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An investigation into the effect of clinical states of infection on pharmacokinetics and pharmacodynamics in ruminant food animal species

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An investigation into the effect of clinical states of infection on pharmacokinetics and pharmacodynamics in ruminant food animal species

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The student author and the program of study committee are solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

This work is dedicated to the food animal patients that inspired it, as well as the veterinary students, house officers, and clients that made the care of those patients possible.

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ABSTRACT

The effects of clinical states of infection on the pharmacokinetics and pharmacodynamics (PK/PD) in ruminant food animal species are not well described. The majority of PK studies utilize healthy animals, and this creates challenges for veterinary professionals treating clinical patients. Knowledge gaps explored are 1) effect of clinical disease on the PK/PD of fentanyl in cattle, 2) effect of pneumonia on the PK/PD of tulathromycin in goats, and 3) the effect of co-administration of ertapenem and an immunomodulator on PK/PD in sheep.

The pharmacokinetics for calves were determined after intravenous dosing of 5 μg/kg fentanyl citrate. The initial PK revealed an increased elimination half-life and mean residence time when compared to other ruminant values. The analytical performance of the assay was extremely sensitive, and as methods become more performant, PK parameters may face the need for adjustment over time.

The PK/PD of fentanyl transdermal patches was determined in healthy and clinical calves. Adverse effects of tachycardia, tachypnea, and hyperthermia were noted in all healthy calves at two variable dosages. Adverse effects were related to the absorption of fentanyl over the initial 4-6 hours, and the absence of acute clinical disease. Clinicians should note that severity of clinical disease may impact PD of fentanyl in calves.

The PK and tissue residue concentration of tulathromycin in goats was determined for healthy (control) and goats with respiratory disease. Volume of distribution was significantly altered by respiratory disease, as well as plasma concentrations. A significantly lower tissue residue concentration was noted in renal tissue from the experimental group. Respiratory disease may alter pharmacokinetics and residues of tulathromycin in meat goats.

Finally the effect *P. aeruginosa* cystitis and an immunomodulator on the PK/PD of ertapenem was determined for sheep. No significant differences in PK or ertapenem concentration were noted. Significant differences in bactiuria were noted between the control and ertapenem with immunomodulator group. Non-antimicrobial adjunctive therapies such as immunomodulators may present promise as a treatment option for severe infections.

The collective results of this work provide clinicians with evidence regarding the effect of clinical states on the PK/PD in ruminant food animal species. These results can be further elucidated for application of precision therapy in the hospitalized ruminant.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Alterations of Pharmacokinetics and Pharmacodynamics Due to Clinical States

Multiple physiologic states in medicine impact pharmacokinetics and pharmacodynamics (PK/PD) in patients and these have been identified to provide guidance to clinicians when treating these patients. Examples of these physiologic states include lactation, pregnancy, age, as well as renal and hepatic impairment. Modric and Martinez identified 6 bovine studies of lactation and 5 bovine studies of lactation altering PK/PD.(1) Pregnancy can alter PK/PD by changing body composition, increasing cardiac output, delaying gastric emptying, decreasing protein binding, and altering the activity of hepatic enzymes.(2) In human medicine these changes require adjusting of doses for drugs such as the aminoglycosides. Lactation can also alter PK/PD through the distribution of milk fat and protein, depending on the state of lactation. Age also has the ability to influence PK/PD, primarily through alterations of volume of distribution, cardiac output, renal clearance, and hepatic clearance.(3) The majority of studies examining the effects of clinical states on PK/PD in veterinary medicine have investigated the effects in small animals. In a review of veterinary studies examining the effects of renal or hepatic impairment on PK/PD Modric et al identified 5 canine studies of hepatic impairment and 21 canine and 2 feline studies for renal impairment, however no bovine studies were identified.(1) While the influence of physiologic states such as pregnancy, lactation, and age are known to influence PK/PD in animals, the effect of clinical states, or presentations unique to patients presented for medical care, especially among ruminant food animals, is less known.

The effect of clinical states of infection on PK/PD in veterinary patients is less understood. While some models exist for examination of individual aspects of clinical

presentation few investigate multimodal factors for disease. For example: a goat with pneumonia can present with significant stress, inflammation and infectious consequences, and require administration of multiple therapeutic agents, however there are few studies that account for all aspects of clinical disease when researched, as most PK studies are performed in healthy animals of fairly homogenous populations with little variation.

