ABSTRACT

KISSELL, LINDSEY, WALTMAN. Factors Influencing the Pharmacokinetics and Milk Elimination of Flunixin in Dairy Cattle. (Under the direction of Dr. Geof Smith).

Drug residues in the food supply are both an economical and human health safety concern. Flunixin (FLU) is a non-steroidal anti-inflammatory drug labeled for use in beef and dairy cattle for the modulation of inflammation in endotoxemia and for the control of pyrexia associated with bovine respiratory disease, endotoxemia and acute bovine mastitis. The United States Department of Agriculture-Food Safety Inspection Service has reported an increasing number of residue violations in meat from dairy cattle resulting in FLU becoming the second most common residue violation behind penicillin in cull dairy cattle (USDA-FSIS, 2005-2010). Currently, milk is not routinely tested for FLU residues; however, because a significant number of FLU tissue residue violations are found in cull dairy cows, a concern exists that the same practices which have lead to tissue residue violations may also be leading to FLU milk residue violations. Therefore, a surveillance study of saleable milk collected from processing plants in the United States was conducted to determine whether violative FLU residues could be found in milk. Five hundred milk samples were analyzed for 5-hydroxy flunixin (5OH), the milk marker residue. Of the five hundred milk samples, 1 sample was positive for 5OH above the tolerance limit. The results of this study indicate that violative FLU residues in milk are possible; however, what may cause a violative milk residue is unknown. Therefore two experiments were conducted to investigate potential situations that could result in violative FLU milk residues.

The first experiment used a crossover design to determine the pharmacokinetics and milk elimination of FLU and 5OH in lactating dairy cows following intravenous,
intramuscular and subcutaneous administration. The results showed differences in several plasma pharmacokinetic parameters following extravascular administration compared to intravenous administration. Using non linear mixed effects modeling, the decrease in 5OH milk concentration versus time was analyzed. The model indicated that both route of administration and rate of milk production were significant covariates. When FLU was administered either intramuscularly or subcutaneously, some cattle had 5OH residues greater than the tolerance limit in the milk at the labeled withdrawal time. Cattle that received FLU by the approved route (intravenous) eliminated the drug before the approved withdrawal time. This study shows that FLU residues identified in milk may be related to administration of FLU by an unapproved route.

The second experiment was a prospective clinical trial where 10 cows with naturally occurring mastitis and 10 healthy paired controls were administered FLU to determine if FLU and 5OH plasma pharmacokinetics and milk elimination differed between healthy and mastitic cows. Analysis of plasma samples indicated a significant reduction in plasma clearance, an increase in the area under the concentration time curve from time zero to infinity, and prolonged mean residence time in diseased versus healthy cows. Analysis of milk samples showed significant differences in FLU and 5OH milk concentrations at various time points. At the 36-hour milk withdrawal time, 8 cows with mastitis had 5OH residues greater than the tolerance limit. FLU residues persisted in the milk up to 60 hours post administration for 3 out of 10 of the mastitic cows. This study provides strong evidence that milk withdrawal times determined in healthy cattle may not be appropriate in cows with clinical mastitis. Our results also suggest that the use of a marker residue, such as 5OH is of
concern since its ratio to parent compound (FLU) changes in the very disease for which it is labeled.

Violative FLU residues in milk from dairy cattle are likely due to multiple factors; primarily extralabel drug use and alterations in clearance as result of a disease process. Therefore, education of veterinarians and farm personnel in proper drug administration and determining a milk withdrawal time under the condition in which a drug is labeled for is critical in the prevention of residue violations.
DEDICATION

To my father, who inspired me to pursue my dreams and to my husband, for supporting me as I work to achieve them.
BIOGRAPHY

Lindsey Kissell was born in Bryn Mawr, Pennsylvania. In May 2006, she received her Bachelor of Science in Animal Science from the University of Delaware. In December 2008, she received her Master of Science in Animal Science and Nutrition from North Carolina State University. In 2008, she began a combined Doctor of Veterinary Medicine and Doctor of Philosophy program at North Carolina State University under the direction of Dr. Geof Smith.
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I would like to extend special thanks to Jim Yeatts for his lab expertise and to the staff at the Lake Wheeler Dairy Education Unit for all their help with my research projects. I would like to thank all the graduate students that I have had the privilege of working with during my time at NCSU, especially, Dr. Meghan Samberg and Dr. Daniela Karadzovska. Lastly, I would like to acknowledge FARAD for providing me funding during this degree process.
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1. INTRODUCTION

Flunixin (FLU) is a nonsteroidal anti-inflammatory drug (NSAID) widely used in veterinary medicine because of its analgesic, anti-inflammatory and antipyretic effects. In cattle it is labeled for the modulation of inflammation in endotoxemia and for the control of pyrexia associated with bovine respiratory disease, endotoxemia and acute bovine mastitis. The extensive use of FLU in dairy cattle has resulted in a high number of violative meat and milk residues causing FLU to be regularly included on the Food Safety Inspection Service’s repeat violator list (FSIS, 2013). There are several factors which could be contributing to the high incidence of violative FLU residues in cattle including: failure to adhere to the recommended withdrawal time, excessive dosage, poor record keeping, disease status affecting drug clearance, or administration of FLU via a non-approved, extravascular route (KuKanich et al. 2005; and Shelver et al. 2013). Just one of these factors alone or in combination may have the potential to result in violative FLU residues after the approved withdrawal time. Drug residues in meat and milk are of concern because they pose a human health safety risk and can result in significant economic losses to the producer.

The overall purpose of this project was to determine whether violative FLU residues are present in milk above the tolerance limit and then identify potential explanations for the presence of these FLU milk residues. To accomplish this objective, a FLU milk surveillance study was first conducted to determine the occurrence of FLU residues in saleable milk throughout the United States. Then a FLU pharmacokinetic and milk elimination study was performed to determine if administering FLU by a non-approved route (intramuscular and subcutaneous) would alter the plasma pharmacokinetics and milk elimination resulting in
milk residues past the 36 hour withdrawal time. Lastly, an experiment comparing the pharmacokinetics and milk elimination of FLU in healthy and mastitic cows was carried out to ascertain if disease status would negatively impact FLU clearance resulting in violative milk residues beyond the withdrawal time. The results of the aforementioned experiments serve to elucidate mechanisms by which violative FLU residues can occur.
2. LITERATURE REVIEW

Pharmacology of NSAIDS

In veterinary and human medicine NSAIDs are widely used for their anti-inflammatory, antipyretic and analgesic effects. During the 1970’s their mechanism of action was elucidated when Vane et al. (1971) demonstrated that prostaglandin production was inhibited by aspirin and indomethacin (Vane et al. 1971). It wasn’t until the early 1990’s that a relationship between the inhibition of cyclooxygenase (COX) derived eicosanoids and the anti-inflammatory effects of NSAIDS were identified (McKellar et al. 1994; Landoni et al. 1995; Lees et al. 1996).

COX enzymes catalyze the conversion of arachidonic acid to prostaglandin H₂ via prostaglandin G₂. Prostaglandin H₂ is subsequently metabolized by enzymes to produce a variety of products such as prostaglandin D₂, prostaglandin E₂, prostaglandin F₂, prostacyclin, and thromboxane A₂ (Figure 2.1)(Livingston, 2000).
Figure 2.1: Arachidonic acid cascade

COX enzymes are primarily located in the endoplasmic reticulum and nuclear envelope of the cell (Smith et al. 1996). Currently, three COX enzymes have been identified: COX-1, COX-2 and COX-3. COX-1 is expressed in the majority of tissues under normal physiological conditions and is often referred to as the constitutive isoform. It is expressed throughout the body in tissues including the stomach, kidney, reproductive tract and blood platelets (Livingston, 2000). It plays an integral role in the maintenance of the gastric mucosa, platelet aggregation and regulation of renal perfusion (Brater, 1998; Halter et al. 2001; Tomlinson and Blikslager, 2003; Brzozowski et al. 2005). COX-2 is thought to be the inducible form whose expression is increased following stimulation by mediators, such as
lipopolysaccharide, and is predominantly expressed at sites of inflammation (Livingston, 2000; and Brzozowski et al. 2005). It has recently been discovered that not all COX-2 enzymes are inducible. Some COX-2 enzymes are constitutively expressed in the brain and kidney, and are crucial for central pain response and renal homeostasis. The expression of COX-2 is upregulated during inflammation in endothelial cells, chondrocytes, fibroblasts, monocytes, macrophages and synovial cells. At these sites, induction of COX-2 results in an increase in prostaglandin E₂ which mediates pain and inflammation (Jones and Budsberg, 2002). COX-1 and COX-2 are structurally distinct proteins, but they have similar active sites for binding to their biological substrate, arachidonic acid, as well as their pharmacological substrate, NSAIDs (Verbeeck and Blackburn, 1983). Recently, a COX-3 isozyme was discovered and is thought to be encoded by the same gene as COX-1, with the difference that COX-3 retains an intron that is not retained in COX-1. The role of COX-3 remains undetermined; however, it is primarily expressed in the central nervous system, suggesting it may be involved in homeostatic functions.

NSAIDs mechanism of action is competitive inhibition of COX enzymes preventing the production of prostaglandins involved in the inflammatory response. Most NSAIDs nonselectively inhibit COX-1 and COX-2 isoforms (Livingston, 2000). In doing so they produce both therapeutic effects and unwanted side effects simultaneously. Inhibition of COX-2 is thought to be responsible for the antipyretic, analgesic and anti-inflammatory effects associated with NSAIDs. Inhibition of COX-1 is thought to be responsible for the undesirable side effects associated with NSAIDs, such as gastric ulceration. It was thought that selective inhibition of COX-2 would produce therapeutic effects without unwanted side
effects and as a result COX-2 selective NSAIDs were developed (Cronstein, 2002). However, use of COX-2 selective NSAIDs resulted in a significant increase in heart attacks and strokes in human patients, which was thought to be due to increased platelet aggregation and vasoconstriction from the inhibition of prostacyclin. Thus several of the COX-2 selective NSAIDs have been removed from the market. Examples of COX-2 selective drugs currently approved for veterinary use are: deracoxib, fibrocoxib, and meloxicam.

Pharmacokinetics of NSAIDs

NSAIDs are weak acids and typically have a low volume of distribution (Curry, 2005), which is usually less than 0.2 L/ kg (Verbeeck et al. 1983; and Brater, 1988). In circulation, NSAIDs are highly bound to plasma protein, primarily albumin, thereby limiting their distribution to extracellular spaces. NSAIDs are primarily metabolized in the liver by hepatic biotransformation and excreted by the kidney (Aitken and Sanford, 1975). There is large variability in half-life among different NSAIDs, which can be attributed to differences in clearance. Some NSAIDs undergo enterohepatic recirculation. Following oral administration, NSAIDs that are highly lipophilic have a large first pass effect compared to NSAIDs with lower lipophilicity, which are less extensively metabolized resulting in a lower first pass effect. NSAIDs usually have good bioavailability following subcutaneous, intramuscular and oral administration. (Verbeeck et al. 1983; Brater, 1988; and Curry et al. 2005).
**Flunixin**

Flunixin (Figure 2.2) is a COX-1, carboxylic NSAID with a pKa = 5.82 (weak acid) (Odensvik, 1995). Flunixin is extensively protein bound (>98%), with only a small portion of the unbound drug available to be active in tissues. Flunixin is metabolized by the liver and undergoes enterohepatic recirculation. Flunixin plasma pharmacokinetic parameters in cattle from published literature are summarized in Appendix A. FLU is approved for use in beef and dairy cattle for the modulation of inflammation in endotoxemia and for the control of pyrexia associated with bovine respiratory disease, endotoxemia and acute bovine mastitis. FLU is labeled for intravenous administration at a dose of 2.2 mg/kg every 24 hours or 1.1 mg/kg every 12 hours for up to 3 days. When administered according to the label instructions the slaughter withdrawal time is 4 days and the milk withdrawal time is 36 hours. The liver is the target tissue for FLU tissue residues and the tolerance is 125 ppb. In milk, the marker residue is a metabolite of FLU, called 5-hydroxy flunixin (5OH) (Figure 2.3) and the tolerance for 5OH in milk is 2 ppb.
Residues

FLU is the only approved NSAID for use in cattle; therefore it is widely used in bovine practice. NSAIDs, such as FLU, are reported to be the second most prescribed class of drugs by dairy veterinarians (Sundlof et al. 1995) and the most frequently administered analgesic in cattle (Fajt et al. 2011). Since 2005, the United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) has reported an increasing number of residue violations in meat from dairy cattle (USDA-FSIS, 2005-2010). This increase in the number of FLU residue violations has lead to FLU becoming the second most common
residue violation behind penicillin in cull dairy cattle (Table 2.1), accounting for 14% of the violations in 2010 (USDA-FSIS). Of the 285 violations reported in 2010, 71% were in cull dairy cows and 21% in bob veal calves. As a result of the increased residue violations the Food and Drug Administration (FDA) released a reminder on the correct use of FLU in cattle (FDA, 2007). From July 2003, to July 2004, FSIS and FDA initiated a FLU cull cow survey to address possible FLU noncompliance and abuse. Prior to this survey, carcasses were only tested for violative drug residues using the FAST test, which detects antimicrobial drugs, but not NSAIDs. This study found that suspect cull dairy cows, which refers to cows having clinical signs or evidence of disease, had significantly higher incidence of violative tissue FLU concentrations (7.04%) compared to healthy appearing dairy cows (0.8%) at slaughter. This study also reported that nearly 20% of cattle that tested positive for antibiotics also tested positive for FLU (Deyrup et al. 2012). As a result of these finding, in 2006 the FSIS began testing all FAST-positive cattle for FLU residues. A study by Wu et al. (2012) used a population pharmacokinetic model to describe FLU disposition and predict tissue residues in cattle. This study reported a longer FLU meat withhold interval was needed than the FDA-approved withdrawal time (Wu et al. 2012). The results of this study are in accordance with the high number of FLU violations reported by the USDA-FSIS and suggest the need to consider FLU depletion in diseased animals in additon to healthy animals, because disease conditions may alter the pharmacokinetics of FLU.
Table 2.1: The number of tissue residue violations from cull dairy cows in the United States from 2006-2010.

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<tr>
<th></th>
<th>2006</th>
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<tr>
<td>Ampicillin</td>
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<td>10</td>
<td>12</td>
<td>8</td>
<td>17</td>
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<tr>
<td>Aminoglycosides</td>
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<td>129</td>
<td>99</td>
<td>86</td>
<td>67</td>
<td>495</td>
</tr>
<tr>
<td>Ceflofur</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>69</td>
<td>126</td>
<td>193</td>
</tr>
<tr>
<td>Flunixin</td>
<td>107</td>
<td>130</td>
<td>259</td>
<td>269</td>
<td>194</td>
<td>959</td>
</tr>
<tr>
<td>Oxytetracycline or Tetracycline</td>
<td>53</td>
<td>44</td>
<td>27</td>
<td>67</td>
<td>44</td>
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<tr>
<td>Penicillin</td>
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<td>359</td>
<td>411</td>
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<td>336</td>
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<tr>
<td>Phenylbutazone</td>
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<td>ND</td>
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<td>Sulfonamides</td>
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<td>Tilmicosin</td>
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<td>13</td>
<td>6</td>
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Data from FSIS National Program Residue Data (“Red Books”)

Currently, milk is not routinely tested for 5OH, the milk marker residue. This may be partially due to older literature, which suggested that FLU residues were not a concern because no residues were detected in milk following intravenous or intramuscular administration (Benitz, 1984; Anderson et al. 1990). However, newer research using more sensitive analytical equipment clearly demonstrates that 5OH and FLU accumulate in milk at concentrations well above the tolerance limit (Feely et al. 2002). Due to the high incidence of FLU tissue residue violations found in cull dairy cows, there is concern that the same practices which have lead to tissue residue violations may also lead to drug residue violations.
in milk. In December 2011, the FDA announced plans to collect and test milk samples across the United States for 5OH residues (FDA 2012). The results of this surveillance study have not been made public as of September 2013.

