ABSTRACT

MILLER, RANEE A. The Effect of Extra-Label Dosing Regimens on Drug Residues and Pharmacokinetics in Non-Lactating and Dry Dairy Cattle. (Under the direction of Dr. Ronald Baynes).

Large animal veterinarians are tasked with choosing efficacious treatments for livestock while being cognizant of costs and violative residues associated with treatment. Veterinarians must also consider the practicality of administration in various production settings. These constraints, coupled with limited labeled pharmaceutical products, force veterinarians to offer creative solutions utilizing extra-label dosing regimens. The increasing use of extra-label drug administration in veterinary medicine highlights the need to better understand its effects on drug residues and pharmacokinetics, particularly in food-producing animals.

The overall objective of this dissertation was to investigate the impact of extra-label dosing regimens on the pharmacokinetics of two different drug classes in non-lactating and dry dairy cattle and calculate appropriate milk withdrawal intervals based on residues present in early lactation. We hypothesized that residues resulting from these extra-label practices would measure below the FDA tolerance levels in milk and pose little to no food safety risks for consumers.

This research employed controlled experimental trials to investigate how deviations from approved label instructions influence drug distribution, elimination, and potential residue persistence in edible animal products. The compounds evaluated in these experiments were a long-acting parasiticide, eprinomectin, and a third-generation cephalosporin, ceftiofur. These compounds were administered to dry and non-lactating dairy cattle at a predetermined interval before the expected calving date. Upon entering lactation, milk samples were collected,

quantified utilizing analytical chemistry techniques, and modeled to produce pharmacokinetic parameters. These results offered comparisons to residues present in milk following label instructions and allowed the calculation of appropriate milk withdrawal intervals required following these dosing strategies.

In conclusion, these novel extra-label dosing regimens offer creative solutions to combat diseases hindering the dairy cattle industry while minimizing the risk of violative residues in milk collected from treated cattle. Results from these studies highlight the necessity for evidence-based dosing guidelines specific to various animal categories. These findings enhance consumer safety while promoting the responsible use of pharmaceuticals in animal agriculture by providing veterinarians and producers with improved guidance based on rigorous pharmacokinetic data to make educated treatment decisions for their dairy herds.

The Effect of Extra-Label Dosing Regimens on Drug Residues and Pharmacokinetics in Non-Lactating and Dry Dairy Cattle

by
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DEDICATION

I dedicate this dissertation to my family and friends, who have been incredibly supportive and understanding of the opportunity costs associated with pursuing a doctoral degree. To my wife, Cassidy, thank you for your unwavering support and empathy. Thank you for waking up at absurd hours to make me breakfast before I left for the dairy, for all the nights you brought me dinner to the lab, for taking on the mental load at home, and for guiding me toward a brighter future when moments felt dark. Thank you for walking this path with me, for your sacrifices, and for being my greatest source of inspiration. To my loyal companions, Lulu, Wrigley, Tiny, and Mr. Kitty, thank you for your unconditional love, patience, and occasional keyboard interference. Whether it was curling up beside me as I wrote or reminding me to take snack breaks (for both of us), you made this journey much less lonely and more joyful. Your presence reminded me to find joy in the little things and keep going even when the journey felt endless. Knowing that I was coming home to all of you after those long days made enduring them significantly easier. To my sisters, thank you for cheering me on and pretending to understand what I'm talking about when I reference all things science. To my parents, grandparents, and in-laws, thank you for teaching me how to be both resilient and kind. Thank you for encouraging me to chase my passions, even at the expense of seeing you all less. Thank you for instilling the drive to dream big while remaining grounded in humility and gratitude. To all my friends, thank you for listening and providing comedic relief when I needed it most. I am forever grateful to you all. This milestone is not just mine but ours.

BIOGRAPHY

Ranee Ashlyn Miller was born and raised in Houston, Texas. In 2019, she earned a Bachelor of Science in Animal Science from Tarleton State University, where she discovered her passion for research in livestock species. Her undergraduate research focused on evaluating alternative feedstuffs for nutritive value in ruminants. While at Tarleton, Ranee was a supplemental instructor for an animal physiology reproduction course and a veterinary technician at a small animal and equine clinic.

Following the experiences gained at Tarleton, Ranee moved to Raleigh, North Carolina, to further pursue a PhD in the Comparative Biomedical Sciences program at North Carolina State University, College of Veterinary Medicine. During her PhD, Ranee researched extra-label drug use and pharmacokinetics in food-producing species. Additionally, she served as a peer mentor and a responder for the Food Animal Residue Avoidance Databank (FARAD).

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Introduction - Overview of Non-Lactating and Dry Cow Physiology and the Effect on Altered Pharmacokinetics Across the Lactation Cycle

1.1 | Introduction

Veterinarians must consider various factors when treating food animals. Ideally, the preferred treatment is effective in treating or preventing disease, has minimal side effects, and reduces the amount of drug residue in edible products from those animals. The treatment they select depends on the medications available to them and what can be feasibly administered by the vet or the producer in different production settings, considering cost and labor demands. This may include labeled medications, although these are often limited for many food animals. This limitation creates a unique opportunity for veterinarians to explore treatment options beyond labeled medications, ultimately leading to extralabel drug use (**ELDU**). The U.S. Food and Drug Administration (**FDA**) defines ELDU as the use of a drug in an animal in a manner that does not comply with the approved labeling. This includes but is not limited to, use in different species, use for indications (such as diseases or other conditions), or use at dosage levels, frequencies, or routes of administration other than those specified on the drug label (21 CFR § 530.20, 2025).

Any deviation from the drug label voids the labeled withdrawal time (**WDT**). The WDT is the period required after the last treatment with the drug, during which the animal cannot be offered for slaughter, and any products from the animal, including milk and eggs, cannot be sold (FDA-CVM, 1993). The duration of the withdrawal period is determined by the time required for drug residues in the animal to decrease to levels that are considered safe. More specifically, this WDT is determined when there is 95% confidence that 99% of treated animals in the reference population will have tissue residues less than the tolerance for that drug (FDA-CVM, 2022). In the U.S., tolerance is the maximum concentration of a drug or its metabolites that can legally remain in a specific edible tissue of a treated animal (21 CFR § 556.3, 2025). The WDT does not mean that the edible product is residue-free, but rather that any finite residues remaining at the time of harvest are below the established tolerance (DeDonder, 2024). In contrast, ELDU necessitates a substantially extended withdrawal interval (**WDI**) before marketing any milk, meat, eggs, or other edible products produced from the treated animal (21 CFR § 530.20, 2025). This WDI is the duration needed after extralabel treatment for drug concentrations to decrease to levels considered safe for human consumption. If a veterinarian chooses to use a drug extralabel, they must establish a WDI backed by appropriate scientific information. A WDI for ELDU will always be longer than a labeled WDT. A short WDI is often desired when treating food animals, as their consumable products can be marketed shortly after treatment.

For veterinarians to make educated ELDU treatment decisions, they must have a strong understanding of drug formulation and its effect on pharmacokinetics (**PK**). PK describes the movement of drugs within the body with four key processes: absorption, distribution, metabolism, and excretion (**ADME**). When deviating from a drug label, it is important to understand and predict how the newly proposed dosing regimen will affect ADME. Without this understanding, violative residues are likely to remain in the edible products collected from treated animals. Violative residues present in these consumable products can pose health risks to consumers and generate food safety concerns for the public. In addition to food safety, therapeutic failure is more likely to occur if one does not account for the onset, intensity, and duration of a drug's effect and how different physiological factors such as age, breed, lactation status, or production class impact drug efficacy and safety.

This review will specifically focus on ELDU in non-lactating and dry dairy cattle. In 2023, U.S. dairy cattle produced over 226 billion pounds of milk (USDA-NASS, 2023). Preventing disease in these cows is important for maintaining and exceeding this level of production to accommodate increasing population demands. This review highlights the significance of understanding drug residues present after ELDU in relation to food safety and regulatory standards. When used responsibly, ELDU is a critical tool for veterinarians to provide therapeutic and, oftentimes, life-saving care for dairy cattle and food animals as a whole. There are key challenges and public health concerns related to drug residues in milk. This review aims to provide information for the purpose of optimizing dosing regimens that accommodate different physiological factors in dairy cattle while preventing violative drug residues in milk and complying with regulatory standards.

1.2 | Regulatory Context and Justification for ELDU

ELDU in the U.S. is allowed under the Animal Medicinal Drug Use Clarification Act (**AMDUCA**). Established in 1994, AMDUCA offers a legal framework and guidelines for ELDU in veterinary medicine, detailed in Title 21 of the Code of Federal Regulations, Part 530 (21 CFR § 530, 2025). AMDUCA requires that a veterinarian-client-patient relationship (**VCPR**) be established for the extralabel use of FDA-approved drugs for therapeutic purposes, particularly when failure to treat could result in suffering or death. Therapeutic purposes encompass treating, controlling, or preventing diseases, excluding production enhancement or performance improvements, such as increasing milk yield or conception rates. Veterinarians caring for food animals may use extralabel methods when no approved animal drug contains the same active ingredient in the necessary dosage form and concentration labeled for such use. An exception arises when a veterinarian determines that the labeled drug for animals is clinically ineffective for its intended use. ELDU requires a significantly extended WDI based on scientific evidence for any consumable product that might be harvested from the treated animal. If such scientific evidence is unavailable, the veterinarian must ensure that the animal and its food products will not enter the human food supply. This requirement aims to prevent or minimize the chance of violative residues in food products resulting from ELDU. In addition to these restrictions, AMDUCA restricts and limits the extralabel use of specific drugs that pose a public health risk due to residues or secondary effects like antimicrobial resistance (**AMR**). Veterinarians must also maintain comprehensive records of ELDU, including drug name, dosage, route, and duration of treatment, alongside details about the treated animal species, the number of animals treated, the condition being addressed, and the estimated WDI for any consumable product the animal(s) could produce. Compliance is particularly crucial for dairy cattle because of the risks associated with drug residues in milk, a significant public health issue. Moreover, the FDA's Pasteurized Milk Ordinance (**PMO**) stipulates that milk must be devoid of drug residues, meaning producers must follow withdrawal guidelines even for extralabel use. Failure to comply can result in milk being classified as adulterated, leading to economic losses and food safety risks.

The primary justification for ELDU in food animals is to address unmet medical needs. In dairy cattle, many conditions, such as infectious diseases and metabolic disorders, lack specifically approved drugs to treat them. For example, flunixin, a nonsteroidal anti-inflammatory drug (**NSAID**), is approved for managing pyrexia related to bovine respiratory disease (**BRD**) and acute bovine mastitis, as well as controlling pain associated with foot rot in replacement dairy heifers under 20 months old and lactating dairy cows. However, while pain management is critical for dehorning, no drugs are explicitly approved for this procedure in the US. Prescribing flunixin for pain relief after a dehorning procedure would be considered ELDU. In addition to going extralabel for indication, approved drugs may not cover the wide range of pathogens encountered with certain diseases. It is imperative that veterinarians have the ability to use drugs in an extralabel manner in these scenarios where suffering or death may occur from failure to treat. For example, mastitis caused by pathogens resistant to standard treatments may require extralabel antimicrobial use. This legal framework ensures that veterinarians have the flexibility to address unmet medical needs while minimizing risks to food safety and public health.

There are numerous resources for veterinarians to access to help guide responsible ELDU. The Center for Veterinary Medicine (**CVM**), a branch of the FDA, regulates food additives and drugs administered to animals and provides regulatory guidance for ELDU (https://www.fda.gov/about-fda/fda-organization/center-veterinary-medicine). The Food Animal Residue Avoidance Databank (**FARAD**) is a national university-based program that assists in calculating scientifically based WDIs for ELDU in all food animal species (http://www.farad.org/). FARAD has a resource, the Veterinarian's Guide to Residue Avoidance Management (**VetGRAM**), that provides resources for approved uses, restrictions, and required WDTs for FDA-approved drugs in food animals (http://www.farad.org/vetgram/search.asp). FARAD and the American Veterinary Medical Association (**AVMA**) also offer educational resources and policy guidelines for ELDU (https://www.avma.org/). The Food Safety Inspection

Service (**FSIS**) enforces animal drug residues established by the FDA in food products and reports these findings in their Red Book (https://www.fsis.usda.gov/).

1.3 | Classifications of Dairy Cattle

A veterinarian's ability to use ELDU in dairy cattle depends on the production classification of the dairy cow being treated. The FDA CVM defines dairy cattle as those raised primarily to produce milk for human consumption and/or to produce offspring that will yield milk or meat (including veal) for human consumption (GFI #191, 2020). Dairy cattle are then broken up into the following subclasses: dairy calves, replacement dairy heifers, dairy bulls, and dairy cows. Replacement dairy heifers are weaned female dairy cattle that have not yet calved and are intended solely for breeding and future milk production. These heifers may also be called non-lactating dairy cattle if they are under 20 months old and have not yet produced milk for human consumption. Once pregnant and approaching calving, they may also be referred to as prepartum heifers. The periparturient period, often called the transition period, is defined as the three weeks before to the three weeks after calving. After calving, they become periparturient dairy cows, which are then classified as lactating cows. Lactating cows can be further subdivided based on their stage of lactation. According to the most recent report from the Holstein Foundation ("Milking and Lactation", 2017), a lactation cycle typically lasts 305 days, with early lactation occurring in the first 100 days postpartum, mid-lactation occurring from day 100 to 200 postpartum, and late lactation occurring after day 200 postpartum. This lactation cycle can vary depending on the cow's demographics and the dairy's management practices. At the end of lactation, cows are dried off, meaning they undergo a resting phase from lactation, and are then classified as dry dairy cows until they enter lactation again. It is important to note that the term “non-lactating dairy cattle” does not include dry dairy cows.

1.3.1 | Physiological Differences Among Dairy Cow Classes and Their Implications for Pharmacokinetics

Dairy cattle experience notable changes in physiology over the course of their productive lifespan. This section of the review will begin by examining changes that occur during the first gestational period in periparturient heifers, then during the first lactation and dry period, and then in the subsequent lactation periods in mature cows. These physiological changes significantly affect the ADME, efficacy, and safety of xenobiotics compounds. The ability of a compound, or drug in this case, to produce a pharmacological or therapeutic effect is directly attributed to its innate physiochemical properties. Examples of these properties include solubility, partition coefficients, ionization, molecular weight, and pH (Akamatsu, 2011). These properties influence the movement of drugs throughout the animal’s body and into edible food products such as milk. High lipid solubility, poor degree of ionization, and less plasma protein binding increase drug partitioning into milk (Mestorino & Errecalde, 2012). The ratio at which this partitioning occurs can be measured as the milk/plasma ratio (**M/P**). The greater the ratio, the more drug residues are present in the milk. After partitioning, the rate and extent to which a drug is eliminated from the body by the mammary gland depends on various factors. Dubbelboer et al. (2023) lists factors such as, but not limited to, blood flow to the mammary gland, the intrinsic ability of the mammary gland to eliminate the drug, binding of the compound to proteins and fats in the milk, differences in milk composition, and milking frequency. For drugs administered parenterally, physiochemical properties, coupled with mammary transport mechanisms, allow them to partition into milk by crossing the blood-milk barrier (**BMB**). The BMB is a semi-permeable membrane that separates the blood supply from milk in the mammary gland to mitigate the exchange of cellular components (Wellnitz & Bruckmaier, 2021). Weak organic bases like macrolides and sulfonamides tend to accumulate in milk in the ionized form after parenteral administration, whereas weak acids like penicillins and cephalosporins partition much less out of systemic circulation and into milk (Mestorino & Errecalde, 2012). Estimating the M/P for concentration-dependent versus time-dependent antimicrobials is important for establishing treatment efficacy.

For drugs administered intramammary (**IMM**), after release from the pharmaceutical formulation, the drug is distributed into the aqueous and lipid fractions of the milk by passive diffusion (Gehring & Smith, 2006; Gruet et al., 2001). A water-soluble molecule will concentrate in the hydrophilic area formed by milk and modified mammary secretions filling the udder cisterns in the central udder compartment. In contrast, a lipid-soluble drug will concentrate in the lipophilic structures of the udder, primarily the membranes. In addition, drug partitioning throughout the mammary gland will greatly depend on what vehicle the IMM drug is in. It has been established that IMM antibiotic efficacy is correlated with how long activity is maintained in the milk, which is often achieved through formulation (Wilson et al., 1986). Mercer et al. (1974) compared penicillin G absorption in blood and persistence in milk in mastitic quarters in an aqueous-based formulation and an oil-based formulation (3.6% aluminum monostearate in peanut oil). Penicillin G administered in the oil-based formulation was detected in the milk 120 hours after infusion compared to only 56 hours for the aqueous-based formulation. The oil formulation allowed penicillin G concentrations to stay above the Minimum Inhibitory

Concentration (**MIC**) of penicillin G for *Staphylococcus aureus* in milk for 72 hours compared to 32 hours for the aqueous-based formulation. While this may benefit treatment efficacy, economic factors necessitate obtaining the best possible cure along with the shortest withdrawal period, allowing for the milk to be marketed again as quickly as possible (Craven, 1987). The challenge for intramammary products is to create a formulation for lactating cows that effectively penetrates the udder while ensuring rapid elimination to minimize milk withdrawal times. This focus shifts when developing dry cow products, as the goal is to formulate solutions that maintain higher drug concentrations throughout the mammary gland parenchyma (Gehring & Smith, 2006).

In dairy heifers, milk duct and milk-secreting tissue development occurs between puberty and their first parturition. Ideally, heifers reach puberty and become pregnant by 14 or 15 months old to calve around 24 months of age. When she becomes pregnant, a rapid increase in mammary gland development occurs and continues into the peak of lactation (Hammond, 1927). Pregnancy is associated with significant remodeling of the cardiovascular, respiratory, renal, gastrointestinal, and endocrine systems. Similarly to dry cows in the late stage of the dry period, prepartum heifers experience metabolic shifts, with changes in hormone levels, blood flow to the mammary gland, and nutrient demands as the body prepares for both fetal development and subsequent lactation. In late pregnancy, plasma volume, extracellular fluid, and total body water greatly increase (Frederiksen, 2001). A few days prior to calving, there is a two to six times increase in the normal rate of blood flow to the mammary gland (Davis & Collier, 1985). The increased blood flow serves to deliver necessary nutrients, hormones, and oxygen to maternal tissues for fetal development and to synthesize milk once lactation begins

(Reynolds et al., 1987). In general, a dam's cardiac output increases greatly throughout pregnancy, contributing to an increase in maternal blood volume, especially renal blood flow (Frederiksen, 2001). Consequently, the glomerular filtration rate (**GFR**) also significantly increases, which leads to an increase in renal clearance and shorter half-lives of renally eliminated drugs (Pacheco et al., 2013). The increase in blood volume, coupled with a decrease in plasma proteins, increases the volume of distribution of hydrophilic drugs and the amount of unbound (active) drugs available due to decreased protein binding availability (Bastian et al., 2017; Schalkwijk et al., 2017). The increase in the volume of distribution reduces plasma concentrations and may decrease the effectiveness of hydrophilic drugs. Furthermore, hepatic metabolic pathways undergo significant changes throughout pregnancy, becoming most evident in late gestation. Pregnancy-related hormones can either upregulate or downregulate liver enzyme activity (Sheffield et al., 2014). This is crucial because some drugs may metabolize more quickly, leading to shorter half-lives and reduced efficacy, while others may metabolize more slowly, resulting in longer half-lives and increased potential toxicity. The effects of pregnancy on hepatic enzyme activity have been well studied in humans; however, data on this in dairy cattle is lacking, as most hepatic changes have been concerned with the adaptations required during the transition period for early lactation when cows adapt from prepartum to postpartum (Anderson, 2005; Gao, 2021; Tsutsumi, 2001; Wadelius et al., 1997).

Upon calving, the heifer transitions into a periparturient dairy cow and enters early lactation, causing another round of extreme metabolic shifts. The transition period is an extremely stressful time for dairy cows due to sudden nutritional, hormonal, immunological, and metabolic changes driven by late pregnancy and early lactation requirements (Akbar et al., 2015; Mezzetti et al., 2021). The metabolic and endocrine responses in the first lactation are likely to be additionally compromised by the partitioning of nutrients towards somatic growth as heifers are still maturing at the time of their first calving (Taylor et al., 2003). **Figure 1.1** displays the relationship between dry matter (feed) intake, milk yield, and body composition in a dairy cow throughout a standard lactation cycle who calves every 12 months. Each stage of lactation necessitates specific management practices, particularly in drug use, as each stage can alter PK profiles. This is particularly important for drugs administered to prepartum heifers or dry dairy cows for predicting drug behavior and managing withdrawal intervals effectively.

