < 0.05. All results are presented as mean ± SD.

Results

As shown in Table 2, the average % plasma protein binding did not vary as calves aged for any of the four drugs. Danofloxacin exhibited a decrease in protein binding at higher concentrations, irrespective of age, but no age-associated differences in binding were observed at any drug concentration. Neither flunixin nor florfenicol showed age or concentration-associated differences in protein binding. However, it is important to note that for flunixin, it was important to conduct the analysis on the basis of free fraction rather than bound to avoid biasing conclusions generated at these very high values of percent protein binding. For example, if we
look at flunixin binding as a function of drug concentrations on day 1, we see that values go from 99.93 (0.05 μg/mL) to 99.45 (1 μg/mL). The ratio of difference in percent bound is 99.45/99.93 = 0.995. The conclusion would be that binding is nearly identical. Conversely, if we covert these values to their corresponding free fraction, the ratio would be 0.55/0.07 = 7.85. Thus there was approximately an 8-fold greater free drug concentrations at 1 μg/mL as compared to 0.05 μg/mL in day 1 animals. Despite this difference, the magnitude of variability in the estimates resulted in the absence of statistically significant differences as a function of age across the range of concentrations tested. Tulathromycin trended towards a significant lower plasma protein binding at 1 day of age.

The age of calves had a significant effect on the concentrations of albumin (P < 0.0001). At 1 day of age, albumin concentrations in calves were significantly lower than that in any of the other age groups, with levels reaching adult values by day 56 days (Figure 1). The average AGP concentrations in plasma initially decreased from day 1 to day 21, and subsequently increased. The highest concentration of AGP occurred at 1 day of age and was lowest by 21 days of age (Table 2; Figure 2). Since AGP is an acute phase protein, the high variability among individual calves was expected.

Discussion

Calves are born relatively hypoalbuminemic. Therefore, small changes in the quantity and binding capacities of specific plasma proteins may lead to differences in total drug distribution as calves mature. There is some disagreement in the published literature with regard to the change in protein concentrations as a function of age in Holstein calves. In one investigation, serum albumin levels were shown to increase from birth to 80 days of age (Knowles et al. 2000). However, other reports indicate a decrease in the total protein from blood
samples until day 14 and increased after 84 days, with increasing albumin but decreasing globulins (Mohri, Sharifi, and Eidi, 2007). The discrepancy between these studies may be due to differences in the assays used to quantify albumin. Knowles et al. used a method utilizing bromcresol green dye, while Mohri et al. did not include their analytical technique. Bromcresol green (BCG) and bromcresol purple dye are commonly used in automatic analyzers to quantify albumin. The dye binds to the positively charged albumin structures. Although both dyes react with albumin, the serum globulins also consist of positively charged residues, and the BCG assay, considered less specific for albumin, has previously been shown to be influenced by some of the serum globulins (Webster, 1975). In the current study, BCG assay was used to quantify albumin, raising the need to consider if the high concentrations of AGP and other serum globulins present in neonates could have falsely increased the albumin levels quantified. However, because albumin concentrations were estimated at concentrations of g/dL while the AGP was measured in μg/mL (nearly 400 μg/mL in the 1 day old neonate), it is concluded that the $10^6$–fold higher concentrations of albumin as compared to AGP would allow for any AGP-associated bias introduced into the age-associated comparisons to be negligible. Calves sampled indicated that the lowest total proteins occurred at 21 days and increased to adult levels by 6 months of age. In newborn humans, AGP concentration is approximately half that of the adult concentration (Lerman et al., 1989). In contrast, higher concentrations were observed in neonatal pigs as compared to adults (Tagawa et al. 1994).

In the current investigation, temporal changes in the concentration of serum proteins were statistically significant between birth and the first 3 weeks of life as well as from 3 weeks to 6 months of age. These changes were more pronounced for AGP as compared to serum albumin. In general, concentrations of AGP peaked at 1 day of age and were lowest at 21 days (Figure 2).
Age-related decreases in AGP concentrations over the first 3 weeks of life have been previously reported in fetal and newborn calves (Orro et al. 2008; Nagy, Tóthová, and Kováč 2014). Increases in acute phase proteins have been attributed to physical stress or trauma during parturition, which is supported by peak concentrations of AGP in humans at 1 day of age (Marchini et al., 2000).

When comparing statistical significance between ages of calves, concentrations of AGP failed to be determined as normally distributed. Therefore, two different statistical tests to compare age and AGP/albumin concentrations were used. The use of non-parametric tests comes with some disadvantages. The cost of fewer assumptions when using nonparametric tests are assumed generally less powerful than their parametric counterparts. For example, these tests may fail to reject our null hypothesis, indicating that AGP concentrations are different in each of the groups of calves, although no significance was found between some groups. Low power is a major issue when the sample size is small in addition to the high variability, as seen with AGP and albumin concentrations. Despite the high variability, temporal changes in the concentration of serum proteins were noted in both AGP and albumin from 1 to 21 days of age and again from 21 to 183 days (6 months) of age.

Samples were removed from the study if the calf exhibited signs of disease. However, this did not negate the possibility of altered concentrations of acute phase proteins due to the presence of subclinical infections or other disease processes not evident at the time of sampling. This might explain several calves in each group that showed very high AGP concentrations at 6 months of age. Disease status of calves has also been shown to affect circulating plasma proteins, such as haptoglobin and serum amyloid, which increase in serum during outbreaks of viral and bacterial diseases, but are present in very low levels in healthy animals (Murata,
Shimada, and Yoshioka 2004; Csilla Tóthová et al. 2011). The impact of disease on the plasma protein binding of different ages of calves requires further investigation.

For most published pharmacokinetic studies, plasma protein binding is determined by pooling plasma from healthy volunteers and spiking the samples with a known drug concentration. Such investigations fail to describe the intrasubject changes in protein binding with age or the intersubject variability associated with this relationship. Results from previous unpublished studies in our lab that evaluated the effect of age on calf plasma protein binding of tulathromycin (Chapter 4) and danofloxacin (Chapter 2) in calves demonstrated differences between the study results, particularly with respect to tulathromycin. The previous estimates of protein binding were based upon plasma was pooled from 5 calves, which was subsequently divided into 1 ml aliquots for the evaluation of a range of drug concentrations. Although this is a typical study design for the in vitro determination of plasma protein binding, it prohibits the estimation of within or between-individual variability. Therefore, in the current investigation, the mean percent protein binding was generated within and across individual calves. In the present study, we observed an increase in the mean % protein binding as tulathromycin concentrations from 0.1 to 1 μg/mL tulathromycin. However, considering the very large standard deviations, particularly at the lowest drug concentrations, this trend may be more a function of variability than actual differences in mean values. In the prior study utilizing pooled samples from 3-week old and 6-month old calves, trends in the opposite direction were observed. For example, at 0.1 and 1 μg/mL, 63% and 39% binding respectively was observed in the 6-month old calves. In the current study, 0.1 and 1 μg/mL tulathromycin protein binding was estimated as 7.83% and 25.03%, respectively. For danofloxacin, plasma concentrations spiked at 0.1 and 1 μg/mL, 53% and 47% respectively were observed in 3-week old calves, and in the
current study, these values for 0.1 and 1 μg/mL were determined to be approximately 65% and 37%. Reasons for these discrepancies are not evident, but the very large variability associated with these observations suggest that at least for some drugs, estimation of protein binding should be generated using a large sample size to obtain a more robust estimate of the mean and the potential variability about that mean value. Differences in the serum composition of albumin from human neonates have been shown to differ from that of adults (Wallace, 1977). The clinical importance of reported alterations is difficult to assess without measuring the drug binding capacities of each albumin component, both of which have not been studied in cattle for any drug. In addition, the potential changes in binding site composition means that different albumin proteins could bind inhibitors, such as bilirubin (Seedher and Kanojia, 2013), with different affinities, leading to competition at binding sites (having relevance both to drug pharmacokinetics as well as to free serum bilirubin concentrations). Since the plasma protein binding of most compounds is reversible, measuring in vivo plasma protein binding in healthy and disease calves will give key insight into the variability in drug pharmacokinetics that can occur across an animal patient population.

By understanding the changes that occur to plasma protein binding as a function of age, evaluation of the unbound/total drug concentration relationships can improve predictions of the possible pharmacokinetic differences that will influence the relationship between administered dose and drug safety and/or effectiveness for pre-ruminating calves. Although age-associated differences in plasma protein binding were not observed with the drugs examined in this study, the differences in albumin and AGP concentrations as a function of calf age indicates the potential for drug protein binding to be an important factor to consider. Without knowledge of the unbound drug concentrations and its relationship to major binding proteins in plasma, we
cannot appreciate the dose-exposure-response relationships that are integral to the safe and effective use of drugs in young calves.

**Conclusion**

The present study was the first to evaluate plasma protein binding in individual calves as they matured. Age has a significant impact on the concentrations of binding proteins, like albumin and AGP, but did not impact the protein binding of the four drugs in this study. These results contribute to the knowledge about the pattern of acute phase and binding proteins in newborn and young calves. An evaluation of changes in drug protein binding that may occur as a result of pathological states, including those occurring in neonatal calves, is a potential topic for future investigations.