Veterinary drugs are widely used in food animal practice to maintain or improve the health of livestock. When properly administered and when appropriate withdrawal times are observed, violative drug residues entering the human food supply should not be an issue. However, when there is noncompliance, food products such as meat and milk, may contain excessive drug residues. This compromises the safety of the food supply and can potentially lead to allergic or toxic reactions in humans as well as drug resistant pathogens (Doyle, 2006). In addition to safety concerns, there are also economic concerns associated with drug residues. Milk residues can result in significant economic loss to the producer. Strict financial penalties and suspension of the producer’s Grade “A” permit are possible outcomes of drug residues detected in milk. Residues in bulk tank milk for one state were estimated to cost over half a million dollars annually (Seymour et al. 1988).

The potential for adverse effects in humans from the consumption of food products containing FLU residues is unknown; however, in general, at therapeutic levels used in humans, NSAIDS can result in anaphylaxis, gastritis and peptic ulcers (Stevenson, 1998).

In general, violative residues can be attributed to: (1) failure to adhere to the recommended withdrawal times, (2) poor record keeping, (3) inadvertently administering the wrong drug or dose, and (4) extra-label drug use without an appropriate withdrawal interval (KuKanich et al. 2005). Frequent FLU residue violations have been attributed to: (1) failure to adhere to the 36-hour withdrawal time, (2) extralabel drug use (doses greater than
mg/kg, and extravascular administration) and (3) disease conditions altering the elimination of FLU rendering the labeled withdrawal time inadequate (KuKanich et al. 2005; Shelver et al. 2013). A recently published population pharmacokinetic study showed that longer tissue withdrawal intervals than the FDA approved withdrawal time for FLU may be necessary, suggesting that using a subset of healthy animals to determine a drug withdrawal time may not be appropriate due to disease conditions altering the pharmacokinetics of FLU. This is the first study to show that covariates, such as health status may alter FLU pharmacokinetics and in part be responsible for the high number of residue violations. (Wu et al. 2012).

Pharmacokinetics of flunixin in ruminants

Cattle

The pharmacokinetics of FLU in ruminant species has been extensively studied beginning in the 1980’s. One of the earliest experiments using FLU in cattle had limited pharmacokinetic data only reporting an elimination half-life, of 3.68 hours. The study found FLU was primarily eliminated through biliary excretion, although a large polar fraction and minor metabolites were also found in urine. Excretion of FLU through milk was negligible and it was concluded a 12 hour withdrawal time would be sufficient (Benitz, 1984). However, this study only measured FLU in milk, not the metabolite, 5OH, which was later determined to be the marker residue in milk (FOI 204). Prior to 1985, FLU was known to be useful for prevention and reversal of adverse effects of endotoxaemia in horses and dogs (Hardee et al. 1985). It was hypothesized that FLU would provide analgesia in cattle and be a
treatment for bovine endotoxemia (Hardee et al. 1985). However, at the time the only pharmacokinetic parameter in cattle known was the elimination half-life. Therefore, Hardee et al. (1985) conducted a study to determine the pharmacokinetics of FLU in cattle. This study found the pharmacokinetics in cattle to be similar to the pharmacokinetics in horses and dogs, with two exceptions. The elimination half-life was significantly longer (8.12 hours) and the volume of distribution was significantly greater in cattle compared to other species. These findings were attributed to differences in tissue penetration, because the calculated clearance was similar across species. The greater distribution in the cow may have resulted from ion trapping and thus been responsible for the prolonged elimination half-life. Although this study did not consider the pharmacodynamics of FLU, it concluded that a dosing regimen of 2.2 mg/kg followed by 1.1 mg/kg intravenously every eight hours would provide analgesia in the cow (Hardee et al. 1985). In 1985, a crossover study was conducted to determine the bioavailability and milk residue depletion of FLU in 9 lactating cows (Cameron et al. 1985). Similar to the previous study, low levels of FLU were found in the milk, of which the highest concentration was 12 ppb, following subcutaneous administration. Again this study only measured FLU in milk, not the marker residue, 5OH. The bioavailability of FLU following subcutaneous and oral dosing was 75% and 53%, respectively (Cameron et al. 1985). To further examine the elimination of FLU in cattle, a radiolabeled study was conducted in 1986 (Lichtenwalner et al. 1986). Unlike the study conducted by Benitz (1984) this study found that FLU was eliminated in equal proportions in feces and urine (Lichtenwalner et al. 1986). This study was the first to report tissue levels of FLU. Of the tissues examined, the liver had the greatest amount of radioactivity and the
slowest depletion rate; therefore the study concluded that the liver should be the target tissue (Lichtenwalner et al. 1986).

In 1990, a study was conducted by Anderson et al. (1990) to determine the pharmacokinetics and milk residues of FLU in healthy cows and cows challenged with intramammary endotoxin as a model of acute coliform mastitis. FLU was best described by a 2-compartment model following intravenous administration and achieved concentrations in the plasma between 10.1 to 19.2 ppm. The elimination half-life was 3.14 hours; which is similar to the elimination half-life reported by Benitz (1984), but significantly shorter than the elimination half-life reported by Hardee et al. (1985). Variations in the elimination half-life of FLU are evident throughout the literature and are primarily due to the length of sample collection. For example, the longer half-life (8.12 hours) reported by Hardee et al. (1985) may be attributed to a longer sample collection period, 22 hour post dose, compared with the 10 hour post dose sample collection period of the Anderson et al. (1990) study. The difference in elimination half-life may also be partially explained by differences in clearance between the two studies. Anderson et al. (1990) reported a mean clearance of 151 mL/kg/hr compared to 90 mL/kg/hr reported by Hardee et al. (1985). Lastly, Anderson et al. (1990) reported the volume of distribution at steady state to be 0.397 L/kg. In the second experiment conducted by Anderson et al. (1990), cows were inoculated with endotoxin via the teat canal in 1 quarter and FLU was administered intravenously at dose of 1.1 mg/kg initially followed by 6 intramuscular doses. Prolonged FLU concentrations in the plasma following intramuscular administration compared to intravenous administration were observed; which can be attributed to absorption following intramuscular administration. The elimination half-
life following intramuscular administration was longer than the half-life following intravenous administration (5.20 vs 3.14 hours) which reflects prolonged absorption following intramuscular administration. No FLU was detected in the milk following intramuscular or intravenous administration. This may be due to the sensitivity of the assay for which the limit of detection was 50 ppb. The authors concluded that because FLU is an acidic drug and highly protein bound (98%), accumulation in milk would not be predicted (Anderson et al. 1990).

One of the first studies to investigate the dose-response relationship for eicosanoid synthesis inhibition by FLU in cattle was Lees et al. (1991). In this study 24 calves were divided into 6 groups. Group 1 received a placebo injection and groups 2 thru 6 had 1% carrageenan solution placed into subcutaneous pouches implanted in the neck and received varying doses of FLU intramuscularly. Both serum thromboxane B₂ and prostaglandin E₂ were significantly reduced with doses between 1.1-6.6 mg/kg of FLU. This study provided some justification for the 1.1 to 2.2 mg/kg dose rate of FLU that had long been the clinical standard (Lees et al. 1991). Several years later a FLU pharmacokinetic/pharmacodynamic model in calves using subcutaneous tissue cages as a model of acute inflammation was conducted to predict biological responses based on FLU plasma drug concentrations (Landoni et al. 1995). Eight calves were used in a crossover design. Plasma, exudate and transudate samples were collected following 2.2 mg/kg of intravenous FLU. A tricompartment model was used to fit data from 4 of the calves, which allowed for a more detailed description of FLU distribution and led to identification of two distribution phases. The remaining 4 calves were best described by a bicompartiment model (Landoni et al. 1995).
The elimination half-life of 6.87 hours was similar to findings by Hardee et al. (1985) and Lees et al. (1991); however, it was much greater than the elimination half-life reported by Benitz (1984) and Anderson et al. (1990). This difference in half-life can again be attributed to length of sample collection in which FLU was detected in the plasma up to 36 hours post dose (Landoni et al. 1995). In the Landoni et al. (1995) study, the volume of the central compartment (Vdc) was small, 0.033 L/kg, which is expected for a drug that is greater than 98% protein bound. The volume of distribution for the terminal elimination phase (Vdarea) was 2.11 L/kg and larger than expected for a drug that is highly protein bound. However, a large Vdarea (2.29 L/kg) had previously been reported in calves (Lees et al. 1991) and attributed to enterohepatic shunting and biliary secretion. FLU concentrations were significantly greater in the inflammatory exudate compared to the plasma and transudate; indicating increased blood flow to inflamed regions and leakage of protein bound FLU into the interstitial space. FLU was found to be a potent inhibitor of exudate prostaglandin E2, serum thromboxane B2, β-glucuronidase, and bradykinin-induced swelling; however, leukotriene B4 synthesis was unaffected. Lastly, this study was the first to report that the FLU plasma concentrations and the biological response were out of synch, indicating a counter-clockwise hysteresis (Landoni et al. 1995).

Until 1995, many of the FLU pharmacokinetic studies used doses of 1.1 mg/kg; however, 2.2 mg/kg was more commonly used in practice. Therefore, Odensvik and Johansson (1995) conducted a study to determine if administering 2.2 mg/kg altered the pharmacokinetics. For this study 1 cow and 1 heifer were administered 2.2 mg/kg FLU intravenously. Following intravenous administration the half-life for both the cow and the
heifer was approximately 4 hours, which is consistent with previously reported values (Anderson et al. 1990; Benitz, 1984). The Vdss was 0.419 and 0.297 L/kg for the cow and heifer, respectively. The Vdss is also consistent with previously reported values (Anderson et al. 1990; Hardee et al. 1985). The Vdarea in the present study was approximately 0.7 L/kg for both the cow and heifer, which is much less than previously reported by Hardee et al. (1985); Landoni et al. (1995). The smaller Vdarea may explain the shorter half-life in the Odensvik and Johansson (1995) study compared to the half-lifes reported by Hardee et al. (1985) and Landoni et al. (1995) of 8.1 and 6.87 hours respectively. Approximately 3 hours after intravenous administration of FLU, a secondary peak was observed; which the authors attributed to enterohepatic recirculation of FLU. A secondary peak was previously reported by Lichtenwalner et al. (1986) and Lees et al. (1991) for FLU. In a second experiment, 2.2 mg/kg of FLU was administered intramuscularly for either 28 (4 times daily, n=3) or 56 doses (twice daily, n=3) to 6 cows (Odensvik and Johansson, 1995). Following multiple intramuscular doses the mean half-life was 26 hours, which is significantly longer than the half-life following a single intravenous dose of FLU. This difference suggests that the rate of elimination is limited by absorption following intramuscular administration.

In dogs and horses, FLU is frequently administered orally, however; in cattle FLU administration has primarily been limited to intravenous and intramuscular routes due to a lack of data following oral administration. Therefore, Odensvik (1995) conducted a study to determine the pharmacokinetics of FLU after a single oral and intravenous administration to heifers. This was a crossover study in which 6 heifers received 2.2 mg/kg orally and intravenously. Following oral administration the half-life was 6.2 hours, which is similar to
half-life values reported following intravenous administration (Landoni et al. 1995; Lees et al. 1991; Hardee et al. 1985; Odensvik and Johansson, 1995). The oral bioavailability was 60%; which is similar to the oral bioavailability (53%) reported by Cameron et al. (1985). A comparison of the pharmacodynamics following oral and intravenous administration showed that oral dosing was as effective at inhibiting prostaglandin biosynthesis as compared to intravenous administration and the pharmacokinetics did not significantly differ between the two routes. The results of this study were the first to show that oral administration of FLU may be an effective alternative to parenteral administration (Odensvik, 1995).

Prior to 1998, FLU was not labeled for use in cattle; therefore all use was considered extralabel. In 1998, FLU was approved for the control of pyrexia associated with bovine respiratory disease and endotoxemia and for control of inflammation in endotoxemia in beef and nonlactating dairy cattle (FOI, 1998). Based on several of the pharmacokinetic studies aforementioned as well as meat residue and toxicity studies, the liver was determined to be the target organ for FLU meat residues. The tolerance limit for FLU in the liver was set at 125 ppb. Using the FDA’s statistical tolerance limit method which predicts with 95% confidence a time when the tissue residue in 99% of the animal population receiving the drug is at or below the tolerance, a 4-day meat withdrawal time was set for FLU (FOI, 1998).

Prior to 2002, few studies had measured FLU milk concentrations and of those studies only 2 had found FLU in the milk (Lictenwalner et al. 1986; Cameron et al. 1985). At the time FLU was not labeled for use in lactating cows; however, it was being used in an extra label manner as a treatment for mastitis. One study used FLU in combination with enrofloxacin to examine the pharmacokinetics of both drugs in cows with experimentally
induced Escherichia coli mastitis. No significant differences in elimination half-life, mean residence time, area under the concentration time curve from zero to infinity (AUC_{0,\infty}), V_{dss} and clearance were found between cows treated with FLU alone and FLU plus enrofloxacin. For both groups of cows (FLU alone and FLU plus enrofloxacin) the pharmacokinetic parameters were similar to those previously reported in the literature. In contrast to Anderson et al. (1990), FLU was found in the milk at very low concentrations. However, this study used a method in which the limit of quantification was 13 ppb which is substantially lower than the 50 ppb limit of quantification used in the Anderson et al. (1990) study.

All of the previous studies where milk samples were collected and analyzed made the assumption that FLU was the primary residue in milk. However, in order for FLU to be approved for use in lactating cows as a treatment for the control of pyrexia associated with acute bovine mastitis, a total residue depletion and metabolism study in dairy cattle needed to be conducted. The main purpose of this study was to identify a marker residue in milk and describe the depletion of the marker residue over time (Feely et al. 2002). A preliminary study indicated that a metabolite of FLU called 5OH was present in milk from cattle treated with radio-labeled FLU. A follow up study was the first to describe the detection of 5OH in cow’s milk and to characterize the milk residue profile (Feely et al. 2002). In this study, 8 cows received 2.2 mg/kg of $^{14}$C labeled FLU once daily for 3 days and milk samples were collected every 12 hours for 9 days. FLU, 5OH and two minor metabolites (4-hydroxy flunixin and 2-hydroxy methyl flunixin) were identified in milk. 5OH was determined to be the major residue in milk at the first milking, 12 hours after the last dose. For the second and third milkings post dose, 5OH was greater than or equal to the amount of FLU present in 9
out of 11 samples. Overall, in 15 out of 19 milk samples the most abundant residue identified was 5OH. Percentage of the total administered dose of FLU that was excreted in milk was less than 0.02% (Feely et al. 2002). The data from this study was used, in part, for the approval of FLU in lactating cows.