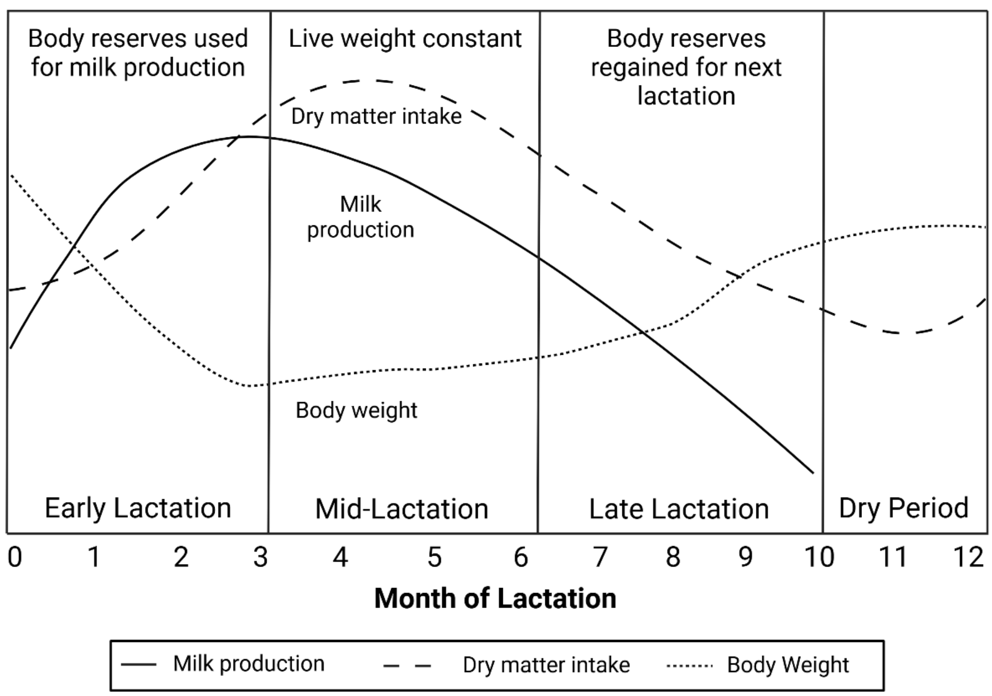

Figure 1.1: Dry matter intake, milk yield, and body weight changes in a dairy cow during her lactation cycle. (Adapted from 'Managing Cow Lactation Cycles' by Moran, 2015, The Cattle Site, https://www.thecattlesite.com/articles). Created in https://BioRender.com.

At the initiation of early lactation, the cow's body weight is the highest it will be throughout the entire lactation cycle. However, fetal growth prior to calving and the onset of lactation, often coupled with decreased dry matter intake, puts prepartum cows in a negative energy balance (**NEB**) that lasts well into early lactation (Herdt, 2000). Depending on the degree of NEB, body fat stores are often mobilized in the form of non-esterified fatty acids (**NEFA**) to keep up with the energy demand from early lactation (Overton & Waldron, 2004). The increased fat stores can serve as a reservoir for lipophilic drugs. These lipophilic drugs may have increased distribution volumes due to this fat mobilization, leading to prolonged drug half-lives and delayed clearance. Fat content in milk is highest immediately after calving, which can also increase the passage of lipophilic drugs into milk (Dubbelboer et al., 2023). Additionally, excess NEFA production is associated with reactive oxygen species (**ROS**) generation, and if there are insufficient quantities of antioxidants present, the cow will face oxidative stress (Sordillo & Aitken, 2009). Excessive NEFA production, followed by oxidative stress, leads to inflammation and immune dysfunction (Abuelo, 2019; Sordillo, 2014). A recent study found that oxidative stress is the primary cause responsible for periparturient diseases in dairy cattle (Ayemele et al., 2021). These periparturient diseases can further alter drug ADME, safety, and efficacy. These alterations will be further discussed in section 1.4.

Cows in early lactation have a rapid increase in milk production and generally reach peak lactation 50-70 days after calving, with higher-producing animals peaking later than lower-producing animals (Waldner, 2002). This increased milk production, coupled with increased blood flow to the mammary gland, may increase drug partitioning into milk. Metabolic shifts in the liver prioritize gluconeogenesis and fat metabolism to meet energy demands in early lactation. This may also alter drug clearance for drugs processed by hepatic enzymes. During this time, it is particularly critical that fresh cows have adequate access to fresh feed to support the metabolic demands associated with peak milk production and to support reproductive performance. This presents a challenge to producers because, at calving, appetite is only about 50 to 70 percent of the maximum at peak feed intake (Stanley et al., 1993). This is due to the growing calf taking up space, reducing rumen volume, and reducing the density and size of rumen papillae during late gestation (Forbes, 1968; Steele et al., 2015). After calving, the rumen expands, and the papillae regrow so that the appetite reaches its full potential around weeks 10-12 (Moreira et al., 2025).

As milk production and dry matter intake are rapidly increasing, the cow's body condition is rapidly declining. This can pose a problem for cows returning to estrous. Dairy cows in good health with adequate body conditioning usually resume estrous cyclicity 65 days postpartum, with multiparous cows (multiple calvings) initiating the onset of estrous cycles more quickly than primiparous cows (calving for the first time) (Santos et al., 2009). Once the appetite is regained, the cow is able to maintain her body weight as she enters mid-lactation. This reduces fat mobilization and reduces the delayed release of lipophilic drugs from fat stores. Thankfully, there is little competition for nutrient use for lactation and the beginning phases of fetal growth since the majority of fetal development occurs during the dry period (Bauman & Bruce Currie, 1980). As the cow's energy balance stabilizes, metabolism does the same, and drug PK can be better predicted. Additionally, this energy balance improves immune response and enhances the cow's ability to respond to infection.

Throughout the lactation cycle, few researchers have specifically studied the impact of milk yield on PK. Berger et al. (2016) found that pregnant cows with high milk yield had significantly greater mammary blood flow volume compared to those with low milk yield, but there was no difference between the two groups when the cows were not pregnant. The authors attributed this to the increased demand for nutrients and oxygen while pregnant. More research evaluated the impact of milk yield on the PK of various IMM drugs utilizing compartmental population models (Grabowski et al., 2018). This study recorded a significant correlation between milk yield and the typical value of udder tissue compartment clearance for IMM ampicillin, penicillin, and cloxacillin, but found no correlation for IMM amoxicillin, cefoperazone, or cefacetrile. This indicates that milk yield affects the distribution and clearance of drugs differently, potentially leading to considerable variation in the level of drug residues in udder tissue. Again, the study attributed these differences to the increase in mammary blood flow in high-yielding cows. Previous studies have indicated a correlation between lower milk yields and extended drug elimination for intramammary pirlimycin (Whittem, 1999) and ceftiofur (Smith et al., 2004). It remains unclear whether this effect is drug-dependent or if milk production has any significant relation to the elimination of systemically administered drugs.

After mid-lactation, cows will enter late lactation, where they are able to regain body conditioning. Late lactation is characterized by declining milk production and preparation for the dry period as physiological demands shift to support the next pregnancy and repair mammary tissues. A decrease in dry matter intake is often observed as gestation progresses (Gionbelli et al., 2016). Additionally, as pregnancy continues and the fetus develops, the enlarging uterus compresses maternal organs like the rumen (Forbes, 1968). The reduction in ruminal capacity can lead to decreased feed intake (Scheaffer et al., 2001; Stanley et al., 1993). The ruminal epithelium of late-pregnant cows transitions to an energy-saving state, which reduces its remodeling and absorptive capacity (Moreira et al., 2021). Progesterone relaxes smooth muscle, slowing gastrointestinal (**GI**) motility and gastric emptying (Alqudah et al., 2022). As a result, the slower GI transit time may enhance the absorption of some orally administered drugs. However, delayed gastric emptying could also affect the timing required to reach peak plasma concentrations. Changes in feed intake and metabolism during pregnancy may impact rumen fermentation and pH (Dunière et al., 2021). Consequently, drugs that rely on microbial metabolism or ruminal pH for activation or absorption (e.g., ionophores) may exhibit altered efficacy.

About 45 to 60 days before calving, cows enter a dry period. This dry period typically begins with the abrupt cessation of milking and can be divided into two phases: the initial dry period and the prepartum period. Since the dry period facilitates healing and prepares cows for their next lactation cycle, it is also an ideal time to administer therapies that could otherwise affect milk residues. However, ELDU in dairy cattle during the dry period poses challenges regarding milk withdrawal times and adherence to regulatory guidelines. During the dry period, the mammary gland regresses, reverting to a non-lactating state known as involution. This involution process may be counteracted due to signals acting on the mammary gland by concomitant pregnancy (Zhao et al., 2019). This involution phase decreases blood flow to the mammary gland in early dry periods, affecting how quickly drugs can reach mammary tissue (Themistokleous et al., 2023). However, the BMB loses some of its integrity and experiences greater permeability at the beginning and end of the dry period (Zhao et al., 2019). The degree of permeability can be determined by quantifying various blood-derived components in milk (Wellnitz & Bruckmaier, 2021). However, to our knowledge, a correlation between blood-derived components and drug residues perfusing into milk has not yet been investigated. As cows progress through the dry period and enter the prepartum period, physiological changes occur similarly to what was discussed previously with prepartum heifers. The mammary gland develops during pregnancy, and blood flow increases in preparation for lactation. However, evidence shows that multiparous females, compared to primiparous females, show an even greater increase in cardiac output and plasma volume expansion (Turan et al., 2008). Upon calving, multiparous cows have greater milk production, less body conditioning, more plasma proteins, and take less time between calving and first ovulation in comparison to their primiparous counterparts (Adrien, 2012). Consequently, drugs may be distributed to the mammary gland more readily in multiparous cows, increasing their presence in colostrum or milk post-calving. Drugs administered during late pregnancy may persist in mammary tissues and appear in milk after calving, raising concerns for food safety. This consideration underscores the importance of timing in managing withdrawal intervals, as regulatory frameworks do not always account for non-standard treatment periods.

In conclusion, dose adjustments are extremely relevant throughout the lactation cycle. Recognizing these physiological changes is crucial for anticipating drug behavior and establishing appropriate withdrawal times to avoid violative residues in milk when dairy cows enter lactation.

1.4 | Preventions and Treatments for Common Diseases in Dairy Cows

In addition to the stage of a cow's lactation cycle, her disease status can also significantly affect ADME and food safety. Changes in hormone levels, metabolic shifts, and body condition influence the cow's immune system and susceptibility to infections. This highlights the importance of prophylactic, metaphylactic, and therapeutic treatments for maintaining health throughout the lactation cycle. Prophylaxis in veterinary medicine refers to any preventative medical treatment given to a herd or flock of animals at risk of a disease outbreak (Bousquet-Melou, 2010). Ideally, prophylactic treatment prevents the disease from developing in that population. Metaphylaxis, a form of prophylaxis, involves treating healthy animals from the same flock or pen that are showing clinical signs of disease. In this case, treating the entire herd when a pathogen poses a high likelihood of disease would serve both prophylactic and therapeutic purposes in controlling the disease and preventing its spread. Therapeutic treatment is administered to an individual animal or herd already diagnosed with a disease. On-label treatments base their WDTs on residue depletion data that have been generated from healthy animals representative of those typically treated with the specific product (DeDonder, 2024). While the WDT is determined statistically, considering drug disposition variability among animals, it may not reflect the physiological differences of diseased dairy cattle.

In 2024, FARAD received 626 submissions, averaging 12 drugs per week, from veterinarians requesting ELDU advice in dairy cattle. In order, the top 10 extralabel drug requests in dairy cattle over 20 months old were for oxytetracycline, penicillin, florfenicol, meloxicam, ceftiofur, flunixin, aspirin, tulathromycin, sodium iodide, and ampicillin trihydrate. These statistics indicate that the majority of ELDU submissions for dairy cattle involve the use of an antimicrobial or NSAID. Common diseases requiring the use of these drug classes in dairy cows include mastitis, metabolic disorders, reproductive disorders, lameness, and respiratory disease. When an animal's health deviates from normal, disease-induced changes in drug PK are bound to occur. Altered blood flow, gastrointestinal emptying, metabolism, renal impairment, and plasma protein binding are just a few examples of how disease status influences an animal's ability to absorb, distribute, metabolize, and eliminate a drug. (Barre et al., 1983). In the following section, common dairy cow diseases, treatments, and PK alterations will be discussed.

1.4.1 | Mastitis

Mastitis, an intramammary infection most often caused by bacterial infection, is the most common and burdensome disease in the dairy cattle industry. It is estimated that 99.7% of all dairy herds experience mastitis, with 25% of cows per herd diagnosed with mastitis annually (USDA-NAHMS, 2014). Cows are most susceptible to mastitis in the weeks leading up to and directly following calving due to metabolic disorders, oxidative stress, and excessive lipid mobilization (Ayemele et al., 2021). To prevent this, most cows are administered IMM antimicrobial therapy after the last milking in late lactation prior to the dry period. Blanket dry cow therapy (**BDCT**) is used in 80% of all dairy herds and 94% of large herds (>500 lactating cows) to treat all quarters of each cow at the end of lactation, regardless of disease status, to reduce the risk of mastitis during the dry period and subsequent lactation (USDA-NAHMS, 2014). Most antimicrobials used for BDCT are mainly formulated for efficacy against Gram-positive cocci providing little to no protection against Gram-negative bacteria. Therapeutic levels of these blanket therapies may only persist for 14-28 days into the dry period and fail to protect the udder during the last trimester (Petzer et al., 2009). **Table 1.1** presents a complete list of IMM antimicrobials currently offered for BDCT in the U.S.

Table 1.1. IMM Antimicrobials Used for BDCT.

Brand Name	Active Ingredient	Specificity	Meat WDT	Milk WDT
Spectramast® DC	Ceftiofur Hydrochloride	Broad-spectrum	16 days	30 day dry period
ToMORROW®	Cephapirin Benzathine	Broad-spectrum	42 days	30 day dry period + 72h post-calving
Dry-Clox®	Cloxacillin Benzathine	Gram-positive	30 days	30 day dry period
Orbenin™-DC	Cloxacillin Benzathine	Gram-positive	28 days	28 day dry period
Quartermaster®	Penicillin G Procaine + Dihydrostreptomycin Sulfate	Gram-positive	60 days	42 day dry period + 96h post-calving
Albadry Plus®	Penicillin G Procaine + Novobiocin Sodium	Gram-positive	30 days	30 day dry period + 72h post-calving

For clinical mastitis diagnosed during lactation, 87.3% of dairy farms in 2014 utilized antimicrobial treatment, with nearly three-quarters of those farms using IMM cephalosporins (first and third generations) as their primary treatment (USDA-NAHMS, 2014). **Table 1.2** provides a complete list of IMM antimicrobials that are currently available for clinical mastitis treatment in the U.S.

Table 1.2. IMM Antimicrobials Used for Clinical Mastitis.

Brand Name	Active Ingredient	Specificity	Administration	Meat WDT	Milk WDT
Spectramast® LC	Ceftiofur Hydrochloride	Broad-spectrum	1 syringe q24h x 8	2 days	72 hours
ToDAY®	Cephapirin Sodium	Broad-spectrum	1 syringe q12h x 2	4 days	96 hours
Amoxi-mast®	Amoxicillin	Gram-positive	1 syringe q12h x 3	12 days	60 hours
Dariclox®	Sodium Cloxacillin	Gram-positive	1 syringe q12h x 3	10 days	48 hours

In addition to antimicrobial treatment for mastitis, Banamine® Injectable Solution (flunixin meglumine) is labeled for lactating dairy cattle at 2.2 mg/kg of body weight once intravenously for the control of pyrexia associated with acute bovine mastitis. This treatment has an associated 4-day meat WDT and 36-hour milk discard. Banamine® Transdermal is also labeled at 3.3 mg/kg of body weight topically for this purpose for lactating dairy cattle and dairy heifers with an associated 8-day meat WDT and 48-hour milk discard. Use of either medication in dry dairy cows or for an indication other than pyrexia would be considered extralabel. An additional extralabel drug that is commonly used along with antimicrobials for mastitis is dexamethasone, a glucocorticoid. Research has demonstrated that glucocorticoids can reduce inflammation caused by mastitis in dairy cows (Sipka et al., 2013). In 2024, dexamethasone ranked alongside ampicillin trihydrate as the tenth most inquired about medication for ELDU in dairy cattle.

Mastitis significantly alters the chemical composition of milk. Typically, during healthy lactation, mammary tight junctions that form the BMB are strongly sealed and form a highly impermeable barrier between milk and the interstitial fluid (Nguyen, 1998). However, mastitis decreases the integrity of the BMB, which causes an increase in vascular permeability. As a result, blood components such as ions, proteins, and inflammatory cells pass from systemic circulation into milk (Bruckmaier et al., 2004; Lehmann et al., 2013; Wellnitz et al., 2016). The physical properties of milk are, therefore, affected, and there is an increase in pH, conductivity, and viscosity (Wellnitz & Bruckmaier, 2021). The increase in pH causes organic bases to partition less and organic acids to partition more into milk. Damage to the BMB and increased vascular permeability enhance the systemic absorption of IMM drugs and potentially facilitate the distribution of drugs into the udder (Smith, 2015). For example, studies have shown that IMM gentamicin, polymyxin B, and cefoperazone sodium are not detectable in the plasma of healthy cows; however, significant systemic absorption was reported in cows with mastitis (Kumar et al., 2010; Sweeney et al., 1996; Ziv & Schultze, 1982). Parenterally administered drugs such as flunixin and meloxicam have also exhibited significantly slower elimination rates in mastitic cows compared to healthy controls (Kissell et al., 2015; Mzyk et al., 2023). These PK changes extend the half-life of milk and may require more time than the approved WDT for labeled medications. They also significantly extend ELDU WDIs to reduce the risk of violative residues.

1.4.2 | Metabolic Diseases

A metabolic disease refers to a condition that affects the body's ability to process and use nutrients, such as carbohydrates, fats, and proteins (Sundrum, 2015). Clinical diseases most related to nutritional management in dairy cattle are ketosis, milk fever (hypocalcemia), retained fetal membranes, metritis, displaced abomasum, and lameness (Oetzel, 2014). Each of these diseases is classified as a 'production disease.' Retained fetal membranes and metritis will be discussed in section 1.4.3. These diseases are most likely to occur during the transition period and are often interrelated. A list of compiled periparturient disease incidence rates is presented in **Table 1.3**.

Table 1.2: Prevalence of metabolic diseases in dairy cattle during the transition period from various published studies. (Adapted from Van Saun & Sniffen, 2014). Data presented as median+(range).

Disease	**Median Incidence Risk (%)**
Milk Fever (Hypocalcemia)	6.5 (0.3-22)
Subclinical Hypocalcemia	2.2 (8-54)
Ketosis	4.8 (1.3-18.3)
Subclinical Ketosis	43 (26-55)
Lameness	7 (1.8-30)
Retained Fetal Membranes	8.6 (1.3-39.2)
Metritis	10.1 (2-37)
Subclinical Metritis	53 (37-74)

Metabolic disease prevalence in dairy herds has increased over the last several decades as cows have undergone intensive genetic selection to prioritize milk yield. The metabolic demand for supporting such high levels of production often results in poor health and infertility (Mulligan & Doherty, 2008). Metabolic disease is most likely to occur in the transition period due to a reduction in feed intake while energy demands are increasing from gestational development and the onset of lactation (Grummer, 1995). For reference, fluctuations in feed intake, milk yield, and body condition are displayed in **Figure 1.1**. This period is characterized by significant endocrine fluctuations and immunosuppression, which increases susceptibility to infection (Mulligan & Doherty, 2008). Ingvartsen et al. (2003) summarized data from 93,000 first-parity and 58,000 third-parity Danish dairy cows, showing that the highest incidence of total disease (including mastitis, ketosis, digestive disorders, and laminitis) occurred from the day of calving until 10 days post-calving. The initiation of milk production requires calcium output from the cow at a time when calcium input from feed is low due to the reduced feed intake. To meet this demand, the cow draws on stored calcium from her bones. Milk fever (hypocalcemia) occurs when blood calcium levels are depleted more quickly than they can be replenished in the blood and bones (DeGaris & Lean, 2008). Treatment for milk fever includes calcium injections or oral supplementation.

When a dairy cow is in NEB after calving, metabolic demands for lactation cause intense adipose mobilization, usually in the form of NEFAs, to counteract the inability to consume enough feed to meet those demands. NEFAs are first used as energy, and then excess amounts are directed to the liver to produce ketone bodies as an alternative energy source to glucose. High serum concentrations of NEFAs and ketone bodies and low concentrations of glucose cause hyperketonemia. Ketosis is the clinical expression of hyperketonemia. Cows that develop hyperketonemia during early lactation face a heightened risk of ketosis, metritis, and displaced abomasum, and they are also less fertile than cows with normal serum ketone body levels (Mann & McArt, 2023). In addition, cows with excessive conditioning are at a higher risk for developing ketosis due to the severity of NEFA and ketone production (Ingvartsen et al., 2003). Treatment for ketosis involves the oral administration of propylene glycol and vitamin B12 in cases of concurrent hypoglycemia. Fatty liver disease is a complication of NEB and ketosis as NEFA uptake by the liver exceeds its ability to process them, resulting in liver lipid accumulation (McArt, 2019). This can decrease liver function, which can affect concurrent drug elimination. Additionally, clinical ketosis has been shown to decrease renal function, which can also affect concurrent drug elimination (Issi et al., 2016).