**Acknowledgments**

This work was supported by the United States Department of Agriculture for the Food Animal Residue Avoidance and Depletion Program. The authors wish to thank Jim Yeatts and Jenna Schirmer for their help with assay development and Dr. Jennifer Davis for her assistance and remarks while working on this manuscript.
Table 5.1 Physiochemical properties of danofloxacin, tulathromycin, florfenicol and flunixin meglumine

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Drug Class</th>
<th>Molecular Weight</th>
<th>Weak Acid/Base</th>
<th>pKa</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>danofloxacin</td>
<td>fluoroquinolone antibiotic</td>
<td>357.37 g/mol</td>
<td>amphoteric</td>
<td>6.22, 9.43</td>
<td>0.77</td>
</tr>
<tr>
<td>tulathromycin</td>
<td>macrolide antibiotic</td>
<td>806.08 g/mol</td>
<td>weak base</td>
<td>8.6, 9.6, 9.9</td>
<td>-1.41</td>
</tr>
<tr>
<td>florfenicol</td>
<td>amphenicol antibiotic</td>
<td>358.21 g/mol</td>
<td>weak base</td>
<td>9.03</td>
<td>-0.12</td>
</tr>
<tr>
<td>flunixin</td>
<td>nonsteroidal antinflammatory drug</td>
<td>296.24 g/mol</td>
<td>weak acid</td>
<td>5.82</td>
<td>4.35</td>
</tr>
</tbody>
</table>
Table 5.2 Average % Plasma Protein Binding for four veterinary drugs used in calves

<table>
<thead>
<tr>
<th>(µg/ml)</th>
<th>Average % Plasma Protein Binding</th>
<th>Days of Age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>danofloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>39.05</td>
<td>6.39</td>
</tr>
<tr>
<td>0.1</td>
<td>66.25</td>
<td>7.56</td>
</tr>
<tr>
<td>0.05</td>
<td>68.37</td>
<td>4.75</td>
</tr>
<tr>
<td>tulathromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38.39</td>
<td>19.83</td>
</tr>
<tr>
<td>0.5</td>
<td>15.73</td>
<td>27.17</td>
</tr>
<tr>
<td>0.1</td>
<td>20.55</td>
<td>6.63</td>
</tr>
<tr>
<td>florfenicol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21.84</td>
<td>11.45</td>
</tr>
<tr>
<td>0.1</td>
<td>21.72</td>
<td>12.65</td>
</tr>
<tr>
<td>0.05</td>
<td>17.25</td>
<td>11.27</td>
</tr>
<tr>
<td>flunixin meglumine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>99.45</td>
<td>0.26</td>
</tr>
<tr>
<td>0.1</td>
<td>99.91</td>
<td>0.08</td>
</tr>
<tr>
<td>0.05</td>
<td>99.93</td>
<td>0.10</td>
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</table>
Table 5.3 Changes of concentrations of total protein, albumin and Alpha₁-acid glycoprotein concentrations in different ages of calves

<table>
<thead>
<tr>
<th>Variables</th>
<th>Age of calves (days)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>7</td>
<td>21</td>
<td>56</td>
</tr>
<tr>
<td>AGP (µg/mL)</td>
<td>Mean</td>
<td>356.3</td>
<td>245.5</td>
<td>72.8*</td>
<td>77.1*</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>213.4</td>
<td>177.1</td>
<td>33.1</td>
<td>42.9</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>Mean</td>
<td>2.67†</td>
<td>0.27†</td>
<td>3.03†</td>
<td>3.68*</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>0.24</td>
<td>0.21</td>
<td>0.21</td>
<td>0.30</td>
</tr>
<tr>
<td>Total Protein (mg/dL)</td>
<td>Mean</td>
<td>6.7</td>
<td>6.1</td>
<td>5.5</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>±SD</td>
<td>0.94</td>
<td>0.68</td>
<td>0.57</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Means not connected by the same superscripts in rows differ significantly (P < 0.0001)
Figure 5.1 Mean (±SD) serum concentrations of albumin in calves sampled during a 3-week period after birth. Mean (±SD) serum concentrations of albumin in calves sampled during a 3-week period after birth ($n = 20$). Significant difference ($P < 0.005$) from 1 day and 21 day old calves and between 21 and 183 days (6 month) old calves using student’s t-test.
Figure 5.2 Mean (±SD) serum concentrations of alpha\textsubscript{1}-acid glycoprotein (AGP) in calves sampled during a 3-week period after birth. Mean (±SD) serum concentrations of alpha\textsubscript{1}-acid glycoprotein (AGP) in calves sampled during a 3-week period after birth ($n = 20$). (*) Significant difference ($P < 0.005$) from 1 day and 21 day old calves and between 21 and 183 days (6 month) old calves using Wilcoxin/Kruskai Wallis test for non-normal data.
References


Chapter 6

Impact of bovine respiratory disease on the pharmacokinetics of danofloxacin and tulathromycin in different ages of calves

2018
Abstract

Pneumonia is one of the most economically important respiratory diseases of calves and the knowledge of the impact of clinical disease on pharmacokinetics (PK) in young calves is limited. This study was undertaken to investigate the efficacy and PK of two antibiotics, tulathromycin and danofloxacin, in two age groups of calves experimentally infected with *Pasteurella multocida*. Both danofloxacin, a fluoroquinolone antibiotic, and tulathromycin, a macrolide antibiotic is approved for the treatment of bovine respiratory disease (BRD). To evaluate potential influences of age and disease on drug distribution and elimination in calves, plasma, interstitial fluid (ISF), and pulmonary epithelial lining fluid (PELF) were collected and analyzed for drug concentrations. Concentrations for both drugs in the PELF were estimated by a urea dilution assay of the collected bronchoalveolar lavage fluids. Age was determined to be a significant covariate for calves administered danofloxacin and tulathromycin for plasma PK parameters. For calves administered danofloxacin, the area under the curve (AUC) in the plasma was lower in 6-month old calves (18.9 ± 12.6 hr* µg/mL) vs. 3-week old calves (32.0 ± 8.2 hr* µg/mL). Clearance (CL/F) of danofloxacin was higher in 6-month old calves. In contrast, tulathromycin plasma concentrations were higher in 6 month old calves and CL/F was higher in 3-week old calves. Age did not significantly influence the ISF concentrations of danofloxacin or tulathromycin in calves with respiratory disease, unlike previous studies which reported higher ISF concentrations of danofloxacin and tulathromycin in 6-month old calves when compared to younger calves. PELF concentrations were higher than plasma and ISF for both danofloxacin and tulathromycin, but did not differ between age groups. Potential reasons for age-related differences on plasma concentration–time profiles and the impact of disease on the partitioning of the drug from the blood to the lungs and the ISF as a function of age are explored.
Introduction

Pneumonia is one of the most economically important respiratory diseases of calves (Ames 1997). Its etiology is complex and can involve viruses, mycoplasmas and bacteria (Bryson 1985). Bacteria, particularly *Pasteurella* species, play an important role in many outbreaks of calf bronchopneumonia (Panciera and Confer 2010). *Pasteurella multocida* (*P. multocida*), a gram-negative bacteria, can increase the severity of the primary lung damage caused by viruses and exacerbate the clinical signs. Furthermore, studies indicate that *P. multocida* can act as a primary pathogen, producing severe acute pneumonia in calves (Praveena et al. 2014; Dagleish et al. 2010). Due to the high prevalence of gram-negative bacteria implicated as significant primary pathogens, the treatment of bovine respiratory disease (BRD) in cattle generally includes the use of Gram-negative spectrum antibiotics.

For this study, these drugs were selected for two reasons. First, they target different essential components of bacterial metabolism. Fluoroquinolones act by inhibiting DNA synthesis by targeting the activity of both DNA gyrase and the topoisomerase IV enzymes. Macrolides inhibit bacterial protein synthesis by targeting various ribosomes and damaging critical bacteria proteins. Second, both drug classes represent different mechanisms of eradication of bacterial infections. Macrolides are considered to be one of the classic bacteriostatic drugs, which prevent bacterial growth, but not killing bacteria. Quinolones are generally “bactericidal” meaning it kills bacteria. In practice, efficacy based solely on these classifications can lead to false assumptions of clinical efficacy, especially if other major PK/pharmacodynamic parameters are ignored. Although the main goal of treatment with either type of antibiotic is to eradicate infection, several host related factors can influence the efficacy of these treatments in calves. Age-related
changes in cattle, including body composition, metabolism and clearance mechanisms as well as the competency of the immune response can affect the efficacy of these drugs.

Fluoroquinolones, such as danofloxacin, have various pharmacologic properties of that contribute to the clinical success of treating BRD. These properties include quick times to maximum concentrations ($T_{\text{max}}$), high maximum plasma concentrations ($C_{\text{max}}$), large volumes of distribution ($V_d, V_d/F$) and excellent penetration in lung fluid with rapid bactericidal activity against commonly isolated gram negative bacterial respiratory pathogens (Giles et al. 1991; Friis 1993; McKellar et al. 1999). Since a majority of bacterial infections take place in the extracellular fluid of tissues, the drug concentration in this compartment determines the efficacy of an antibiotic.

Macrolide antibiotics, including tulathromycin, accumulate in leukocytes and bronchial secretions, and are present at concentrations in lung tissues that markedly exceed concentrations in the plasma (Nowakowski et al. 2004). Clinical disease and inflammation has been shown to influence the PK of tulathromycin. In pigs with induced respiratory infections, tulathromycin concentrations in tissues and the elimination half-life increased as compared to that observed in healthy controls (Bladek et al. 2015). Tilmicosin, another macrolide used in veterinary medicine, has been shown to accumulate in significantly higher concentrations in the lungs of rats with respiratory disease than healthy rats, although serum concentrations were not different between the two groups (Modric, Webb, and Davidson 1999). However, the increase in pulmonary concentrations of macrolides in the presence of disease is not a universal phenomenon as other macrolides, such as erythromycin, have shown a decrease in penetration into the lung tissue of calves with clinical respiratory disease (Burrows 1985).
Clinical disease has also been shown to alter the PK of other pharmacological compounds in cattle. In some situations, these PK changes may be linked to alteration in Phase I metabolizing enzymes and subsequent disease-associated changes in drug clearance (Martinez and Modric, 2010). Disease-associated changes in drug metabolism may or may not be confounded with age-associated differences in the enzyme maturation process, indicating a need to evaluate the distribution and PK of danofloxacin and tulathromycin in disease states in calves if we are to consider use of these compounds in pre-ruminating calves (Gorden et al. 2016; Kissell et al. 2015). Since these drugs are intended for use in calves over a large age range, it is imperative to understand the impact of infection and inflammation on the distribution of tulathromycin and danofloxacin in different age calves.