In 2004, a supplement was added to the Freedom of information summary for FLU. This supplement allowed for the use of FLU in lactating dairy cattle for the existing indication of “the control of pyrexia associated with bovine respiratory disease and endotoxemia and for the control of inflammation in endotoxemia”. It also included a new indication “for the control of pyrexia associated with acute bovine mastitis”. This supplement showed that FLU was effective at controlling pyrexia associated with acute bovine mastitis. Using the data from Feely et al. (2002), 5OH was established as the marker residue in milk (FOI, 2004). To determine the tolerance limit for 5OH in milk, the ratio of the mean marker to total residue was calculated. In 17 individual milk samples the ratio was 0.18. This ratio was rounded to 0.2 and multiplied by the safe milk concentration, 10 ppb, to give a tolerance limit of 2 ppb for 5OH in milk (FOI 2004). To establish a milk withdrawal time for FLU a study was conducted in which 27 Holstein dairy cows were administered FLU intravenously once daily for 3 days (Ngoh et al. 2003). The 27 cows were separated into 3 groups by milk production. Group 1 consisted of 9 cows with an average milk production of greater than 14.4 kg/milking. Group 2 consisted of 9 cows with an average milk production greater than 8.7 kg/milking but less than 14.4 kg/milking and Group 3 consisted of 9 cows with an average milk production of less than 8.7 kg/milking. Twelve hours after the last dose of FLU, the average concentration of 5OH in the milk was 20.9 ppb. Twenty-four hours after
the last administered dose, the average concentration of 5OH in the milk had depleted to 3.4 ppb and by 48 hours post dose 5OH concentrations in milk had depleted to less than 0.5 ppb for 24 out 25 cows. For groups 2 and 3 no residues above the tolerance limit were detected in the milk 36 hours post dose; however, for group 1 residues above the tolerance limit were present in the milk 36 hours post dose for 1 out 9 cows (Ngoh et al. 2003). Based on the FDA’s statistical tolerance limit method a milk discard period of 36 hours was determined for FLU. The animals used for determination of the FLU milk withdrawal time were strictly healthy cows, which raises the question would the milk elimination of FLU be similar between healthy and diseased animals?

In 2006, a study was conducted by Szprengier-Juszkiewicz et al. (2006) to determine whether 5OH was the appropriate milk marker residue, because previous studies had reported that 50% of the total residues (C\textsuperscript{14}) were residues other than 5OH (Feely et al. 2002; and Boner et al. 2003) and up to 75% of the total FLU was found as a β-glucuronide derivative (Rupp et al. 1995). This study showed that a large percentage of FLU is present as β-glucuronide in cow milk, which cannot be extracted by traditional analytical methods if enzymatic deconjugation is not performed. Using a metabolite, 5OH, as the marker residue circumvents the need for enzymatic deconjugation when analyzing milk samples; however, it may be more appropriate to use the sum of the parent compound and the primary metabolite as the marker residue for milk (Szprengier-Jusziewicz et al. 2006).

Over the last two decades the pharmacokinetics of FLU following intravenous administration has been extensively studied. However, few studies sought to investigate the pharmacokinetics following intramuscular or subcutaneous administration, with the
exception of Anderson et al. (1990) and Odensvik and Johannson (1995). Recently, there has been increasing interest in administering FLU via extravascular routes in combination with antimicrobials. In 2009, Resflor Gold®, a flofenicol and flunixin injectable solution was approved for use in beef and non-lactating dairy cattle. This drug is labeled for subcutaneous administration and with FLU being dosed at 2.2 mg/kg. For drug approval, pharmacokinetic studies administering FLU subcutaneously were performed. The elimination half-life and mean residence time for FLU when administered subcutaneously were prolonged compared to the elimination half-life and mean residence time reported in the literature following intravenous administration. This finding may have been due to prolonged absorption influencing FLU elimination. The $\text{AUC}_{0\rightarrow\infty}$ ($14.4 \mu g/mL/kg$) was similar to the $\text{AUC}_{0\rightarrow\infty}$ reported in the literature. A total residue depletion study was also performed using $^{14}$C-FLU administered subcutaneously to determine the total radioactive residue at the injection site. Similar to intravenous administration, FLU was eliminated in both the urine (34.2%) and feces (56.6%) and the tissue with the greatest total radioactive residue was the liver followed by the kidney. Based on the total residue ratio at the injection site the study concluded that a 4 day meat withdrawal would still be adequate for subcutaneously administered FLU; however, because Resflor Gold® also contains florfenicol which has a slower depletion, a withdrawal time of 38 days was necessary (FOI 2009).

A few years later the pharmacokinetics of FLU following intramuscular administration was studied to determine if subcutaneous and intramuscular routes were bioequivalent. Using a cross-over study design, 2.2 mg/kg of FLU was administered intramuscularly and subcutaneously. The elimination half-life, and mean residence time were
greater following subcutaneous administration compared to intramuscular administration. In contrast the area under the curve from zero to the last sampled time point and maximal plasma concentration were greater following intramuscular administration compared to subcutaneous administration. The elimination half-life following subcutaneous administration was 7.46 hours, which is similar to the elimination half-life reported in the Freedom of Information Act for Resflor Gold® (9.5 h) (Lacroix et al. 2011). This study showed that the plasma concentration vs time profiles for FLU following intramuscular and subcutaneous administrations were similar, but differed from the pharmacokinetics following intravenous administration due to absorption following extravascular administration.

**Drug residues in milk**

The use of drugs in dairy cows can often result in the occurrence of residues in milk. Drug residues in milk have become a particular topic of interest, especially with respect to FLU milk residues. Although some data describing the depletion of FLU in milk is published there is some controversy over whether a FLU glucuronide is transferred into milk. Therefore Jedziniak et al. (2013) conducted a study to investigate levels of residues of FLU and 5OH in milk of cows treated with FLU and to compare the results with the ones obtained after treating the same milk samples with β-glucuronidase. For this study, 6 cows received 2.2 mg/kg of FLU intravenously once daily for 3 days. Milk samples were collected twice daily and analyzed twice, first with enzymatic hydrolysis and afterward without enzymatic hydrolysis. FLU and 5OH were rapidly eliminated from the milk and only a few samples collected 12 hours after dosing had concentrations of 5OH above the maximum residue level.
of 40 ppb. FLU concentrations were lower than 5OH concentrations in milk. Mean FLU concentrations in milk samples treated with β-glucuronidase were 7.34 ppb vs 1.88 ppb for untreated milk samples. This suggests that FLU conjugated with glucuronic acid is present in milk of cows administered intravenous FLU. This study was the first to confirm that a FLU glucuronide metabolite is present in milk; however, despite this finding 5OH remains the main metabolite of FLU in milk (Jedziniak et al. 2013).

To date, the most recently published FLU pharmacokinetic study in cattle characterized the plasma pharmacokinetics of FLU and 5OH in beef cattle following intravenous and subcutaneous administration (Shelver et al. 2013). Following intravenous administration the elimination half-life, AUC_{0\rightarrow\infty}, mean residence time and clearance are similar to values previously reported in the literature (Benitz, 1984; Anderson et al. 1990; and Odensvik and Johannson, 1995). Following subcutaneous administration the elimination half-life was 6.3 hours, which is similar to the elimination half-life reported by Lacroix et al. (2011). For 5OH, the maximal plasma concentration was 755 ppb and 155 ppb following intravenous and subcutaneous administration, respectively. The time to maximal concentration for 5OH following intravenous and subcutaneous administration was 0.19 hours and 1.75 hours, respectively. The short time to maximal concentration and high maximal plasma concentration following intravenous administration compared to subcutaneous administration was expected because following intravenous administration metabolism of FLU to 5OH would reach equilibrium quickly, followed by rapid elimination of 5OH. Following subcutaneous administration, there is a greater lag time for the conversion of FLU to 5OH due to the absorption of FLU. The elimination curves for FLU and 5OH
were parallel suggesting 5OH is more rapidly eliminated than the FLU. This study was the first to report the pharmacokinetics of both FLU and 5OH in cattle.

*Other ruminant species*

Currently, there are no NSAIDs approved for use in ruminant species other than cattle. When a situation arises where extra-label use of an NSAID in a ruminant would be justified by the Animal Medicinal Drug Use Clarification Act; FLU is the preferred NSAID because it is labeled for use in cattle. Studies evaluating the pharmacokinetics of FLU in non-bovine ruminants are limited. One of the first pharmacokinetic studies conducted was in sheep in which FLU was administered both intravenously and intramuscularly (Welsh et al. 1993). Following intravenous administration, FLU plasma concentrations were best described by a tri-exponential equation. This differs from kinetics studies in cattle where FLU plasma concentrations are best fit using a 2-compartment model. The mean elimination half-lives in sheep for intravenous doses of 1.0 mg/kg and 2.0 mg/kg were 3.83 and 3.43 hours, respectively, which are comparable to the range of elimination half-lifes reported in cattle (3.1-8.1 hours). The clearance following intravenous administration was 42 mL/h/kg, which is less than the range of clearances reported in cattle (90-200 mL/h/kg). This may be due to the model used to fit the plasma concentration vs time curve in the sheep study or possibly sheep eliminate FLU slower than cattle. Following intramuscular administration FLU was rapidly absorbed and maximal plasma concentration occurred less than 45 minutes post dose. The bioavailability following intramuscular administration was 70%; which is similar to the 76% bioavailability reported by Anderson et al. (1990). A study conducted by
Cheng et al. (1998) investigated the pharmacokinetics of FLU in plasma, exudate and transudate using a tissue cage subcutaneous acute inflammatory model in sheep. The clearance was in agreement with the previous study using sheep; but less than clearance values that have been reported for cattle. The elimination half-life of 2.42 hours was less than the half-life reported previously in sheep, as well as the elimination half-life reported in cattle. Cheng et al. (1998) reported Vdss to be 0.116 L/kg in sheep; which is much less than the range of Vdss reported in cattle (0.297-0.782 L/kg). FLU plasma concentrations rapidly declined following administration; however, distribution and elimination from the tissue cage exudate and transudate were very slow despite extensive extravascular penetration. The extensive extravascular penetration suggests low plasma protein binding for FLU in sheep in contrast to cattle where FLU is more than 98% protein bound (Odensvik and Johannson, 1995). However, plasma protein binding was not measured in the Cheng et al (1998) study. The extensive distribution and slow elimination into tissue cage fluids in sheep is therapeutically advantageous as it indicates FLU reaches extravascular sites.

A study conducted using llamas where 2.2 mg/kg of FLU was administered intravenously also reported a slower clearance compared to cattle, but similar to sheep. As a result of the reduced clearance a much larger AUC$_{0-\infty}$ was observed. The elimination half-life of FLU in llamas was 1.47 hours, which is much shorter than the elimination half-life reported for both cattle and sheep. The Vdss was also much smaller in llamas (0.03 L/kg) compared to cattle, which may have been due to differences in water metabolism affecting the distribution of FLU (Navarre et al. 2001).
In contrast to the pharmacokinetics in sheep and llamas, the pharmacokinetics of FLU in goats is similar to cattle. Following intravenous, intramuscular and oral administration, a crossover study using goats reported similar AUC$_{0\rightarrow\infty}$, clearance, mean residence time, Vd$_{ss}$, and elimination half-life values to reported cattle values (Konigsson et al. 2003).

These studies indicate that the pharmacokinetics of FLU can greatly vary among ruminant species with cattle and goats having similar pharmacokinetics, but dissimilar to sheep and llamas. The pharmacokinetic differences among ruminants may suggest that extrapolation of dosing regimens and withdrawal times from cattle data to other ruminant species may not be appropriate.

To summarize, FLU is a weak acid with a high degree of protein binding (>98%) in cattle. The unbound portion of the drug readily partitions into tissues exhibiting a Vd$_{ss}$ reported in the literature between 0.297 to 0.782 L/kg. The Vd$_{area}$ has been reported to be 0.685 to 2.11 L/kg. The large discrepancy in Vd$_{area}$ reported across studies may be a result of extended drug elimination from a deep compartment. FLU is eliminated through urinary and biliary excretion and there is evidence of enterohepatic recirculation following intravenous dosing. A wide range of clearance values has been reported in cattle, from 90 to 200 mL/kg/h. Variation in the elimination half-life following intravenous administration has also been reported ranging from 3.14 to 8.12 hours. This may be attributed to the length of sample collection, the sensitivity of the assay and variations in clearance. Following extravascular administration, FLU is rapidly absorbed; however, the mean transit time and elimination half-life have been reported to be slower compared to intravenous administration, which may
be due to prolonged absorption influencing elimination. FLU has been shown to persist in inflammatory tissues with anti-inflammatory properties that extend beyond the time when FLU is detected in the plasma. This indicates a counterclockwise hysteresis is associated with FLU. Therefore, using the terminal elimination half-life to predict drug plasma concentration is likely to underestimate the concentration of FLU remaining at the site of action as well as the duration of action.

**Disease induced alterations in pharmacokinetics**

The main goal of drug administration in veterinary medicine is to treat diseased animals. Part of the drug approval process for veterinary medicines requires efficacy studies to be conducted in diseased animals and pharmacokinetic studies to be conducted in healthy animals. These studies provide the basis for the development of dosage regimens and determination of a withdrawal time, assuming no changes in the dose-effect relationship and pharmacokinetics in diseased animals. This implies that the pharmacokinetic behavior of a drug remains the same in diseased and healthy animals. However, diseased states can profoundly alter the pharmacokinetic behavior of a drug (Lohuis et al. 1991; Jha et al. 1996; Gips and Soback, 1999; Rao et al. 2000; Ismail and El-Kattan, 2007; Lucas et al. 2009). The most profound differences in pharmacokinetic responses are generally associated with hepatic, renal and cardiovascular disease, but other processes such as inflammation, endotoxemia and stress can also significantly alter a drug’s absorption, distribution, metabolism, and elimination (Martinez and Modric, 2010). In ruminants much of the literature has focused on describing the effect of disease on the pharmacokinetics of various...
antimicrobials. For example, differences in pharmacokinetics were noted between febrile and afebrile goats administered norfloxacin. The clearance was significantly reduced in febrile goats compared to afebrile goats (Jha et al. 1996). Similarly, a 47% reduction in enrofloxacin clearance was observed in febrile goats following an intravenous injection of endotoxin (Rao et al. 2000). There was also a reduction from 28.8% to 8.5% in the metabolic conversion of enrofloxacin to ciprofloxacin in febrile goats; which is likely responsible for the reduced clearance. As a result of the reduction in clearance; the elimination half-life and mean residence time were prolonged (Rao et al. 2000). In another study where febrile goats were administered marbofloxacin, both Vdss and clearance were significantly reduced compared to healthy animals. Consequently, mean residence time was significantly greater in febrile goats (Waxman et al. 2003). A study conducted by Lucas et al. (2009) found that mammary health status had an influence on the pharmacokinetics of azithromycin. Quarters with subclinical mastitis caused by *Staphylococcus aureus* had significantly lower drug clearance from the mammary gland, a greater milk elimination half-life and longer mean residence time in milk for azithromycin (Lucas et al. 2009). Differences in drug pharmacokinetics have been described for oxytetracycline in cows with theileriosis (Kumar and Malik, 1999). Following intramuscular administration, infected cattle had significantly prolonged absorption, elimination half-life, mean residence time, AUC0–∞ and bioavailability as compared to oxytetracycline administration in healthy cows. Another example is theophylline where in a field trial, 5 out of 20 calves with respiratory disease died after administration whereas all 20 calves treated with a placebo survived (McKenna et al. 1989). A subsequent study showed calves with pneumonia had significantly higher plasma concentrations of theophylline as
compared to healthy calves (Langston et al. 1989). Likewise, a greater secretion of ceftriaxone into milk was also noted in cows with endometritis compared to the control cows following intravenous administration (Kumar et al. 2010).