Ketosis and milk fever can increase the risk of displaced abomasum (**DA**) in cows (Massey et al., 1993; Zwald et al., 2004). DA is most common in the first few weeks after calving as the abomasum becomes displaced to account for the growing calf during gestation (Doll et al., 2009). After calving, the abomasum must move back to its normal position, but this movement increases the risk of further displacement. The abomasum fills with gas and rises to the top of the abdomen, where it can either become trapped between the rumen and abdominal wall (left-displaced DA; most common) or above the omasum (right-displaced DA). This displacement to the right increases the risk of the abomasum twisting and consequently cutting off blood and nutrient supply. Treatment typically requires surgery to reposition the abomasum to its normal position. Perioperative sedatives, analgesics, and antimicrobials are often administered for this surgery. Earnest (2021) describes using a combination of ketamine, butorphanol, and xylazine to sedate prior to a DA surgery, lidocaine to block before cutting, ampicillin or ceftiofur for post-operative antimicrobial treatment, and flunixin for post-operative pain and inflammation. A survey of Canadian vets summarized that post-operative antimicrobial administration was favored and more common compared to intra-operative or pre-operative antimicrobials (Chicoine et al., 2008). Intra-operative antimicrobials are typically administered intraperitoneal (**IP**) (intra-abdominal). Penicillin and oxytetracycline were the most used antimicrobials in each perioperative period. In addition, prokinetic agents, like erythromycin, have been shown to stimulate, coordinate, and restore GI motility and increase abomasal emptying rate, milk production, and rumen contraction frequency in the immediate postoperative period in cows undergoing surgical correction of LDA (Wittek et al., 2008). Each medication requires a greatly extended WDI for meat and milk.

Another cause of DA is subacute ruminal acidosis (**SARA**) (Olson, 1991). SARA has been reported to be prevalent in 19% of early lactation and 26% of mid-lactation dairy cows in the US. (Mulligan & Doherty, 2008). Early lactation cows are at higher risk due to a reduced absorptive capacity of the rumen, poorly adapted rumen microflora, and the rapid introduction of high-energy dense diets (Dirksen et al., 1985), while cows at peak feed intake face an increased risk due to the greater amount of acids produced in the rumen (Oetzel, 2005). There is no specific treatment for SARA, but the secondary conditions caused by SARA may be treated as needed.

SARA has also been implicated in the cause of laminitis which results in pain and impaired mobility (Enemark et al., 2002; Oetzel, 2000). Laminitis is common around the time of calving due to diet and housing changes (Bergsten, 2003). Primiparous cows are also more susceptible to laminitis in comparison to multiparous cows (Vermunt & Greenough, 1996). Laminitis is inflammation of the laminae and corium, but lameness, in general, is very common around the time of calving. Causes for lameness can include foot rot, sole ulcers, and digital dermatitis. Treatment may include topical (tetracycline, oxytetracycline, copper sulfate, silver sulfadiazine) and systemic antimicrobials (penicillin, oxytetracycline, sulfonamides) and NSAIDs (flunixin or ketoprofen) (Moore et al., 2001; Shearer et al., 2015). It is important to note that laminitis is usually a consequence of metabolic disorders or poor animal husbandry, which should be addressed in addition to these therapies. Coetzee and colleagues (2014) concluded that topical treatment with either tetracycline or oxytetracycline derivatives was likely to result in detectable residues in plasma and milk, but concentrations were well below actionable levels. Tissue concentrations were not evaluated in this study.

1.4.3 | Reproductive Disorders

As mentioned earlier, retained fetal membranes (placenta) and metritis (uterine infection) are associated with poor nutritional management and metabolic dysfunction caused by reduced immunity during the transition period. Retained placenta has been correlated with elevated NEFA levels and decreased feed intake in the pre-partum period, and the most effective preventative strategies are those linked to good periparturient health and immunity (LeBlanc, 2008). Treatment for retained placenta typically includes administration of oxytocin to stimulate uterine contractions, along with supportive care to prevent secondary infections. Retained placenta and metritis can occur individually, but retained placenta is considered a significant risk factor for cows developing metritis (Sandals, 1979). The Merck Veterinary Manual (Roberts, 2024) lists three labeled treatment options for metritis: 1) ceftiofur hydrochloride (2.2 mg/kg, IM, every 24 hours for 5 days), 2) ceftiofur crystalline free acid (6.6 mg/kg, SC in the fat pad behind the ear twice, 72 hours apart), and 3) oxytetracycline (11 mg/kg, IV or SC, every 24-48 hours for 4 days). Extralabel procaine G penicillin and ampicillin trihydrate are also commonly administered in addition to NSAIDs. Both ceftiofur treatments require no milk discard, but oxytetracycline and the extralabel treatments require milk WDIs. Additionally, metritis is often treated with intrauterine antimicrobials such as oxytetracycline. Kaneene and colleagues (1986) observed that the severity of metritis had no difference in the maximum retention time of oxytetracycline. However, primiparous cows had significantly shorter retention times compared to their multiparous counterparts. FARAD recommends testing for residues after intrauterine administration because clearance can be erratic with vast individual variability when given by this route (Black et al., 1979).

In conclusion, metabolic diseases in dairy cattle during the transition period are abundant and interrelated. Ultimately, preventing metabolic and reproductive disorders in dairy cattle relies primarily on appropriate nutritional and management alterations across the lactation cycle. This is imperative to increase animal welfare, decrease economic loss, and account for drug residues in animal products that are influenced by disease-induced alterations to PK.

1.4.4 | Parasitic Diseases

Although not often considered a disease specific to dairy cows, the prevalence of internal and external parasites among dairy herds across the U.S. and throughout the world has been reported to be significant and detrimental to body composition, reproduction, milk production, and animal welfare (Gross et al., 1999; Sanchez et al., 2004). While the growth of dairy production and the non-use of pasture has drastically reduced pasture-dependent parasitic diseases, parasites still remain an underestimated problem in dairy operations (Gazzonis et al., 2022). Internal parasites are primarily present in the GI tract, respiratory tract, and liver. External parasites include flies, lice, mites, and ticks. In addition to the previously mentioned negative effects, external parasites have been identified as vectors for spreading mastitis-associated bacteria between cows (Sommer et al., 2024). Gazzonis and colleagues (2022) measured parasitic burden and associated risk factors for GI parasitic infections in 495 animals across 19 farms in northern Italy. They discovered at least one GI parasite in 51.7% of the enrolled animals and observed a significant impact of production category on parasitic burden. Heifers and dry cows showed higher infection risk than lactating cows and calves. They determined that farm management variables like smaller herd sizes, closer proximity to other farms, straw bedding, feeding hay in addition to silage, and allowing cows pasture access were predictors of a higher parasitic burden on these dairy farms. Biosecurity measures such as the control of incoming and outcoming vehicles and manure removal were predictors of a lower parasitic burden. Parasitic burden is also greater in warmer climates, especially tropical and subtropical climates (Grisi et al., 2014).

To address this issue, deworming protocols are often integrated into dairy cow management systems, including those for heifers and lactating cows. On organic farms, ivermectin, moxidectin, and fenbendazole are allowed for emergency use only in replacement dairy heifers but are not allowed for slaughter stock, during lactation, or in the last trimester of gestation in breeding stock (Sorge et al., 2015). On conventional farms, labeled drugs, including oral albendazole or levamisole, and injectable moxidectin, levamisole, or ivermectin, are available for heifers prior to breeding age. Injectable eprinomectin and an injectable doramectin-levamisole combination product are also labeled in heifers less than 20 months old. Topical eprinomectin is labeled for lactating dairy cattle, and topical moxidectin is labeled for use in all dairy production classes. Neither topical formulations of moxidectin nor eprinomectin require a milk WDT.

1.5 | Public Health Implications of Drug Residues

Drug residues in food products resulting from ELDU in food-producing animals can pose several significant public health risks. When it comes to extralabel usage, certain drugs may have narrow safety margins. Residues, if ingested, could potentially lead to toxic effects, particularly in vulnerable populations such as children, the elderly, or immunocompromised individuals. In addition to general toxicity, even low levels of drug residues, such as beta-lactam antibiotics or other allergens, may trigger allergic reactions in sensitive individuals (Patterson & Stankewicz, 2023). The consumption of antimicrobial drug residues has been linked to various toxic effects such as transfer of antibiotic-resistant bacteria to humans, immunopathological effects, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, reproductive disorders, bone marrow toxicity, and even anaphylactic shock in humans (Darwish et al., 2013; Nisha, 2008). AMR acquired from consuming residues can compromise the effectiveness of antibiotics used in human medicine, making infections harder to treat (NARMS Executive Report, 2005). Public health concerns may prompt changes in regulatory guidelines, which can influence how veterinarians approach ELDU and how producers manage animal treatments. To increase public safety, AMDUCA places stipulations on ELDU in food animals. These stipulations prohibit and restrict the use of certain drugs to reduce the risk of violative residues in edible products (21 CFR § 530.41, 2025).

Residues exceeding the established tolerance can lead to food safety violations. These violations can lead to penalties for veterinarians and producers. Major reasons for residues in cattle include 1) not following the directions for the correct treatment or dosage of drugs to be administered; 2) failure to adhere to the appropriate meat and milk withdrawal times after treating cattle; 3) lack of written records for animal treatment; 4) inadequate or improper animal identification; 5) extralabel or illegal drug use (utilizing a drug not approved for the animal being treated); or 6) unintentionally administering a drug in a manner different from what is indicated on the label (Smith, 2015). This not only poses a direct risk to consumers but also results in economic losses due to discarded products and trade restrictions. Public exposure to cases of drug residue violations can erode consumer confidence in the safety of dairy and meat products, affecting market dynamics and public health policies. Ensuring compliance with drug residue regulations often requires extensive testing and monitoring, increasing costs for producers and regulators. The PMO requires all bulk milk pickup tankers to be sampled and analyzed for animal drug residues before milk can be processed (NMDRD 2024 Annual Report). Any milk that is found positive is rejected for human consumption. In the 2024 fiscal year, 3,668,308 milk samples were analyzed for animal drug residues. Of these samples, 301 (0.008%) were positive for a drug residue. The antimicrobial classes being tested were amphenicols, beta-lactams, sulfonamides, and tetracyclines. Beta-lactams had the highest percentage of milk residue violations in comparison to other antimicrobial classes. Positive samples in 2024 led to 10 million pounds of milk being properly disposed of and out of the human food supply.

In addition to milk testing, USDA FSIS inspects meat, poultry, and egg products for chemical residues through various sampling methods. Primarily, this includes surveillance sampling and inspector-generated sampling. Surveillance sampling is the scheduled sampling of specified slaughter subclasses at the time of slaughter after a carcass has passed antemortem inspection. In the 2023 fiscal year, 8 analytical methods were used to screen for approximately 250 different veterinary drugs, pesticides, and environmental contaminants (USDA-FSIS Annual Sampling Summary Report, 2023). For surveillance sampling in 2023, FSIS inspectors collected 7,254 muscle, kidney, and liver tissue samples. Out of the samples collected, 30 tested positive for residue violations, one of which was in a dairy cow for sulfadimethoxine and one in a heifer for piperonyl butoxide. The number of samples taken for surveillance purposes is typically chosen to provide a 95% probability of detecting at least one violation, assuming 1% of the animal population contains residues above safe concentrations (DeDonder, 2024).

In comparison, inspector-generated sampling occurs when an inspector suspects that animals presented for slaughter may have violative levels of chemical residues based on clinical signs or herd history. In 2023, dairy cows accounted for approximately 56% of all residue violations from inspector-generated sampling. Ceftiofur represented roughly 48% of dairy cow residue violations. Dairy cows had 170 samples with violative residues out of 68,771 samples collected (0.25%), 91 of which were attributed to ceftiofur. Heifers had nine samples containing violative residues out of 3,374 samples collected (0.27%), four of which were due to ceftiofur. Food from animals identified with violative residues of veterinary drugs or pesticides does not enter the food chain. There are repercussions for the producer and, in cases involving prescription drugs, for the veterinarian when violative residues are found (DeDonder, 2024).

1.6 | Establishing a WDI after ELDU

In instances where FARAD is unavailable to help determine an appropriate WDI after ELDU or in the case of accidental exposure, it is important for a veterinarian to have a basic understanding of how to establish a WDI. All WDTs for labeled drugs and WDIs for ELDU are based on the drug's elimination half-life in the particular food product (tissues, milk, eggs, and honey) (DeDonder, 2024). Half-life is the length of time required for the concentration of a drug to decrease to half of its starting dose in the body (Hallare & Gerriets, 2023). **Table 1.4** shows the influence of elimination half-life on the time required to eliminate a drug.

Table 1.3: The influence of elimination half-life on the time required to eliminate a drug.

Number of Half-lives	Dose eliminated (%)
1	50
2	75
3	87.5
4	93.8
5	96.9
6	98.4
7	99.2
8	99.6
9	99.8
10	99.9

Drugs with longer terminal elimination half-lives take longer for their residues to deplete to below the established tolerance and will consequently require a longer withdrawal. Half-life (**$t_{1/2}$**) is typically measured in hours (h) and determined by the following relationship:

$$\mathbf{t_{½} = 0.693 \times (Vd / Cl)}$$

Clearance (**Cl**) is the volume of blood from which a drug is removed per unit of time (L/h), referring to the irreversible elimination of the drug from the body (DeDonder, 2024). Volume of distribution (**Vd**) relates the total amount of drug in the body to the concentration of drug in the plasma at a given time (Toutain & Bousquet-Mélou, 2004). Vd can be represented as the following equation:

Vd (L) = Amount of drug in the body (mg) / Plasma concentration of drug (mg/L)

A drug with a high Vd tends to leave the plasma and enter the extravascular compartments of the body, meaning that a higher dose of a drug is required to achieve a given plasma concentration. On the contrary, a drug with a low Vd tends to remain in the plasma, meaning a lower dose of a drug is required to achieve a given plasma concentration (Mansoor & Mahabadi, 2023).

If there is no additional information, WDIs can be estimated based on a drug's half-life. For instance, if it is necessary to administer an animal twice the recommended dosage, it is assumed that the elimination half-life remains unchanged, so doubling the administered dose will extend the depletion time by one additional half-life. Therefore, the WDT should be prolonged by one half-life (Riviere, 1991). Another example, as seen in **Table 1.4**, uses the 10 half-life rule since 99.9% of the drug is eliminated after 10 half-lives. However, half-lives are typically calculated using serum or plasma concentration data and may not accurately represent tissue or milk residues at harvest. If the elimination half-life differs in tissues compared to plasma, then the 10 half-life rule may not hold true. It is best to use tissue half-life data, although this is often unavailable. Additionally, even if 99.9% of the drug is eliminated, there could still be enough present to trigger a violation, especially if the drug is not approved for the food animal species being treated. Furthermore, sections 1.3.1 and 1.4 touched on PK parameter alterations based on production or disease status. This is another instance where caution should be used when using half-life methods to estimate WDIs because the elimination half-life used for the calculation may not reflect the actual elimination rate in the animal being treated. It is best advised that veterinarians consult with FARAD prior to ELDU where data is limited.

Alternatively, the half-life multiplier (**HLM**) method explained by Gehring et al. (2004) may be a better way to calculate a WDI since tissue half-life data is often unpublished or unavailable to the average person. The HLM method estimates the number of tissue half-lives within the labeled WDT. The fewer half-lives presumed to be contained in the WDT (i.e., the smaller the HLM), the longer the estimated tissue half-life will be. Since the extended WDI is estimated by adding a multiple of the tissue elimination half-life to the WDT, a smaller HLM results in a more conservative estimate. A HLM of three was determined to be the most conservative and practical in comparison to HLMs of four or five or safety factors of 5-10%. For example, oxytetracycline has a 28 day labeled WDT. If this is divided by a HLM of three, the estimated tissue half-life is 9.3 days. Like the previous example, doubling the administered dose would require one additional estimated tissue half-life. This would make the estimated WDI to be 38 days.

With the uncertainty involving WDI calculations, commercially available drug residue screening tests should be used to verify the absence of violative residues in edible products. Since the mammary gland is constantly changing in milk volume, blood flow, and drug elimination/degradation, there will be variability in collected milk samples (Dubbelboer, 2023). FARAD recommends confirming the lack of milk residues with two consecutive negatives prior to adding the milk to the bulk tank. Rapid assay tests are listed on FARAD's website (https://cafarad.ucdavis.edu/RapidAssay/rapidassay.aspx). It is important to note that occasionally, tests are not sensitive enough to detect levels of drug residues below FDA-established tolerances. It is ultimately up to the veterinarian to compare the screening test sensitivity to FDA-established tolerances and account for that discrepancy in their recommended WDI. If the veterinarian cannot calculate a WDI or test for restudies due to a lack of data or testing resources, it is their responsibility to ensure that the treated animal and its food products will not enter the human food supply (21 CFR § 530.20, 2025).

1.7 | Conclusions

In summary, while ELDU can be essential for addressing unmet medical needs in dairy cattle, it must be managed carefully to minimize the risk of drug residues in food products and therapeutic failure in treated animals. The public health implications, from antimicrobial resistance to potential adverse reactions, highlight the importance of adhering to WDIs, using pharmacokinetic data to inform dosing, and employing robust residue monitoring and regulatory oversight to protect consumer safety. In addition, while extralabel regimens are a useful tool for preventing and treating disease in dairy cattle, they cannot compensate for poor management in production settings. When treating dairy cattle with ELDU, it is vital that veterinarians consider the different physiological factors, such as health status, lactation status, or production class, that could impact drug efficacy, safety, and residue deposition in food products.

1.8 | References

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CHAPTER 2

The Pharmacokinetics of Subcutaneous Eprinomectin in Plasma and Milk in Dry Dairy Cattle

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2.1 | Abstract

Parasitic infections in dairy cattle reduce herd immunity, milk production, and conception rates. This leads to higher production costs, compromised animal welfare, and increased interest in extra-label drug use. The extra-label use of anthelmintics poses food safety risks for consumers since appropriate withdrawal intervals in milk have yet to be established. Although topical eprinomectin has no milk withdrawal time, more research is needed to determine the residues present in milk after subcutaneous administration. This study aimed to characterize the pharmacokinetics of injectable eprinomectin in dry dairy cows. We hypothesized that, when given at the labeled dose, eprinomectin residues in dry dairy cattle would be below the FDA milk tolerance at the onset of lactation. Plasma was collected daily from thirteen mature dairy cattle for seven days post-administration, followed by periodic samples for 90 days. After calving, milk was collected daily until 90 days. Eprinomectin concentrations were measured using HPLC-fluorescence detection. The maximum eprinomectin concentration in plasma and milk was approximately 36 ng/mL 43 hours after administration and 3 ng/mL at the onset of lactation, respectively. The low eprinomectin levels in milk collected from these lactating dairy cattle suggest that administering eprinomectin at dry-off is unlikely to result in violative residues. However, subcutaneous eprinomectin in lactating dairy cattle would be hard to justify unless there is evidence that the approved topical formulation is clinically ineffective.

2.2 | Introduction

Parasitic infections in dairy cattle affect production performance by reducing milk yield, milk quality, and lowering contraception rates (Sanchez et al., 2004). Treating dairy cattle with anthelmintics can help mitigate these losses by increasing dairy production and average daily gain, improving animal welfare, and maintaining a healthy herd (Rashid et al., 2022). However, only a limited number of anthelmintic drugs are licensed for use in lactating animals (Imperiale and Lanusse, 2021). This raises concerns about the development of anthelmintic resistance from the overuse of these products and the potential for increased extra-label drug use (**ELDU**). The ELDU of anthelmintics generates food safety risks for consumers as suitable milk withdrawal intervals have not yet been established (Canton et al., 2021). There is growing pressure from consumers to grant production animals with pasture access due to perceived benefits in environmental conservation, animal welfare, and health (Stampa et al., 2020; Bousquet-Mélou et al., 2021). However, with greater pasture access, dairy cattle will experience greater susceptibility to increased parasitic burden.

Currently, only two topical macrocyclic lactone products, eprinomectin and moxidectin, are approved for use in lactating dairy cattle. Topical eprinomectin is a broad-spectrum anthelmintic used for the treatment and control of internal and external parasites (Eprinex, Boehringer Ingelheim Animal Health). This formulation has a zero-day milk discard time, and the label claims up to 99.9% efficacy. However, topical eprinomectin has been shown to only provide 7-21 days of protection from reinfection depending upon the parasite species, and previous studies show a rapid decline in the efficacy of pour-on macrocyclic lactones in comparison to injectable formulations (Gasbarre et al., 2015). Topical anthelmintics have also been shown to differ in absorption and efficacy depending on group housing conditions and licking behavior within a herd (Laffont et al., 2001). Although this product has a zero-day milk discard time, the lack of residual efficacy is a potential concern for producers. Alternatively, an eprinomectin extended-release injectable parasiticide is approved for use in beef or dairy cattle 20 months of age or younger (Longrange, Boehringer Ingelheim Animal Health). This formulation has provided up to 150 days of efficacy for parasitic control in beef cattle depending on the parasite species. The extent of efficacy is attributable to the polymer matrix that remains beneath the skin, allowing for a slow release of eprinomectin over time (Avgoustakis, 2005). This long duration of action has been shown to provide considerable advantages for production parameters in heifers compared to the topical formulation with a much shorter duration of action (Dudley and Smith, 2020). However, this long-duration formulation has no published pharmacokinetic data and no label approval for use in mature, adult dairy cattle. FARAD has received several questions about the usage of this product in cattle, which is why its application was explored in this particular scenario.