Studies involving pain, stress, inflammation and infectious disease.

Inflammation and infectious disease can alter PK/PD through alterations in drug transport as well as metabolizing enzyme activity. Multiple mechanisms have been elucidated for these phenomena. These clinical states alter drug transport via modulation of the solute carriers(4) and the ATP-cassette binding drug transporters, an example being P-glycoprotein.(5) Proinflammatory cytokines such as interleukin 1β, interleukin 16, and tumor necrosis factor are involved in this transporter protein inhibition.(1) Processes of infection and inflammation can also increase plasma concentration and the potential for toxicity of drugs, an example being phenylbutazone in febrile greyhounds.(6) Martinez and Modric identified 12 bovine studies of inflammation and 14 bovine studies of infection altering PK/PD.(1) In humans inflammation and pain in patients with rheumatoid arthritis will lead to over expression of central opioid receptors, leading to potentially altered PD in these patients.(7)

Less understood is the effect of pain from clinical states on PK/PD in ruminant food animals. There are two primary forms of pain described: nociceptive pain and neuropathic pain. Nociceptive pain occurs from stimuli reaching harmful thresholds on the sensory nerve fibers. These can be from thermal, mechanical, or chemical stimuli. Mechanical and thermal pain is commonly described for processing procedures for ruminant food animals, such as tail docking or dehorning. There are instances of nociceptive pain causing altered PK in ruminants. For

example, Kleinhenz et al has demonstrated that pain from hot iron dehorning increased the plasma half-life of transdermally administered flunixin in calves.(8)

Multiple mechanisms are understood regarding the specifics of inflammation and infection influencing PK/PD in ruminants. Sepsis has been demonstrated to alter the pharmacodynamics of fentanyl in septic sheep, as septic subjects had significant hemodynamic changes with renal perfusion reduced 60% when compared to controls.(9) While no studies identify altering of PK/PD of tulathromycin in cattle, however in pigs infected with *Actinobacillus pleuropneumoniae,* tulathromycin demonstrated both a slower elimination half-life as well as a longer drug persistence when compared to healthy pigs (10). This could be due to the accumulation of tulathromycin in inflammatory cells which would accumulate in infected tissues(11, 12), the presence of three functional groups which allow for ionization at multiple pH ranges due to a dissociation constant (PK_a) between 8-9.6(11), or the potential increased volume of distribution of infected pigs.(13) This increased volume of distribution in pneumonic animals has been noted in ruminants, as goats undergoing an experimentally induced Pasteurellosis were noted to have an increased volume of distribution for doxycycline compared to uninfected controls.(14)

Studied involving co-administration of multiple therapeutic agents.

One concern that is not well documented in food animal medicine is the effect of concurrent drug administration or co-administration on the effect of PK/PD in ruminant food animal species. There are several examples in the literature of co-administration altering, not altering or partially altering PK/PD in ruminant food animals.

There are cases where the co-administration of multiple drugs appears to have no effect on PK in ruminant food animals. Gorden et al demonstrated that co-administration of flunixin

meglumine and ceftiofur hydrochloride did appear to cause a drug interaction that alters pharmacokinetics in healthy dairy cows.(15) Similarly, another study identified no differences in pharmacodynamics and no biologically significant differences in pharmacokinetics when calves were simultaneously administered carprofen and oxytetracycline.(16) In goats similar findings have been reported as administration of marbofloxacin with tolfenamic acid did not alter the PK profile of marbofloxacin(17), and this finding has been repeated in calves.(18, 19) These studies do have a common thread in that other than the administration of multiple drugs at the same time, no other clinical state such as disease or pain was investigated.

However, this observance of co-administration not affecting PK is not always the case in ruminant food animals as co-administration of piperonyl butoxide and albendazole in goats altered the pharmacokinetics of albendazole due to the modulation of cytochrome P450 by piperonyl butoxide, resulting in increased albendazole concentrations.(20) This mechanism could be potentially exploited clinically as the persistence of albendazole led to an increased fecal egg count reduction in the co-administered goats. In sheep co administration of verapamil with ivermectin has led to significant changes in the pharmacokinetic parameters of ivermectin, and this is most likely due to the effect of verapamil on P-glycoproteins.(21) Also noted in sheep is the co-administration of ketoconazole increasing plasma concentrations of ivermectin, and this mechanism is also thought to be due to P-glycoprotein inhibition by ketoconazole.(22) In alpacas, it has been hypothesized and proven i*n vitro* that co administration of doxycycline with ivermectin or doramectin may lead to increased cerebrospinal concentrations of the avermectins due to inhibition of P-glycoproteins at the blood-brain-barrier.(23) These mechanisms need to be studied more closely to allow for potential therapeutic strategies to be developed due to altered pharmacokinetics from co-administration.