Differences in pharmacokinetics and milk elimination of drugs have also been observed for intramammary preparations used to treat mastitis. Mastitis produces physical and chemical changes both in the milk and the mammary gland itself that have the potential to alter distribution and elimination of drugs through the mammary gland (Gehring and Smith, 2006). Inflammation of the mammary gland leads to vascular permeability changes that often enhance systemic absorption and perhaps distribution of drugs into the udder. For example gentamicin is not detected in the plasma following intramammary administration in normal quarters, however the drug is well absorbed in cows with mastitis (Sweeney et al. 1996). Similarly in studies using polymyxin B, the drug was not found in the blood or untreated quarters following intramammary administration in normal cattle; however significant systemic absorption was seen in cows with experimentally induced coliform mastitis (Ziv and Schultze, 1982). Lastly, a study using an intramammary preparation of cefoperazone sodium reported significantly greater systemic drug absorption, milk half life and mean residence time in cows with subclinical mastitis compared to healthy controls (Cagnardi et al. 2010).

Literature describing the effect of mastitis on the pharmacokinetics of NSAIDs is limited. Even fewer studies have tried to address disease-induced pharmacokinetic changes in regards to their impact on milk or tissue residues. A study evaluating the influence of Escherichia coli endotoxin-induced mastitis on the pharmacokinetics of the NSAID,
carprofen, found a significant reduction in systemic clearance, prolonged elimination half-life, and increased milk carprofen concentrations in mastitic cows compared to the healthy controls following intravenous administration (Lohuis et al. 1991). Carprofen concentrations in milk from healthy cows were below the limit of detection at all times points; however, carprofen could be detected in the milk of mastitic cows 45 hours after administration (Lohuis et al. 1991). One explanation for the greater milk carprofen concentrations in mastitic cows may be increased vascular permeability resulting in exudation of serum albumin proteins into milk; which carprofen would have been bound to because it is an acidic drug (Oliver and Calvinho, 1995). Because carprofen is not labeled use for in lactating cows in the United States it impossible to assess the impact of prolonged plasma elimination half-life on the meat withdrawal time or make assumptions about whether the increased milk carprofen concentrations would have resulted in a violative milk residue. Lastly, a study conducted by Wu et al. (2012) used a population pharmacokinetic model to assess FLU pharmacokinetics while simultaneously accounting for the effect of concomitant pathophysiological, environmental, demographic and genetic variables. The study found a longer meat withdrawal interval was necessary for FLU than the FDA approved withdrawal time, suggesting a need for pharmacokinetic studies to be performed in both healthy and diseased animals (Wu et al. 2012). Animals in which a disease process has altered either distribution or clearance deserve increased attention to ensure complete drug withdrawal (Riviere, 2011). Since pharmaceutical companies must conduct trials to demonstrate the efficacy of various drugs for treating a specific disease or condition during the approval
process, it seems logical that pharmacokinetic and residue studies could be done using the same animals or under similar conditions.
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3. OCCURRENCE OF FLUNIXIN RESIDUES IN BOVINE MILK SAMPLES FROM THE USA

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Occurrence of flunixin residues in bovine milk samples from the USA

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5-Hydroxy-flunixin concentrations in milk samples were quantified by two commercially available screening assays — CHARM® and enzyme-linked immunosorbent assay (ELISA) — to determine whether any concentrations could be detected above the tolerance limit of 2 ng g⁻¹ from different regions in the United States. Milk samples came from large tanker trucks hauling milk to processing plants, and had already been screened for antibiotics. Positive results for flunixin residues based on a screening assay were confirmed by ultra-HPLC with mass spectrometric detection. Of the 500 milk samples analysed in this study, one sample was found to have a 5-hydroxy-flunixin concentration greater than the tolerance limit. The results of this study indicate that flunixin residues in milk are possible. Regulatory agencies should be aware that such residues can occur, and should consider incorporating or expanding flunixin screening tests as part of routine drug monitoring in milk. Larger studies are needed to determine the true prevalence of flunixin residues in milk from other regions in the United States as well as different countries.

Keywords: flunixin; milk residue; UPLC-MS; CHARM®; milk; food safety

Introduction

Flunixin (FLU) is a non-steroidal anti-inflammatory drug (NSAID) approved in the United States for use in beef and dairy cattle for modulation of inflammation in endotoxemia and for the control of pyrexia associated with bovine respiratory disease and acute bovine mastitis. FLU is labelled for intravenous administration at a dose of 2.2 mg kg⁻¹ every 24 h or 1.1 mg kg⁻¹ every 12 h for up to 3 days. The slaughter withdrawal time is 4 days following the last injection and the milk withdrawal time is 36 h. Since 2005, the US Department of Agriculture – Food Safety Inspection Service (USDA-FSIS) has reported an increasing number of residue violations in meat from dairy cattle (USDA-FSIS 2005–2010). This increase in the number of FLU residue violations has lead to FLU becoming the second most common residue violation behind penicillin in calf dairy cattle (USDA-FSIS). In milk, the marker residue is a metabolite of FLU, called 5-hydroxy flunixin (5OH) and the tolerance for 5OH in milk is 2 ng g⁻¹. Milk samples in the United States are not routinely tested for 5OH (National Milk Drug Residue Data Base Fiscal Year 2011 Annual Report). However, due to the significant number of FLU tissue residues violations found in calf dairy cows, there is concern that the same practices which have led to tissue residues may also lead to drug residues in milk. For example, a previous study by Kissell et al. (2012) found that extra-vascular administration of FLU to dairy cattle resulted in 5OH concentrations in milk above the 2 ng g⁻¹ tolerance at the 36-h withdrawal time. Therefore, our objective was to conduct a surveillance study of saleable milk collected from processing plants in the United States to determine whether violative residues could potentially be found in milk that had already passed antibiotic screening tests.

Materials and methods

This surveillance study was designed to allow farms to participate anonymously. Thus, the farm of origin for milk samples is not known, only the location of the processing plants. A total of 500 samples were collected from eight different processing plants in different regions of the United States. Plants were located in California, Colorado, New Mexico, Ohio, Tennessee, Indiana and Utah. However, these were all large processing plants and received milk from multiple states. Some tanker loads represented milk from a single large dairy, while other tankers represented milk from multiple dairies that had been commingled together. Samples were collected in sterile 25 ml tubes straight from the milk tanker. All milk samples had already been screened for antibiotics but had not undergone any processing (i.e. pasteurisation or homogenisation).

Milk samples were frozen at ~20°C prior to shipment. Samples were shipped on ice overnight to North Carolina...
State University and upon arrival were immediately frozen at -20°C until further analysis was performed. All 500 milk samples were analysed for SOH using two different approved screening tests: the CHARMM® Flunixin test (Charm Sciences, Inc., Lawrence, MA, USA) and the Alert Flunixin Assay (Neogen Corporation, Lansing, MI, USA). Each milk sample was run using both assays and positives were confirmed using a UPLC-MS detection method.

**CHARMM® Flunixin and β-lactam test**

The CHARMM® Flunixin and β-lactam test is a combination receptor assay and antibody assay utilising lateral flow technology that detects SOH in the milk at a concentration of 2 ng g⁻¹ or greater. Frozen milk samples were thawed and then centrifuged at 2300g for 10 min 300 µl of supernatant were applied to the CHARMM® FLUSLAB test strip and incubated in the CHARMM® ROSA incubator for 8 min. Following incubation, test strips were read immediately using the CHARMM® ROSA reader.

**Neogen® Alert Flunixin Assay**

The Neogen® Alert Flunixin Assay is a competitive direct enzyme-linked immunosorbent assay (ELISA) that detects SOH in milk at a concentration of 2 ng g⁻¹ or greater. A total of 20 μl of the positive control, the negative control and the unknown milk sample was added to different wells in the test strip. A total of 180 μl of the drug-enzyme conjugate was then added to each well followed by gentle shaking of the plate to mix each well. The plate was covered using plastic film and allowed to incubate at RT for 45 min. Following incubation the liquid was discarded from the wells and each well was washed with 300 μl of wash buffer. A total of three washings was performed on each well. A total of 150 μl of K-blue substrate was added to each well followed by gentle shaking of the plate. The plate was incubated at RT for 30 min with periodic gentle shaking of the plate. After 30 min, 50 μl of stop solution were added to each well and results were read visually.

**UPLC-MS**

Milk samples testing positive by either the CHARMM® Flunixin and β-lactam assay or the Neogen® Alert Flunixin Assay were quantified by UPLC-MS for SOH. For SOH milk extraction, 0.5 ml of milk and 1.5 ml of 0.5% citric acid in acetonitrile were combined in a centrifuge tube. Samples were sonicated for 5 min and then centrifuged for 10 min at 2300g. The supernatant was loaded on a Supelco Hybrid SPE-phospholipid cartridge (Sigma-Aldrich, St. Louis, MO, USA). The eluate from the cartridge was collected and placed in a 55°C TurboVap® LV evaporator (Zymark Corporation, Hopkinton, MA, USA) to dryness under a 20 psi stream of nitrogen, reconstituted in 300 µl of mobile phase and filtered through a 0.22-µm nylon syringe filter. The injection volume was 5 µl. Concentrations were derived by comparing peak areas of the samples with those of an external standard curve made from spiked milk samples put through the sample clean-up process. The Agilent UPLC-MS (Waters Corporation, Milford, MA, USA) consisted of a HSS T3 column (1.8 µm, 2.1 x 100 mm) and filter disc. The mobile phase was acetone–0.1% acetic acid in water (68:32 v/v). The EMD 100 was a single quadrupole mass spectrometer run in ESI+ mode. Ion with a mass-to-charge ratio of 313.0 was used for the quantification of SOH. Column temperature was 30°C and sample temperature was 4°C. Run times were 2.2 min. The LOQ was determined as 10 times the standard deviation (SD) of six blank samples. The LOD was determined as three times the SD of six blank samples. The LOD and LOQ for SOH in milk were 0.001 and 0.002 µg ml⁻¹, respectively; the linear range for milk was 0.002-1 µg ml⁻¹. Relative standard deviations (RSDs) for both inter-day and intra-day were <15% at all concentrations.

**Results and discussion**

Of the 500 milk samples tested for the presence of SOH residues, one sample was found to have a SOH concentration greater than the tolerance limit using both screening methods. This milk sample was confirmed positive for SOH using UPLC-MS. The concentration of SOH in the milk sample was 42 ng g⁻¹. This concentration is substantially greater than the 2 ng g⁻¹ tolerance limit set forth by the USDA. A SOH milk residue of this magnitude is unlikely to occur frequently due to dilution of milk containing SOH residues with non-contaminated milk. Some plausible explanations for the high concentration of SOH in the milk tanker load are: (1) prolonged FLU milk elimination as a result of prolonged absorption when FLU is administrated via a non-labelled route (intramuscular or subcutaneous) (Kissell et al. 2012); (2) milking a large number of cows treated with FLU into the bulk tank 12-24 h after treatment; (3) milking FLU-treated cows into the bulk tank shortly after FLU administration (less than 12 after FLU administration); (4) milking cows administered doses of FLU greater than the label dose into the bulk tank; (5) the volume of milk the tanker truck collected on that particular day was less than an average tanker truck load volume of 28 390 L (7500 gallons); and (6) drug disposition may differ between healthy cows and cows with mastitis, which is the primary indication for FLU use in dairy cattle in the United States. Milking of treated cows into the bulk tank prior to the withdrawal time primarily occurs due to improper identification of a treated animal (Booth & Harding 1986; McElwain et al. 1991). These possible explanations for the high concentration of SOH found in milk tanker load
are not necessarily equivalent in their potential to cause violative SOH milk residues, thus the most likely cause for the high concentration of SOH in the tanker truck milk load is a combination of several of the explanations aforementioned.

In this relatively small survey of 500 milk samples we found a positive violation rate of 0.2%. From 2003 to 2011, the percentage of milk tanker samples that had a violative residue associated with any drug in the United States ranged from 0.032% to 0.11% (National Milk Drug Residue Data Base Annual Reports 2003–2011). This suggests that SOH residues may be found in the milk almost as often as other potential drug residues. Milk used for drug residue testing is sampled from tanker truck loads and not the individual cow. Therefore, an individual cow with SOH concentrations greater than the tolerance limit of 2 ng g⁻¹ could have contributed to the tanker truck load; however, as a result of dilution the whole tanker load tests negative for SOH.

To quantify the number of cows with SOH milk residues required to make a tanker truck load of milk test positive at the tolerance level (2 ng g⁻¹), a hypothetical calculation is presented. For this scenario an average tanker truck load of 28390 L (7500 gallons) of milk was used. The average milk yield per cow per day is assumed to be 38 L (10 gallons) of milk. The concentration of SOH in one cow’s daily milk yield was assumed to be 114 ng g⁻¹ (Freedom of Information 2004). This concentration was determined using data from the Freedom of Information Summary (FOI) for Banamine® in which the highest concentration of SOH in the milk from one cow was 142 and 87 ng g⁻¹, 12 and 24 h after FLU administration. Therefore, a concentration of 114.5 ng g⁻¹, which is the average of 142 and 87 ng g⁻¹, was used for the following calculation. Figure 1 presents calculations indicating the number of cows required to make a normal (28390 L or 7500 gallon) tanker truck load of milk test positive for SOH residues at the 2 ng g⁻¹ tolerance limit when excreting SOH in their milk at a concentration of 114.5 ng g⁻¹.

In this scenario, a tanker truck load of milk may test positive for SOH residues when 14 cows administered FLU at the labelled dose are milked into the bulk tank at 12 and 24 h after FLU administration instead of observing the label 36-h milk withhold time. This is just one scenario of how individual cows cause a tanker truck to test positive. Antibiotic drugs such as FLU are reported to be the second most prescribed class of drugs by dairy veterinarians (Sundlof et al. 1995) and the most frequently administered analgesic in cattle (Fajt et al. 2011). Because FLU is the only approved NSAID for use in dairy cattle in the United States, it is regularly used on dairy farms and treatment of 14 or more cows at any one time is not uncommon. Thus, the potential for FLU residues in milk tanker truck loads is plausible and warrants further investigation to identify the relative risk. FLU is not currently one of the residue tests that is routinely performed in the United States at milk processing plants, according to the National Milk Drug Residue Data Base annual reports for the past 10 years. This may be partially due to previous literature, which suggested that FLU did not distribute into milk following intravenous or intramuscular administration, and therefore residues are not a concern (Benitz 1984; Anderson et al. 1990). However, these studies were performed more than 10 years prior to the identification of the maker residue, SOH, therefore only FLU was measured in milk in the literature aforementioned. In addition, the fact that FLU was not detected in milk in the study performed by Anderson et al. (1990) may reflect the sensitivity of the assay as the LOD was 50 ng g⁻¹. Newer research using more sensitive analytical equipment clearly demonstrates that SOH and FLU accumulate in milk at concentrations well above the tolerance limit (Feely et al. 2002; Kissell et al. 2012).

\[
\text{Volume of milk containing 114.5 ng g}^{-1} \text{ to test positive for SOH at tolerance level of 2 ng g}^{-1} = \frac{(28390 \text{ litres of milk}) \times (2 \text{ ng g}^{-1})}{(114.5 \text{ ng g}^{-1} \text{ of SOH in the milk/cow/day})} = 495.9 \text{ litres of milk}
\]

\[
\text{Estimated number of cows treated with flunixin} = \frac{(495.9 \text{ litres of milk})}{(38 \text{ litres of milk/cow/day})} = 14 \text{ cows}
\]

Figure 1. Presents calculations indicating the number of cows required to make a normal (28390 L or 7500 gallon) tanker truck load of milk test positive for SOH residues at the 2 ng g⁻¹ tolerance limit when excreting SOH in their milk at a concentration of 114.5 ng g⁻¹.
Conclusions
Although this study only sampled a small fraction of milk delivered to processing plants, it demonstrates that illegal FLU milk residues may occur in the dairy industry and may necessitate future FLU milk testing at dairy processing plants using a rapid assay. Therefore, emphasis on observing the appropriate drug withdrawal time, route of administration and labeled dosage is critical to the prevention of residue violations.