This study aimed to provide new pharmacokinetic data utilizing the dry period in dairy cows with the goal of benefiting food safety, animal welfare, producers' economic interests, and educated treatment decisions for veterinarians. The objective of this study was to determine the elimination kinetics of eprinomectin when administered subcutaneously in dry dairy cattle. We hypothesized that a single subcutaneous, label dose of 1 mg/kg eprinomectin would result in drug concentrations falling below the FDA milk tolerance level by the time of post-calving lactation.

2.3 | Materials and Methods

2.3.1 | Animals and Housing

The study was approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #21-172). Inclusion criteria for enrollment included cows with no history of anthelmintic administration in the previous 4 months, were multiparous, and were about to begin a 60-day dry-off period. Prior to enrollment, all cows were determined to be pregnant (by rectal palpation), healthy based on physical examination, and had a negative California Mastitis Test in all working quarters. Fourteen mature, adult dairy cows (Jersey, n= 5; Holstein, n= 9; weight range: 463-873kg) were enrolled in this study. The cows were sourced and group-housed at the North Carolina State University Dairy Educational Unit. After a final milking, the enrolled cows were administered dry-cow therapy (Spectramast DC, Zoetis Animal Health) and a teat sealant (Lockout, Boehringer Ingelheim Animal Health) at one syringe/quarter. The cows were fed once daily, provided water *ad libitum*, and had pasture access for grazing throughout the duration of the study.

2.3.2 | Dosing and Sample Collection

Following the last milking before dry-off, each cow received a single dose of subcutaneous eprinomectin (5% sterile solution) at a dose of 1 mg/kg in the front of the right shoulder. Doses exceeding 10 mL were divided between two injection sites.

Blood sample collection time points were extrapolated from previous studies in dairy cattle (Soll et al., 2013). Heparinized blood samples (10 mL) were collected by tail venipuncture using a vacutainer needle prior to drug administration (0 hr) and then at 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, 35, 49, 63, 77, and 91 days. Plasma was separated by centrifugation at 3500 x *g* for 10 minutes at 4°C and equally divided into two cryovials. Once the cows calved, milk samples were collected every 12 hours for the first 7 days and then once daily until 91 days post-drug administration. Milk sample collection time points were taken based on the established milking frequency at the dairy unit. The cows were milked in a side-by-side parallel parlor by a milking claw. A milk meter ("Turn&Mix" Sampler Metatron, GEA Farm Technologies) collected a measured amount of milk throughout the entirety of milking, and a 15 mL aliquot was taken immediately from this collection to provide a representative sample of the total milking volume. Leftover milk was vacuumed out of the system, and a clean milk meter was used for every collection. If treated cows were milked in the same stall, there was a small chance for cross-contamination from residual milk left in the milking claw or hoses, but careful attention was paid to avoid this from happening. Between milkings, the entire milking system was thoroughly cleaned during the rinsing cycle. Blank milk was taken from the bulk tank prior to the start of the study to be used for method development and spiking solutions during sample analysis. Eprinomectin was not used at this dairy, so there was no concern for contaminated blank milk from the bulk tank. Milk samples were divided into three cryovials. All samples were placed in a -20°C freezer until the study's completion and moved to a -80°C freezer until they could be analyzed.

2.3.3 | Drug Concentration Analysis

Eprinomectin concentrations in all samples were determined by high-performance liquid chromatography with fluorescence detection (**HPLC-FL**) by modifying a method used previously in alpacas (Pollock et al., 2017). The analytical method for determining eprinomectin in plasma and milk was validated before use in this study. The HPLC-FL system consisted of a Waters

Alliance 2965 HPLC Separations Module System with a Waters 2475 HPLC Multi Fluorescence Detector set at an excitation wavelength of 355 nm and emission wavelength of 465 nm. The LC column used was an XBridge C18 3.5 µm, 4.6x100 mm HPLC Column with an XBridge BEH C18 3.5 µm, 3.9x5 mm Vanguard precolumn (Waters Corp.) kept at a constant temperature of 30°C. The mobile phase consisted of 87% acetonitrile and 12.9% ultra-pure water with the addition of 0.1% trifluoroacetic acid. The flow rate was 1 mL/min and was delivered in an isocratic mixture. The retention time for eprinomectin using this method and equipment was approximately 6 minutes. Eprinomectin USP reference standard (300 mg, MilliporeSigma) was dissolved in 100% HPLC-grade acetonitrile to achieve a 1 mg/mL stock solution concentration. From this solution, further dilutions were made in acetonitrile to prepare spiking solutions for plasma and milk. The spiking solutions were made fresh weekly. The reference standard, stock solution, and spiking solutions were tightly sealed, protected from light, and stored at -20°C.

In glass tubes, 10 µL of the spiking solutions were added to 990 µL of blank bovine plasma to create a calibration curve consisting of one blank and seven calibration standards ranging from 0.625 ng/mL-100 ng/mL. To these tubes, 1000 µL of acetonitrile was added, vortexed briefly, and then centrifuged at 3500 x *g* for 10 minutes at 4°C. The supernatant was decanted, and 1000 µL was transferred to a clean glass tube and dried under 100% air at 55°C for 30 minutes. Plasma samples were reconstituted in 100 µL of a 1:1 acetonitrile:1-methylimidazole solution and vortexed. Then, 100 µL of a 7:3 acetonitrile:trifluoroacetic anhydride solution was added to the samples and vortexed. The acetonitrile:1-methylimidazole and acetonitrile:trifluoroacetic anhydride solutions were made fresh daily. The derivatized plasma samples were filtered with an EZFlow hydrophilic PVDF 13 mm 0.22 µm syringe filter

(Foxx Life Sciences) into HPLC total recovery vials (Waters Corp.), and 50 µL was injected for analysis.

For milk sample analysis, the same method for plasma sample analysis was used except that the calibration curve had a different concentration range, a solid phase extraction technique was added for additional cleanup, and each milk sample was analyzed in triplicate. The milk calibration curve consisted of one blank and six calibration standards ranging from 1.25 ng/mL-50 ng/mL. After centrifugation, the milk supernatant was diluted with ultra-pure water and loaded into Oasis PRiME HLB 3 cc (60 mg) Extraction Cartridges (Waters Corp.) onto a positive pressure manifold (Biotage). These samples were washed with 2000 µL of 95:5 water:methanol and eluted with 2000 µL of 90:10 acetonitrile:methanol into clean glass tubes. The samples were dried down, reconstituted with derivatizing reagents, syringe filtered, loaded into HPLC total recovery vials (Waters Corp.), and 50 µL was injected into the instrument for analysis.

The chromatograms were integrated with a computer program (Empower 3 Software, Waters Corp.) which quantified the unknown concentrations in both matrices.

Calibration, quality control, and blank samples were made fresh at the start of each run. The quality control samples were made by spiking blank plasma or milk with eprinomectin concentrations at 10 ng/mL and 50 ng/mL. Quality control samples for plasma and milk were within 7.5 and 9.4% of the nominal value, respectively, and the relative standard deviation was ≤15.7 and ≤10.8%, respectively. All calibration curves were linear for both matrices with an average $R^2 > 0.99$. The limit of quantification (**LOQ**) and the limit of detection (**LOD**) for eprinomectin in both plasma and milk were 2.5 ng/mL and 1.25 ng/mL, respectively.

Plasma and milk intraday precision and accuracy were obtained on the same day using four different concentrations repeated five times each at 0.625, 0.75, 1.25, and 25 ng/mL and 1.25, 2.5, 5, and 25 ng/mL, respectively. Plasma interday precision and accuracy were obtained by measuring five different concentrations on seven different days at 2.5, 10, 25, 50, and 100 ng/mL. Milk interday precision and accuracy were obtained by measuring six different concentrations on 22 different days at 1.25, 2.5, 5, 10, 25, and 50 ng/mL, respectively. Plasma intraday precision was 5.3 to 15.9%, with an accuracy of 97.7 to 125.3%. Milk intraday precision was 2.0 to 13.1%, with an accuracy of 91.1 to 102.6%. Plasma interday precision and accuracy for 10 to 100 ng/mL standards were 2.2 to 15.6% and 91.3 to 102.2%, respectively. Plasma interday precision and accuracy for the 2.5 ng/mL standard were 40.0% and 105.6%, respectively. Milk interday precision and accuracy for standards of 2.5 to 50 ng/mL were 1.4 to 18.2% and 92.8 to 101.2%, respectively. Milk interday precision and accuracy for the 1.25 ng/mL standard were 34.1% and 105.1%, respectively.

2.3.4 | Pharmacokinetic and Statistical Analysis

A noncompartmental analysis of eprinomectin concentration versus time profiles was performed with analytical software (Phoenix Win-Nonlin 8.0, Certara Inc.). The pharmacokinetic analysis of milk was performed on the average concentration value of the 3 replicates to account for any variability present in the raw milk samples. The noncompartmental analysis of plasma was conducted on points for the initial concentration peak only (approx. 49 days post-drug administration). All analyses were conducted on observed values that were above the LOQ (2.5 ng/mL) for plasma and above the LOD (1.25 ng/mL) in milk. The linear log trapezoid method calculated the area under the concentration–time curve from time zero to infinity

(**AUC0→∞**; day × ng/mL). Other parameters determined were peak concentration (**Cmax**; ng/mL), time to reach peak concentration (**Tmax**; day), and the terminal half-life (**T1/2**; day). This study could not calculate absolute bioavailability as an accompanying intravenous dose was not administered. Therefore, the apparent clearance and volume of distribution per fraction absorbed are not reported.

The mean pharmacokinetic parameters were compared between breeds using the Mann-Whitney U-test based on the normality of data distribution. All statistical analyses were performed using R Statistical Software (v4.3.1; R Core Team, 2023).

2.4 | Results

No tissue site reactions were observed in the cows after subcutaneous eprinomectin administration. One Holstein cow was eliminated from the study due to spontaneous abortion shortly after dosing. Another Holstein cow was eliminated from the study due to insufficient quantities of plasma and milk samples for processing after they were spilled. A total of 12 cows (Jersey, n= 5; Holstein, n= 7) were used for plasma analysis. One Holstein cow's milk data was removed from analysis due to contracting metritis after calving, so a total of 11 cows (Jersey, n= 5; Holstein, n= 6) were used for milk analysis.

2.4.1 | Plasma

The plasma concentration-time curves and pharmacokinetic parameters following a single subcutaneous dose of eprinomectin at 1 mg/kg are summarized in **Figure 2.1** and **Table 2.1**. The observed values that fell below the LOQ (2.5 ng/mL) were removed before analysis. This study only reports pharmacokinetic parameters in plasma from the initial peak which lasted until approximately 49 days after dosing. The initial mean maximal plasma concentration (Cmax ± SD) for Holstein and Jersey cows in this study reached 37.52 ± 1.30 ng/mL at 1.74 ± 0.06 days (Tmax) and 33.34 ± 1.37 ng/mL at 1.89 ± 0.06 days, respectively. There may be apparent breed differences, but the analysis suggests there are no statistically significant differences in eprinomectin depletion in plasma (**Figure 2.1**). There were no significant differences in T1/2, Cmax, Tmax, AUCinf, AUClast, and MRT between the two groups (**Table 2.1**).

2.4.2 | Milk

Breed milk concentration-time curves following a single subcutaneous dose of eprinomectin at 1 mg/kg are shown in **Figure 2.2**. The median dry period for all cows was 65 days (Holstein, 66 days; Jersey, 64 days) with a range of 53-71 days. Eprinomectin concentrations were not present above the FDA milk tolerance for cattle of 12 ng/mL in any milk sample at the start of lactation or thereafter (**Figure 2.2**). Drug concentration analysis quantified most milk samples to be below the LOQ (2.5 ng/mL). Therefore, subsequent milk samples collected past seven days in lactation were excluded from analysis. At the time of calving, 87% of milk samples fell below the LOQ in milk. Therefore, only Cmax and Tmax parameters are reported, and the observed values that fell below the LOD (1.25 ng/mL) were removed before analysis. The mean maximal milk concentration (Cmax ± SD) for Holstein and Jersey cows in this study reached 2.67 ± 1.27 ng/mL at 0.63 ± 0.06 days in lactation (Tmax) and 2.39 ± 1.33 ng/mL at 0.59 ± 0.06 days in lactation, respectively. The milk pharmacokinetic parameters and milk yield data are summarized in **Table 2.2**. The milk yield data is an average of all milkings for the first seven days in lactation and does not accurately depict the increase in milk yield throughout the course of the study given these cows were in early lactation.

The Holstein cow that contracted metritis after calving had detectable levels of eprinomectin above the LOQ for the first seven days in lactation and reached a maximum concentration of approximately 6.90 ng/mL 144 hours into lactation. Her observed values are excluded from our analyses because she was not a member of our target, healthy population.

2.5 | Discussion

This study is the first to report on the pharmacokinetic parameters of subcutaneous eprinomectin in dry dairy cattle. Plasma and milk have a maximum eprinomectin concentration of approximately 36 ng/mL 43 hours after administration and 3 ng/mL at the onset of lactation, respectively.

In this trial, the subcutaneous formulation of eprinomectin was not used per the label directions as it was administered to dry dairy cattle, a production class not listed on the label. On average, 90% of the milk samples in this study within 48 hours were free of residues according to our LOQ of 2.5 ng/mL. If the Holstein cow with metritis whose milk sample concentrations remained above the LOQ for the first seven days of lactation was removed from the data set, 97% of the milk samples were free of residues by 48 hours. The milk Cmax and Tmax parameters reported in this study are less accurate due to our decision to exclude only values that fell below our LOD instead of our LOQ. This decision was made to still report the milk concentration-time profile and emphasize the lack of residues present at the onset of lactation. The use of injectable eprinomectin in mature dairy cattle instead of the approved topical formulation would be considered prohibited ELDU under the Animal Medicinal Drug Use Clarification Act (**AMDUCA**) if used for convenience. FARAD does not encourage prohibited ELDU and conducted this research to provide veterinarians with pharmacokinetic data to make educated treatment decisions. We highly recommend veterinarians to request extra-label advice at http://www.farad.org/ prior to use in dairy cattle and abide by AMDUCA's requirement that there is evidence that shows the approved topical formulation is clinically ineffective for its intended use.

The mean plasma concentration profiles of eprinomectin in dairy cows measured in the current study are lower than those measured in lactating dairy cows, nonlactating dairy cows, and crossbred cows (Bos indicus x Bos taurus) (Baoliang et al., 2006; Aksit et al., 2016; Nascimento et al., 2020) yet higher than those measured in beef cows (Soll et al., 2013). The differences among these studies may be explained by the various eprinomectin formulations administered or individual animal variability, such as body condition score and physiology across the classes of production animals. A study conducted in canines supported the conclusions that three macrocyclic lactones (ivermectin, moxidectin, and eprinomectin) show decreased clearance, increased volume of distribution, and longer terminal half-lives in obese patients when compared to their lean counterparts (Bousquet-Mélou et al., 2021). This is supported by the known pharmacokinetics of lipophilic drugs and their affinity for adipose tissue. This may explain the pharmacokinetic differences between dairy and beef cattle breeds as dairy breeds historically have less body conditioning and altered metabolism, but more research needs to be conducted to evaluate the effect of disease, breed, and production parameters on eprinomectin elimination kinetics.

Eprinomectin was chosen for evaluation in this study because it has substantially reduced distribution in milk (Imperiale and Lanusse, 2021) and offers a large margin of safety for human consumers due to its mechanism of action. Eprinomectin consists of a 90:10 mixture of two homologous components, B1a and B1b. The B1a component is the marker residue in all matrices. Previous studies have reported that the milk-to-plasma concentration ratio for eprinomectin in cattle ranges from 0.12 to 0.14 and remains constant for the duration of eprinomectin clearance (Alvinerie et al., 1999). This ratio is valuable as it estimates the amount of drug distributed into the milk after it is transferred out of systemic circulation. Applying the 0.14 partitioning ratio to the current study's plasma Cmax, Holstein and Jersey cows dosed during lactation instead of at dry-off would have an estimated milk Cmax of 5.25 ± 1.36 ng/ml and 4.67 ± 1.49 ng/mL, respectively. Anthelmintic treatment in heifers during the growing period has been shown to increase average daily gain, milk production, and reproduction parameters (DesCôteaux et al., 1999; Volk et al., 2019; Dudley and Smith, 2020). It is easy to assume that administering anthelmintics to prepartum dairy cows during a time of suppressed immunity would contribute the most to increasing production parameters during freshening. However, studies have shown that production parameters such as milk yield increase the most when dairy cows are treated in mid-lactation (Gross et al., 1999). Due to the concern of violative residues after ELDU of subcutaneous eprinomectin in lactating dairy cattle, mid-lactation application is currently impractical for this formulation of eprinomectin.

There were multiple limitations in this study. The sampling duration, sample size, and lack of an efficacy component were the main limitations. Injectable eprinomectin exhibits a biphasic peak in plasma drug concentration, but this study did not ultimately capture the secondary plasma peak due to insufficient sampling duration. The secondary mean maximal plasma concentration has been shown to have reached approximately 30% of the initial mean maximal plasma concentration around 90 days in beef cattle when sampling continued 160 days after drug administration (Soll et al., 2013). It is unknown if there is also a biphasic peak in milk drug concentration and what the secondary mean maximal milk concentration value would be. It is recommended to have a minimum of 20 animal subjects for dry-cow studies (FDA, 2015). However, after evaluation to meet the study's inclusion criteria, only 14 subjects were available for enrollment at the dairy farm, 12 of which were used for plasma analysis and 11 for milk analysis. The cows were representative of commercial dairy practices but were not randomly selected as it was not appropriate for the pharmacokinetic assessment or practical for the extensive sampling with minimal animal availability. This study lacked an efficacy component however, previous publications indicate that plasma concentrations for avermectins ranging between 0.5 and 2 ng/mL would represent the minimal drug concentration required for optimal nematocidal activity (Lifschitz et al., 1999). Mean plasma concentrations for Holstein and Jersey cows remained above 1 ng/mL for the entire study duration. One exception was that Jersey cows had an average concentration that fell between our LOQ (2.5 ng/mL) and our LOD (1.25 ng/mL) on day 63 post-administration, which makes this quantity less accurate. Regarding an ectoparasite comparison, it has been documented that a plasma concentration for eprinomectin in cattle of at least 8 ng/mL is the necessary threshold for tick control (Lifschitz et al., 2016). More importantly, it was shown that this concentration was necessary for the length of tick development (21-27 days). Plasma concentrations for Holstein and Jersey cows remained above 8 ng/mL for at least 14 days, but concentrations dropped below the threshold level between days 14 and 21. This may contribute to larval reinfestation and concerns for resistance. This study did not assess parasitic resistance to eprinomectin, but there is growing concern that extended-release formulations may expedite the development of resistance by increasing selection pressure for resistant parasites (EMA, 2017).

In conclusion, low eprinomectin concentrations in milk collected from these dairy cattle in post-calving lactation could indicate a novel method for anthelmintic treatment without the concern for violative residue risks for consumers. Still, they will require a prolonged withdrawal interval to comply with a zero-tolerance limit for ELDU. In addition, while this extended-release product may seem advantageous to use due to its low concern for violative residues, we encourage veterinarians and producers to utilize labeled products first and look to extended-release formulations as a last resort to minimize the formation of resistant parasites. FARAD does not encourage prohibited ELDU and conducted this research to provide veterinarians with pharmacokinetic data to make educated treatment decisions. Using subcutaneous eprinomectin in lactating dairy cattle would be hard to justify unless there is evidence that the approved topical formulation is clinically ineffective for its intended use.

Author Contributions

REB and DMF conceived and designed the study. DMF, RAM, TSM, and JLH collected samples and data. RAM developed the analytical method and performed sample analysis. RAM performed pharmacokinetic modeling. All authors were involved in the drafting and revising of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest for the present study.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement

The study was approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #21-172).