In the United States, danofloxacin is approved for treatment of BRD associated with *Mannhemia haemolytica* (*M. haemolytica*) and *P. multocida* when administered as a single dose of 8 mg/kg, SC. Tulathromycin is approved for treatment of BRD associated with *M. haemolytica*, *P. multocida*, and *Histophilus somnus* (single dose of 2.5 mg of tulathromycin/kg, SC). The purpose of the study reported here was to compare the concentrations of danofloxacin and tulathromycin in plasma, interstitial fluid (ISF) and pulmonary epithelial lining fluid (PELF) of calves treated with these antimicrobial drugs after challenge with *P. multocida*. By evaluating drug kinetics in affected calves of two different ages (3 weeks and 6 months), the concomitant influence of disease and age on the distribution and PK could be examined. A comparison of these results to outcomes previously reported in normal healthy 3 week and 6-month-old calves provides insights into whether disease influences the age-associated differences in the PK of these two compounds.
Materials and Methods

Animals

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee. Weaned and milk-fed male Holstein calves were bought from a local dairy herd. Eighteen milk-fed Holstein calves, 2-3 weeks of age, weighing between 45.5-70.5 kg were purchased and trailered to the treatment facility. Calves were housed in groups of two, fed commercial milk replacer twice a day, and allowed access to water and calf starter (Milk Specialties, Inc., Eden, MN) throughout the study. Eighteen weaned calves, 6 months of age and weighing between 188-281 kg at time of study, were purchased from a local dairy herd. The calves were group housed indoors on a concrete floor bedded with wood shavings, fed grass hay and allowed free access to water throughout the study. None of the calves had any previous history of disease or antibiotic administration and had normal physical examinations prior to start of the study. All calves were euthanized at the end of the study.

Experimental Infection

All calves were subjected to the physical stress of a two-hour trailer ride prior to being inoculated with the pathogen. On day 0, all calves were inoculated with field isolates of *P. multocida* from confirmed clinical cases via Collison nebulizer (Jorgenson Labs Inc. Loveland, CO). This device produces single-cell droplets, < 3 μm in diameter, delivered using a head mask fitted with an inhalation and exhalation port placed over the nostrils and mouth. The nebulizer delivered 10 mL of bacterial suspension containing approximately $1 \times 10^9$ CFUs and the time of exposure lasted 20-25 min. Calves were monitored throughout nebulization with vitals taken every hour post nebulization to monitor clinical progress. The bacteria used in this study were shown to be susceptible to danofloxacin and tulathromycin. All susceptibility testing in our
laboratory was conducted according to CLSI standards. Three isolates from clinical field strains of *P. multocida* were re-steaked from glycerol stocks onto trypticase soy agar supplemented with 5% sheep blood and incubated at 37°C overnight. The next day, a single colony was selected and inoculated into 100 mL of typtic soy broth. The broth was enriched for 18-20 hours at 37°C. After enrichment, a 1 mL aliquot was removed and serially diluted ten-fold, spot plated onto blood agar plates, and incubated to determine the starting concentration. The remaining enrichment was used to challenge calves.

**Clinical Score Assessment**

Each calf was assessed using a clinical score sheet from the University of Wisconsin Calf Respiratory Scoring System (McGuirk 2008). Briefly, this 12-point system assigns a score based on rectal temperature, nasal discharge, and eye and ear scores. Each criterion is graded on a 0–3 scale, with 0 being normal and 3 being the highest severity. Calves were enrolled into the trial that scored ≥ 5 after nebulization. The score of each calf was noted prior to induction and every 24 hours after drug administration.

**Thoracic Ultrasound**

Ultrasonography was performed using an 8.5 MHz linear probe that was directly applied on the thorax after 70% isopropyl alcohol had been sprayed. All calves were scanned on each side of the thorax for the presence of abnormal ultrasonographic findings and scored based on previously validated scoring system (Ollivett et al. 2015). Lungs were scanned prior to nebulization and every 24 hours throughout the study.

**Drug Administration and Blood Collection**

All calves were weighed on a digital scale on the morning of the study commencement for determination of the administered dose. Upon arrival, calves were restrained for intravenous
catheter placement. The area where the catheter was to be placed was clipped and aseptically prepared. Using sterile technique, a 14 G x 3.25 inch catheter (Angiocath™, BD, Franklin Lakes, NJ, USA) was inserted into the right jugular vein, an extension set was then attached and both were sutured in place using a 2-0 monofilament suture. Catheters were flushed three times a day using 6 mL of heparinized saline (10 units/ml). Depending on group assignment, a single SC injection of danofloxacin (8 mg/kg) (Advocin™; Zoetis, Florham Park, NJ, USA) or tulathromycin (2.5 mg/kg) (Draxxin™, Zoetis, Florham Park, NJ, USA) was administered to each calf in the neck per label instructions.

For calves administered danofloxacin, blood samples were collected from the jugular catheter and transferred to lithium heparinized tubes at time 0 (pretreatment), 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, and 144 hours post administration of the drug. For calves administered tulathromycin, blood samples were taken at 0, 0.25, 0.5, 1, 3, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, 216, 228, 240, 252, 264, 276, 288, and 300 hours post administration. These samples were stored on ice until centrifugation at approximately 3500 x g for 20 min to separate plasma. The plasma samples were stored at -80°C until analysis.

**Interstitial Fluid Collection**

All calves were implanted with SC ultrafiltration interstitial fluid (ISF) probes on the side of the neck opposite the site of the SC drug injection (BASI Inc, West Lafayette, IN). Each probe contained three semi-permeable loops connected to a non-permeable tube that extended outside the animal and attached to a 3 ml no additive plastic vacutainer tube. This tube provided negative pressure for fluid collection through small pores in the probe membranes. These pores allowed for the movement of water, electrolytes and low molecular weight molecules (<30,000
Da) to pass into collection tube. This pore size excludes large molecules such as proteins, protein bound drugs, and cells. Probes were placed with calves under sedation with xylazine [Rompun® Injectable (20mg/ml); Bayer Animal Health Division] at a dose of 0.05-0.1 mg/kg in the cervical neck muscles. Probes were placed twenty-four hours prior to the start of the trial to allow adequate time for equilibration with body fluids. One probe was inserted into each calf subcutaneously in the area cranial to the scapula. All probes were placed through a small stab incision using an introducer needle provided by the company. The ISF was collected at time 0 (pretreatment), 2, 4, 6, 8, 10, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, and 144 h after SC administration of danofloxacin. For tulathromycin, ISF was collected at time 0 (pretreatment), 3, 4, 6, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, 216, 228, 240, 252, 264, 276, 288, and 300 hours. Since each ISF sample represents fluid collection over a certain amount of time (i.e. not instantaneous sampling), a lag time was calculated based on the length of the tube and fluid collected over time for each sample. The fluid collected was frozen at -80°C until analysis.

**Lung Fluid Collection**

To determine drug concentrations in the PELF, a BAL was performed using a method described previously (Poulsen and McGuirk, 2009). Briefly, BALs were performed in all 3-week old calves using a sterilized, flexible 10 French X 36 inch catheter with a 3-cc balloon cuff and in ruminating calves a 24 French X 59 inch catheter was used (Mila International, Inc. Medical Instrumentation for Animals, Florence KY). At each time point, the calf was restrained and the head and neck of the calf were extended to facilitate passage of the sterile BAL catheter. The BAL catheter was introduced into the ventral meatus of the nose through which it was advanced down the trachea until it was wedged in a terminal bronchus. Repeated coughing was used as an
indicator of appropriate placement. In the wedged position, the balloon cuff was inflated to create a seal and the catheter was held firmly in place while the guide-wire was removed. At each time point, 100 ml of sterile saline were infused into the lungs. Immediately after the infusion, negative pressure was applied to aspirate fluid. The volume of fluid that was retrieved ranged from 4 to 75 ml of clear to mildly turbid foamy fluid. The fluid sample was placed into a sterile collection tube, the total amount recorded and placed on ice until centrifugation. The BAL samples were centrifuged at 300 x g for 10 min and supernatant fluid was separated from cell pellet and frozen at -80°C until analysis.

Drug Analysis

Total (protein bound and free) plasma and BAL fluids concentrations were determined as total tulathromycin and danofloxacin. ISF concentrations were solely the unbound concentrations of the two compounds.

Plasma and BAL fluids were analyzed by either high-performance liquid chromatography (HPLC) with fluorescence detection (danofloxacin) or by ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) (tulathromycin) following solid phase extraction. The ISF was injected directly onto the HPLC/UPLC. Danofloxacin was extracted from plasma and BAL supernatant using a modified enrofloxacin extraction method described previously by Davis et al (2007). For tulathromycin, plasma samples for UPLC-MS/MS analysis were pretreated by mixing 500-μL of plasma with 500-μL of 4% phosphoric acid, and vortexing for 10 seconds. The 1 mL pretreated sample was then loaded onto Oasis 3 cc (60 mg) PRiME HLB cartridges (Waters Corporation) and pulled through with a vacuum at ~3 psi. Each sample was then washed with 1 mL of 5:95 (v:v) methanol:water. Samples were then eluted from the cartridge using 400-μL of 60:40 (v:v) acetonitrile:water with 0.1% formic acid.
The collected liquid was then transferred to a vial and 5-μL aliquot was analyzed by the UPLC-MS/MS.

Validation standards were prepared over a linear range for each matrix (plasma, ISF, sodium chloride 0.9% (as a substitute for PELF), mobile phase) and were used to construct calibration curves. These standards were validated over the range 0.001-5.0 μg/mL in fortified (spiked) blank plasma, BAL and ISF with danofloxacin or tulathromycin (reference drug standards for both danofloxacin and tulathromycin were provided by Zoetis) to validate the HPLC analysis.