Acknowledgements
The authors would like to express their appreciation to the Dairy Farmers of America for providing them with the tanker load milk samples.

References

4. PLASMA PHARMACOKINETICS AND MILK RESIDUES OF FLUNIXIN AND 5-HYDROXY FLUNIXIN FOLLOWING DIFFERENT ROUTES OF ADMINISTRATION IN DAIRY COWS

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Plasma pharmacokinetics and milk residues of flunixin and 5-hydroxy flunixin following different routes of administration in dairy cattle

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ABSTRACT

The objective of this study was to determine if the plasma pharmacokinetics and milk elimination of flunixin (FLU) and 5-hydroxy flunixin (SOH) differ following intramuscular and subcutaneous injection of FLU compared with intravenous injection. Twelve lactating Holstein cows were used in a randomised crossover design study. Cows were organized into 2 groups based on milk production (<20 or >30 kg of milk/d). All cattle were administered 2 doses of 1.1 mg of FLU/kg at 12-h intervals by intravenous, intramuscular, and subcutaneous injections. The washout period between routes of administration was 7 d. Blood samples were collected from the jugular vein before FLU administration and at various time points up to 36 h after the first dose of FLU. Composite milk samples were collected before FLU administration and twice daily for 5 d after the first dose of FLU. Samples were analyzed by ultrahigh-performance liquid chromatography with mass spectrometry detection. For FLU plasma samples, a difference in terminal half-life was observed among routes of administration. Harmonic mean terminal half-lives for FLU were 3.42, 4.48, and 5.30 h for intravenous, intramuscular, and subcutaneous injection, respectively. The mean bioavailability following intramuscular and subcutaneous dosing was 84.5 and 104.2%, respectively. The decrease in SOH milk concentration versus time after last dose was analyzed with the nonlinear mixed effects modeling approach and indicated that both the route of administration and rate of milk production were significant covariates. The number of milk samples greater than the tolerance limit for each route of administration was also compared at each time point for statistical significance. Forty-eight hours after the first dose, SOH milk concentrations were undetectable in all intravenously injected cows; however, one intramuscularly injected and one subcutaneously injected cow had measurable concentrations. These cows had SOH concentrations above the tolerance limit at the 36-h withdrawal time. The high number of FLU residues identified in all dairy cows by the United States Department of Agriculture Food Safety Inspection Service is likely related to administration of the drug by an unapproved route. Cattle that received FLU by the approved (intravenous) route consistently eliminated the drug before the approved withdrawal times; however, residues can persist beyond these approved times following intramuscular or subcutaneous administration. Cows producing less than 20 kg of milk/d had altered FLU milk clearance, which may also contribute to violative FLU residues.

Key words: flunixin, residues, pharmacology

INTRODUCTION

Flunixin (FLU) is a nonsteroidal antiinflammatory drug (NSAID) licensed for use in beef and dairy cattle for modulation of inflammation in endotoxemia and for the control of pyrexia associated with bovine respiratory disease and acute bovine mastitis. Flunixin is labeled for intravenous administration at a dose of 2.2 mg/kg every 24 h or 1.1 mg/kg every 12 h. The slaughter withdrawal time is 4 d following the last injection and the milk withdrawal time is 36 h. Although FLU is only approved for intravenous administration, intramuscular and subcutaneous administrations are common routes of extra-label drug use in dairy cattle due to their ease of administration (FDA, 2004). However, altering the route of administration for convenience is not an appropriate reason for extra-label drug use according to the Animal Medicinal Drug Use Clarification Act (AMDUCA; FDA, 1996). Because intramuscular and subcutaneous administration of FLU are not approved in the United States, milk-withholding times have not been established. In addition, limited data is available that describes the pharmacokinetics of FLU after intramuscular or subcutaneous administration in cattle, which is necessary to provide guidance on withdrawal times following intramuscular and subcutaneous administration.

Altering the route of administration, formulation, and dose can affect the rate of elimination of a drug,
resulting in violative residues (KuKanich et al., 2005; Gehring et al., 2006). The United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) Red Book from 2005 through 2010 reported an increasing number of residue violations in meat from dairy cattle (USDA-FSIS, 2005–2010). In the last 5 yr, FLU has become the second most common residue violation behind penicillin in culled dairy cattle (USDA-FSIS, 2005–2010). Currently, milk is not tested for 5-hydroxy FLU (5OH), the marker residue for FLU in milk. However, in December 2011, the FDA announced plans to collect and test milk samples across the United States for 5OH residues. Because a significant number of FLU tissue residue violations are found in culled dairy cows, a concern exists that the same practices that lead to tissue residues might also be leading to drug residues in milk. This increase in violative FLU residues may be associated with administration of this drug by an extra-label route. The administration of FLU by intramuscular or subcutaneous routes may prolong drug elimination and result in milk concentrations of 5OH to be greater than the tolerance level of 2 μg/kg after 36 hr. Flunixin is metabolized to 5OH in the liver and is excreted in both urine and feces; however, 0.002 to 0.0124% of the administered dose is excreted as 5OH in milk (Lichtenwalner et al., 1986; FDA, 2004). Our hypothesis was that if FLU is administered via an extra-label route, then the plasma half-life and milk residues following intramuscular or subcutaneous injection may persist longer than the plasma half-life and milk residues following intravenous administration. Therefore, the primary objective of this study was to examine the plasma pharmacokinetics and milk residues of FLU and 5OH in lactating dairy cattle following administration by intravenous, intramuscular, and subcutaneous routes.

**MATERIALS AND METHODS**

This study was approved by the North Carolina State University (Raleigh) Institutional Animal Care and Use Committee.

**Animals**

Twelve lactating Holstein cows weighing between 545 and 670 kg were used in a randomized crossover design study. Cows were organized into 2 groups based on milk production. Group A consisted of 6 cows producing less than 20 kg of milk per day. Group B consisted of 6 cows producing greater than 30 kg of milk per day. Prior to the start of the trial, intravenous catheters were aseptically placed in the jugular vein.

**Experimental Design**

Flunixin was administered intravenously, intramuscularly, and subcutaneously in a randomized crossover design at a dose of 1.1 mg/kg, given in 2 doses at a 12-h interval. After 7-d washout periods, each group received FLU via each of the other routes (intravenous, intramuscular, or subcutaneous), so that by the study completion, each of the 12 cows had received FLU intravenously, intramuscularly, and subcutaneously. For the intravenous group, FLU was administered into the contralateral jugular vein to which the jugular catheter had been placed. All intramuscular and subcutaneous injections were given in the neck.

**Blood and Milk Sampling**

Blood samples were collected from the jugular catheter into heparinized tubes before FLU administration and at 0.25, 0.5, 1, 2, 4, 8, and 12 hr after the first dose of FLU. Blood samples were also collected 12 and 24 hr after the second dose of FLU. Blood samples were centrifuged at 1,690 × g for 10 min at −15°C; plasma was collected and frozen at −20°C until analysis of plasma FLU and metabolite concentrations. Prior to FLU administration, 5 mL of foremilk was manually collected from each quarter of every cow. Composite milk samples were collected using a Metatron sampler (Westfalia Surgo Inc., Naperville, IL). Composite milk samples (50 mL) were collected at 1.5 and 12 hr after the first dose of FLU and 12, 24, 36, 48, 72, 84, and 96 hr after the second dose of FLU. Milk samples were immediately frozen at −20°C until analysis.

**Sample Measurements**

Flunixin and 5OH concentrations were quantified by ultra-HPLC with mass spectrometric detection. For plasma sample extraction, plasma samples were thawed, and 0.3 mL of plasma was combined with 0.9 mL of 0.5% citric acid in acetonitrile. Samples were sonicated for 5 min and then centrifuged for 10 min at 3,500 × g. The supernatant was loaded on a Supelco Hybrid SPE-phospholipid cartridge (Sigma-Aldrich, St. Louis, MO). The eluate from the cartridge was collected and placed in a 55°C TurboVap LV evaporator (Zymark Corp., Hopkinton, MA) to dryness under a 20-psi (137.9-kPa) stream of nitrogen, reconstituted in 300 μL of mobile phase, and filtered through a 0.22-μm nylon syringe filter. Injection volume was 5 μL. Concentrations were derived by comparing peak areas of the samples to those of an external standard curve made from spiked plasma samples put through the
sample cleanup process. For FLU and 5OH milk extraction, 0.5 mL of milk and 1.5 mL of 0.5% citric acid in acetonitrile were combined in a centrifuge tube, and the same process described previously for plasma was used for extraction and quantification.

The Acquity ultra performance liquid chromatography (UPLC)-MS system (Waters Corp., Milford, MA) consisted of an HSS T3 column (1.8-µm particle size, 2.1 × 100 mm) and filter disc. The mobile phase was acetonitrile:0.1% acetic acid in water (65:35 vol/vol). The evaporative mass detector (EMD) 100 was a single quadrupole mass spectrometer run in positive electrospray ionization (ESI+) mode. Ions with mass-to-charge ratios of 297.0 and 313.0 were used for quantification of FLU and 5OH, respectively. The column temperature was 30°C and sample temperature was 4°C. Run times were 2.2 min. The limit of quantification (LOQ) was determined as 10 times the standard deviation of 6 blank samples. The limit of detection (LOD) was determined as 3 times the standard deviation of 6 blank samples. The LOD and LOQ for FLU and 5OH in plasma were 0.01 and 0.02 µg/mL, respectively, and the linear range was from 0.02 to 20 µg/mL. The LOD and LOQ for FLU and 5OH in milk were 0.001 and 0.002 µg/mL, respectively, and linear range for milk was 0.002 to 1 µg/mL. Relative standard deviations for both interday and intraday were <15% at all concentrations.

Analysis

A noncompartmental analysis of FLU and 5OH plasma concentration versus time profiles was performed with Phoenix pharmacokinetic modeling software (Pharsight Corp., St Louis, MO). For all routes of administration, the area under the plasma concentration-time curve from time zero to infinity (AUC0-∞) and the area under the first moment curve (AUMC) were calculated by the linear trapezoidal rule for the first dose only. The rate constant (λ1), associated with the terminal elimination phase, was estimated by means of linear regression of the terminal phase of the log concentration versus time profile, and the corresponding terminal half-life (t1/2,λ1) for intravenous, intramuscular, and subcutaneous routes of administration were calculated. The rate constant also was used to extrapolate AUC0-∞ and AUMC from the time of the last observed concentration to infinity for all routes of administration. For intravenous administration, the volume of distribution at steady state (Vdss) and volume of distribution for the terminal elimination phase (Vdτ,τ) were calculated. The AUC0-∞ and AUMC were used to calculate clearance (CL), and mean residence time for intravenous administration. For extravascular routes of administration, AUC0-∞ and AUMC were used to calculate mean transit time (MTT), mean absorption time (MAT), and bioavailability (F).

Statistical Analysis

All values are expressed as mean ± standard deviation, with the exception of t1/2,λ1, which is expressed as the harmonic mean ± standard deviation. Pharmacokinetic parameters for FLU and 5OH in plasma, and 5OH milk concentrations were compared by a one-way ANOVA with the Tukey test, where P < 0.05 was considered statistically significant. Mean transit times, MAT, and F were compared using a Student's t-test, where P < 0.05 was considered statistically significant. For comparison of the number of 5OH milk samples greater than the 2 µg/kg tolerance limit following intravenous, intramuscular, and subcutaneous administration, the differences in frequency of detectable concentrations at each time point among the routes of administration were evaluated with the Cochran Q test. For the test, the milk concentrations of 5OH at each time point were coded as 0 for undetectable and 1 for greater than the tolerance limit. The statistical analyses were performed with SAS software (SAS Institute Inc., Cary, NC). The decrease in 5OH milk concentration versus time after last dose was analyzed with the nonlinear mixed-effects modeling approach as implemented with Phoenix (Pharsight Corp.) to determine if variability in the rate was significantly affected by either route of administration or rate of milk production.

RESULTS AND DISCUSSION

Plasma

The objective of this study was to determine if the plasma pharmacokinetics and milk elimination of FLU and 5OH in lactating dairy cattle would differ following intramuscular and subcutaneous injection of FLU as compared with intravenous administration. Table 1 presents the plasma pharmacokinetic parameters after intravenous, intramuscular, and subcutaneous dosing. Following intravenous administration, mean FLU plasma concentrations decreased from 8.8 to ≤0.1 µg/mL by 12 h after the first dose (Figure 1). Following intramuscular administration, the mean observed peak plasma concentration (Cmax) was 2.2 ± 0.56 µg/mL, and the observed time to maximum concentration (Tmax) occurred 0.25 to 0.5 h after injection. Following subcutaneous administration, Cmax was 1.33 ± 0.65 µg/mL and Tmax was observed 0.25 to 2 h after dosing. Twenty-four hours after the second dose, 50% of the cows that received FLU intramuscularly or subcutaneously had detectable FLU plasma concentrations.
however, FLU was not detected in the plasma of any of the cows 24 h after intravenous administration (data not shown).