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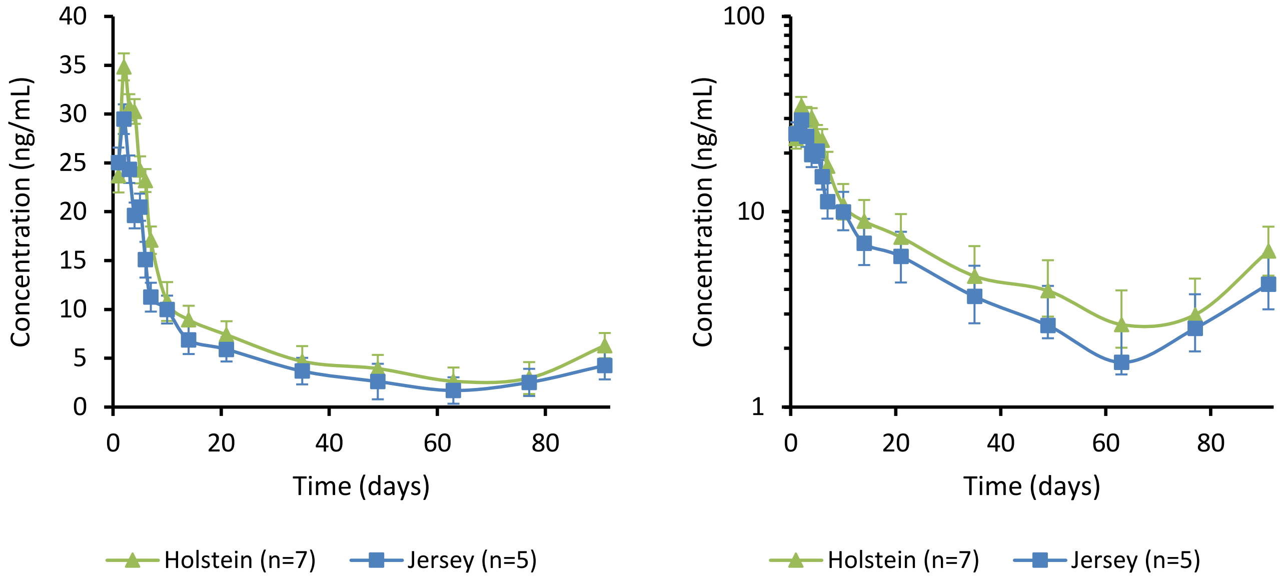

Figure 2.1. Plasma concentration versus time profile (linear scale axis, left; log scale axis, right) following a single 1 mg/kg dose of subcutaneous eprinomectin (geometric mean ± SD).

Table 2.1. Plasma pharmacokinetic (PK) parameters* of eprinomectin based on noncompartmental PK analysis following a single 1 mg/kg dose of subcutaneous eprinomectin to dry dairy cattle (geometric mean (%CV))[1].

Subjects	T1/2 (day)	Tmax (day)	Cmax (ng/mL)	AUClast (day × ng/mL)	AUCinf (day × ng/mL)	AUCextrap (%)	MRTinf (day)
Holstein (n=7)	35.80 (45.88)	1.74 (42.31)	37.52 (26.41)	486.59 (21.87)	709.31 (25.83)	27.89 (48.35)	43.06 (42.93)
Jersey (n=5)	22.05 (77.04)	1.89 (41.24)	33.34 (32.26)	335.61 (37.59)	466.20 (40.31)	26.18 (35.03)	29.08 (43.83)
All Cows (n=12)	26.90 (63.16)	1.80 (40.05)	35.72 (28.19)	416.81 (34.12)	583.46 (37.40)	25.49 (45.45)	35.54 (43.46)

[1]T1/2 = terminal half-life of drug; Tmax = time to maximum concentration; Cmax = maximum plasma concentration; AUClast = area under the concentration-time curve from time zero to time of the last measurable concentration; AUCinf = area under the concentration-time curve of total exposure of drug to the body; AUCextrap = percentage of the area under the concentration-time curve extrapolated from the last observed time point; MRTinf = mean residence time of total exposure of drug to the body.

*Values shown are for the initial plasma peak only (approx. 49 days post-drug administration).

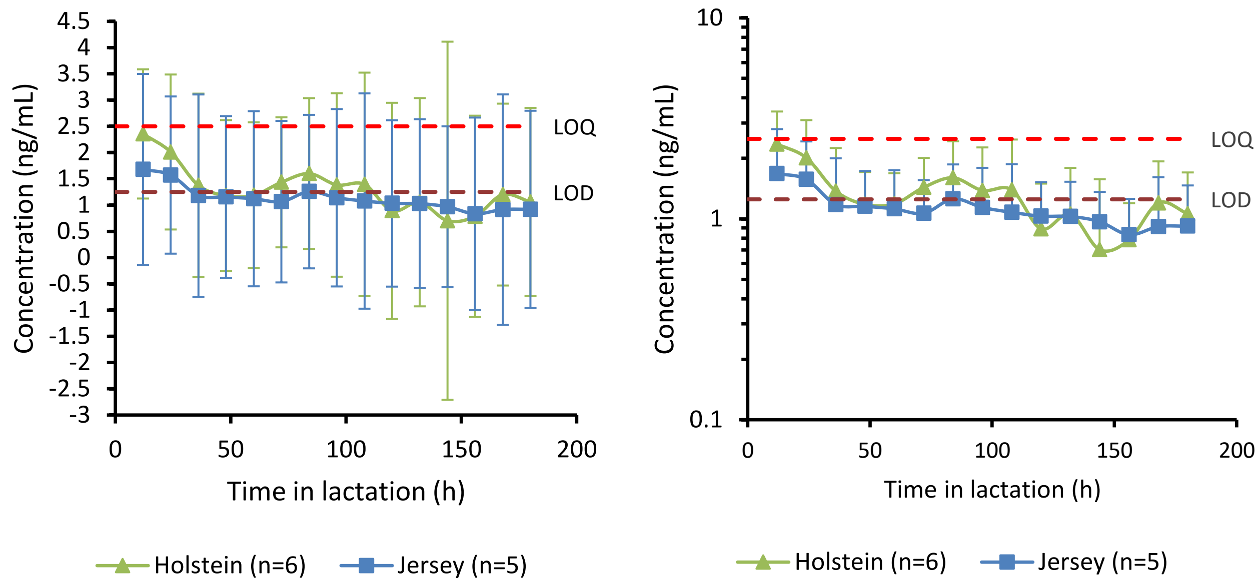

Figure 2.2. Milk concentration versus time profile (linear scale axis, left; log scale axis, right) following a single 1 mg/kg dose of subcutaneous eprinomectin (geometric mean ± SD). Negative values for the lower SD could not be plotted on the semi-logarithmic scale.

LOQ= limit of quantification (2.5 ng/mL); LOD= limit of detection (1.25 ng/mL).

Table 2.2. Milk pharmacokinetic (PK) parameters of eprinomectin based on noncompartmental PK analysis following a single 1 mg/kg dose of subcutaneous eprinomectin to dry dairy cattle (geometric mean (%CV))[1].

Subjects	Tmax (day)	Cmax (ng/mL)	Milk Yield (kg)
Holstein (n=6)	0.63 (36.97)	2.67 (23.93)	17.19 (±4.08)
Jersey (n=5)	0.59 (35.72)	2.39 (29.08)	13.02 (±3.12)
All Cows (n=11)	0.62 (34.44)	2.55 (25.11)	15.42 (±4.23)

[1]Tmax = time to maximum concentration; Cmax = maximum plasma concentration; Milk yield = average amount of milk collected per milking for the first seven days in early lactation.

CHAPTER 3

Investigating the Pharmacokinetics and Efficacy of Intramammary Ceftiofur Hydrochloride in Prevention of Udder Inflammation in Non-Lactating Dairy Heifers

Pending Acceptance in the *Journal of Veterinary Pharmacology and Therapeutics*
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3.1 | Abstract

Mastitis is the most burdensome concern for the dairy cattle industry. Antimicrobials are often prophylactically administered to dairy cows at dry-off to reduce the risk of intramammary infection during the dry period and subsequent lactation. Mastitis incidence has increased in dairy heifers after calving, leading to extralabel drug use of various dry cow products, including intramammary ceftiofur hydrochloride. However, the pharmacokinetics and efficacy of this application have yet to be studied. This study aimed to compare the pharmacokinetics and efficacy following no treatment, a non-antimicrobial teat sealant, or a single dose of intramammary ceftiofur given at 21 or 14 days before expected calving. We hypothesized that milk collected following dosing would contain drug residues below the FDA tolerance of 100 ng/mL by calving, and heifers within the ceftiofur treatment groups would have lower somatic cell counts than heifers in the teat sealant and non-treatment control groups. Following treatment or no treatment of 24 prepartum heifers, milk samples were collected until 21 days after calving. Somatic cell counts and ceftiofur concentrations were assessed utilizing a cell counter and UPLC/MS detection, respectively. Ceftiofur administration did not demonstrate significant differences in reducing somatic cell counts between groups by days 7, 14, or 21. For heifers treated 14 and 21 days prior to calving, milk had a maximum ceftiofur concentration of 8.14±6.24 ng/mL and 4.20±5.07 ng/mL 48 hours into lactation, respectively. The minimal ceftiofur concentrations in milk collected from these heifers indicate that administration of ceftiofur 14 or 21 days before calving is unlikely to lead to violative residues.

3.2 | Introduction

Mastitis is inflammation of the mammary gland, typically caused by bacterial infection, that poses a significant economic and welfare concern in dairy cattle, including prepartum heifers (USDA-ARS, 2021). While it is commonly associated with lactating cows, mastitis can also affect heifers before their first calving. This condition often results in impaired mammary development, reduced milk production, and an increased risk of chronic infections throughout the animals' productive lifespan (Akers & Nickerson, 2011; Oliver & Sordillo, 1988; Puerto et al., 2021). Prior to calving, environmental pathogens are primary culprits, as young animals are often exposed to these bacteria in housing environments, making them more susceptible to bacterial colonization and infection (Fox, 2009). Prepartum cows and heifers are most susceptible to mastitis in the weeks leading up to and following calving due to metabolic disorders, oxidative stress, and excessive lipid mobilization (Ayemele et al., 2021; Laliotis et al., 2020; Mezzetti et al., 2021). Following calving, postpartum heifers are additionally challenged by contagious pathogens spread from infected cows through contact with contaminated milking equipment (Fox, 2009).

Preventing mastitis in dairy heifers involves a combination of management practices and environmental controls. Keeping heifer housing areas clean and dry reduces the environmental bacterial load and decreases the likelihood of exposure to mastitis-causing pathogens (De Vliegher et al., 2012). Proper nutrition is also essential for supporting a strong immune response in heifers, which aids in reducing their susceptibility to infection (Compton et al., 2007). Numerous studies have also shown varying degrees of efficacy for non-antibiotic teat sealants used in prepartum heifers, as these create a physical barrier that protects the teat canal from bacterial invasion (Larsen et al., 2021; Nickerson et al., 2020; Parker et al., 2007). Vaccination against pathogens such as *Staphylococcus aureus* and *Escherichia coli* can be a proactive approach to preventing mastitis in heifers. While vaccines may not entirely prevent infection, they can reduce the severity and duration of clinical mastitis cases, thereby limiting the overall impact on milk production and animal welfare (Ismail, 2017). Unfortunately, mastitis can still occur in herds that practice the highest standards of animal husbandry. Therefore, producers find it crucial to investigate alternative methods for preventing or controlling mastitis in heifers.

Currently, there are no labeled treatment options available for mastitis in heifers, which leads to the extra-label use of antimicrobials, and this generates food safety risks for consumers. Blanket dry cow therapy (**BDCT**) is a commonly used strategy in which each cow is administered an intramammary antimicrobial at the end of lactation, regardless of disease status, to reduce the risk of mastitis during the dry period and subsequent lactation. Ceftiofur hydrochloride, a cephalosporin antibiotic, is used extensively in dairy cattle to treat existing clinical mastitis infections as well as for BDCT (USDA NAHMS, 2014). While ceftiofur is not used in human medicine, cephalosporin drugs are critically important for treating various human diseases. This leads to concerns that their overuse may contribute to increased antimicrobial resistance (**AMR**) and reduced antibiotic effectiveness in livestock and human medicine. To combat this, the FDA limits the use of cephalosporin antibiotics in livestock and prohibits producers from using them in particular extra-label applications (21 CFR § 530.41, 2024).

The Food Animal Residue Avoidance Databank (**FARAD**), a U.S. Department of Agriculture-supported national food safety program, has received numerous inquiries about the use of intramammary ceftiofur hydrochloride in heifers. FARAD's mission is to assist producers and veterinarians in preventing or mitigating illegal or harmful residues of drugs, pesticides, biotoxins, and other chemical agents that may contaminate foods of animal origin (Riviere et al., 2017). In addition to the risks posed by ceftiofur regarding AMR, the 2023 Milk & Dairy Beef Drug Residue Prevention Manual and the 2023 National Milk Drug Residue Database Annual Report states that it has the highest percentage of tissue residue violations in dairy cull cows, and beta-lactams, in general, have the highest percentage of milk residue violations in comparison to other antimicrobial classes. While there are several drugs used extralabel in heifers, this paper specifically focuses on the lactating cow formulation of ceftiofur hydrochloride (Spectramast LC, Zoetis Animal Health) due to its significant regulatory concern. Pretreating heifers to minimize the impact of subclinical mastitis (**SCM**) at calving is a relatively common practice, but not much is known about how close to calving antimicrobials can be administered without impacting food safety. Most studies assessing antimicrobial use in prepartum heifers have focused on formulations intended for lactating cows. We believe this is due to the fact that replacement heifers do not have a designated dry period, as they have not previously lactated. Additionally, dry cow therapies may only persist for 14-28 days into the dry period and fail to protect the udder during the late dry and early lactation periods (Petzer et al., 2009). To address this gap in coverage, producers might consider lactating cow formulations closer to calving for their heifers instead of dry cow formulations. Producing replacement heifers requires a significant investment, making intramammary ceftiofur hydrochloride an attractive option for dairy producers due to its effectiveness against the major pathogens that cause mastitis and its established use on farms. However, there is no published pharmacokinetic data for this application, and therefore, no data to establish an appropriate withdrawal interval for periparturient heifers. Therefore, evaluating this treatment's efficacy and risk of violative residues is crucial.

Milk somatic cell count (**SCC**) measurements are used as an indicator of milk quality and as a marker to monitor the prevalence of mastitis in dairy herds (Alhussien & Dang, 2018). An elevated level of milk SCC has a direct correlation to the severity of intramammary infection (**IMI**), reduces the quality of raw milk, and decreases profitability as it is less suitable for processing and sale (Sharma et al., 2011). In the European Union, China, New Zealand, Australia, Switzerland, and Canada, the legal bulk tank SCC limit is $300\text{-}400 \times 10^3$ cells/mL; in South Africa and Brazil, 500×10^3 cells/mL; and in the US, 750×10^3 cells/mL (Alhussien & Dang, 2018). However, it is generally accepted that a SCC exceeding 200,000 cells/mL indicates cows suffering from a bacterial infection or SCM (Petzer et al., 2017).

This study aimed to determine ceftiofur hydrochloride elimination in milk in periparturient heifers and to evaluate its efficacy in antimicrobial-treated versus non-antimicrobial-treated groups. We hypothesized that milk collected from postpartum heifers after a single intramammary dose of 125 mg of ceftiofur hydrochloride in each quarter, administered 14 or 21 days before calving, would contain drug residues below the FDA's tolerance level by the time of calving or shortly thereafter. Additionally, we hypothesized that heifers in the antimicrobial treatment groups would demonstrate a decreased association with subsequent infection by measuring lower SCC values compared to those in the non-treatment and teat sealant control groups.

3.3 | Materials and Methods

3.3.1 | Animals and Housing

The study was approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #21-172). The sample size was determined by the FDA's recommendation that at least 20 prepartum animals be enrolled in a milk residue study (FDA, 2015) while maintaining an equal number of animals in each group and accounting for an anticipated 15% drop-out rate. Investigators were not blinded to the treatment groups of the heifers. Inclusion criteria for enrollment included prepartum heifers who had not been administered any antibiotic treatment 30 days prior to the start of the study, functional mammary quarters that passed udder inspection (redness, swelling), and palpation (firmness, pain, heat). Twenty-four prepartum, Holstein-Jersey cross-bred heifers were enrolled for use in this study, each expected to calve 14 or 21 days from the initial treatment date. Using a parallel study design, all treatments were decided based on heifer and farm labor availability 14 or 21 days prior to calving, so heifers were not randomly allocated to treatment groups. The heifers were sourced and group-housed on pasture at the North Carolina Department of Agriculture (**NCDA**) Cherry Research Station. The heifers were fed a total mixed ration (**TMR**) twice daily, provided water *ad libitum*, and had pasture access for grazing throughout the duration of the study. All heifers received two coliform mastitis vaccines (J-VAC, Boehringer Ingelheim Animal Health) approximately 60 days prior to calving and 7 days post-calving. All heifers received two neonatal calf diarrhea vaccines (Scourguard 4KC, Zoetis Animal Health) approximately 60 days and 21 days prior to calving. All heifers were dewormed (Cydectin (moxidectin) Pour-On, Elanco Animal Health) 7 days post-calving.

3.3.2 | Dosing and Sample Collection

The heifers received one of the four following intramammary treatments (**Figure 3.1**): **TS**, teat sealant (n= 6); **14**, Spectramast LC treatment and teat sealant 14 days prior to the expected calving date (n= 6); **21**, Spectramast LC treatment and teat sealant 21 days prior to the expected calving date (n= 6); **NT**, no treatment (n= 6). Prior to treatment, all teats were scored on a 1-3 scale based on the outward appearance of the health of the teat. This scale was adapted from a previous study in prepartum heifers (Booth et al., 2016). A score of 1 was given for teats that appeared healthy with no abrasions or scabbing. A score of 2 was given for teats with residual damage from healing scabs. A scale of 3 was given for teats that appeared unhealthy with fresh scabs. Lacteal secretions (2-3 mL) were also collected from each quarter of every heifer for future testing before treatment. Each teat end was scrubbed with a 70% alcohol-soaked pad starting on the teats farthest away from the administrator before lacteal secretion collection and dosing.

The antimicrobial-treated heifers (groups 14 and 21) received 125 mg ceftiofur hydrochloride lactating-cow therapy (Spectramast LC, Zoetis Animal Health) intramammary from one 10 mL syringe/quarter. These heifers were treated using a partial-insertion technique starting on the teats closest to the administrator. Ceftiofur was massaged into each quarter to distribute the suspension into the milk cistern. Treated heifers then received 4 g of a sterile, non-antibiotic internal teat sealant (Lockout, Boehringer Ingelheim Animal Health) containing 60% bismuth subnitrate administered intramammary at one syringe/quarter. For group TS, this internal teat sealant was administered intramammary as the sole therapy. For sealant administration, the teat was pinched where it joined the base of the udder, and the teat sealant was applied starting on the closest teat to the administrator. All contents of the syringe were deposited into the teat, and careful attention was paid to not massage this into the udder. Heifers in the NT group only had their teats scored and lacteal secretion samples collected. TS and NT procedures were performed 21 days prior to the expected calving date. All heifers were moved to a close-up pen to be watched in the weeks leading up to calving.

Once the heifers calved, milk samples were collected every 12 hours for the first 7 days and then once daily on days 14 and 21 post-treatment. Milk samples were collected for 21 days to measure treatment efficacy when the heifers were most susceptible to acquiring mastitis. Milk sample collection time points were taken based on the established milking frequency at the dairy unit. The heifers were ushered into a side-by-side herringbone parlor where they could be identified while in a milking stall. All teats were dipped in a germicidal solution allowing at least 30 seconds of contact time. Teats were then dried with a clean paper towel starting with teats farthest away from the collector. Each quarter had a foremilk sample stripped before an aliquot of approximately 3 mL was collected from each quarter. Approximately 15 mL was collected as a composite sample into a sterile 15 mL conical tube (Globe Scientific, Mahwah, NJ). The heifers were then milked as they usually would by a milking claw, and a post-dipping germicidal solution was applied before releasing them back to pasture. The antimicrobial-treated heifers (groups 14 and 21) were allowed to move out of the waste milk group and into the milking herd once each heifer received a negative antibiotic screening test (Delvotest SP-NT, dsm-firmenich). Milk samples were stored in cryovials. All samples were placed in a -20°C freezer until the study's completion and moved to a -80°C freezer until they could be analyzed.

3.3.3 | Somatic Cell Count Analysis

In this study, the lacteal secretions, as well as a subset of all composite milk samples, were used for SCC analysis to determine the quantity of leukocytes present per mL of sample. At each collection time point, milk was analyzed for SCC utilizing a DeLaval Cell Counter (**DCC**) (DeLaval International AM, Tumba, Sweden), an electronic quantitative cow-side cell counter. The analysis took place immediately after sample collection, and the measured SCC was recorded.

3.3.4 | Drug Concentration Analysis

Milk samples were analyzed by ultra-performance liquid chromatography with mass spectrometry detection (**UPLC/MS**) to determine the active concentrations of ceftiofur hydrochloride and its metabolites as previously described (Smith et al., 2004). Ceftiofur is rapidly metabolized to the active metabolite, desfuroylceftiofur, in cattle. The assay converts all ceftiofur and desfuroylceftiofur conjugates to a single stable derivative, desfuroylceftiofur acetamide (**DCA**). The analytical method for determining DCA in milk was validated before use in this study.