There is also the possibility for co-administration of drugs to partially alter the pharmacokinetics. In the case of enrofloxacin administered to goats with probenecid, some of the pharmacokinetic parameters of the parent drug were not altered, whereas the metabolite, ciprofloxacin did display altered elimination half-life, area under the curve, and mean residence times.(24) This is most likely due to the properties of probenecid in reducing active tubular secretion of the kidney decreasing the excretion of the metabolite.(25) Similar activity has been noted in the case of probenecid's action on cefuroxime's PK in calves.(26) In sheep co-administration of diclofenac and enrofloxacin did not change volume of distribution or mean residence time, however it did increase area under the curve, elimination half-life, and bioavailability.(27) These changes are presumed to be due to the inhibition of cytochrome P450 enzymes by enrofloxacin. These circumstances perhaps dictate the most need for further investigation as this type of interaction could pose a therapeutic benefit or failure.

One exciting new approach to pharmacotherapy involves immunomodulators or agents that stimulate the immune system. One of the promises of this drug class is that by improving the immune response to infection other therapy could be applied in a more precise manner, or not at all. Several examples have recently been utilized in veterinary medicine including mycobacterium cell wall fraction stimulants for calf (28) and equine (29, 30) use, as well as the DNA-based immunostimulant currently approved for use in bovine respiratory disease.(31) The DNA-based immunostimulant is a cationic lipid/bacterial plasmid DNA liposome, which activates the *stimulator of interferon genes* (STING) pathway to then activate *interferon response factor* 3 (IRF3).(32) This modulation of the immune system will decrease both lung lesions as well as death loss in cattle undergoing a respiratory disease challenge.(31) As a non-antibiotic adjunctive therapy for respiratory disease, this immunomodulator may serve as a

useful therapy for concurrent of infections that rapidly develop antimicrobial resistance. However, there is currently no information in the literature describing what effect, if any, that these immune system stimulators may have on the pharmacokinetics of antibiotics. These effects need to be explored, as a potential drug interaction may alter antimicrobial pharmacokinetics in a manner that may alter drug efficacy when these immunomodulators are used in ruminant food animals.

Importance of Comparative Pharmacokinetics in US Ruminant Food Animal Referral Practice

The majority of labelled drugs for ruminant food animal species in the United States are labelled for cattle and most of these label indications are for respiratory disease, hoof rot, pyrexia, parasitism and inflammation. While at the current time there are over four hundred individual dugs labelled for cattle in the US(33), there are only 26 specific drugs labelled for use in sheep(34), and only 15 separate labelled drugs for goats.(35) This limitation of therapeutic tools in the toolbox of practitioners will often lead to extra-label drug use. Extra-label use will normally follow under one or more of multiple circumstances such as: (1) administration of the drug to a different species than on the label, (2) administration of a different dose or volume than on the label, (3) administration via a different route or interval than labelled as well as (5) for different durations or indications than labelled.(36) Food animal practitioners will routinely use drugs extralabelly in the treatment of clinical animals, provided they can meet the clarifications of the Animal Medical Drug Use Clarification Act (AMDUCA). Under AMDUCA in the US a practitioner may pursue ELDU treatment if the five conditions are met: the veterinarian is licensed, an animal or human FDA approved drug is used, data exists to establish an adequate withdrawal time, the drugs are not used in an ELDU manner in feed, and the use of the drug is only for therapeutic purposes where suffering or death may result from failure to treat. This issue