The percent coefficient of variation (%CV) for inter and intra-day danofloxacin recovery averaged 10.7% (with an average recovery of 95.9%) over the validated range. The limit of quantification (LOQ) was 5 and 10 ng/ml and the limit of detection (LOD) was 1 and 5 ng/mL for plasma and BAL, and ISF respectively.

For tulathromycin, for each assay matrix, calibration curves were constructed spanning over the range of 5–1000 ng/mL. The R² values for the calibration curves were 0.99. Intra and inter-day %CV were less than 20%, and the accuracy ranged from 102 to 106%. The LOQ was 5 ng/mL, with a precision of 8% and an accuracy of 105%.

*Danofloxacin/Tulathromycin Concentrations in PELF*

Estimation of the amount of PELF sampled by BAL fluid was performed using the urea dilution method as described previously in cattle (Giguère, S. et al. 2011). Urea nitrogen concentrations in serum and BAL fluid was determined by use of a urea test kit (Urea test kit; Sigma Chemical, St Louis, MO, USA) and the absorbance values measured by use of a spectrophotometer.

*Pharmacokinetic Analysis*
A compartmental PK analysis was generated using Phoenix 64, Certara, Princeton, NJ, USA. The influence of age and calf respiratory score on primary and secondary PK parameters was determined using a nonlinear mixed-effect model (Phoenix® WinNonlin/NLME, Version 1.3 Certara). For both danofloxacin and tulathromycin, the population base model was fitted as a multiplicative two-compartment model parameterized by clearance. Model selection was based upon the precision of parameter estimates, goodness-of-fit plots (e.g., residual plots) and statistical significance between models using lowest log-likelihood ratio (-2LL) values obtained in the software. A preliminary compartmental analysis was conducted with Phoenix WinNonlin to obtain the initial estimates for the parameters of the basic model (i.e. no covariates).

For both danofloxacin and tulathromycin, a determination assessment of the contribution of age and respiratory score to calf pharmacokinetics was assessed by improvement in the objective function following the addition of each term to the base model. A box plot of effect of the covariate (age and respiratory score) on each parameter showed that clearance (CL) expressed as a function of bioavailability (CL/F) was the parameter most likely affected by age. Age was added to the base model as a categorical covariate (where 3-week old calves = 0 and 6 month old calves = 1), and its effect on the PK parameters, the apparent volume of distribution (Vd) expressed as a function of bioavailability (Vd/F) and CL/F was evaluated using a likelihood ratio test. A P-value \( \leq 0.05 \) was considered to be significant. For both tulathromycin and danofloxacin, age was found to significantly improve model predictions for CL/F (P < 0.001), but not Vd/F. After age was determined to improve the model, this covariate remained in the final model. Total respiratory score was determined to not be a significant covariate irrespective of calf age. All PK parameters, except for elimination half-life (T½), were reported as a
The individual animal PK values were calculated, and the descriptive statistics (geometric mean ± SD) were reported.

Statistical comparisons of PK parameters from the compartmental analysis and measured clinical outcomes of each age group were performed with the Kruskal Wallis test for nonparametric data using SigmaPlot (Systat Software, Inc, San Jose, CA, USA); P-values of \( \leq 0.05 \) using a two tailed test were considered statistically significant. The normality assumption was tested for each variable set with the Shapiro–Wilk W-test, which is the preferred method for testing the normality of data when the sample size is small (Ghasemi & Zahediasl, 2012). PELF concentrations for all groups were averaged at all-time points. Compartmental PK parameters were compared statistically for both groups.

**Results**

Jugular catheters and ISF probes were well tolerated. During some sampling time intervals, fluid was not collected into the ISF probe, so ISF data is missing for these time points. One 3-week old calf (enrolled in the group administered tulathromycin) died 8 hours post nebulization. Necropsy results attributed the cause of death to significant pulmonary edema due to high endotoxin levels and *P. multocida* was cultured from lungs and joints. During BAL collection, harvested fluid volumes varied considerably.

**Clinical Health Scores and Thoracic Ultrasound Scores**

The mean clinical health and ultrasound scores, as well as rectal temperatures throughout the study are presented in Table 2 (danofloxacin group) and Table 4 (tulathromycin group). Prior to drug administration, the clinical health score and thoracic ultrasonographic score did not differ significantly between the two age groups. All calves prior to administration of either study drug
attained a respiratory disease score ≥ 5. Scores were monitored throughout the study period.
Total respiratory scores decreased throughout the study in both groups.

Danofloxacin Plasma

The fitted PK parameters of danofloxacin following a single 8 mg/kg SC danofloxacin in 3-week and 6-month old calves with respiratory disease are shown in Table 1 and Figure 1. A chart comparing non-compartmental (observed) parameters and compartmental parameters are available in Table 5a,b for comparison. Danofloxacin demonstrated a Vd/F of 3.1 L/kg and 3.5 L/kg in 3-week old calves and 6-month old calves respectively. 3-week old calves had a significantly lower mean CL/F (262.9 ml/hr/kg) as compared to 6-month old calves (516.3 ml/hr/kg). However, these age-related differences in clearance were not reflected in plasma terminal elimination half-life (T½) values. The 3-week old calves presented with T½ similar harmonic mean (±harmonic SD) values of 34.45 ± 15.5 hours while that of the 6-month-old calves were 34.11 ± 20.1 hours. The area under the curve (AUC) was significantly greater in the 3-week old calves as compared to older calves. (Table 1). After SC administration, the maximum danofloxacin concentration in plasma (Cmax) reached a value of 2.4 μg/mL for 3-week old and 3.6 μg/mL in 6-month old calves. The mean time to maximum concentration (Tmax) was 2.4 and 2.0 h, respectively. ISF:PlasmaTotal ratios are reported for danofloxacin in Figure 6. Although ratios were variable, there was a trend for lower ISF:PlasmaTotal ratios in 3-week old calves as compared to 6-month old calves.

Danofloxacin Interstitial Fluid

Using an in vivo ultrafiltration technique to collect ISF in repeated samples allowed for the monitoring of unbound drug disposition over time and was less invasive than tissue biopsies.
Although some samples were missed due to occlusion of the probe, these devices were well tolerated and collected between 0.05 μL and >2 mL of fluid per sampling time for drug analysis.

The mean (±SD) concentration of danofloxacin in ISF samples from both groups of calves are shown in Figure 2. The $T_{\text{max}}$ for ISF fluid occurred later than in plasma irrespective of calf age. There was no difference in concentrations between the two groups of calves.

*Danofloxacin Pulmonary Epithelial Lining Fluid*

Average PELF concentrations from the BAL samples are shown in Figure 3. The maximum concentration in PELF was noted to occur at the first sampling time (2 h post administration) in both groups, with estimated PELF concentrations far exceeding the concentrations seen in plasma and ISF. High variability in drug concentrations in PELF was seen in both groups of calves. 3-Week old showed trends for higher concentrations of danofloxacin in PELF as compared to 6-month old calves.

*Tulathromycin Plasma*

The PK parameters and plasma concentrations of tulathromycin in two different ages of calves affected by respiratory disease following SC administration of 2.5 mg/kg once are shown in Table 3 and Figure 4 respectively. Following SC administration, tulathromycin demonstrated a $V_d/F$ of 27.9 L/kg and 14.7 L/kg in 3-week old calves and 6-month old calves respectively. The $CL/F$ values estimated for the 3-week old calves (343.3 ml/hr/kg) was significantly greater than that estimated in the 6-month old calves (126.5 ml/hr/kg). The harmonic mean plasma $T_{\frac{1}{2}} ±$ harmonic SD was longer in 6-month old calves ($114.9 ± 74.2$ hours) as compared to that observed in the 3-week old calves ($98.7 ± 52.6$ hours). The AUC was significantly lower in the 3-week old calves as compared to that estimated in the older calves (Table 3). After SC administration, the maximum tulathromycin concentration in plasma ($C_{\text{max}}$), reached a value of
0.49 ± 0.26 μg/mL for 3-week old and 0.54 ± 0.53 μg/mL for the 6-month old calves. The corresponding mean T<sub>max</sub> values were significantly later in 6-month old calves (3.4 h) than in the 3-week old calves (0.6 h). ISF:Plasma<sub>Total</sub> ratios are reported for tulathromycin in Figure 6. Although ratios were variable, there was a trend for higher ISF:Plasma<sub>Total</sub> ratios in 3-week old calves as compared to 6-month old calves.

*Tulathromycin Interstitial Fluid*

The mean (±SD) concentration of tulathromycin in ISF samples from both groups of calves are shown in Figure 6. The T<sub>max</sub> for ISF fluid occurred later than in plasma irrespective of calf age. Despite 6-month old calves showing increased variability in ISF concentrations as compared to 3-week old calves, there was no difference in concentrations between the two groups of diseased calves at any time point.

*Tulathromycin Pulmonary Epithelial Lining Fluid*

Average PELF concentrations from the BAL samples are seen graphically in Figure 7. The maximum concentration in PELF was noted to occur at 3 h post administration for 3-week old calves and around 12 hours in 6-month old calves, with estimated PELF concentrations far exceeding the concentrations seen in plasma and ISF at all time points. High variability in drug concentrations in PELF was seen in both age groups of calves administered tulathromycin.

**Discussion**

One of the challenges to address was identifying an appropriate approach for analyzing the study data and the impact of the covariates (age and disease) on the PK of danofloxacin and tulathromycin in calves was selecting an appropriate approach for the data analysis. On the one hand, compartmental models are a simplification of the complex processes influencing the dose-exposure profiles and therefore are subject to bias due to model-misspecification. On the other
hand, much information is lost through the use of simple non-compartmental (NC) methods. Moreover, for compounds such as tulathromycin (long residence time in the body although relatively rapid disappearance from the plasma) NC methods are inadequate for the questions that we are asking in this investigation. Therefore, the results of both methods of analysis are presented. Covariate analysis was subsequently generated using a NLME approach based upon the initial parameter estimates obtained using a two-compartmental model. Data collected in this study was analyzed by both methods.