A difference in the terminal \( t_{1/2A} \) for FLU was observed among routes of administration. The \( t_{1/2A} \) of FLU in plasma following subcutaneous administration was significantly longer (\( P < 0.05 \)) compared with the \( t_{1/2A} \) following intravenous administration. The prolonged \( t_{1/2A} \) observed following subcutaneous administration may be due to delayed absorption affecting the terminal phase. Because the last time point sampled was 12 h after injection, a greater likelihood exists of seeing an effect on absorption. The \( t_{1/2A} \) in the current study was also shorter than the \( t_{1/2A} \) (7.46 h) reported by Lacroix et al. (2011) following subcutaneous administration of FLU. This difference may be attributed to a shorter sample-collection period in the current study (12 h after dosing) compared with the 60 h post-dosing sample time of the Lacroix et al. (2011) study. The MTT and MAT did not differ significantly (\( P = 0.08 \) and \( P = 0.09 \)) across routes of administration in the current study; however, significant differences in absorption between intramuscular and subcutaneous routes has been reported in the literature (FDA, 2010). One limitation of the current study was that a large percentage (up to 32%) of AUC\(_{0-\infty} \) was extrapolated for several of the cows receiving FLU by intramuscular and subcutaneous administration. Because AUC\(_{0-\infty} \) was used to calculate MTT, MAT, and \( P \), a greater degree of variability exists in those parameters. This limitation was unavoidable because giving the entire dose of FLU (2.2 mg/kg) in a single injection may have resulted in tissue necrosis from intramuscular administration (Pyörälä et al., 1999; Smith et al., 2008); therefore, the dose was divided into 2 doses of 1.1 mg/kg and samples could only be collected up to 12 h after the first dose before administration of the second dose. In the present study, the mean plasma \( t_{1/2A} \) for FLU after intravenous dosing was 3.42 h, which corresponds with that found in previous studies (Benita, 1984; Anderson et al., 1990; Odensvik and Johansson, 1995; Jaraczewski et al., 2008; Abo-El-Soud and Al-Anani, 2011; Wu et al., 2012). However, other studies have reported \( t_{1/2A} \) (5.2-8.12 h) much longer than the 3.42 h \( t_{1/2A} \) in the current study (Hardee et al., 1985; Landoni et al., 1995; Odenvik, 1995; Rantala et al., 2002). This variability may be

### Table 1. Flunixin plasma pharmacokinetic parameters after intravenous, intramuscular, and subcutaneous dosing

<table>
<thead>
<tr>
<th>Parameter</th>
<th>i.v.</th>
<th>i.m.</th>
<th>s.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_{1/2A} ) (h)</td>
<td>3.42 ± 0.08*</td>
<td>4.26 ± 1.77**</td>
<td>5.59 ± 2.47**</td>
</tr>
<tr>
<td>( \lambda ) (h⁻¹)</td>
<td>0.216 ± 0.09**</td>
<td>0.175 ± 0.09**</td>
<td>0.151 ± 0.06**</td>
</tr>
<tr>
<td>AUC(_{0-\infty} ) (µg.h/mL)</td>
<td>7.29 ± 2.72</td>
<td>7.36 ± 3.3</td>
<td>8.29 ± 4.98</td>
</tr>
<tr>
<td>AUC(_{0-h} ) (µg.h/mL)</td>
<td>7.63 ± 2.66</td>
<td>6.37 ± 2.93</td>
<td>6.30 ± 2.34</td>
</tr>
<tr>
<td>CL (L/h per kilogram)</td>
<td>150.6 ± 43.1</td>
<td>143.6 ± 8.2</td>
<td>140.5 ± 5.4</td>
</tr>
<tr>
<td>( V_d ) (L/kg)</td>
<td>0.254 ± 0.14</td>
<td>0.244 ± 0.143</td>
<td>0.244 ± 0.143</td>
</tr>
<tr>
<td>( V_{ss} ) (L/kg)</td>
<td>0.78 ± 0.391</td>
<td>0.78 ± 0.391</td>
<td>0.78 ± 0.391</td>
</tr>
<tr>
<td>MTT* (h)</td>
<td>2.80 ± 0.89</td>
<td>5.51 ± 2.57</td>
<td>7.58 ± 3.44</td>
</tr>
<tr>
<td>MAT* (h)</td>
<td>2.71 ± 2.54</td>
<td>4.78 ± 3.17</td>
<td>8.45 ± 2.89</td>
</tr>
<tr>
<td>P (%)</td>
<td>104.2 ± 37.2</td>
<td>104.2 ± 37.2</td>
<td>104.2 ± 37.2</td>
</tr>
</tbody>
</table>

*Means within a row with different superscripts differ (\( P < 0.05 \)).

\( t_{1/2A} \) = harmonic mean elimination half-life; \( \lambda \) = rate constant associated with the terminal elimination phase; AUC\(_{0-\infty} \) = total area under the curve; AUC\(_{0-h} \) = area under the curve from 0 to the last observed concentration; CL = clearance; \( V_d \) = volume of distribution at steady state; \( V_{ss} \) = volume of distribution in terminal elimination phase; MTT = mean transit time; MAT = mean absorption time; P = bioavailability. All results are expressed as mean ± SD, except \( t_{1/2A} \), which is expressed as harmonic mean ± SD.

*Mean residence time for i.v. administration.

*\( P < 0.05 \).

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explained by the time points used for determination of $t_{1/2\text{MA}}$. In a study by Landoni et al. (1995), time points up to 36 h were used to calculate $t_{1/2\text{MA}}$, resulting in a $t_{1/2\text{MA}}$ of 6.87 h. In the current study, mean AUC$_{0-\infty}$ following intravenous dosing at 1.1 mg/kg was half the AUC$_{0-\infty}$ values reported in the literature, in which 2.2 mg/kg was administered intravenously (Anderson et al., 1990; Odenstvik and Johansson, 1995). The CL calculated in the current study is similar with findings in the literature (Hardoe et al., 1985; Anderson et al., 1990; Landoni et al., 1995; Odenstvik, 1995; Odenstvik and Johansson, 1995; Rantala et al., 2002). The Vd$_{ma}$ and Vd$_{u}$ in the current study were greater than expected for a drug that is highly (99%) protein bound; however, the values were consistent with the literature (Hardoe et al., 1985; Anderson et al., 1990; Landoni et al., 1995; Odenstvik, 1995; Odenstvik and Johansson, 1995; FDA, 1998; Rantala et al., 2002). Both Vd$_{ma}$ and Vd$_{u}$ were at the lower end of the range of values reported in the literature because of the large percentage of AUC$_{0-\infty}$ that was extrapolated in the current study. Multiple studies have described FLU with a multi-compartment pharmacokinetic model and have reported peripheral distribution despite high protein binding of FLU (Anderson et al., 1990; Odenstvik and Johansson, 1990; Buur et al., 2006).

The mean F following intramuscular and subcutaneous dosing was 84.5 and 104.2%, respectively. Variability in F was noted in this study and may again be due to the large percentage of AUC$_{0-\infty}$ that was extrapolated. However, the F reported in the current study for intramuscular administration corresponds to the F (79%) reported by Anderson et al. (1990) in cattle and F (97%) reported by Königsson et al. (2003) in goats.

Table 2 presents SOH plasma $t_{1/2\text{MA}}$ following intravenous, intramuscular, and subcutaneous dosing. Regardless of route of administration, plasma SOH concentrations were greatest between 0.25 to 0.5 h after FLU administration. A significant difference between plasma FLU and SOH $t_{1/2\text{MA}}$ was noted, with SOH $t_{1/2\text{MA}}$ being shorter than FLU $t_{1/2\text{MA}}$ following intravenous administration of FLU. The shorter $t_{1/2\text{MA}}$ for SOH, compared with the $t_{1/2\text{MA}}$ for FLU, was most likely due to an inadequate concentration-versus-time profile for SOH in plasma caused by samples being below the LOD within 4 h after the first dose and, thus, the $t_{1/2\text{MA}}$ reflected the distribution phase rather than the terminal elimination phase (Bonate and Howard, 2004).}

### Milk

The nonlinear mixed effects modeling of the decrease in SOH milk concentrations over time indicated that both the route of administration ($P < 0.001$) and rate of milk production ($P < 0.1$) were significant covariates. In this study, cows producing less than 20 kg of milk/d eliminated SOH slower than cows producing greater than 30 kg of milk/d. Research describing the effect of milk production on drug elimination is limited, especially for systemically administered drugs. However, several studies have shown a correlation between low milk production and prolonged drug elimination for some intramuscular drugs (Mercer et al., 1976; Whittem, 1990; Smith et al., 2004; Gehring and Smith, 2006; Stockler et al., 2009). One study with 21 different commercially available Intramuscular products found that cows producing less than 9 kg of milk/d were more likely to have prolonged withholding times than higher-producing cows (Mercer et al., 1970). However, level of milk production may only partially explain variations in excretion rates as several high-producing cows were reported to have slow drug elimination (Mercer et al., 1970; Lainese et al., 2012). Table 3 presents SOH milk concentrations and the number of cows with milk concentrations greater than the tolerance limit of 2 μg/kg following intravenous, intramuscular, and subcutaneous administration at various sampling times. At each time point, SOH milk concentrations were compared by route of administration. Mean SOH milk concentrations 1.5 h after injection for intravenous, intramuscular, and subcutaneous injections were 0.044, 0.029, and 0.02 μg/mL, respectively, indicating that FLU was rapidly metabolized to SOH and entered the milk shortly after FLU administration. Following intramuscular and subcutaneous FLU administration, mean SOH milk concentrations at 1.5 h were significantly less than SOH milk concentrations following intravenous FLU administration. The difference in mean SOH milk concentrations between intravenous and extravascular routes can be attributed to the lower plasma concentrations, which resulted from differences in absorption of FLU. Differences ($P < 0.05$) in SOH milk concentrations were also detected 12 h after the first dose, but not 12 h after the second dose. The number of milk samples where SOH could be detected above a con-

### Table 2: 5-Hydroxy-5-Fluorotryptamine plasma elimination half-life ($t_{1/2\text{MA}}$, harmonic mean ± SD) after intravenous, intramuscular, and subcutaneous dosing.

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>i.v.</th>
<th>i.m.</th>
<th>s.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2\text{MA}}$ (h)</td>
<td>1.44 ± 0.041$^a$</td>
<td>6.0 ± 0.51$^b$</td>
<td>7.01 ± 2.22$^a$</td>
</tr>
</tbody>
</table>

$^a$Means within a row with different superscripts differ ($P < 0.05$).
Table 3. Milk concentrations (μg/mL) of 5-hydroxy fumisin (mean ± SD) and number of positive samples (samples greater than 2 μg/kg) following intravenous, intramuscular, and subcutaneous administration of fumisin

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Concentration</th>
<th>Number of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>l.v.</td>
<td>l.m.</td>
</tr>
<tr>
<td>1.5</td>
<td>0.044 ± 0.02A</td>
<td>0.021 ± 0.021B</td>
</tr>
<tr>
<td>12</td>
<td>0.020 ± 0.008A</td>
<td>0.020 ± 0.018B</td>
</tr>
<tr>
<td>36</td>
<td>0.005 ± 0.008B</td>
<td>0.004 ± 0.003B</td>
</tr>
<tr>
<td>48</td>
<td>0.000 ± 0.002B</td>
<td>0.002 ± 0.002B</td>
</tr>
</tbody>
</table>

°Means within a row with different superscripts differ (P < 0.05).

CONCLUSIONS

The high number of FLU residues identified in culled dairy cows by the USDA-FSIS is likely related to administration of the drug by an unapproved route. Cattle that received FLU by the approved route (intravenous) consistently eliminated the drug before the approved withdrawal times; however, residues can persist beyond these approved times following intramuscular or subcutaneous drug administration. Education of veterinarians and farm personnel in proper drug administration is critical in the prevention of milk residue violations.

ACKNOWLEDGMENTS

Funding for this project was supported in part by the Food Animal Residue Avoidance and Depletion Program (Raleigh, NC).

REFERENCES


5. COMPARISON OF FLUNIXIN PHARMACOKINETICS AND MILK ELIMINATION IN HEALTHY COWS AND COWS WITH MASTITIS.
Abstract

Objective-To determine if the pharmacokinetics and milk elimination of flunixin and 5-hydroxy flunixin (5OH) differed between healthy and mastitic cows.

Design- Prospective clinical trial

Animals- 20 lactating Holstein cows (10 cows with naturally occurring mastitis and 10 healthy paired controls)

Procedures- Cows with clinical mastitis and matched controls received flunixin intravenously, ceftiofur intramuscularly and an intramammary antibiotic (cephapirin or ceftiofur). Blood samples were collected at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 36 hours after flunixin administration. Composite milk samples were collected at 0, 2, 12, 24, 36, 48, 60, 72, 84, and 96 hours after flunixin administration. Plasma and milk samples were analyzed by ultra-high-pressure liquid chromatography with mass spectrometric detection.

Results- For flunixin plasma samples, differences in AUC\textsubscript{0\rightarrow\infty}, clearance and mean residence time were observed between groups. Significant differences in flunixin and 5OH milk concentrations were observed at various time points. At the 36-hour milk withdrawal time, 8 cows with mastitis had 5OH residues greater than the tolerance limit. Flunixin residues persisted in the milk up to 60 hours post administration for 3 out 10 of the mastitic cows.

Conclusions and Clinical Relevance- The pharmacokinetics and elimination of flunixin and 5OH in milk from diseased cows differed from healthy cows resulting in violative drug residues. This may partially explain the high number of violative flunixin residues that have been reported in beef and dairy cattle. This study also raises questions as to whether determining meat and milk withdrawal times in healthy animals are appropriate.
**Abbreviations**

- **5OH**: 5-hydroxy flunixin
- **AUC\(_{0-\infty}\)**: Area under the plasma concentration-time curve from time zero to infinity
- **AUMC**: Area under the first moment curve
- **LOD**: Limit of detection
- **LOQ**: Limit of quantification
- **MRT**: Mean residence time
- **NSAID**: Non-steroidal anti-inflammatory drugs
- **t\(_{1/2\lambda z}\)**: Terminal elimination half-life
- **V\(_{dss}\)**: Volume of distribution at steady state
- **V\(_{darea}\)**: Volume of distribution for the terminal elimination phase

Flunixin is a NSAID approved for use in beef and dairy cattle for the modulation of inflammation in endotoxemia and for the control of pyrexia associated with bovine respiratory disease and acute bovine mastitis. When administered according to the label instructions the milk withdrawal time is 36 hours. In milk the marker residue is a metabolite of flunixin, called 5OH and the tolerance for 5OH in milk is 2 ppb. Non-steroidal anti-inflammatory drugs such as flunixin are reported to be the second most prescribed class of drugs by dairy veterinarians\(^1\) and the most frequently administered analgesic in cattle.\(^2\)
Because flunixin is the only approved NSAID for use in dairy cattle in the United States and is routinely used on dairy farms, there is a significant potential for violative residues. Although flunixin is regularly used on dairy farms, milk samples in the United States are not routinely tested for 5OH residues.³ This may be partially due to older literature, which suggested that flunixin residues were not a concern, since residues were not detected in milk following IV or IM administration.⁴⁻⁵ However, more recent research with more sensitive analytical equipment clearly demonstrated that 5OH and flunixin accumulate in milk at concentrations well above the tolerance limit.⁶⁻⁷

A recent flunixin surveillance study which sampled 500 tanker truck loads of milk found 1 out the 500 milk samples was positive for 5OH above the tolerance limit.⁸ This study demonstrated that illegal flunixin milk residues do occur in the dairy industry, although the cause of these illegal residues remains unknown. Some possible explanations for violative flunixin milk residues are: (1) not observing the milk withdrawal time (2) extralabel use of flunixin; such as altering the dose, route of administration, frequency and duration of treatment and (3) altered milk elimination as a result of a disease process. Several studies have reported alterations in plasma pharmacokinetics of drugs in diseased animals when compared with their healthy counterparts.⁹⁻¹⁸ A recently published study found a correlation between low milk production and prolonged flunixin milk elimination.⁷ Since mastitis often results in a significant drop in milk production¹⁹ violative flunixin residues may be prevalent in milk from mastitic cows. Since 2005, the United States Department of Agriculture-Food Safety Inspection Service has reported an increasing number of flunixin residue violations in meat from dairy cattle.²⁰ This increase in the number of flunixin violations has led to
flunixin becoming the second most common residue violation behind penicillin in cull dairy cattle.\textsuperscript{20} These residue violations in meat have primarily been attributed to extralabel use of flunixin.\textsuperscript{21} However, delayed plasma clearance due to a disease process may result in prolonged residues in meat and contribute to the high number of flunixin tissue violations. Therefore the objective of this study was to determine if the plasma pharmacokinetics and milk elimination of flunixin and 5OH differed between healthy cows and cows with clinical mastitis.

**MATERIAL AND METHODS**

**Animals**-This study was approved by the North Carolina State University Institutional Animal Care and Use Committee. Twenty lactating Holstein cows from a single dairy in North Carolina, weighing between 545 and 676 kg, were used in a case control design study. Ten cows were identified having naturally occurring mastitis as defined by the abnormal appearance of milk, with a red or swollen mammary gland. Some of the cows had systemic signs of diseases while others did not. Ten healthy cows were then matched to the cows with mastitis using parity, days in milk and milk production to serve as control cows. Within 2 hours of the cow being diagnosed with clinical mastitis, an intravenous catheter was aseptically placed in the jugular vein. Heart rate and rectal temperature were recorded for each cow and 5 mL of milk was aseptically collected from each mastitic quarter for aerobic culture prior to treatment.