A 500 µL aliquot of raw milk was added to 5 mL acetonitrile and centrifuged for sample cleanup. The supernatant was evaporated to dryness, reconstituted in 2 mL of ultra-pure water, and 5 mL of 0.4% w/v dithioerythritol in borate buffer (pH, 9.0; prepared by dissolving 19 g of boric acid and 3.7 g of potassium chloride in 1 L of ultra-pure water) was added. The samples were incubated in a 50℃ water bath for 30 minutes and then cooled in room-temperature water. Three milliliters of 14% w/v iodoacetamide in phosphate buffer (pH, 9.0; prepared by dissolving 3.4 g of potassium phosphate in 1 L of ultra-pure water) was added, and the samples were incubated in the dark at room temperature for 30 minutes. The pH was adjusted to between 2.5 and 2.6 with a 5% phosphoric acid solution, placed in the refrigerator for at least one hour, and then loaded onto Oasis PRiME HLB 3 cc (60 mg) Extraction Cartridges (Waters Corporation, Milford, MA) in a positive pressure manifold (Biotage). The samples were washed with 2 mL of 95:5 water:methanol, eluted with 2 mL of 90:10 acetonitrile:methanol, and evaporated to dryness. The sample was then reconstituted in 250 uL 15:85 ACN:H2O, filtered with an EZFlow hydrophilic PVDF 13 mm 0.22 µm syringe filter (Foxx Life Sciences) into a max recovery vial (Waters Corporation, Milford, MA), and 5 uL was injected into the instrument for analysis.

Milk concentration analysis was performed on a Waters Acquity Ultra Performance Liquid Chromatography coupled to a Waters Acquity QDa mass spectrometer detector. The instrument was set to Single Ion Recording (**SIR**) of 487.0528 m/z using electrospray ionization in the positive ion mode (**ESI+**). The cone and capillary voltages were 15 V and 0.8 V, respectively. A Waters Acquity UPLC BEH C18 1.7 µm (2.1 mm x 100 mm) column with a corresponding VanGuard Pre-Column (2.1 mm x 5 mm) (Waters Corporation, Milford, MA) was used for the separations. A gradient was used for the mobile phase. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The flow rate was 0.4 mL/min. The gradient was programmed as follows: from 0 to 1 minute, the mobile phase composition was 90% A and 10% B; from 1 to 2.5 minutes, the composition changed linearly to 5% A and 95% B then held there until 3.5 minutes; finally, back to 90% A and 10% B from 3.51 to 5 minutes.

The chromatograms were integrated with a computer program (MassLynx Software V4.1, Waters Corporation, Milford, MA) which quantified the unknown concentrations in all samples. The DCA concentration range was 2.5 ng/mL to 100 ng/mL with an average correlation coefficient, R^2, of 0.99. The limit of detection (**LOD**) and the limit of quantification (**LOQ**) for DCA in milk was 2.5 ng/mL and 5 ng/mL, respectively. Intraday precision and accuracy were obtained on the same day using three different concentrations (2.5, 5, and 10 ng/mL) repeated four times each. Intraday precision was 1.5 to 15.2%, with an accuracy of 97.4 to 115.2%. Interday precision and accuracy were obtained by measuring six different concentrations (2.5, 5, 10, 25, 50, and 100 ng/mL) across 10 days. Interday precision was 4.4 to 16.3%, with an accuracy of 84.8 to 108.4%.

3.3.5 | Statistical Analysis

Repeated measures ANOVA with the Geisser-Greenhouse non-sphericity assumption followed by Tukey's multiple comparisons test was used to compare the mean somatic cell counts between treatment groups. Fisher's exact test was used to compare the prevalence of SCM over time for all treatment groups. All tests were chosen based on the normality of data distribution. All statistical analyses were performed using GraphPad Prism Software (V10.4.0 for Windows, Boston, MA).

3.4 | Results

No adverse reactions were observed in the heifers after intramammary ceftiofur hydrochloride administration. The median time (and range) from study enrollment to calving for groups TS, NT, 14, and 21 was 20.5 days (14-29), 17 days (12-27), 13 days (9-16), and 19.5 days

(15-22), respectively. Upon calving, four heifers received dexamethasone for the treatment of udder edema. Of the four, three heifers belonged to the non-antimicrobial treatment groups (TS, 1; NT, 2) and one belonged to the antimicrobial treatment group, 21. The heifer belonging to group 21 needed assistance during parturition. Two cases of clinical mastitis were briefly observed from one heifer in the NT group and one in the 14 group. Both heifers had visible changes in milk appearance (clotted and discolored milk). The heifer's milk in the NT group presented this way for only one milking the day after calving while the heifer's milk in the 14 group presented poor milk appearance for three consecutive days before spontaneously recovering. All 24 heifers were used for milk analysis.

3.4.1 | Teat scores

All heifers had four, functional mammary quarters at the time of enrollment. With the exception of one heifer in the NT group, all animals received teat scores of 1 (healthy appearance with no abrasions or scabbing). The heifer in the NT group had one teat that scored a 2 (residual damage from healing scabs). Due to the consistency of teat appearance, teat scores were not evaluated for their effect on the efficacy of treatment or antimicrobial milk residues.

3.4.2 | Somatic Cell Count

Unfortunately, all pre-treatment lacteal secretions and some milk samples collected soon after calving could not be analyzed as they were too thick to flow through the DCC properly. The DCC had a measuring range of 10,000 to 4,000,000 cells/mL. No milk sample exceeded 4,000,000 cells/mL, but samples that measured below the lower limit of quantification were given a fixed SCC value of 10,000 cells/mL. **Figure 3.2** compares mean SCC measurements at 7, 14, and 21 days post-calving. There may be apparent treatment efficacy differences, but the analysis suggests there were no statistically significant differences ($p<0.05$) in SCC measurements among the different treatment groups or over time within the same treatment groups. **Table 3.1** depicts the prevalence of SCM among heifers in all treatment groups out to 21 days post-calving. SCM was confirmed based on SCC measurements exceeding 200,000 cells/mL. There were no statistically significant differences ($p<0.05$) in SCM prevalence among the different treatment groups.

3.4.3 | Milk Residues

Figure 3.3 displays milk concentration-time curves for groups 14 and 21 following antimicrobial treatment with intramammary ceftiofur hydrochloride at one syringe/quarter. Ceftiofur concentrations, measured as DCA, were not present above the FDA milk tolerance for cattle of 100 ng/mL in any milk sample. Drug concentration analysis showed that 87% and 94% of milk sample concentrations fell below our LOQ of 5 ng/mL for groups 14 and 21, respectively. A noncompartmental analysis (**NCA**) of ceftiofur concentration versus time profiles for the antimicrobial-treated heifers performed with analytical software (Phoenix Win-Nonlin 8.0; Certara Inc.) was attempted. However, not enough values were measured above our LOQ to accomplish this. Due to the unreliability of our data, the NCA was not performed, and only approximate concentration values were reported. The mean peak milk concentration (**Cmax**±SD) for groups 14 and 21 in this study reached 8.14±6.24 ng/mL and 4.20±5.07 ng/mL 48 hours into lactation (**Tmax**), respectively.

3.5 | Discussion

This study is the first to report on the efficacy and milk residue profile of 125 mg intramammary ceftiofur hydrochloride lactating cow formulation in prepartum dairy heifers. On average, 87% and 94% of all milk samples fell below our LOQ of 5 ng/mL for groups 14 and 21, respectively, demonstrating that the risk of violative residues from this practice is low (**Figure 3.3**). Milk had a maximum DCA (ceftiofur equivalents) concentration of approximately 8.14±6.24 ng/mL and 4.20±5.07 ng/mL 48 hours into lactation for groups treated 14 or 21 days prior to expected calving, respectively. We did not find significant differences in SCC among the different treatment groups or within the same treatment groups over time. It is important to note that a cow's SCC in milk will typically be higher immediately following calving due to the production of colostrum (Barkema et al., 1999). The mean SCC for groups 14, 21, and NT was below the SCM threshold of 200,000 cells/mL at days 14 and 21 post-calving, but group TS exceeded this.

Prior research has evaluated the efficacy of intramammary lactating cow therapy administered to prepartum heifers. Oliver et al. (1992, 2003) observed significant cure rates in heifers treated with sodium cloxacillin, or cephapirin sodium compared with untreated controls after infusion at 7 to 14 days prior to calving. They also found a significant reduction in SCC and an increase in milk production over the first lactation. Middleton et al. (2005) observed a greater cure rate for heifers treated with pirlimycin 10 to 14 days prior to calving compared with untreated controls, but a reduction in SCC was not consistent and no effect on milk yield was observed. Borm et al. (2006) observed greater cure rates in heifers treated with cephapirin sodium 10 to 21 days prior to calving compared with controls but found no effects on SCC or milk production.

Similarly, others have evaluated the efficacy of intramammary dry cow therapy administered to prepartum heifers. Nickerson et al. (2020) administered the dry cow formulation of ceftiofur hydrochloride (500 mg) two months before the heifers were expected to calve. They concluded that no treatment was just as beneficial as administering dry cow therapy, teat sealant, or a combination of the two if the quarters were uninfected at the time of treatment. However, most heifers had at least one infected quarter at the time of enrollment. For these infected quarters, dry cow therapy was most successful (100%) at curing the infection upon entering early lactation followed by combination therapy (96.1%) and sole teat sealant therapy (85.7%) in comparison to no treatment ($p<0.001$).

There are several limitations to this study. Four individuals administered treatment and scored teats; this introduced potential variability. Heifers were also not randomly allocated to treatment groups, as this would have required a large number of dosing days. Instead, heifers were grouped based on proximity to calving and allocated to treatment groups that aligned best with the availability of farm staff on dosing days. This introduced potential selection bias. A randomized controlled trial is preferred. Because there was substantial variability present in SCC measurements within each treatment group, more animals would have to be enrolled in future studies to detect a significant difference between them, if one exists. The sample size for this study was selected to address the risk of violative residues, which likely led to too small of a sample size to demonstrate differences in SCC. To prevent this moving forward, power analyses should be conducted prior to the start of a study to adequately detect meaningful differences in treatment efficacy and residue concentrations. Additionally, our cell counter could not quantify the pre-treatment lacteal secretions collected from each heifer due to their high viscosity. It would have been beneficial to compare pre-calving and post-calving SCC across the different treatment groups. The lacteal secretion samples should be diluted in future studies with a suitable buffer to accurately measure SCC. This study was conducted at a small-scale, pasture-based dairy, which was beneficial for evaluating the effect of these environmental conditions but may not represent heifer housing at large-scale dairies. It would also be preferable to collect representative samples of the total milking volume at each collection time point, but the dairy did not have a way to do this. Instead, quarter milk samples had to be collected, which may not be the most accurate representation of residues present at that time.

In the future, conducting a bacteriological or metagenomic analysis to detect causative and potentially resistant pathogens would be beneficial. Identifying these pathogens could aid in creating tailored control and treatment strategies for mastitis in prepartum heifers. In 2008, the FDA placed prohibitions on the use of cephalosporins in food animals due to concerns about the movement of foodborne bacteria between livestock and humans and evidence of cross-resistance among drugs in the cephalosporin class (Davis et al., 2009). Assessing AMR after ceftiofur's use in this application would be beneficial. It may also be appropriate to evaluate the efficacy and pharmacokinetics of the dry-cow formulation of ceftiofur hydrochloride, which contains 500 mg per syringe, instead of the 125 mg used in this study at various time points prior to calving. Due to the higher dose in the dry cow formulation, additional residue studies should be performed.

In the U.S., the extralabel use of third-generation cephalosporins is prohibited (Davis et al., 2009) with some exceptions under the Animal Medicinal Drug Use Clarification Act (**AMDUCA**). It is important to note that the use of the lactating ceftiofur hydrochloride formulation evaluated in this study or the dry cow formulation in prepartum heifers would be considered prohibited in the U.S. There are other countries where the use of third-generation cephalosporins in food-producing animals is completely prohibited (i.e. the Netherlands), while in other countries, extralabel use is freely permitted. It is imperative that veterinarians and dairy producers follow the drug use laws of their country to ensure that antimicrobials are being used in a safe and legal manner. FARAD does not encourage prohibited extralabel drug use and conducted this research to explore the potential for violative residues in milk collected from treated heifers. With this pharmacokinetic data, FARAD is now able to improve its guidance for U.S. veterinarians and producers in establishing evidence-based milk withdrawal recommendations, specifically for accidental exposures. Additionally, this research serves to encourage veterinarians on a global scale to make educated and legal treatment decisions for their dairy herds based on their local regulations. For the sake of antimicrobial stewardship, it is encouraged that antimicrobials other than third-generation cephalosporins be considered as primary interventions with supported justification (susceptibility testing, SCC, California Mastitis Test (**CMT**), etc.) wherever possible if prophylactic treatment is desired for use in prepartum heifers.

In conclusion, intramammary ceftiofur hydrochloride administered at least 14 days before the anticipated calving date poses little to no food-safety risks. Rules and regulations concerning legal and prohibited drug use should be followed in each country.

Author Contributions

GS, REB, DMF, JLH, and RAM conceived and designed the study. RAM collected samples and data. RAM developed the analytical method and performed sample analysis. RAM performed pharmacokinetic modeling. All authors were involved in the drafting and revising of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest for the present study.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethics Statement

The study was approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC #21-172).

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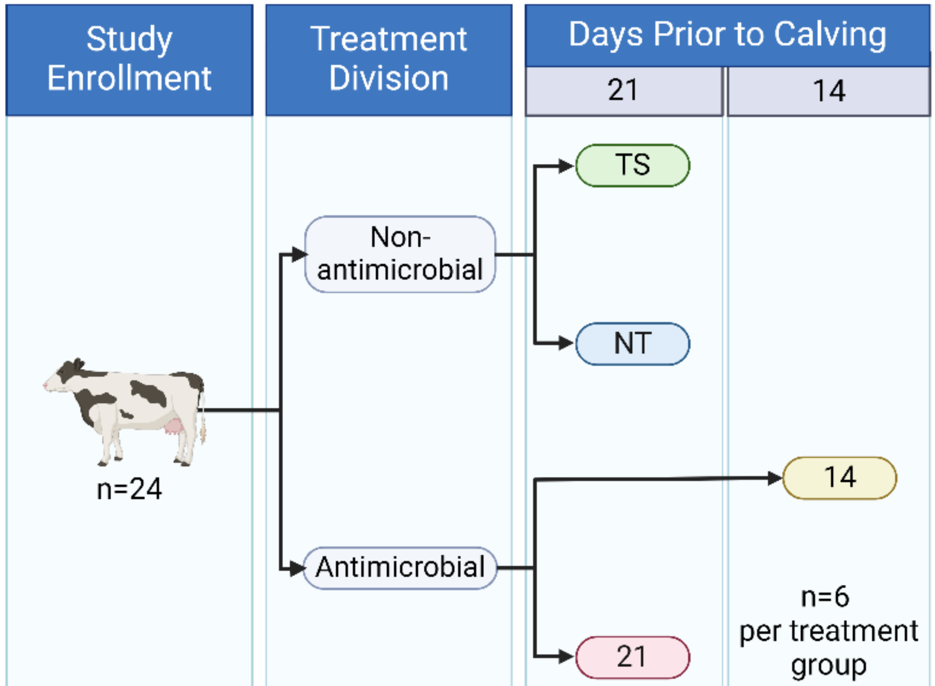

Figure 3.1 | Experimental design schematic for treatment enrollment[1].

Created in https://BioRender.com.

[1]TS, heifers received only internal teat sealant (n= 6); NT, heifers received no treatment (n= 6); 14, heifers received intramammary ceftiofur hydrochloride and internal teat sealant 14 days prior to expected calving (n= 6); 21, heifers received intramammary ceftiofur hydrochloride and internal teat sealant 21 days prior to expected calving (n= 6).

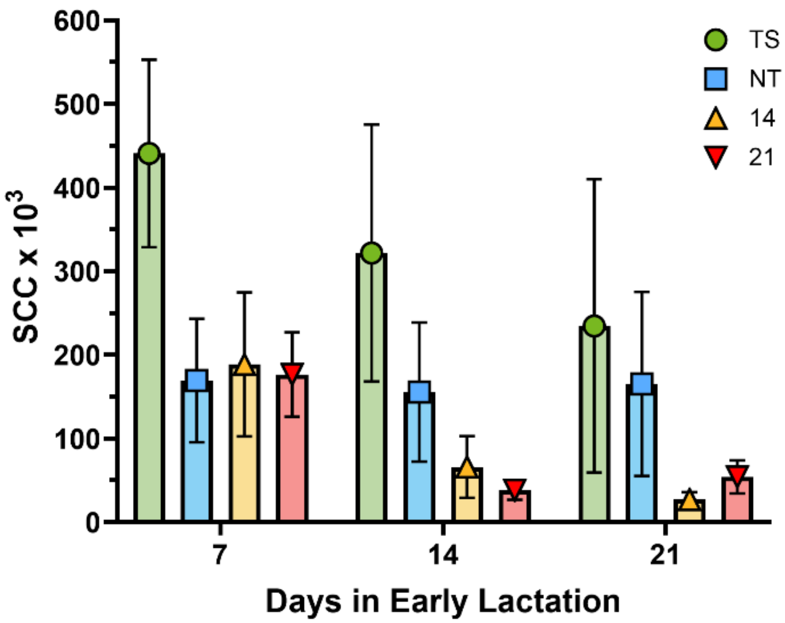

Figure 3.2 | SCC measurements in early lactation from heifers among different treatment groups[1] (mean±SEM).

[1]TS, heifers received only internal teat sealant (n= 6); NT, heifers received no treatment (n= 6); 14, heifers received intramammary ceftiofur hydrochloride and internal teat sealant 14 days prior to expected calving (n= 6); 21, heifers received intramammary ceftiofur hydrochloride and internal teat sealant 21 days prior to expected calving (n= 6).

Table 3.1 | Prevalence of SCM* in early lactation from heifers among different treatment groups[1].

Time (d)	NT % (n)	TS % (n)	14 % (n)	21 % (n)
1	100 (1/1)	100 (1/1)	66 (2/3)	100 (3/3)
2	100 (5/5)	80 (4/5)	60 (3/5)	33 (2/6)
3	50 (3/6)	83 (5/6)	83 (5/6)	50 (3/6)
4	50 (3/6)	83 (5/6)	50 (3/6)	50 (3/6)
5	33 (2/6)	66 (4/6)	33 (2/6)	66 (4/6)
6	33 (2/6)	66 (4/6)	33 (2/6)	33 (2/6)
7	33 (2/6)	66 (4/6)	33 (2/6)	33 (2/6)
14	17 (1/6)	50 (3/6)	17 (1/6)	0 (0/6)
21	17 (1/6)	17 (1/6)	0 (0/6)	0 (0/6)

[1]TS, heifers received only internal teat sealant (n= 6); NT, heifers received no treatment (n= 6); 14, heifers received intramammary ceftiofur hydrochloride and internal teat sealant 14 days prior to expected calving (n= 6); 21, heifers received intramammary ceftiofur hydrochloride and internal teat sealant 21 days prior to expected calving (n= 6).

*Subclinical mastitis (SCM) confirmed based on SCC measurements exceeding 200,000 cells/mL.

n represents milk samples collected from each heifer that could properly flow through the DCC.