*Respiratory Disease Model*

Pneumonia is an important health problem in calves. In the current investigation, the calves showed clinical and physiological signs of respiratory disease after nebulization with the targeted bacterial pathogens. Changes in clinical parameters included an increase in animal heart rate, nasal and ocular discharge, and an increase in body temperature. During our clinical evaluation of these animals, we concluded that the thoracic ultrasound scores associated with disease resulting from use of the induction model shared some (although not identical to) the clinical and pathological manifestation of natural disease processes. The anterior–ventral lobar distribution of natural and experimental BRD is well recognized in cattle and that same pattern was noted on thoracic ultrasounds in the current experiment. Similar to previous disease models, the distribution of recognizable lesions in lung tissue is not uniform. The distribution of pathological processes may be influenced by the architecture of the lung parenchyma. Gravitational forces may account for some of the variability in the lesions noted after disease induction.
The concentration of *P. multocida* ranged from $1 \times 10^9$ to $4 \times 10^9$ but was not found to correlate with more severe clinical signs. A similar result was reported in experiments using lambs where differences of 3 logs in *M. haemolytica* A2 aerosols did not result correlate with the severity of the pneumonia (Gilmour, 1984). This failure to determine any obvious correlation suggests that additional physiological factors can significantly contribute to the expression and severity of the BRD.

More severe clinical signs of respiratory disease were observed in 3-week old calves. Several pathophysiological changes could explain the more severe clinical signs in young calves as compared to 6-month old calves, including higher local bacteria dose in airways, lack of mature immunologic response and differences in physiological barriers in the alveoli-capillary membrane. All calves in this study received the same volume of nebulized bacteria. Young calves have smaller diameter airways, potentially leading to higher concentration of bacteria locally. The immune response in lungs of young calves in general is slower, less efficient, and much less focused than in older patients (Batista et al. 2012), which could lead to an infection that is less likely to be localized due to poor inhibition by the host defenses. Tissue damage from bacteria could predispose young calves to damage of the alveolar-capillary membrane and therefore an increase in membrane increased permeability. Bacteria and toxins in theory can cross this barrier, leading to endotoxemia, which has been shown to have critical effects on drug distribution and PK (Agrawal, Singh, and Jayachandran 2001). After birth, the formation and maturation of functional alveoli of the lungs that form the alveolar-capillary membrane continue, and immaturity of the barrier could explain more severe clinical disease in 3-week old calves (Burri, 2006). Patients with acute pneumonia have demonstrated depressed myocardial function.
and dehydration from extracellular fluid shifts, leading to a decrease blood volume and effectively a decrease in cardiac output.

**Danofloxacin**

We investigated the impact of experimental BRD on the PK and distribution of subcutaneously administered danofloxacin in two different ages of calves. Several authors have described plasma concentration-time relationships as well as determined concentrations in lung tissue, bronchial secretion and bronchial epithelium for danofloxacin in mature calves (with and without the presence of BRD), (Apley and Upson, 1993a; Friis, 1993; Giles et al., 1991). However, in our study, we further considered these relationships as a function of calf age. The results of the present investigation, as well as those of previous studies performed in healthy calves (Mzyk et al, 2017), were consistent with a two-compartment open model (Figure 1). In an earlier plasma PK studies in healthy 6-month old beef calves, observed plasma C\text{max} values ranged between 1.2 and 2.2 µg/mL. In the current study involving diseased calves, the range of plasma C\text{max} were higher (1.1 – 10.4 µg/mL for the 6-month old calves and 1.6 – 4.2 µg/mL for the 3-week old calves). Thus, there is a trend suggesting that higher C\text{max} values in diseased animals. Importantly, age appeared to be more important than disease in terms of the peak concentrations.

Maximum danofloxacin concentrations in the bronchial secretions of ruminating calves infected with BRD was reported to occur by other investigators at approximately 2 hours post administration. The corresponding peak concentrations were about 6.9 µg/mL, with T\text{max} values of about 2 hrs in the bronchial secretions (TerHune et al. 2005). Similarly in our study, the T\text{max} in diseased 3-week and 6-month old calves in PELF averaged 2.4 and 2.0 hours. The C\text{max} in PELF reached higher concentrations in 3-week old calves (4.54 µg/ml) as compared to 6-month old calves (1.61 µg/ml). Common respiratory pathogens, including *M. haemolytica* and *P.*
*multocida*, are extracellular organisms, so determining concentrations at the site of infection in PELF may be more clinically relevant than lung tissue concentrations.

With regard to the plasma profiles, Apley and Upson (1993a) previously noted that the presence of BRD could influence the T$_{1/2}$ of danofloxacin. They showed that crossbred steer calves with acute pneumonia had a plasma T$_{1/2}$ of approximately 6.3 h (In the current study, we observed plasma T$_{1/2}$ values of 30-hr for 3-week old calves and 29-hr for 6-month old calves).

As observed in our previous studies in healthy calves, the longer time for depletion in the plasma concentrations reflected our ability to capture a second depletion phase. As compared to that reported in other investigations, the longer T$_{1/2}$ estimated from the data generated in the present study may be attributed to the interdependence of the drug pKa, lipophilicity, metabolism pathways and elimination rate. It would be expected that as clearance decreases, (owing to disease processes), T$_{1/2}$ would increase. However, this relationship only holds true when the disease process does not simultaneously decrease the Vd/F. For lipophilic drugs like danofloxacin, age (as well as pathophysiological shifts in body water and fat distribution) can be expected to influence the calculated T$_{1/2}$, and any decrease in drug available in its unionized state due to pneumonia-induced respiratory acidosis (a change in the pH of the plasma and in other body tissues) could lead to a decrease in drug lipophilicity. This decrease in lipophilicity would decrease the movement of drug into the tissue compartment, thereby reducing the volume of distribution, and counteracting the effects of the decrease in drug clearance. In that regard, there is evidence that inflammation and disease can affect the acid-base exchange within the lung fluid (Fischer et al., 2002). Using a bronchoscopically-directed pH electrode, Bodem et al., (2983) showed that as compared to that observed in non-infected bronchi, the presence of pneumonia can significantly lower the pH of the infected bronchi. Acidification of the PELF can also
negatively impact the innate immune responses of the respiratory tract and impair the host response to pathogens and inflammation (Ng et al., 2004). Since both danofloxacin and tulathromycin have multiple pKa values, the acidification of the PELF could potentially lead to an increase in tissue trapping, and an increase in local lung concentrations. However, this trapping would still need to overcome the impact of acidosis-associated changes in plasma pH. Thus, it is not surprising to find higher inter-individual variability in the PK of diseased as compared to normal healthy animals.

Total clearance of drugs is governed by blood flow to all eliminating organs, the unbound fraction and intrinsic clearance mechanisms. Unlike intrinsic clearance, which represents the ability of an organ to clear unbound drug and is typically unaffected by plasma protein binding (Benet and Hoener, 2002; Toutain and Bousquet-Melou, 2002; Stern et al., 2016), total drug clearance and total plasma drug concentrations can be influenced by the extent of plasma protein binding. Plasma protein binding in previous studies demonstrated minor differences between that observed in healthy 3-week versus 6-month old calves (Mzyk et al. 2017). In disease, widely variable levels of a drug binding protein, alpha1-acid glycoprotein (AGP), can modify the total drug distribution of basic drugs (Murata et al., 2004). For this discussion, the authors are specifically referring to changes in total drug concentrations and not to changes in the distribution of free drug concentrations.

The distribution of free drug concentrations will change only if the drug has a very large volume of distribution (such that the concentrations of drug in the plasma have negligible contribution to the overall Vd) and if the protein binding in the tissue differs from that in the blood. Based upon the article by Benet and Hoener, volume of distribution (V):

\[ V = \frac{F_u}{F_{uT}} \times V_T + V_p \]
depends on the fraction unbound in plasma ($F_u$), the fraction unbound in tissue ($F_{uT}$), the volume of tissue ($V_T$), and the volume of plasma ($V_P$). If the change in $F_u$ in blood and tissue are the same, then $V_T$ will not change and the overall volume of distribution will not change. It should be noted that other than albumin, there is very limited data quantifying tissue levels of AGP in cattle and therefore we will not consider the potential impact of changes in AGP on the free fraction in tissues and plasma.