**Experimental design**- One dose of flunixin\textsuperscript{a} was administered intravenously in the contralateral jugular vein to which the catheter had been placed at a dose of 2.2 mg/kg to all cows. All cows also received ceftiofur\textsuperscript{b} at a dose of 1 mg/lb every 24 hours for 3 days. For
intramammary antibiotic treatment, cows were divided into two groups. Groups 1 and 2 each consisted of 10 cows: 5 cows with mastitis and 5 paired controls. Group 1 cows received intramammary cepha
drin 1 syringe (10 mL) every 12 hours for 3 days. Group 2 cows received intramammary cef
tiofur 1 syringe (10 mL) every 24 hours for 3 days.

**Blood and Milk sampling**- Blood samples were collected from the jugular catheter in heparinized tubes prior to flunixin administration and at 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 36 hours after administration. Blood samples were centrifuged at 1690 \( \times g \) for 10 minutes at a temperature of 4°C; plasma was harvested and frozen at -20°C until analysis of flunixin and metabolite concentrations. Prior to flunixin administration, 5 mL of foremilk was manually collected from each quarter of every cow to serve as baseline composite sample. Composite milk samples were collected during milking with a sampling device. Composite milk samples were collected at 2, 12, 24, 36, 48, 72, 84, and 96 hours after flunixin administration. Milk samples were immediately frozen at -20°C until analysis.

**Sample measurements**- Flunixin and 5OH concentrations were quantified in both plasma and milk by ultra-high-pressure liquid chromatography with mass spectrometric detection. For plasma sample extraction, plasma samples were thawed, and 0.3 ml of plasma was combined with 0.9 ml of 0.5% citric acid in acetonitrile. Samples were sonicated for 5 minutes and then centrifuged for 10 minutes at 3500 \( \times g \). The supernatant was loaded on a solid phase extraction cartridge. The eluate from the cartridge was collected and placed in a 55°C evaporator to dryness under a 20 psi stream of nitrogen, reconstituted in 300 \( \mu L \) of mobile phase and filtered through a 0.22-\( \mu m \) nylon syringe filter. Injection volume was 5 \( \mu L \). Concentrations were derived by comparing peak areas of the samples to those of an
external standard curve made from spiked plasma samples put through the sample cleanup process. For flunixin and 5OH milk extraction, 0.5 mL of milk and 1.5 mL of 0.5% citric acid in acetonitrile were combined in a centrifuge tube, and the same process described previously for plasma was used for extraction and quantification.

The ultra-high-pressure liquid chromatography with mass spectrometric detection consisted of a HSS T3 column (1.8 um, 2.1 X 100mm) and filter disc. The mobile phase was acetonitrile: 0.1% acetic acid in water (68:32 v/v). The EMD 100 was a single quadrupole mass spectrometer run in ESI+ mode. Ions with mass-to-charge ratios of 297.0 and 313.0 were used for quantification of FLU and 5OH, respectively. Column temperature was 30°C and sample temperature was 4°C. Run times were 2.2 minutes. The LOQ was determined as 10 times the standard deviation of 6 blank samples. The LOD was determined as 3 times the standard deviation of 6 blank samples. The LOD and LOQ for flunixin and 5OH in plasma were 0.01 µg/mL and 0.02 µg/mL, respectively, and the linear range was 0.02 to 30 µg/mL. The LOD and LOQ for flunixin and 5OH in milk were 0.001 µg/mL and 0.002 µg/mL, respectively, and linear range for milk was 0.002 to 1 µg/mL. Relative standard deviations for both inter-day and intra-day were < 15% at all concentrations.

**Pharmacokinetic analysis** - A non-compartmental analysis of flunixin plasma concentration vs. time profiles was performed with pharmacokinetic modeling software. The AUC_{0-\infty} and the AUMC were calculated by the linear trapezoidal rule. The AUC_{0-\infty} and AUMC were used to calculate clearance, and MRT. The rate constant, lambda z, associated with the terminal elimination phase, was estimated by means of linear regression of the terminal phase of the log concentration vs. time profile and used to calculate the corresponding t_{1/2z} for
control and mastitic cows. Lambda z also was used to extrapolate AUC\(_{0,\infty}\) and AUMC from the time of the last observed concentration to infinity. Lastly, V\(_{dss}\) and V\(_{darea}\) were calculated for flunixin.

**Statistical analysis** - The statistical analyses were performed using software. All values are expressed as mean ± standard deviation, with the exception of t\(_{1/2z}\), which is expressed as the harmonic mean ± standard deviation. Pharmacokinetic parameters for flunixin in plasma were compared by a Student’s t-test, where \(P < 0.05\) was considered statistically significant. Flunixin and 5OH milk concentrations were compared using a repeated measures ANOVA where \(P < 0.05\) was considered statistically significant.

**RESULTS**

**Cows** - Ten cows with clinical mastitis were identified and enrolled on the trial between May and August of 2012 along with 10 cows without clinical mastitis (Table 1). Mean heart rate of the mastitic cows was significantly different from the control; however, no difference in rectal temperature was noted between the two groups (Table 1). Milk culture results from 8 of the 10 mastitic cows indicated a *Klebsiella pneumoniae* infection, and the remaining 2 cows had an *Escherichia coli* infection.

**Plasma** - Following flunixin administration the mean observed peak plasma concentration was 18.35 ± 5.40 μg/mL and 24.45 ± 8.22 μg/mL for control cows and cows with mastitis, respectively. Flunixin was present in the plasma of cows with mastitis up to 24 hours after administration, which is 12 hours longer than flunixin was present in the plasma of healthy cows (Figure 1). The clearance for the cows with mastitis was significantly reduced compared to clearance for the healthy cows (Table 2). The AUC\(_{0,\infty}\) for the mastitic cows
was more than twice the AUC\(_{0-\infty}\) for the control cows and was significantly different between the two groups (Table 2). Likewise, the MRT differed significantly, with the MRT of the mastitic cows being longer compared to the control cows. Both the plasma t\(_{1/2λz}\), V\(_{\text{area}}\) and V\(_{\text{ss}}\) were not significantly different between the healthy and diseased cows.

**Milk residues**—Milk residues of 5OH above the tolerance limit were present in milk samples from 8 of the mastitic cows at the labeled withdrawal time (36 hours post dose) and up to 48 hours post dose in 3 of the mastitic cows (Table 3). However, no 5OH residues above the tolerance limit were present in the milk of the control cows longer than 24 hours after flunixin administration (Figure 2). Milk concentrations of 5OH were significantly different between control cows and cows with mastitis for both the 2 and 12 hour samples, with the mastitic cows having lower 5OH concentrations in their milk at both time points (Table 3).

Flunixin residues persisted in the milk up to 60 hours post dose in some of the mastitic cows; however, for control cows, flunixin residues were only present in the milk up to 24 hours post dose (Figure 2). Flunixin milk concentrations were significantly different between groups at all time points with the mastitic cows having substantially greater flunixin concentrations in their milk compared to control cows (Table 4).

**DISCUSSION**

The objective of this study was to determine if the plasma pharmacokinetics and milk elimination of flunixin and 5OH in lactating dairy cattle would differ between cows with naturally occurring mastitis and paired control cows without mastitis. The significant
difference in AUC\(_{0\rightarrow\infty}\) between the two groups was due to a higher observed maximum plasma concentration and the presence of flunixin in the plasma out to 24 hours for the mastitic cows. The clearance calculated in the current study for the healthy cows is similar with findings in the literature.\(^5,7,22-25\) The reduced clearance in the mastitic cows may be due to alterations in hepatic drug metabolism.\(^9,26,27\) This is similar to findings in literature for another NSAID, carprofen, in which clearance was reduced by 40% in mastitic cows compared to healthy control cows.\(^9\) Inflammation and infection have been shown to greatly impact drug metabolism and protein binding,\(^28\) which may also contribute to alterations in clearance. The significant difference in MRT between the groups can be attributed to the difference in AUC\(_{0\rightarrow\infty}\) aforementioned because AUC\(_{0\rightarrow\infty}\) was used to calculate MRT. In the present study, the mean plasma t\(_{1/2λz}\) for flunixin following intravenous dosing corresponds with that found in previous studies.\(^4,5,7,23-25,29,30\) The Vd\(_{\text{area}}\) and Vd\(_{ss}\) were also consistent with values reported in the literature;\(^5,7,23-25,29,30\) however, they were greater than expected for a drug that is highly (99%) protein bound.

The high number of flunixin tissue residue violations in dairy cattle aforementioned may in part be due to disease-induced alterations in pharmacokinetics, particularly alterations in clearance of a drug. Many studies have reported changes in pharmacokinetics as a result of inflammation.\(^9-18\) One study in horses found significantly greater concentrations of several NSAIDs in inflamed tissues compared to healthy tissues.\(^31\) Likewise an increased partition of both ceftiofur and erythromycin into infected bovine tissue chambers compared to healthy bovine tissue chambers has been reported.\(^32,33\) In rabbits it has been shown that inflammation can increase capillary permeability thus altering flunixin partitioning into tissue.\(^15\) This study
also reported a significantly lower clearance and longer elimination half-life in endotoxemic rabbits compared to healthy controls.\textsuperscript{15} A recently published surveillance study found suspect cull dairy cows, which refers to cows having clinical signs or evidence of disease, had a significantly higher incidence of violative tissue flunixin concentrations than healthy appearing dairy cows at slaughter.\textsuperscript{34} The high number of flunixin tissue residues identified in culled dairy cattle may be related to mastitis-induced alterations in drug clearance.

Milk concentrations of 5OH in the control cows at each time point were similar to milk concentrations in previous studies.\textsuperscript{6,7,35} The persistence of 5OH residues in milk beyond the labeled withdrawal time may in part be related to a drop in milk production that occurred as a result of the mastitis.\textsuperscript{19} Research describing the effect of milk production on drug elimination is limited, especially for systemically administered drugs. However, several studies have shown a correlation between low milk production and prolonged drug elimination for some intramammary drugs.\textsuperscript{36-40} A previous study in our laboratory found that milk production was a significant covariate for the depletion of 5OH milk concentrations over time. In that study, cows producing less than 20 kg of milk/day eliminated 5OH slower than cows producing 30 kg of milk/day.\textsuperscript{7} In the current study all of the mastitic cows produced less than 6 kg of milk/day while on trial. Prior to being diagnosed with clinical mastitis these cows were producing 29.9 ± 4.3 kg of milk/day. The persistence of 5OH residues in milk beyond the labeled withdrawal time may also be due to binding of 5OH to inflamed mammary tissue. A study examining the pharmacokinetics and pharmacodynamics of flunixin in calves demonstrated that the drug binds to inflammatory tissue and achieves high concentrations in inflammatory exudate.\textsuperscript{23} The accumulation of flunixin in inflamed
tissue and slow clearance from exudate may partially explain why 5OH residues were present in milk up to 60 hours post dose.

Flunixin concentrations in the milk of control cows were comparable to concentrations reported in the Freedom of Information Summary.\(^{35}\) The significantly greater concentration of flunixin in the milk from mastitic cows was unexpected. Several factors may have contributed to this finding. One explanation may be due to disruption of the blood-milk barrier. Both *Klebsiella pneumoniae* and *Escherichia coli* are known to increase mammary vascular permeability and cause breakdown of the blood-milk barrier.\(^{41}\) This may have resulted in leakage of flunixin from the blood into the milk prior to hydroxylation by the liver. Since all cases of mastitis in this study were caused by coliform bacteria, it is unclear whether the increase in milk flunixin concentrations would be as pronounced with other causes of mastitis (ie. Gram-positive organisms). Another potential explanation for the significantly greater flunixin concentrations in milk from mastitic cows may be related to impaired hepatic metabolism as a result of mastitis. Disease states have been shown to impair hepatic metabolism of drugs resulting in altered clearance of the parent compound. Similarly, the long persistence of flunixin residues in the milk out to 60 hours post dose may also be related to the drop in milk production associated with mastitis as well as flunixin binding to inflamed mammary tissue aforementioned.

The parenteral use of ceftiofur in this study to treat clinical mastitis represents an extralabel use of this drug. It was standard protocol on this farm to administer ceftiofur intramuscularly to all cattle that had mastitis and exhibited moderate to severe signs of systemic disease (particularly if milk production dropped significantly). A previous study
found that intramuscular administration of ceftiofur to severe cases of coliform mastitis reduced the proportion of cases that resulted in death by culling. Although there has been a recent prohibition on the extralabel use of cephalosporins in the United States, the Federal Register states that these drugs can still be used for non-labeled indications as long as the labeled dose and duration is followed. The dose of 1 mg/lb every 24 hours for 3 days follows the label accordingly and would not represent an illegal use of this drug. Control cows (without mastitis) also received three doses of parenteral ceftiofur so that they would be treated in exactly the same manner as the mastitic cows. Administration of antibiotics to one group and not the other could potentially affect the pharmacokinetics of flunixin. Intramammary cephapirin is labeled for administration every 12 hours. The use of this drug every 12 hours for 3 days represented an extralabel use. Farm protocol at the beginning of this trial was to administer intramammary cephapirin every 12 hours until the milk returned to normal. Due to our need to have a consistent duration of therapy for all cows on the study, the farm elected to use intramammary cephapirin for 6 total treatments (every 12 hours for 3 days) in all cows regardless of their duration of clinical mastitis. Towards the middle of the trial, the farm elected to switch intramammary therapy to ceftiofur because the vast majority of cases had been caused by coliform bacteria. Intramammary ceftiofur was given once a day for 5 days, which followed label directions. Treatment of only the mastitic cows with antimicrobials could have potentially resulted in a drug-drug interaction, which may have altered the pharmacokinetics and confounded comparisons between healthy and mastitic cows. Therefore, the same treatment regimen that was administered to the mastitic cows was also administered to the control cows.
The presence of drug residues in milk is a primary concern for the dairy industry. Milk residues from NSAIDs can result in significant economic losses to the producer and pose a potential health hazard to the consumer. Strict financial penalties and suspension of the producer’s Grade “A” permit are possible outcomes of drug residues detected in milk. To prevent economic losses to the producer it is imperative that a labeled withdrawal time is accurate under the condition in which the drug is administered. A problem arises when there is discord between the labeled withdrawal time, which is calculated using data from healthy animals, and the time it takes for diseased animals to eliminate the drug.

A drug withdrawal time is calculated by performing the statistical tolerance limit procedure on residue data from the depletion curve of the drug residue in milk or the target tissue. The FDA procedure predicts with 95% confidence a time when the milk or tissue residue in 99% of the animal population receiving the drug is at or below tolerance. Part of the approval process for veterinary drugs requires pharmacokinetics studies to be conducted in healthy animals. One assumption of the approval process is there will be no change in the drug’s pharmacokinetics when administered to diseased animals versus healthy animals. However, diseased states can profoundly alter the pharmacokinetic behavior of a drug.