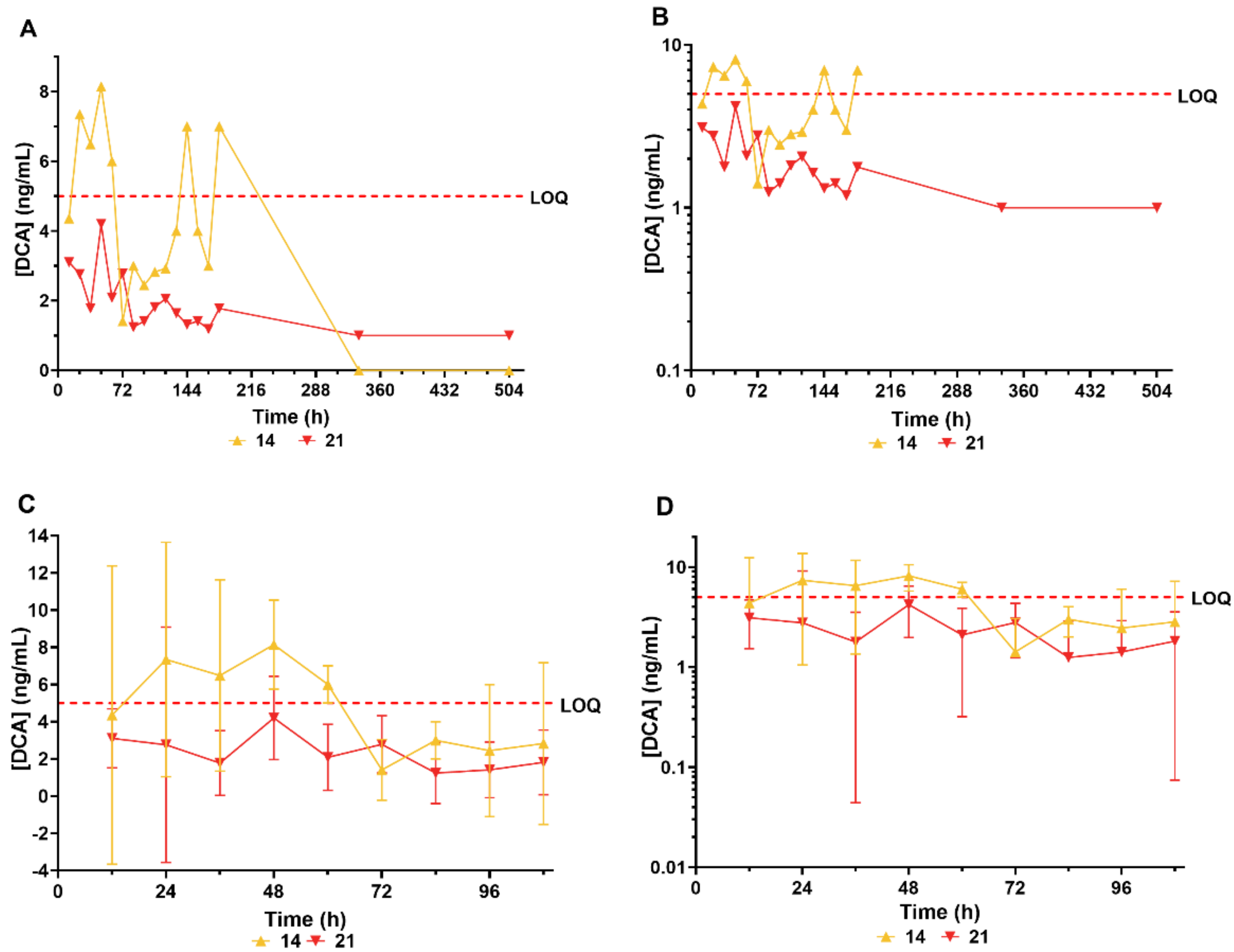

Figure 3.3 | Post-calving milk concentration versus time profile (**A** and **C**; linear scale axis, **B** and **D**; log scale axis) following intramammary ceftiofur hydrochloride to prepartum heifers either 14 (n=6) or 21 (n=6) days from expected calving. Panels **A** and **B** demonstrate DCA concentrations over the extent of the entire study (geometric mean). Panels **C** and **D** demonstrate DCA concentrations 96 hours into lactation when postpartum heifers were most likely to be moved out of the dump group and into the milking group (geometric mean±SD).

LOQ, limit of quantification (5 ng/mL). The US tolerance for DCA in cow milk is 100 ppb.

CHAPTER 4

Conclusions of Current Work and Future Directions

4.1 | Conclusions of Current Work

This dissertation investigated the impact of prophylactic extralabel dosing regimens on the pharmacokinetics of two different drug classes in non-lactating dairy heifers and dry dairy cattle. We specifically chose to study these dairy production classes because their non-lactating status served as an opportunity to advantageously administer therapies that would otherwise require milk withdrawals if given during lactation. However, these therapies have not been studied in non-lactating dairy cows, and therefore, PK data has not been generated until now. This lack of PK data makes establishing appropriate milk withdrawal intervals impossible, violating a significant ELDU stipulation outlined by AMDUCA. Adhering to these regulatory guidelines is imperative to minimize the risk of violative residues in milk that could penalize the veterinarian of record or enter the human food supply.

Our first study (Chapter 2) focused on the elimination kinetics of injectable eprinomectin, a long-acting parasiticide, in dry dairy cattle. When administered at the beginning of the dry period, 60 days prior to the expected calving date, eprinomectin concentrations were not present above the FDA milk tolerance for cattle (12 ng/mL) in any milk sample at the start of lactation or thereafter. Furthermore, 87% of all measured milk samples fell below our quantification limit (**LOQ**) of 2.5 ng/mL.

Our second study (Chapter 3) focused on the elimination kinetics of IMM ceftiofur hydrochloride, an antimicrobial, in prepartum dairy heifers. When administered 14 or 21 days prior to the expected calving date, ceftiofur concentrations (measured as desfuroylceftiofur acetamide (**DCA**)) were not present above the FDA milk tolerance for cattle (100 ng/mL) in any milk sample. Drug concentration analysis showed that 87% and 94% of milk sample concentrations fell below our LOQ of 5 ng/mL for groups 14 and 21, respectively.

Through these studies, we supported our hypotheses that residues from these extra-label practices would measure below the FDA tolerance levels in milk and pose little to no food safety risks for consumers. This research emphasized the importance of evidence-based withdrawal intervals to ensure consumer safety. In addition, both studies advance the ability to support dairy cow health prior to calving when the animal is most susceptible to infection. Supporting dairy cows during this time increases their well-being and potential to be productive in the milking herd.

4.2 | Future Directions

While this dissertation has advanced our understanding of the impact of prophylactic extralabel dosing regimens on PK and resulting drug residues, several important questions remain that warrant further investigation. The following sections will explore some of these questions in further detail.

4.2.1 | Dry and Lactating Cow Therapy During the Dry Period

The most common use of prophylactic drug use in dairy cattle is for blanket dry cow therapy (**BDCT**) to reduce the risk of mastitis during the dry period and subsequent lactation. BDCT is widely accepted as standard practice because mastitis is the most common and costly disease in U.S. dairy cattle, with annual losses exceeding $2 billion (USDA-ARS, 2021). However, international politics are pressuring the animal health industry to restrict the use of antimicrobials to reduce the risk of transmitting antimicrobial resistance (**AMR**) to humans. For example, the EU banned BDCT under Regulation 2019/6 in 2022 to reduce the risk of AMR (European Parliament and the Council of the European Union, 2019). In addition, the World Health Organization (**WHO**) published *guidelines on use of medically important antimicrobials in food-producing animals* (2017), which recommends reducing the overall use of antimicrobials in animals. More specifically, the WHO strongly recommends an overall reduction in the use of all medically important antibiotics in food-producing animals, including complete restriction of antibiotics used for growth promotion and disease prevention without diagnosis. In addition, it is recommended that medically important antimicrobials that are not currently used in food production should not be used in the future, including in food-producing animals. These guidelines note that although this recommendation only pertains to the use of medically important antimicrobials in food-producing animals, the WHO believes it should apply to all antimicrobial uses in food-producing animals. If the FDA adopted the WHO recommendation, newly developed antimicrobials not currently authorized for use in food-producing animals could not be utilized in the future. This discourages drug sponsors from developing new treatments for animal diseases. Therefore, it is essential to further investigate ways to optimize dosing strategies for pharmaceuticals that are currently available to veterinarians.

The consequences of this opposition could result in the development of new therapeutic strategies and new cow-side diagnostic tests to allow for targeted treatment. An example of future development could involve conducting additional dry period PK studies to determine milk residues present at the onset of lactation. These studies could optimize dosing regimens while moving away from BDCT and towards selective dry cow therapy (**SDCT**). With SDCT, dairy cows are only treated with antimicrobials based on their risk of having an intramammary infection (**IMI**) at the time of dry-off. As mentioned in section 1.4.1, 80% of all U.S. dairy herds and 94% of large herds (>500 lactating cows) utilized BDCT with long-acting antimicrobials as standard practice in 2014 (USDA-NAHMS, 2014). Of these dairies, only 9% and 6% based their decision to use BDCT on somatic cell count (**SCC**) or history of clinical mastitis, respectively. There have been promising results from recent studies evaluating the use of SDCT and the incidence of mastitis post-calving (Lipkens et al., 2023; Rowe et al., 2020; Vasquez et al., 2018). However, some dairies are apprehensive about moving towards SDCT driven by culture-based diagnostics due to costs and lack of labor and skill for sampling (Vasquez et al., 2018). Even with producer apprehensiveness, it is reasonable to predict that BDCT use will face massive opposition, and dairies will be pressured to move toward SDCT in the near future to decrease the overall use of antimicrobials in dairy cattle.

Limiting access to new antimicrobials in veterinary medicine could result in the repeated use of existing antimicrobials. This is counterintuitive to the judicious use of antimicrobials, but it may be the only option if new therapies are not allowed for use in food animal medicine. Even if additional PK studies are conducted during the dry period, therapeutic levels of these dry-cow therapies (**DCT**) may only persist for 14-28 days into the dry period and fail to protect the udder during the late dry and early lactation periods (Petzer et al., 2009). To address this gap in coverage, the incorporation of lactating-cow therapy (**LCT**) along with DCT in dry cows has been evaluated for its protection against mastitis during early lactation. For example, Philpot (1984) reported a 68% reduction in clinical mastitis during the first week of lactation when cows received both DCT at the end of lactation and LCT at parturition. However, Pankey and colleagues (1982) reported only a marginal increase in efficacy using DCT-LCT combination therapy in comparison to sole DCT against *Staphylococcus aureus* IMIs. While not statistically significant, both DCT-treated groups outperformed the sole LCT treatment. All treatment groups outperformed the control group that received no treatment. These two studies were the only found that incorporated DCT-LCT combination therapy. Considering their age and the advancements made since then, it may be worthwhile to explore DCT-LCT combination therapy in dry cows diagnosed with mastitis or at higher risk of developing it before calving. DCT-LCT combination therapy could still be considered for SDCT if the producer or herd veterinarian could provide reasonable justification using a targeted approach with SCC, animal history, or culture-based diagnostics. DCT-LCT combination therapy would also have to make economic sense for the producer.

As for prepartum replacement heifers, the use of either DCT or LCT has been evaluated individually as it would not make sense to do both, considering they have never entered lactation and do not have the need for a specialized dry period. DCTs that have been evaluated for use in heifers include, but are not limited to, penicillin-dihydrostreptomycin, cephapirin benzathine, cloxacillin, penicillin-novobiocin, tilmicosin, ceftiofur hydrochloride, and cephalonium (Nickerson et al., 2020; Owens et al., 1991; Owens et al., 1994; Owens et al., 2001; Sampimon & Sol, 2005; Trinidad et al., 1990). Each study resulted in significantly greater cure rates of treated heifers compared to untreated control heifers. Overall, Nickerson (2009) advocated for the treatment of heifers during pregnancy with DCT because the cure rate is higher than during lactation, there is no milk loss during therapy, the risk of antibiotic residues is minimal, SCC at calving is reduced, and milk production is increased by approximately 10% in successfully treated animals in some herds. However, Sampimon and Sol (2005) concluded that nonlactating cow therapy was only beneficial in farms with a high prevalence of heifer mastitis for increasing milk production. The optimal time for administering DCT to heifers was also evaluated by comparing treatment efficacy in each trimester (Owens et al., 2001). The authors concluded that there were no significant differences in treatment efficacy among the three trimesters during gestation. However, after treatment in the group infused during the third trimester, fewer new *S. aureus* infections occurred, indicating that treatment during this time could reduce the incidence of new IMI in early lactation.

LCTs that have been evaluated for use in heifers typically two to three weeks before calving include, but are not limited to, sodium cloxacillin, cephapirin sodium, penicillin-novobiocin, and pirlimycin hydrochloride (Borm et al., 2006; Oliver et al., 1992; Oliver et al., 1997; Oliver et al., 2003; Oliver et al., 2004). While treatment still significantly resulted in greater cure rates, there was conflicting evidence about whether LCT treatment had an effect on reducing SCC or increasing milk production. Additionally, while the use of LCTs has shown treatment effectiveness, critics have argued that waiting until this time to treat chronic *S. aureus* mastitis might be too late, and it may not be warranted as a universal strategy for mastitis management (Nickerson, 2009).

Although the use of DCT or LCT therapy has proven effective in preventing or treating IMI in dry cows and prepartum heifers, reducing antimicrobial use in dairy cows remains a key focus for regulatory agencies despite the low prevalence of AMR among udder pathogens (Bengtsson et al., 2009; Call et al., 2008; Cameron et al., 2016). As discussed previously, DCT has been shown to be more effective in preventing mastitis while decreasing the risk of violative residues in comparison to prepartum LCT. Additionally, the European Food Safety Agency Panel on Biological Hazards has concluded that the risk of shedding AMR bacteria in newborn calves fed colostrum does not increase when dams receive antimicrobial DCT, provided that the interval between drying off and calving exceeds the antimicrobial withdrawal period (EFSA Panel on Biological Hazards et al., 2017). This risk has not been evaluated for prepartum LCT use. Furthermore, the impacts of SDCT on AMR development and spread are not completely understood, as studies investigating direct relationships between antimicrobial DCT and AMR are scarce. Although correlations between DCT and AMR on dairy farms have been noted, more research is needed to comprehend the potential emergence, persistence, and transfer of AMR genes to humans as a consequence of DCT. Antibiotics continue to be a vital tool for ensuring the health and welfare of food-producing animals. Ultimately, policy decisions regarding the restriction of antibiotics in dairy cattle should be based on a careful evaluation of possible unintended consequences compared to the benefits observed from DCT usage in production environments. Further studies should be conducted to assess an SDCT approach alongside DCT, LCT, or combination therapy and their influence on treatment outcomes and AMR development to support policy development and decision-making.

4.2.2 | New Therapy Formulations

Considering the world's perception of antibiotic use in food-producing animals, it is in the best interest of pharmaceutical companies, producers, and veterinarians to explore novel formulations of currently available DCTs to preserve their effectiveness for as long as possible. New intramammary formulations incorporating nanotechnology appear to be very promising in veterinary medicine. Nanoparticles may serve as drug-delivery systems that allow for the delivery of drug molecules directly into target cells (Neculai-Valeanu et al., 2021). This increases treatment efficacy by increasing intracellular uptake which is particularly important in combating *S. aureus* mastitis as it resides within udder phagocytes (Craven & Anderson, 1984). Nanoparticles such as silver, copper, zinc oxide, and chitosan have demonstrated in vitro antibacterial activity against *S. aureus*, *Streptococcus agalactiae*, *Escherichia coli*, and *Klebsiella pneumoniae* (Abd El-Aziz et al., 2021; Hozyen et al., 2019; Orellano et al., 2021; Rivera Aguayo et al., 2020). More so, research has shown synergistic effects against bacteria when antimicrobials are used in combination with nanoparticles (Aabed & Mohammed, 2021; Adeniji et al., 2022). An additional benefit of nanoparticles is that their mechanism of action enables the use of low doses of antimicrobials which could potentially reduce side effects, withdrawal times, and residue risks in food-producing animals (El-Sayed & Kamel, 2020; Osama et al., 2020). It is also extremely difficult for bacteria to develop resistance to nanoparticles since they can act on multiple targets or by multiple mechanisms (Zhao & Jiang, 2013). This is still an emerging area of study in veterinary medicine, but its application in DCT has the potential to be an effective strategy for both prophylactic and therapeutic use.

While the SDCT approaches mentioned in section 4.2.1 decrease the amount of antimicrobials used at dry-off, producers are still almost solely relying on antimicrobials to prevent new IMI during freshening. It is essential to explore other non-antimicrobial solutions to reduce the incidence of mastitis in both dry and lactating cows. Along with examining alternative formulations of currently available DCTs, there is also the potential to use non-antimicrobial alternatives for promoting growth and preventing disease in dairy cattle. One alternative to antimicrobials is bacteriophage therapy, which utilizes bacteriophages (viruses) to infect bacteria. Bacteriophages have a high specificity for pathogens of interest which is advantageous for not disrupting the host's microbiota but disadvantageous as a single bacteriophage is only effective against a limited number of bacterial strains (Nilsson, 2014). This means that different bacteriophages would be necessary to treat all bacteria associated with infection (Baker et al., 2018). Several bacteriophages have been identified for therapy against mastitis-associated pathogens, including *S. aureus*, *S. agalactiae*, *E. coli*, *Streptococcus uberis*, and *K. pneumoniae* (Guo et al., 2021; Shan et al., 2020; Titze et al., 2020; Vander Elst et al., 2020; Zhao et al., 2021). However, resistance has been documented in a high percentage of bacteriophage therapy studies, and methods to monitor or prevent this are still in development (Oechslin, 2018). Additionally, their use is limited in livestock due to their instability and need for special storage conditions (Sharun et al., 2021).

Another alternative to antimicrobials is the use of phytotherapy which utilizes plants and their extracts for medicinal treatment. Plant extracts have been incorporated into various pharmaceutical formulations, but their reported ability to cure or prevent mastitis is very limited and variable (Mullen et al., 2014; Pinedo et al., 2013). Additionally, phytochemicals face instability and degradation and are insoluble in certain solutions (Kamiloglu et al., 2021). Similarly, nutraceutical supplementation is another option to decrease antimicrobial usage while still providing adequate care. A previous study utilized IMM treatment with 25-hydroxyvitamin D3 (**25(OH)D3**) against induced mastitis in cows and showed significantly lowered bacterial counts in milk, reduced symptoms of mastitis, and decreased loss in milk production in comparison to the control group (Lippolis et al., 2011). Interestingly, serum levels of 25(OH)D3 did not differ between treatment groups, which highlights that it may be vital for nutraceutical supplements to be administered in anatomical locations that are naturally low in concentrations, such as the mammary gland, to best influence the early course of mastitis.

New therapy formulations utilizing nanotechnology or bacteriophage therapy are not currently available for labeled use in dairy cattle. As a result, their extralabel use would be difficult to justify under AMDUCA, and establishing a scientifically sound withdrawal interval would be challenging due to limited or no residue data in meat or milk from treated animals. While phytotherapy and nutraceutical supplementation have less of an impact on residues, their effectiveness against mastitis still requires further analysis.

In addition to advancing therapies for mastitis, parasiticide formulations are being refined to address resistance in livestock. Most commonly used anthelmintics fall into one of four chemical classes: 1) Benzimidazoles (e.g., fenbendazole), 2) imidazothiazoles (e.g., levamisole), 3) tetrahydropyrimidines (e.g., pyrantel), and 4) macrocyclic lactones (e.g., ivermectin), which include avermectins and milbemycins (Selzer & Epe, 2021). These compounds operate similarly, indicating that resistance to one compound may correlate with resistance to other members of its class, known as class effect. (Prichard & Geary, 2019; Vercruysse et al., 2018). One method to combat this is the use of parasiticides that control both endo- and ectoparasites, known as endectocides, offering advantages due to their broad-spectrum efficacy against these parasites. Recent advancements have been made in developing products that combine active ingredients to achieve this effect, including registered drugs like Valcor® (from Zoetis). Valcor® is the first prescription cattle dewormer with two active ingredients (doramectin and levamisole) in one product. Valcor® is an injectable approved for use in beef cattle aged two months or older and in replacement dairy heifers under 20 months of age, with a 15 day meat withdrawal period. Its effectiveness and the potential for meat or milk residues in lactating or dry dairy cows have not been assessed, but it would be beneficial to evaluate this in future studies.

An additional advancement in parasiticide formulations comes from the latest class of ectoparasiticides, isoxazolines. Isoxazolines address the resistance encountered by other parasiticides through their novel mechanism of action (Long, 2018; Weber & Selzer, 2016). As of now, the four U.S.-marketed isoxazoline products for dogs and cats are Nexgard® (afoxolaner, from Boehringer Ingelheim AH), Bravecto® (fluralaner, from MSD AH), Simparica® (sarolaner, from Zoetis), and Credelio® (lotilaner, from Elanco). Although combination products are available for dogs and cats, there are currently no isoxazoline products approved for use in livestock in the U.S. Exzolt® (from MSD AH), a fluralaner pour-on formulation available in Australia, is labeled in beef cattle and in replacement dairy heifers prior to first mating, with a 38 day meat withdrawal period. Exzolt® is also labeled in poultry in Europe as a medicated water treatment, with a zero day egg discard and a 14 day meat withdrawal period. FARAD has received numerous inquiries about the extralabel use of fluralaner in swine and poultry. Unfortunately, there is a lack of PK data regarding isoxazoline products to determine withdrawal intervals for all consumable products in food-producing species. As a result, their use is discouraged in the U.S. as it would necessitate significantly extended withdrawal intervals to account for the lack of tolerance values in food products. However, it can be expected that isoxazoline products will be available for livestock in the near future. Meanwhile, conducting PK residue studies for isoxazoline products in U.S. cattle is worth exploring, as producers and veterinarians will focus on novel products to combat parasitic resistance in their herds.