The effect of age on danofloxacin CL/F values was significant. Assuming a similar fraction of drug absorbed from the injection site, CL/F values in 3-week old calves with respiratory disease (262.9 mL/kg/hr) was lower than in healthy 11-13 week old calves (578 mL/kg/h) or in healthy 3-week old calves (Sarasola et al. 2002; Mzyk et al. 2017). Maturation differences of CL/F values, as well as changes in blood flow and pathophysiology of clinical diseases in calves less than three weeks of age may impact the intrinsic clearance and elimination of danofloxacin. As compared to healthy 3-week old calves, 3-week old calves with respiratory disease had higher AUC values (23.2 µg*hr/mL and 32.1 µg*hr/mL, respectively) (Mzyk et al. 2017). Danofloxacin is eliminated both by hepatic and renal clearance. Severe infection is associated with a decrease in organ blood flow. Renal blood flow is affected by any changes in cardiac output (Langenberg et al., 2005). Thus, the observed lower CL/F values could be attributed, at least in part, to a reduction in the renal elimination of unchanged drug in diseased 3-week old calves. The observed lower total drug CL/F values reported in 3-week old calves with respiratory disease could potentially lead to higher total drug exposure to danofloxacin. It should be noted that this same pattern was not observed in healthy vs diseased 6-month old calves (15.2 µg*hr/mL and 18.9 µg*hr/mL respectively). Thus, disease appeared to have a greater influence on the PK of the very young versus the mature calves.
In our previous studies performed in healthy calves, the ISF tended to be somewhat greater in the 3-week versus 6-month old calves, but the ratio of ISF/unbound plasma concentrations were markedly higher in the 6-month old versus 3-week old animals. In the current investigation, penetration of danofloxacin into the ISF did not differ significantly between the two age groups at any time point. However, a trend was noticed in the ISF:PlasmaTotal ratio where there were differences noted in the shapes of the ratios in 3-week old calves vs 6-month old calves as a function of time (Fig. 6). For the 3-week old calves, the ratios peaked at 24 h but their values remained lower than those seen in the 6-month old calves. In contrast, the ratios continued to decline over the duration of sampling period in 6-month old calves. While the reason for this disparity cannot be determined, we cannot exclude the possibility of age-associated differences in disease effects on plasma protein binding. Such age-associated difference could reflect the greater severity of disease seen in the 3-week versus 6-month old calves. If severity of disease is linked with respiratory acidosis, then a greater lowering of the blood and pulmonary pH in the very young animals could lead to greater drug ionization and therefore greater affinity for the proteins in the blood and in the bronchial fluids. Such differences would have gone undetected in our previous study where we could find no difference in plasma protein binding as a function of age in healthy calves.

While these results are provocative, it is important to recognize several limitations to interpreting the ISF concentrations in calves with respiratory disease as compared to work previously published ISF data in healthy calves by other authors. First, previous studies using ultrafiltration probes in calves changed the sampling tube every 24 hours instead of every 12 hours, as was done in this study. Studies performed by our lab (data not shown) indicated that maximum fluid collected/hour occurred when the collection tubes were changed every 12 h.
instead of every 24 h. Second, the method used for capturing the ISF reflects an averaging of concentrations over a collection period. Accordingly, when estimated at the time of fluid sampling, the measured concentration will exceed the actual concentration of drug in the ISF at any given moment in time. Thus, the use of ultrafiltration tends to overestimate ISF concentrations at a point in time.

In the current investigation, danofloxacin PELF concentrations decreased considerably by 24 hours after administration, but remained higher than those seen in the plasma and the ISF. Danofloxacin concentrations in PELF collected from healthy calves showed lower concentrations in samples taken at 24 hours as compared to calves with induced respiratory disease at the same time points. Concentrations in PELF from calves with respiratory disease was significantly higher at 2 and 12 hours in 3-week versus 6 month-old calves. However, disease did not appear to influence the hr 2 PELF danofloxacin concentrations in the 3-week old calves $[5.4 \pm 6.5 \, \mu g/mL$ and $5.6 \pm 3.4 \, \mu g/mL$ in healthy and respiratory disease calves respectively (Mzyk et al, 2017)]. However, at 24 hours, healthy 3-week old calves had lower danofloxacin concentrations ($0.62 \pm 0.86 \, \mu g/mL$) when compared to calves with respiratory disease ($2.3 \pm 5.4 \, \mu g/mL$). This could possibly have been of function of danofloxacin accumulation within the bronchial mucosa and lung tissues (Friis, 1993). Similar disease effects were not observed at hr 24 in the 6-month old calves $[3.6 \pm 1.7 \, \mu g/mL, 1.6 \pm 0.89 \, \mu g/mL$ in healthy and diseased calves, respectively (Myzk et al., 2017)].

An additional finding was that although concentrations of danofloxacin in the PELF were highly variable across both groups of calves, on the average, they were higher in the 3-week old calves as compared to that of the 6-month old calves at all-time points sampled. Although mean values differed, the magnitude of variability, particularly in diseased calves, rendered it difficult
to draw conclusions. Rather, it would appear that the primary challenge was that of experimental
whereby there was greater difficulty in obtaining reliable sampling of PELF in neonates as
compared to mature calves. Furthermore, the sampling method used in this study may be
overestimating fluoroquinolone concentrations in PELF (Foster et al. 2017). Pathophysiological
changes to the lung parenchyma and blood flow to affected lung lobes may also contribute to the
observed high variability. Data from calves with no areas of pulmonary consolidation indicated
that blood flow was significantly lower in the caudodorsal position of the left lung and in the
caudodorsal and cranioventral positions of the right lungs as compared to other pulmonary
locations (Apley and Upson, 1993b). Since the BAL sampling technique used in our study was
performed without the aid of a video bronchoscope, the sampling of a specific lobe was not
feasible, which may have contributed to the sampling of multiple lobes (healthy and/or
consolidated) across the various sampling occasions.

The measurement of the danofloxacin in ISF and PELF is ideal as most respiratory
pathogens seen in calves are extracellular bacteria. Collectively, these findings, along with
previous studies in healthy calves, suggest that age and disease both impact danofloxacin
concentrations in plasma, ISF and PELF. By measuring the drug concentration in the active sites
of infection, a more accurate conclusion of clinical efficacy can be determined for specific
populations of calves. However, even if that is not feasible, our results show that comparative
blood levels were able to identify differences in drug PK as a function of age and disease.

*Tulathromycin*

Plasma concentrations of macrolides such as tulathromycin in cattle are in general
considerably low relative to the MIC of the extracellular respiratory pathogens for which they are
used. A better measure of clinically relevant information would include measuring free/unbound
drug concentrations at the site of infection. Studies of respiratory tract infections have demonstrated a role for white blood cells in the delivery of drug to the infection site. This is of importance for drugs, like tulathromycin, which concentrate in white blood cells and accumulate in lung tissue (Villarino et al., 2013; Drusano, 2005).

We observed that irrespective of age, plasma tulathromycin concentrations were relatively low as compared to its concentrations in the PELF, was absorbed within less than 1 hr of dosing but declined slowly after SC administration (Fig. 4). The median T$_{1/2}$ was similar across both ages groups (116.5 (3-week old) and 91.6 hours (6-months old)). However, when compared to healthy calves, the mean T$_{1/2}$ was nearly twice as long in the diseased calves [98.7 hrs vs 67.6 hr in diseased versus healthy 3-month old calves and 114.9 versus 44.4 hr in diseased versus healthy 6-month old calves (Mzyk et al., 2018)]. Thus, disease markedly affected the duration of systemic tulathromycin exposure.

For macrolides, the use of blood levels for the assessment of dose when treating BRD does not directly mirror drug concentrations at the site of action. Unbound concentrations of tulathromycin in the ISF were therefore considered as a method for evaluating the movement of unbound drug from plasma throughout the body. Consistently, in healthy and diseased calves, concentrations in ISF were lower than were the plasma concentrations and were nearly superimposable across the two age groups. However, unlike that seen in healthy calves where the ISF/total plasma concentration ratios tended to be higher in the 6 month old calves during the initial sampling times, tulathromycin concentrations in the ISF were similar in 6-month old calves as compared to 3-week old calves with respiratory disease across the duration of the evaluation, suggesting that there was a greater ability of the drug to partition out of the blood of the diseased 3-week vs 6 month-old calves. Accordingly, the tulathromycin ISF: total plasma
ratios tended to be higher in the 3-week versus 6-month old calves. While this interstudy difference could be due to the shorter sampling time duration in diseased calves (every 12 hours) vs healthy calves (every 24), other potential causes for this difference need to be considered. For example, in calves with respiratory disease, there is a potential for ionization in the ISF and PELF, due to respiratory acidosis and acidification of the PELF. We know that respiratory disease was more severe in the 3-week versus 6-month old calves. Therefore, although we did not evaluate plasma or ISF pH, a difference as a function of age and disease need to be considered. Tulathromycin has multiple pKa values, and the acidification of the PELF could potentially lead to an increase in drug concentrations in the ISF and PELF due to tissue trapping.

Several techniques have been described for estimating the PELF of macrolides including tissue homogenates, BAL, and bronchial microsampling (Winther 2012; Foster et al. 2017). In contrast, the use of tissue homogenates does not allow for a differentiation between intracellular vs. extracellular drug concentrations or for the binding of drug to the tissues, therefore overestimating the active concentration of macrolides at the site of infection (Mouton and Theuretzbacher, 2008). PELF is a reflection of the concentrations of the drug available at the site of bovine respiratory infections. Measurement of active/free drug in this compartment allows for the evaluation of drug exposure, and potential improvement of dosing strategies in different ages of cattle affected with BRD.

Tulathromycin concentrations in PELF were higher than concurrent plasma concentrations at all sampling times. PELF concentrations in diseased cattle did not differ between 3-week old and 6-month old calves and were typically similar in healthy and diseased calves. Given these limitations and uncertainties, PELF values should be considered as a rough approximation of pulmonary drug concentrations. However, this does not support concerns that
concentrations estimated in healthy animals will be biased as compared to that in animals with BRD.

One 3-week old calf (Calf #34) administered tulathromycin after induction with aerosilized P. multocida demonstrated clinical signs of septicemia/endotoxic shock, including decreased heart rate, loss of suckle reflex, dehydration and cold extremities. Evaluating the calf’s plasma concentration profile revealed $C_{\text{max}}$ concentrations 5x the average for other calves in the three-week-old group. The AUC was approximately double the reported mean. The CL/F for this calf was approximately 172 ml/hr/kg, about half of the reported mean for all 3-week old calves with respiratory disease. Other calves enrolled on this study showed signs of respiratory disease, but not septicemia/endotoxemia. Unlike typical BRD, septicemia indicates bacteria in the bloodstream. Sepsis-induced liver hypoperfusion may result in a decreased clearance for high extraction ratio drugs (De Paepe et al. 2002). These PK changes are consistent with previous studies evaluating the impact of fever and sepsis on PK parameters which demonstrated decreased clearance values and increased maximum plasma concentrations (Agrawal, Singh, and Jayachandran 2001; Altan et al., 2017).