The most profound differences in pharmacokinetic responses are generally associated with hepatic, renal and cardiovascular disease, but other processes such as inflammation, endotoxemia and stress can also significantly alter a drug’s absorption, distribution, metabolism, and elimination. Although there is limited data on the effects of disease on the pharmacokinetics of drugs in cattle, there are several examples that suggest the practice of using healthy animals for establishment of drug withdrawal periods may not be appropriate.
For example in the late 1970’s a study demonstrated that following the infusion of several different intramammary antibiotics, only minimal concentrations were found in the kidneys and liver of healthy cows. Detectable concentrations of antibiotics were only found in liver and kidney for 24 hours and residues were not detected in the meat of these cattle. In contrast, antibiotic concentrations in the tissues of mastitic cows were much higher and persisted for a longer period. Differences in drug pharmacokinetics have been described for oxytetracycline in cows with theileriosis. Following intramuscular administration, infected cattle had significantly prolonged absorption and $\frac{1}{2}t_{\alpha}\lambda$, MRT, $\text{AUC}_{0,\infty}$, and bioavailability as compared to oxytetracycline administration in healthy cows. Another example is theophylline where in a field trial, 5 out of 20 calves with respiratory disease died after administration whereas all 20 calves treated with a placebo survived. A subsequent study showed calves with pneumonia had significantly higher plasma concentrations of theophylline as compared to healthy calves. Likewise, a greater secretion of ceftriaxone into milk was also noted in cows with endometritis compared to the control cows following intravenous administration. Lastly, a population pharmacokinetic model showed a longer meat withdrawal interval was needed for flunixin than the FDA approved withdrawal time, suggesting a need for pharmacokinetic studies to be performed in both healthy and diseased animals.

Mastitis produces physical and chemical changes both in the milk and the mammary gland itself that have the potential to alter distribution and elimination of drugs through the mammary gland. Inflammation of the mammary gland leads to vascular permeability changes that often enhance systemic absorption and perhaps distribution of drugs into the
udder. For example, gentamicin is not detected in the plasma following intramammary administration in normal quarters, however the drug is well absorbed in cows with mastitis.\(^{52}\)

Similarly in studies using polymyxin B, the drug was not found in the blood or untreated quarters following intramammary administration in normal cattle, however significant systemic absorption was seen in cows with experimentally induced coliform mastitis.\(^{53}\) A study evaluating the influence of *Escherichia coli* endotoxin-induced mastitis on the pharmacokinetics of the NSAID, carprofen, found a significant reduction in systemic clearance, prolonged \(t_{1/2}\), and increased milk carprofen concentrations in mastitic cows compared to the healthy controls following intravenous administration.\(^9\) Lastly, a study using an intramammary preparation of cefoperazone sodium reported significantly greater systemic drug absorption, milk half life and MRT in cows with subclinical mastitis compared to healthy controls.\(^{54}\)

This study provides strong evidence that milk withdrawal times determined in healthy cattle may not be appropriate in cows with clinical mastitis. Our results also suggest that the use of a marker residue, such as 5OH is of concern since its ratio to parent compound (FLU) changes in the very disease for which it is labeled. Since pharmaceutical companies must conduct trials to demonstrate the efficacy of various drugs for treating a specific disease or condition during the approval process, it seems logical that pharmacokinetic and residue studies could be done using the same animals or under similar conditions.

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\(^{a}\) Banamine®, Merck Animal Health, Summit, NJ.

\(^{b}\) Naxcel® Sterile Powder, Zoetis, Kalamazoo, MI.
c ToDAY®, Boehringer Ingelheim Vetmedica, St. Joseph, MO.

d Spectramast® LC Sterile Suspension, Zoetis, Kalamazoo, MI.

e Metatron Sampler, Westfalia Surge, Naperville, IL.

f ® Hybrid SPE-phospholipid cartridge, Sigma-Aldrich, St. Louis, MO.

g TurboVap® LV evaporator, Zymark Corporation, Hopkinton, MA.

h Acquity, Waters Corporation, Milford, MA.

i Phoenix WinNolin, Version 1.3x, Pharsight Corporation, St Louis, MO.

j SAS, version 9.1, SAS Institute Inc, Cary, NC.
Figure 5.1: Flunixin plasma concentration vs. time profile for control cows and cows with mastitis.
Figure 5.2: Flunixin and 5OH milk concentrations (ppb) for control and mastitic cows.
**Table 5.1**: Summary of age, lactation number, days in milk, milk production history, rectal temperature and heart rate for control and mastitic cows included in this study (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Cows</th>
<th>Mastitic Cows</th>
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<tr>
<td>Age of cow (years)</td>
<td>4.8 ± 1.5</td>
<td>4.9 ± 1.8</td>
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<tr>
<td>Current Lactation Number</td>
<td>2.8 ± 0.9</td>
<td>3.0 ± 1.5</td>
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<td>Days in milk</td>
<td>153 ± 73</td>
<td>142 ± 74</td>
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<tr>
<td>Milk production prior to study (kg/day)</td>
<td>33.4 ± 3.8</td>
<td>29.9 ± 4.3</td>
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<tr>
<td>305-day mature equivalent</td>
<td>22,335 ± 4,466</td>
<td>17,940 ± 4,092</td>
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<tr>
<td>Rectal temperature</td>
<td>101.0 ± 1.1</td>
<td>102.0 ± 1.6</td>
</tr>
<tr>
<td>Heart rate (BPM)</td>
<td>74.2 ± 10.4 (^\text{a})</td>
<td>93.6 ± 10.7 (^\text{b})</td>
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</table>

\(^{\text{a-b}}\) Means within a row with different superscripts differ \((P < 0.05)\)
**Table 5.2:** Flunixin plasma pharmacokinetic parameters in control cows and cows with mastitis (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Parameter</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>( t_{1/2\lambda} ), h</td>
<td>3.68 ± 1.97</td>
</tr>
<tr>
<td>( \lambda ), h(^{-1})</td>
<td>0.24 ± 0.14</td>
</tr>
<tr>
<td>AUC(_{0-\infty}), µg(\cdot)h/ml</td>
<td>19.82 ± 6.68(^a)</td>
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<tr>
<td>AUC(<em>{0-t</em>{\text{last}}}), µg(\cdot)h/ml</td>
<td>19.09 ± 6.43(^a)</td>
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<tr>
<td>Cl, mL/h/kg</td>
<td>120.15 ± 31.70(^a)</td>
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<tr>
<td>Vd(_{\text{ss}}), L/kg</td>
<td>0.237 ± 0.136</td>
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<tr>
<td>Vd(_{\text{area}}), L/kg</td>
<td>0.644 ± 0.423</td>
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<tr>
<td>MRT, h</td>
<td>2.00 ± 0.98(^a)</td>
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</tbody>
</table>

\( t_{1/2\lambda} = \) harmonic mean elimination half-life; \( \lambda = \) rate constant associated with the terminal elimination phase; AUC\(_{0-\infty} = \) total area under the curve; AUC\(_{0-t_{\text{last}}} = \) area under the curve from 0 to the last observed concentration; Cl = clearance; Vd\(_{\text{ss}} = \) volume of distribution at steady state; Vd\(_{\text{area}} = \) volume of distribution in terminal elimination phase; MRT = mean residence time;

\(^a-b\)Means within a row with different superscripts differ \((P < 0.05)\)
**Table 5.3:** 5-hydroxy flunixin milk concentrations (ppb) for control cows and cows with mastitis (mean ± standard deviation).

<table>
<thead>
<tr>
<th>Time post flunixin dose (hours)</th>
<th>Control Cows</th>
<th>Mastitic Cows</th>
</tr>
</thead>
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<tr>
<td>2</td>
<td>12.2 ± 7.04$^a$ (n=7)</td>
<td>5.04 ± 4.19$^b$ (n=10)</td>
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<tr>
<td>12</td>
<td>22.2 ± 6.23$^a$ (n=10)</td>
<td>10.5 ± 5.79$^b$ (n=10)</td>
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<td>24</td>
<td>5.76 ± 2.90 (n=6)</td>
<td>5.83 ± 2.56 (n=10)</td>
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<tr>
<td>36</td>
<td>&lt;LOQ</td>
<td>3.75 ± 1.78 (n=8)</td>
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<tr>
<td>48</td>
<td>&lt;LOQ</td>
<td>3.53 ± 0.96 (n=3)</td>
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$^ab$Means within a row with different superscripts differ ($P < 0.05$)

**Table 5.4:** Flunixin milk concentrations (ppb) for control cows and cows with mastitis (mean ± standard deviation).

<table>
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<tr>
<th>Time post flunixin dose (hours)</th>
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<th>Mastitic Cows</th>
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<tr>
<td>2</td>
<td>4.91 ± 3.52$^a$ (n=6)</td>
<td>224.0 ± 232.2$^b$ (n=10)</td>
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<td>16.77 ±16.69$^a$ (n=10)</td>
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<td>24</td>
<td>3.18 ± 0.367$^a$ (n=4)</td>
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<td>48</td>
<td>&lt;LOQ</td>
<td>11.37 ±15.69 (n=7)</td>
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<td>60</td>
<td>&lt;LOQ</td>
<td>13.02 ± 10.93 (n=3)</td>
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$^ab$Means within a row with different superscripts differ ($P < 0.05$)
REFERENCES


determination of flunixin in bovine plasma and pharmacokinetics after single and

26. Abdullah AS, Baggott JD. Influence of induced disease states on the disposition kinetics

phagocytophila) and trypanosomiasis (Trypanosoma brucei) on the pharmacokinetics of

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32. Clarke CR, Bourne DW, Lauer AK, et al. Distribution of intramuscularly administered
erthromycin into subcutaneous tissue chambers before and after inoculation with

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6. SUMMARY AND FUTURE DIRECTION

Anti-inflammatory drugs such as FLU are reported to be the second most prescribed class of drugs by dairy veterinarians (Sundlof et al. 1995) and the most frequently administered analgesic in cattle (Fajt et al. 2011). FLU has been extensively used in bovine practice since its approval in cattle 15 years ago. During this time period its use has resulted in a number of violative residues in meat from cull dairy cows, yet the cause of these residues had not been determined. The assumption is that violative residues occur as a result of extralabel drug use, but other causes cannot be ruled out. Although there is much concern about FLU residues in meat, little attention has been given to FLU residues in milk. This may be due in part to older literature which found no FLU residues in milk; however, the assays used at the time were less sensitive than what is available today and these studies only analyzed milk samples for the parent compound (FLU) not the milk marker residue (5OH) (Benitz 1984; and Anderson et al. 1990). Newer research using more sensitive analytical equipment clearly demonstrates that 5OH and FLU accumulate in the milk at concentrations above the tolerance limit (2 ppb) (Feely et al. 2002). The FDA is vigilant in monitoring milk for antibiotic residues, but other drugs, such as FLU, that are often used on farms are not routinely screened for in milk (National Milk Drug Residue Data Base Fiscal Year 2011). In December 2011, the FDA announced plans to collect and test milk samples across the United States for a variety of drugs including FLU. However, the results of the drug milk surveillance study have not been announced as of September 2013.

To determine if violative FLU residues could potentially be found in saleable milk in the United States a milk surveillance study was conducted. Of the 500 tanker truck samples
collected and analyzed with one sample testing positive for 5OH above the tolerance limit of 2 ppb. This represents a positive violation rate of 0.2% which suggests that FLU residues may be found in milk as often as other drug residues. This study demonstrated that illegal FLU milk residues do occur in the dairy industry and may necessitate testing milk for violative FLU residues at dairy processing plants.

To investigate potential situations which may lead to violative FLU milk residues, two experiments were conducted to replicate scenarios that may routinely occur on a dairy farm. The first experiment sought to characterize the plasma pharmacokinetics and milk elimination of FLU and 5OH following extra-label administration. Although FLU is only approved for intravenous administration; intramuscular and subcutaneous routes are common routes of extra-label drug use in dairy cattle due to their ease of administration. However, altering the route of administration for convenience is not an appropriate reason for extra-label drug use according to AMDUCA (AMDUCA). Altering the route of administration resulted in significant differences in some of the plasma pharmacokinetic parameters, such as elimination half-life. The prolonged elimination half-life following extravascular administration is of particular interest because it is the parameter commonly used to estimate withdrawal intervals (KuKanich et al. 2005; and Riviere, 2011). Using non linear mixed effects modeling both the route of administration and the rate of milk production were found to be significant covariates for the depletion of 5OH milk concentrations over time. Lastly, this study found that at the 36 hour milk withdrawal time, cows which had been administered FLU extravascularly had violative residues in the milk. This study underscores the importance of proper drug administration for the prevention of milk residue violations.
A drug withdrawal time is calculated by performing the statistical tolerance limit procedure on residue data from the depletion curve of the drug residue in milk or the target tissue. The FDA procedure predicts with 95% confidence a time when the milk or tissue residue in 99% of the animal population receiving the drug is at or below tolerance. Part of the approval process for veterinary drugs requires pharmacokinetics studies to be conducted in healthy animals. One assumption of the approval process is there will be no change in the drug’s pharmacokinetics when administered to diseased animals versus healthy animals. However, diseased states can profoundly alter the pharmacokinetic behavior of a drug (Lohuis et al. 1991; Jha et al. 1996; Gips and Soback, 1999; Rao et al. 2000; Ismail and El-Kattan, 2007; and Lucas et al. 2009). A problem arises when there is discord between the labeled withdrawal time, which is calculated using data from healthy animals, and the time it takes for disease animals to eliminate the drug. Therefore, a second experiment was designed to determine if there was a difference in the pharmacokinetics and milk elimination of FLU and 5OH in healthy cows and cows with naturally occurring mastitis. This experiment showed that the plasma pharmacokinetics and milk elimination were indeed different between healthy and diseased cows. Diseased cows had almost a 50% reduction in clearance and as a result the $AUC_{0-\infty}$ and mean residence time were significantly greater in mastitic cows compared to healthy cows. FLU and 5OH milk elimination was also altered in diseased cows, resulting in violative residues in the milk up to 12 hours after the approved withdrawal time. Surprisingly, a change in the type of residue present in the milk was noted. In healthy cows administered FLU, the primary drug residue in the milk was the metabolite of FLU, 5OH. However, in diseased cows, at every milk sampling, FLU concentrations were greater
than 5OH concentrations. The FLU residues persisted in the milk longer (up to 60 post dose) than 5OH residues. The high concentration and persistence of FLU in the milk of diseased cows suggests that 5OH may not be the appropriate milk marker residue in mastitic cows. This may partially explain the high number of violative FLU residues that have been reported in dairy cattle and provides strong evidence that milk withdrawal times determined using healthy cows may not be appropriate in cows with clinical mastitis.

Both of the experiments aforementioned provide evidence that FLU residues in milk can occur under conditions of extravascular administration and administration to cows with clinical mastitis. However, what remains unknown is how the pharmacokinetics and milk elimination of FLU will be altered when these two situations occur simultaneously. Furthermore, no studies have addressed these two situations in the context of FLU meat residues. Thus one potential area of research is a FLU population pharmacokinetic study. Using a population pharmacokinetic approach one could investigate and correlate sources of variability among the target patient population. In addition a measurement of variability among the target population could be determined. This type of study would allow for analysis of data from a variety of data sets, including studies where the data is sparse or unbalanced. The population approach would help determine whether the current FLU meat and milk withdrawal times set by the FDA would be sufficient in preventing violative residues in subsets of patients, such as diseased animals. A population pharmacokinetic model could also be used to predict accurate meat and milk withdrawal intervals when FLU is administered in an extra-label manner. Lastly, to expand on the correlation between altered pharmacokinetics in diseased animals and violative drug residues, future research could be
directed to evaluate other classes of drugs whose pharmacokinetics are altered by health
status and may have a high likelihood of testing positive for violative residues even after the
approved withdrawal time is observed.
7. REFERENCES


Appendix A

Flunixin plasma pharmacokinetic parameters in cattle summarized from published literature
Table 1: Flunixin plasma pharmacokinetic parameters in cattle summarized from published literature.

<table>
<thead>
<tr>
<th>Author</th>
<th>Animal</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th># doses</th>
<th>t1/2 (h)</th>
<th>Sample collection period (h)</th>
<th>Vdss (L/kg)</th>
<th>Vdarea (L/kg)</th>
<th>Vdc (L/kg)</th>
<th>Cl (ml/kg/h)</th>
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