4.2.3 | PK Modeling

Data collected in drug studies in livestock can be used to generate PK models. PK models describe the relationship between the dose administered to an individual and the resulting concentrations inside various body compartments (Pesenti et al., 2022). Several types of PK models have been detailed, showcasing significant differences in structure, complexity, and effort needed for model development and application. Each model serves a different purpose in advancing our understanding of drug behavior within the target species. Physiologically based pharmacokinetic (**PBPK**) models are a type of PK model in which the compartments reflect the anatomical structure of the organism (Nestorov, 2003). By incorporating anatomical and physiological data, PBPK models provide detailed insights into how drugs are absorbed, distributed, metabolized, and excreted among various tissues, including milk. These models account for species-specific physiological parameters and chemical (drug)-specific parameters to describe these processes. PBPK models offer great extrapolation capabilities across species, exposure routes, and doses, but they require large amounts of data to perform well (Zhuang & Lu, 2016). Ideally, PK models in food-producing animals, like dairy cattle, are generic enough to be able to predict the behavior and withdrawal intervals for several compounds (Dubbelboer et al., 2023). Previous PBPK models have described the passage of oxytetracycline, penicillin, cefuroxime, cephapirin, pirlimycin, and flunixin into the mammary gland and milk of dairy cows (Li et al., 2018; Tardiveau et al., 2022; Woodward & Whittem., 2019; Woodward et al., 2020). The descriptions of drug diffusion to and from milk varied greatly between these models as this was dependent on the model construction. Unfortunately, few sources compile physiological parameters that are useful for PBPK models, and many fluctuations in parameters remain unknown throughout the lactation cycle or during pregnancy. FARAD offers PBPK modeling parameters in beef and dairy cattle, swine, chickens, turkeys, sheep, and goats at https://cafarad.ucdavis.edu/pbpk/ (Lin et al., 2020; Li et al., 2021; Wang et al., 2021).

In addition to PBPK models, population pharmacokinetic (**PopPK**) models quantify inter-animal variability related to the time course of drug concentrations to optimize dosing regimens and predict withdrawal times (Serrano-Rodríguez et al., 2023). Currently, PBPK models are used more frequently in veterinary medicine than PopPK models for research publications, but they are not approved for use by FDA CVM. In the future, refining these models by incorporating the multitude of changes associated with different physiological states (i.e., lactation stages, pregnancy, etc.) would greatly benefit the ability to describe or predict drug activity within dairy cows. Making these improvements can be expected to enhance the precision of dosing recommendations and risk assessments and reduce drug residues in dairy products.

4.3 | Final Remarks

Prophylaxis extends beyond drug use and includes farm management strategies to keep dairy cows and replacement heifers healthy. Prophylactic ELDU is a vital tool to accomplish this, but no amount of drug use will compensate for poor farm management. Generating extralabel PK data is necessary for producers and veterinarians to make informed decisions for their herds. Further exploration of current dairy cow and replacement heifer drug regimens, novel formulations, and the use of PK modeling will advance our knowledge of drug behavior and residue chemistry. All of which positively affect judicious antimicrobial use, animal welfare, treatment success, and heightened food safety.

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APPENDICES

Appendix A: Chapter 2 Supplemental Materials

NC STATE UNIVERSITY College of Veterinary Medicine

Eprinomectin (EPR) in Plasma on HPLC- FL

Updated January 17, 2023

Dr. Ronald Baynes Laboratory
Center for Chemical Toxicology and Pharmacokinetics
Department of Population Health and Pathobiology

Determination of EPRINOMECTIN in Plasma by HPLC

A: 0.1% TFA in H_2O
B: 90:10 H_2O:MeOH
C: MeOH
D: ACN

HPLC SYSTEM
Detection: FLUO; Ex = 355, Em = 465
Column: SB-C18 3.5 µm (4.6 x 100) PN: 861953-902

Mobile Phase:

	130 mL DDH20 =	13%
	870 mL Acetonitrile=	87%
	1 mL Trifluoroacetic acid (TFA) =	0.1%

Have a bottle of ACN and a bottle of DDH2O/TFA and use program to inject isocratic mixture.
Run mobile phase through HPLC system for at least 35 minutes to allow for system equilibration.

Preparation of Stock Solution:
Make up a stock solution of 1 mg/mL (1,000 µg/mL) (0.001 g) of eprinomectin by weighing out 2 to 3mg and dissolving in an equal volume of 100% Acetonitrile. Sonicate if necessary to dissolve. Label and store in a tightly sealed vial and keep in refrigerator. Light Sensitive.

Preparation of Acetonitrile (ACN): 1-Methylimidazole solution: (Make up fresh daily under hood)
1:1 ACN to 1-Methyl. Use filter tips. Mix well. Cover. Need approx. 5mL for SC + two cows + QCs (need 6mL if using a repeater pipette)

Preparation of Acetonitrile (ACN): Trifluroacetic Anhydride Soln: (Make up fresh daily under hood)
7:3 ACN to TFAA. Use filter tips. Mix well. Cover. Need approx. 5mL for SC + two cows + QCs (need 6mL if using a repeater pipette)

Preparation of Spiked Standards for Calibration:
Make up solutions for spiking plasma for calibration curve according to the following table.
Use disposable screw top tubes. Label appropriately.

Label for spiking solution	Amount of 1 mg/ml (1,000 µg/ml) stock solution for Eprinomectin	Amount of 100% ACN	Final Concentration of spiking solution Eprinomectin	
10.0	10.0 µL	990 µL	10.0 µg/mL	(10,000 ppb)
5.0	500 µL of the 10.0 µg/mL	500 µL	5.0 µg/mL	(5,000 ppb)
2.5	500 µL of the 5 µg/mL	500 µL	2.5 µg/mL	(2,500 ppb)
1.0	400 µL of the 2.5 µg/mL	600 µL	1.0 µg/mL	(1,000 ppb)
0.50	500 µL of the 1.0 µg/mL	500 µL	0.50 µg/mL	(500 ppb)
0.25	500 µL of the 0.50 µg/mL	500 µL	0.25 µg/mL	(250 ppb)
0.125	500 µL of the 0.25 µg/mL	500 µL	0.125 µg/mL	(125 ppb)
0.075	30 µL of the 2.5 µg/mL	970 µL	0.075 µg/mL	(75 ppb)
0.0625	500 µL of the 0.125 µg/mL	500 µL	0.0625 µg/mL	(62.5 ppb)

Vortex all stocks as you make them

Preparation of Spiked Mobile Phase or Plasma for Calibration

Using clean 16 x 100 borosilicate amber glass, spike plasma as follows. Vortex briefly after adding spiking solutions.

Volume of Plasma or MP 990 µL	Amount of Spiking Soln. 10 µL	Final Concentration of Eprinomectin (vf= 1.0 mL)	
990 µL	10.0 µg/mL	0.1 µg/mL	(100 ppb)
990 µL	5.0 µg/mL	0.05 µg/mL	(50 ppb)
990 µL	2.5 µg/mL	0.025 µg/mL	(25 ppb)
990 µL	1.0 µg/mL	0.01 µg/mL	(10 ppb)
990 µL	0.25 µg/mL	0.0025 µg/mL	(2.5 ppb)
990 µL	0.125 µg/mL	0.00125 µg/mL	(1.25 ppb)
990 µL	0.075 µg/mL	0.00075 µg/mL	(0.75 ppb)
990 µL	0.0625 µg/mL	0.000625 µg/mL	(0.625 ppb)
1000 µL	Blank	Blank	

Blank= 1 mL plasma or mobile phase

Preparation of Plasma Samples/Controls using Liquid Phase Extraction (LPE):

1. Add 1000 uL 100% Acetonitrile to each tube of spiked plasma and/or plasma sample
2. Vortex each tube
3. Centrifuge tubes at 3000 rpm for 10 minutes
4. Preheat pressure manifold to 55º while tubes spin down (press preheat)
5. Take 1 mL of supernatant and transfer to new glass tubes
6. Dry down supernatant under 100% Air for 30 minutes (stop preheat, turn air on, choose program, press run)
    a. Only have necessary rows on
7. Make derivatizing reagents under hood while samples dry down
8. Set up HPLC

Derivatize dried samples: Add reagents to each tube under hood:

1. Add 100 µL of the ACN:1-Methyl. Soln.
2. Vortex 30 seconds
3. Add 100 µL of the ACN:TFAA Soln. (sample will turn dark yellow once this is added)
4. Vortex 30 seconds
5. Set pipette to 250 µL to draw up entire sample. Filter derivatized sample through 0.22 um Foxx Hydrophilic PVDF membrane EZFlow syringe filter into HPLC total recovery vials
    a. Dispose of glass tubes in glass waste and put syringes/tips in labeled waste bag
6. Load vials onto tray to inject
7. Once the tray is loaded, the settings are as follows:
    a. Lamp setting = Ex. 355, Em. 465
    b. Pump setting = 1.0 mL/min
    c. Injection setting = 50 µL
    d. Temp = 30º
    e. Retention Time = 5.5 – 6.0 min

Check waste container often

NC STATE UNIVERSITY College of Veterinary Medicine

Eprinomectin (EPR) in Milk on HPLC- FL

Updated January 20, 2023

Dr. Ronald Baynes Laboratory
Center for Chemical Toxicology and Pharmacokinetics
Department of Population Health and Pathobiology

Determination of EPRINOMECTIN in Milk by HPLC

A: 0.1% TFA in H_2O
B: 90:10 H_2O:MeOH
C: MeOH
D: ACN

HPLC SYSTEM

Detection: FLUO; Ex = 355, Em = 465
Column: SB-C18 3.5 µm (4.6 x 100) PN: 861953-902

Mobile Phase: 130 mL DDH_20 = 13%
870 mL Acetonitrile= 87%
1 mL Trifluoroacetic acid (TFA) = 0.1%

Have a bottle of ACN and a bottle of DDH_2O/TFA and use program to inject isocratic mixture.
Run mobile phase through HPLC system for at least 35 minutes to allow for system equilibration.

Preparation of Stock Solution:

Make up a stock solution of 1 mg/mL (1,000 µg/mL) (0.001 g) of eprinomectin by weighing out 2 to 3mg and dissolving in an equal volume of 100% Acetonitrile. Sonicate if necessary to dissolve. Label and store in a tightly sealed vial and keep in refrigerator. Light Sensitive.

Preparation of Acetonitrile (ACN): 1-Methylimidazole solution: (Make up fresh daily under hood)

1:1 ACN to 1-Methyl. Use filter tips. Mix well. Cover.

Preparation of Acetonitrile (ACN): Trifluroacetic Anhydride Soln: (Make up fresh daily under hood)

7:3 ACN to TFAA. Use filter tips. Mix well. Cover.

Preparation of Spiked STANDARDS for Calibration:

Make up solutions for spiking milk for calibration curve according to the following table.
Use disposable screw top tubes. Label appropriately.

Label for spiking solution	Amount of 1 mg/ml (1,000 µg/ml) stock solution for Eprinomectin	Amount of 100% ACN	Final Concentration of Spiking Solution Eprinomectin
10.0	10.0 µL	990 µL	10.0 µg/mL (10,000 ppb)
5.0	500 µL of the 10.0 µg/mL	500 µL	5.0 µg/mL (5,000 ppb)
2.5	500 µL of the 5 µg/mL	500 µL	2.5 µg/mL (2,500 ppb)
1.0	400 µL of the 2.5 µg/mL	600 µL	1.0 µg/mL (1,000 ppb)
0.50	500 µL of the 1.0 µg/mL	500 µL	0.50 µg/mL (500 ppb)
0.25	500 µL of the 0.50 µg/mL	500 µL	0.25 µg/mL (250 ppb)
0.125	500 µL of the 0.25 µg/mL	500 µL	0.125 µg/mL (125 ppb)

Vortex all stocks as you make them

Preparation of Spiked Mobile Phase or Milk for Calibration
Using clean 16 x 100 borosilicate amber glass, spike as follows. Vortex briefly after adding spiking solutions.

Volume of Milk or MP 990 µL	Amount of Spiking Soln. 10 µL	Final Concentration of Eprinomectin (vf= 1.0 mL)
990 µL	5.0 µg/mL	0.05 µg/mL (50 ppb)
990 µL	2.5 µg/mL	0.025 µg/mL (25 ppb)
990 µL	1.0 µg/mL	0.01 µg/mL (10 ppb)
990 µL	0.50 µg/mL	0.0050 µg/mL (5 ppb)
990 µL	0.25 µg/mL	0.0025 µg/mL (2.5 ppb)
990 µL	0.125 µg/mL	0.00125 µg/mL (1.25 ppb)
1000 µL	Blank	Blank

Blank= 1 mL milk or mobile phase

Preparation of Milk Samples/Controls using Liquid Phase and Solid Phase Extraction (LPE & SPE):

1. Add 1000 uL 100% Acetonitrile to each tube of spiked milk and/or milk sample
2. Vortex each tube
3. Centrifuge tubes at 3000 rpm for 10 minutes
4. Preheat pressure manifold to 55^{o} while tubes spin down (press preheat)
5. Take 1 mL of supernatant and transfer to new glass tubes
6. Dilute supernatant with 1mL of H_2O
7. Set up Positive Pressure Manifold. Turn on nitrogen gas (make sure psi is ~80)
8. Pre-condition Oasis PRiME HLB 3cc extraction cartridges with 1 mL of MeOH, then with 1 mL of H_2O
    a. Waste reservoir sits in metal stand and is then placed beneath metal cartridge holder
    b. Turn on maximum flow to dry the bedding material
9. Load 2mL of diluted samples (aim for 1 drip/3 seconds)
10. Wash with 2 mL of 95:5 H_2O:MeOH
11. Remove waste reservoir and place the collection stand in the platform with new glass tubes
12. Elute with 2 mL of 90:10 ACN:MeOH
13. Dry down samples under 100% Air for 15 minutes (stop preheat, turn air on, choose program, press run)
    a. Only have necessary rows on
14. Make derivatizing reagents while samples dry down
15. Set up HPLC

Derivatize dried samples: Add reagents to each tube:

1. Add 100 µL of the ACN:1-Methyl solution
2. Vortex
3. Add 100 µL of the ACN:TFAA solution (sample will turn dark yellow once this is added)
4. Vortex
5. Set pipette to 250 µL to draw up entire sample. Filter derivatized sample through 0.22 um Foxx Hydrophilic PVDF membrane EZFlow syringe filter into HPLC total recovery vials (not max recovery!)
    a. Dispose of glass tubes in glass waste and put syringes/pipette tips in labeled waste bag
6. Load vials onto tray to inject.
7. Once the tray is loaded, the settings are as follows:
    a. Lamp setting = Ex. 355, Em. 465
    b. Pump setting = 1.0 mL/min
    c. Injection setting = 50 µL
    d. Temp = 30^{o}
    e. Retention Time = 5.5 – 6.0 min

Check waste container often

Thaw milk night before in fridge

Appendix B: Chapter 3 Supplemental Materials

Desfuroylceftiofur (DCA) - Ceftiofur Metabolite in Milk on UPLC/MS

Updated June 21, 2024

Dr. Ronald Baynes Laboratory
Center for Chemical Toxicology and Pharmacokinetics
Department of Population Health and Pathobiology

Adapted from Delta Dise's protocol for DCA in Plasma/Jim Yeatt's protocol for DCA in milk

Solutions:

Extraction Solution

Chemical	Weight/Volume	Notes
Dithioerythritol (DTE)	0.4 g	
Borate Buffer	100 mL	***Only make up the day it will be used**

Borate Buffer

Chemical	Weight/Volume	Notes
Boric Acid	19 g	
Potassium Chloride	3.7 g	
ddH_2O	1 L	pH to 9.0 using Sodium Hydroxide

IOD Solution

Chemical	Weight/Volume	Notes
Iodoacetamide (IOD)	14 g	
Phosphate Buffer	100 mL	***Only make up the day it will be used**

Phosphate Buffer

Chemical	Weight/Volume	Notes
Potassium Phosphate	3.4 g	
ddH_2O	1 L	pH to 9.0 using Sodium Hydroxide

SPE Washing Solution

Chemical	Volume	Notes
ddH_2O	95 mL	
Methanol	5 mL	

SPE Eluting Solution

Chemical	Volume	Notes
Acetonitrile	90 mL	
Methanol	10 mL	

Phosphoric Acid Solution

Chemical	Volume	Notes
Phosphoric Acid	5 mL	
ddH_2O	95 mL	

Reconstitution Solution

Chemical	Volume	Notes
Acetonitrile	15 mL	
ddH_2O	85 mL	

Stock Solution - Makes 1mg/mL (1,000 ug/mL)
Weigh out 1-2 mg of Ceftiofur Hydrochloride (Millipore Sigma 32422-100 mg). Add an equal volume of methanol and vortex to dissolve the powder. Keep refrigerated and away from light (use an amber vial or wrap in aluminum foil).

Spiking Solutions

Label	Volume of 1 mg/mL stock solution	Volume of ddH_2O	Final Concentration
E-10	250 uL	750 uL	250 ug/ml
E-9	100 uL	900 uL	100 ug/ml
E-8	50 uL	950 uL	50 ug/ml
E-7	25 uL	975 uL	25 ug/ml
E-6	10 uL	990 uL	10 ug/ml
E-5	5 uL	995 uL	5 ug/ml
E-4	10 uL of E-10	990 uL	2.5 ug/ml
E-3	10 uL of E-9	990 uL	1 ug/ml
E-2	10 uL of E-8	990 uL	0.5 ug/ml
E-1	10 uL of E-7	990 uL	0.25 ug/ml

Standard Solutions:

Volume of Whole Milk	Add 5 uL of each of the following spiking solution	Final concentration (final volume = 1.0 mL)
495 uL	E-6	0.10 ug/mL (100ppb)
495 uL	E-5	0.050 ug/mL (50ppb)
495 uL	E-4	0.025 ug/mL (25ppb)
495 uL	E-3	0.010 ug/mL (10ppb)
495 uL	E-2	0.0050 ug/mL (5.0ppb)
495 uL	E-1	0.0025 ug/mL (2.5ppb)
500 uL	--------	0 ug/mL

Milk Processing

1. Thaw milk samples/blank completely
2. Pre-heat evaporator to 50℃
3. Add 500 uL milk samples/blank to 15 mL conical tubes. Spike standard curve
4. Add 5 mL acetonitrile
5. Centrifuge milk at 3500 RPM for 15 minutes, at a temperature of 4℃
6. Transfer supernatant to a clean glass tube and dry down completely at 50℃ (~30min)
7. Reconstitute samples in 2 mL ddH_2O and transfer samples into 15 mL conical tubes

Derivatization Procedure

1. Preheat a water bath to 50℃
2. Prepare just enough Extracting Solution and IOD Solution for the number of samples to be run
3. Add 5 mL of Extraction Solution to the sample. Vortex
4. Incubate samples for 30 minutes at 50℃ in water bath
5. Cool samples to room temperature in room temperature water (should take a few minutes)
6. Add 3 mL of IOD Solution to each tube. Vortex
7. Incubate at room temperature in the dark for 30 minutes (place under a box)
8. Adjust the pH of each sample to 2.5-2.6 using 5% phosphoric acid solution
9. Place samples in the refrigerator for at least 1 hour

Sample Extraction

1. Turn on the Nitrogen tank and begin preheating the evaporator to 50℃
2. Prepare a clean 16 x 100 borosilicate glass tube for each sample
3. Load manifold with Waters Oasis Extraction cartridges (3cc/HLB Prime) for each sample
4. Load samples into cartridges (will take 3 rounds)
5. Turn on the positive pressure slowly until sample drips about 1 drip/3 seconds
6. Once all samples have drained completely, add 2 mL of SPE Washing Solution to each cartridge and repeat step 5
7. Replace the collection tray with glass tubes. Add 2 mL of SPE Eluting Solution to each cartridge. Turn on the positive pressure manifold slowly. You will need much less pressure to elute the sample
8. Transfer glass tubes to the evaporator. Turn on the air supply to the evaporator. Dry samples completely (should take ~20 minutes at 50℃)
9. Reconstitute samples in 250 uL 15:85 ACN:ddH_2O
10. Filter each sample into a max recovery vial using a 0.22 um syringe filter
11. Samples can be stored in the refrigerator for a day or two before running

Instrument Conditions

Use the following setting for Waters Acquity UPLC/MS Single Quad:

- Project file: RAM_DCA.PRO
- MS file: DCA
- Inlet file: DCA
    a. Be sure to set the appropriate plate format in the inlet method (bed layout: OA-ANSI-48Vial2mLHolderQDA)
- Mobile phase solvents:
    a. A1: 0.1% formic acid in water
    b. B1: 0.1% formic acid in acetonitrile
    c. Gradient:

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	initial	0.400	90	10	initial
2	1.00	0.400	90	10	6
3	2.50	0.400	5	95	6
4	3.50	0.400	5	95	6
5	3.51	0.400	90	10	6
6	5.00	0.400	90	10	6

- Run time: 5.00 min
- Retention time: 1.97 min
- Wash solvent: Magic Mix (1:1:1:1 water:acetonitrile:methanol:isopropyl alcohol)
- Purge solvent: 90:10 water:acetonitrile
- Injection volume = 5 uL
- Column temperature: 35 ℃
- Sample temperature: 22 ℃
- Guard column: ACQUITY UPLC BEH C18 VanGuard Pre-column, 130Å, 1.7 µm, 2.1 mm X 5 mm
- Column: ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm
- Single Quad settings:
    a. ESI positive mode
    b. Capillary voltage (kV):
    c. Cone (V): 15
    d. Formula: C16H18N6O6S3
    e. Mass (m/z): 487.0528 (1+)
    f. Mass type: monoiso