Summary

Data about the influence of disease on the pharmacology of drugs in pediatric calves are limited. The available studies mainly concentrate on plasma pharmacokinetics in healthy cattle, and largely ignore the effects of age and disease. Extrapolation of study results done in healthy adult cattle is difficult due to large variability in age-effects. In addition, studies in healthy animals fail to examine the underlying pathophysiological conditions and the pharmacokinetic characteristics of the drug concerned. Information on the effect of BRD is critical to estimate the extent of changes in pharmacokinetic parameters, facilitating efficacious drug use in calves.
The present study indicates that age, as well as disease, can affect the plasma pharmacokinetics of danofloxacin and tulathromycin in calves. However, it is important to recognize that therapeutic success is not only a function of drug exposure but also of the ability of the patient to launch an effective antibacterial response. The latter was not considered in this study, but we did observe that given the same mg/kg dose of danofloxacin and of tulathromycin, therapeutic success was maintained following artificial infection of both the 3-week and 6 month old calves.

While the overall influence of age appeared to be well characterized irrespective of whether one considered healthy or diseased animals for tulathromycin, this did not seem to be the case for danofloxacin. Macrolide antibiotics differ from danofloxacin in mechanism of action and PK parameters. Previously, age-associated differences in PK of tulathromycin of healthy calves included higher Vd/F in 6-month olds, as well as lower CL/F in 3-week old calves. Given the variability in data generated for tulathromycin, we conclude that these age-associated differences can be applied to both healthy and diseased calves. Clearly, this implies a need to consider both age and disease on a drug-by-drug basis and a need to better understand the effect of age and disease on the PK processes such as drug metabolism, transporter activity, and tissue perfusion.

Acknowledgments

The authors want to sincerely thank Dr. Ginger Hobgood, Dr. Derek Foster, Hannah Sylvester, and Janelle Wiser for their help in completing the PK studies. The authors also want to express their utmost gratitude to the staff of NC State’s College of Veterinary Medicine Lab Animal Resources Team, especially Maria Stone and Mallory McKinlay for their guidance and patience.
Table 6.1 PK parameters for danofloxacin in 3-week old vs. 6-month old calves with respiratory disease

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Estimate</th>
<th>Stderr</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>T(_{MAX})</td>
<td>h</td>
<td>2.2</td>
<td>0.9</td>
<td>60.8</td>
</tr>
<tr>
<td>C(_{MAX})</td>
<td>µg/mL</td>
<td>3.0</td>
<td>1.4</td>
<td>79.4</td>
</tr>
<tr>
<td>(\theta V)</td>
<td>L/kg</td>
<td>2.9</td>
<td>0.26</td>
<td>9.1</td>
</tr>
<tr>
<td>(\theta V2)</td>
<td>L/kg</td>
<td>1.22</td>
<td>0.24</td>
<td>19.7</td>
</tr>
<tr>
<td>Cl</td>
<td>L/kg/h</td>
<td>0.26</td>
<td>0.017</td>
<td>6.9</td>
</tr>
<tr>
<td>Cl2</td>
<td>L/kg/h</td>
<td>0.03</td>
<td>0.0045</td>
<td>13.5</td>
</tr>
<tr>
<td>T(_{1/2}\ast)</td>
<td>h</td>
<td>29.5</td>
<td>2.2</td>
<td>40.7</td>
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</table>

<table>
<thead>
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<th>Parameter</th>
<th>3-Week Old Calves</th>
<th>6-Month Old Calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(_{inf})</td>
<td>hr*µg/ml</td>
<td>29.8*</td>
</tr>
<tr>
<td>CL/F</td>
<td>ml/hr/kg</td>
<td>268.6*</td>
</tr>
<tr>
<td>T(_{1/2})</td>
<td>hr</td>
<td>35.8</td>
</tr>
<tr>
<td>C(_{max})</td>
<td>µg/mL</td>
<td>2.2</td>
</tr>
<tr>
<td>T(_{max})</td>
<td>hr</td>
<td>2.0</td>
</tr>
<tr>
<td>V(_d)/F</td>
<td>L/kg</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Population values from NLME model and median and range of PK parameters from compartmental analysis after single S.C. injection of 8 mg/kg danofloxacin in 3-week-old vs. 6-month-old calves with induced respiratory disease with *P. multocida*.  
* Indicates significantly different by t-test (P < 0.01).
Table 6.2 Mean Respiratory scores and rectal temperatures for danofloxacin in 3-week old vs. 6-month old calves
Ultrasound scores are median scores.

<table>
<thead>
<tr>
<th></th>
<th>Rectal Temperature</th>
<th>Respiratory Score</th>
<th>Ultrasound Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3-Week Old Calves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to Induction</td>
<td>101.7 °F</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>0 hr</td>
<td>102.0 °F</td>
<td>6.5</td>
<td>1</td>
</tr>
<tr>
<td>24 hr</td>
<td>102.3 °F</td>
<td>3.7</td>
<td>2</td>
</tr>
<tr>
<td>72 hr</td>
<td>101.9 °F</td>
<td>2.9</td>
<td>2</td>
</tr>
<tr>
<td>144 hr</td>
<td>101.9 °F</td>
<td>2.5</td>
<td>2</td>
</tr>
<tr>
<td><strong>6-Month Old Calves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to Induction</td>
<td>100.7 °F</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>0 hr</td>
<td>102.6 °F</td>
<td>6.3</td>
<td>1</td>
</tr>
<tr>
<td>24 hr</td>
<td>101.6 °F</td>
<td>2.8</td>
<td>1</td>
</tr>
<tr>
<td>72 hr</td>
<td>101.4 °F</td>
<td>2.2</td>
<td>1</td>
</tr>
<tr>
<td>144 hr</td>
<td>101.2 °F</td>
<td>0.8</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 6.1 Mean (±SD) plasma concentrations of danofloxacin administered by subcutaneous route at doses of 8 mg/kg in 3-week and 6-month old calves with induced respiratory disease from *P. Multocida*
Figure 6.2 Mean interstitial fluid ± SD concentrations after single S.C. injection of 8 mg/kg danofloxacin in 3-week old and 6-month old calves
Figure 6.3 Individual PELF concentrations of danofloxacin administered by subcutaneous route at doses of 8 mg/kg in 3-week and 6-month old calves with induced respiratory disease from *P. Multocida*. Lines represent average concentration over time for 3-week old calves (solid line) and 6-month old calves (dashed line).
### Population Values – Final NLME Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Estimate</th>
<th>Stderr</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{MAX}}$</td>
<td>h</td>
<td>2.0</td>
<td>1.6</td>
<td>109.7</td>
</tr>
<tr>
<td>$C_{\text{MAX}}$</td>
<td>µg/mL</td>
<td>1.3</td>
<td>0.9</td>
<td>81.5</td>
</tr>
<tr>
<td>$\theta V$</td>
<td>L/kg</td>
<td>5.7</td>
<td>0.9</td>
<td>46.9</td>
</tr>
<tr>
<td>$\theta V2$</td>
<td>L/kg</td>
<td>19.6</td>
<td>2.9</td>
<td>71.5</td>
</tr>
<tr>
<td>Cl</td>
<td>L/kg/h</td>
<td>0.19</td>
<td>0.29</td>
<td>62.9</td>
</tr>
<tr>
<td>Cl2</td>
<td>L/kg/h</td>
<td>0.34</td>
<td>0.44</td>
<td>70.6</td>
</tr>
<tr>
<td>$T_{1/2}^*$</td>
<td>h</td>
<td>100.3</td>
<td>7.0</td>
<td>69.8</td>
</tr>
</tbody>
</table>

### 3-Week Old Calves vs. 6-Month Old Calves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3-Week Old Calves</th>
<th>6-Month Old Calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>Median</td>
<td>Range</td>
</tr>
<tr>
<td>CL/F</td>
<td>hr*µg/ml</td>
<td>6.7*</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>ml/hr/kg</td>
<td>373.3*</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>hr</td>
<td>116.5</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>µg/mL</td>
<td>0.4</td>
</tr>
<tr>
<td>V&lt;sub&gt;d/F&lt;/sub&gt;</td>
<td>hr</td>
<td>0.5*</td>
</tr>
<tr>
<td>V&lt;sub&gt;d/F&lt;/sub&gt;</td>
<td>L/kg</td>
<td>25.9</td>
</tr>
</tbody>
</table>

Population values from NLME model and median and range of PK parameters from compartmental analysis after single S.C. injection of 2.5 mg/kg tulathromycin in 3-week-old vs. 6-month-old calves with induced respiratory disease with *P. multocida*.

* Indicates significantly different by t-test ($P < 0.05$).
Table 6.4 Mean Respiratory scores and rectal temperatures for tulathromycin in 3-week old vs. 6-month old calves
Ultrasound scores are median scores.

<table>
<thead>
<tr>
<th></th>
<th>Rectal Temperature</th>
<th>Respiratory Score</th>
<th>Ultrasound Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Week Old Calves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to Induction</td>
<td>102.7 °F</td>
<td>3.25</td>
<td>1.5</td>
</tr>
<tr>
<td>0 hr</td>
<td>102.9 °F</td>
<td>7.4</td>
<td>2</td>
</tr>
<tr>
<td>24 hr</td>
<td>102.1 °F</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>48 hr</td>
<td>101.7 °F</td>
<td>2.9</td>
<td>1</td>
</tr>
<tr>
<td>72 hr</td>
<td>101.7 °F</td>
<td>2.9</td>
<td>1</td>
</tr>
<tr>
<td>96 hr</td>
<td>102.3 °F</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>144 hr</td>
<td>102.2 °F</td>
<td>2.6</td>
<td>1.5</td>
</tr>
<tr>
<td>6-Month Old Calves</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior to Induction</td>
<td>102.1 °F</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>0 hr</td>
<td>102.9 °F</td>
<td>6.6</td>
<td>1</td>
</tr>
<tr>
<td>24 hr</td>
<td>102.3 °F</td>
<td>3.7</td>
<td>1</td>
</tr>
<tr>
<td>48 hr</td>
<td>101.9 °F</td>
<td>3.6</td>
<td>1</td>
</tr>
<tr>
<td>72 hr</td>
<td>101.9 °F</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>96 hr</td>
<td>101.6 °F</td>
<td>2.4</td>
<td>2</td>
</tr>
<tr>
<td>144 hr</td>
<td>101.1 °F</td>
<td>0.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Figure 6.4 Mean (±SD) plasma concentrations of tulathromycin administered by subcutaneous route at a dose of 2.5 mg/kg in 3-week and 6-month old calves with induced respiratory disease from *P. Multocida*
Figure 6.5 Mean interstitial fluid ± SD concentrations after single S.C. injection of 2.5 mg/kg tulathromycin in 3-week old and 6-month old calves
Figure 6.6 Individual PELF concentrations of tulathromycin administered by subcutaneous route at doses of 2.5 mg/kg in 3-week and 6-month old calves with induced respiratory disease from *P. Multocida*. Lines represent average concentration over time for 3-week old calves (solid line) and 6-month old calves (dashed line).
Table 6.5 Observed (noncompartmental) and fitted (compartmental) parameters for danofloxacin (A) and tulathromycin (B).

### A)

<table>
<thead>
<tr>
<th></th>
<th>3-Week Old</th>
<th></th>
<th>6-Month Old</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>AUC hr*µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed</td>
<td>33.1</td>
<td>8.5</td>
<td>21.7</td>
<td>14.1</td>
</tr>
<tr>
<td>Fitted</td>
<td>32.1</td>
<td>8.2</td>
<td>18.9</td>
<td>12.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; µg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed</td>
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<td>1.2</td>
<td>3.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Fitted</td>
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<td>1.2</td>
<td>3.6</td>
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<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; µg/mL</td>
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<td></td>
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<tr>
<td>Observed</td>
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<td>1.2</td>
<td>2.0</td>
<td>1.5</td>
</tr>
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</tr>
<tr>
<td>Fitted</td>
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### B)

<table>
<thead>
<tr>
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<th>6-Month Old</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>AUC hr*µg/ml</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed</td>
<td>9.8</td>
<td>7.6</td>
<td>23.4*</td>
<td>11.4</td>
</tr>
<tr>
<td>Fitted</td>
<td>11.3</td>
<td>6.5</td>
<td>25.0</td>
<td>6.7</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; µg/mL</td>
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<td></td>
<td></td>
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<tr>
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<td>1.5</td>
<td>1.4</td>
<td>1.0</td>
<td>0.5</td>
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<tr>
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<td>0.26</td>
<td>0.54</td>
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<td></td>
<td></td>
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<tr>
<td>Observed</td>
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<td>0.6</td>
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<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; hr</td>
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<td>127.7</td>
<td>92.9</td>
<td>72.9</td>
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<td>Fitted</td>
<td>98.7</td>
<td>52.6</td>
<td>114.9</td>
<td>74.2</td>
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Figure 6.7 ISF:Plasma<sub>Total</sub> Ratios for danofloxacin
Figure 6.8 ISF:Plasma\textsubscript{Total} Ratios for tulathromycin
References


Infected with Mannheimia (Pasteurella) Haemolytica.” Antimicrobial Agents and Chemotherapy 46 (9): 3013–19.


Chapter 7

Summary and Future Directions

The application of pharmacokinetic knowledge to pediatric patients requires an understanding of the maturation process from neonate to adult. Cattle undergo anatomical, physiological and biochemical changes as they age that may affect the pharmacokinetics of drugs. Currently, there is a lack of understanding of how factors like weight, body composition and ontological development of metabolic pathways influence drug disposition in cattle. In the field of veterinary medicine, where these age groups mature much more rapidly than human pediatric patients, these differences require further study.

There were two main goals of the research presented in this thesis. The first one was to determine if age was a significant variable affecting the pharmacokinetics and distribution of two major classes of antimicrobials in healthy calves. This was evaluated through pharmacokinetic studies, as well as through the quantification of changes in plasma proteins and protein binding of several drugs in different ages of calves. The second goal was to determine the impact of disease on drug distribution in different ages of calves. By achieving these goals, our studies successfully described the impact of age and disease on key pharmacokinetic variables in calves. These results can be useful for designing critically needed studies in neonatal calves to determine effective and judicious treatment regimens for common calfhood diseases.

The first drug evaluated was the fluoroquinolone antibiotic, danofloxacin. Fluoroquinolones have several pharmacologic properties that make them ideal for the treatment of BRD. These include relatively quick absorption, high plasma concentrations, low plasma-protein binding and large volumes of distribution into tissue and extracellular fluid (Terhune et al., 2005). This class of drugs has potent \textit{in vitro} bactericidal activity against
common respiratory pathogens found in calves, including *M. haemolytica* and *P. multocida* (Walker, 2000).

For the pharmacokinetic studies we performed with danofloxacin, we were able to determine that age impacted several pharmacokinetic parameters in young healthy calves. For calves administered a label dose of danofloxacin, the time to maximum concentration in the plasma was delayed in 3-week old calves (3.1 h) versus ruminating calves (1.4 h). Total body clearance was significantly lower in 3-week old calves. Six-month-old calves maintained higher ISF/plasma concentration ratios throughout the study period as compared to that observed in younger calves. In addition, these studies demonstrated high but variable concentrations in PELF, indicating that high concentrations are found in lung fluid regardless of age, which is critical when treating extracellular respiratory pathogens.

The second drug evaluated was the macrolide antibiotic, tulathromycin. Macrolide antibiotics are known to accumulate in leukocytes and bronchial secretions, penetrating into lung tissues at high concentrations which contributes to the efficacy of the antibiotic against susceptible pathogens (Nowakowski et al. 2004). Studies presented here determined that irrespective of age, plasma tulathromycin concentrations were relatively low. SC administration was associated with rapid absorption and a subsequent slow decline from plasma. The younger calves had statistically significantly lower CL/F and Vd/F values as compared to that of the 6-month old calves. Across age groups, a significant difference was not detected in the terminal elimination half life, although there was an observed trend for a longer T\(_{1/2}\) in the 3-week old (67.6 hours) versus 6-month old (44.4 hours) calves. The T\(_{\text{max}}\) values tended to occur earlier in 3-week versus 6-month old calves. Large variability in PELF concentrations was noted in both
groups of healthy calves, although both groups maintained PELF concentrations higher than plasma at all time points.

Most studies report pharmacokinetic parameters derived from the total plasma concentrations over time, but neglect to report the more clinically relevant unbound concentrations. The binding of drugs to plasma proteins tends to be lower in pediatric human patients (McNamara and Alcorn 2002) although prior to this research, this has not been examined in young calves. Age-related changes in plasma protein binding could lead to differences in distribution and clearance of some veterinary drugs depending on its extraction ratio and distributional characteristics. Since the unbound drug is pharmacologically active and free to move out of the vasculature and into the site of action, a higher unbound fraction may increase drug distribution to the rest of the body, particularly in situations when the drug is highly protein bound in the plasma and where it can concentrate in peripheral tissues. Additionally, for low extraction ratio drugs, the unbound drug is cleared faster from the body.

Based upon the findings in our studies, it was evident that both total and free drug concentrations need to be considered before rendering conclusions regarding the impact of protein binding characteristics on the pharmacokinetics of a compound.

We performed an in vitro study to determine what influence, if any, increasing age has on the composition of plasma proteins, as well as the binding of commonly used drugs to those plasma proteins in cattle. Albumin concentrations were lowest at 1 day and over 56 days. Significant decreases in alpha\textsubscript{1}-acid glycoprotein were seen up to 21 days. The percent of protein binding was determined for the antibiotics danofloxacin, tulathromycin, and florfenicol, as well as for the NSAID flunixin meglumine. Danofloxacin demonstrated non-linear protein binding at high concentrations but this was not affected by age. Significant differences were not
detected in percent protein binding for tulathromycin, florfenicol or flunixin meglumine over different concentrations or at different ages. Findings from these calves suggests that, despite marked changes in alpha1-acid glycoprotein and albumin concentrations over time, age is not an important factor in the % binding of these drugs to plasma proteins in vitro.

Although administering drugs to healthy calves allowed for a thorough understanding of key pharmacokinetic differences in normal animals, this does not mimic the clinical situation where drugs are administered to sick animals. To investigate the influence of pathophysiological changes in calves with respiratory disease, respiratory disease was induced with a bovine isolate of *P. multocida*, prior to treatment with either danofloxacin or tulathromycin in both young and older calves. Non-linear mixed effects modeling showed that age, but not clinical respiratory score, is a significant covariate for the depletion of danofloxacin and tulathromycin concentrations over time. The results of this study highlight the importance of age-related changes in calves that could influence the PK/PD target, the dose-effectiveness or dose-safety relationship, and potentially drug depletion and residue levels in treated calves.

All of the aforementioned studies provide evidence that age can influence key pharmacokinetic parameters in healthy and diseased cattle. However, what remains unknown is how pharmacokinetics and pharmacodynamics are altered when naturally occurring disease in different ages of calves. Thus, one potential area of future research is a population pharmacokinetic study using naturally occurring respiratory disease. By utilizing a population pharmacokinetic approach, future investigations could identify other sources of variability amount the target population including breed, gender, and potentially pathogen exposure (Momper, Bradley, and Best 2016). This approach could also be expanded to evaluate other classes of drugs whose pharmacokinetics may be influenced by disease and age. This knowledge
would then continue to develop rational, scientifically based recommendations for future clinical field trials seeking regulatory approvals, that account for pharmacokinetic and pharmacodynamic population variables that impact therapeutic success of drugs used to treat bovine respiratory disease.
References