is further compounded in referral facilities, such as the Food Animal and Camelid Hospital (FACH) at Iowa State University. To provide context for these clinical scenarios for food animal veterinarians one could consider a recent case of the treatment of dairy goats for rattlesnake envenomation.(37) All of the goats in the case series were treated with the non-steroidal anti-inflammatory drug flunixin meglumine for inflammation. Flunixin meglumine did not at the time, nor does it at the current time, have a label for caprine use. However, it is labelled for bovine use and there are multiple studies that suggest that intravenous flunixin meglumine has comparable pharmacokinetics in the goat as the bovine(38-40), as well as pharmacodynamic research to indicate that it inhibits thromboxane B2 in caprine blood.(41) For this example since the veterinarians were licensed, an approved food animal drug was administered, it was not administered via feed, and was used to alleviate inflammation from the rattlesnake envenomation, this ELDU would be appropriate. There are multiple other legal indications for extra label use of flunixin in food animals, such as inflammation from parasite migration(42), pre-operative pain management(43-45), as well as the treatment of the inflammatory processes from aspiration pneumonia(46, 47). These examples demonstrate the importance of extra-label drug use in food animal practice for clinicians.

Currently pharmacokinetic studies are conducted with small populations of animals, typically 4-6, and these animals are often fairly homogenous as far as age, breed, production status and weight.(48) This can create challenges for the clinician as clinical states such as pain, disease or concurrent drug administration have the ability to alter PK/PD. Further compounding this issue is that there is currently a lack of studies reporting the differences in pharmacokinetics based on analytical sensitivity. For example, when trying to make decisions regarding extrapolations of PK for a patient, it is challenging to determine if a study with a lower limit of

quantification of 10 ng/mL will provide the same results as a similar study conducted at with a lower limit of 1 ng/mL. There is an urgent need for more information of the effect of the clinical states on PK/PD for ruminant food animals to guide drug usage by veterinarians as well as the effect of analytical sensitivity on PK parameters themselves.

Review of the Clinical Pharmacology of Fentanyl, Tulathromycin, and Ertapenem in Ruminant Food Animal Species

The goal of this work was to further explore the effect of clinical states on PK/PD in ruminant food animals. To investigate this goal three studies were conducted in three separate ruminant species. 1) Currently no guidelines exist for the pharmacodynamics of fentanyl with respect to clinical disease in the bovine; therefore, the effect of pain and clinical disease on PK/PD of fentanyl transdermal patches in calves was investigated. This study also contributed the PK of fentanyl to bovine practice as this has not been previously reported. A secondary goal of this study was to examine the effect of analytical sensitivity on pharmacokinetic parameter generation to aid ruminant practitioners when comparing pharmacokinetic studies performed with different analytical methodologies. 2) Recently the swine literature demonstrated altered pharmacokinetics of tulathromycin in pigs with *Actinobacillus pleuropneumoniae* respiratory disease.(10) Tulathromycin has multiple properties that would make it ideal for use in caprine respiratory disease; however, no studies have explored this. Therefore, the effect of *Pasteurella multocida* respiratory disease on the pharmacokinetics and tissue residue concentration of tulathromycin in meat goats was researched. 3) Currently there are no descriptions to guide food and fiber animal practitioners on any alterations in PK/PD from co-administration of immunomodulators with beta lactam antimicrobials. There are also no ovine models of catheter-

associated urinary tract infections or studies describing the use of ertapenem in sheep. Therefore, the effect of co-administration of ertapenem and a *STING* activator immunomodulator was explored in an ovine model of urinary catheter associated cystitis due to *Pseudomonas aeruginosa*.

Pharmacology of Fentanyl

Fentanyl is a synthetic μ receptor opioid agonist that is commonly used to provide analgesia in veterinary species. Similar to opioid analgesics such as morphine and butorphanol, fentanyl acts on μ-opioid receptors located primarily in the central and peripheral nervous system, as well as the intestines. However, opioid receptor activity can vary from drug to drug in the commonly utilized opioids. For example: morphine is a primary μ opioid agonist and butorphanol is a partial opioid agonist with activity as an agonist for the kappa receptor and weak μ receptor antagonist activity. Morphine is commonly used in comparisons of potency amongst opioids, with butorphanol being recognized as 4-7 times as potent as morphine, and fentanyl approximately 100 times more potent than morphine.

Pharmacokinetics of fentanyl.

The properties of fentanyl allow for fast activity after administration. Fentanyl is highly lipid soluble and as such will rapidly transit the blood-brain barrier. After an initial intravenous administration, plasma concentration decreases quickly due to redistribution of fentanyl to tissues such as muscle and fat.(49) Fentanyl is primarily metabolized by hepatic cytochrome P450 3A enzymes to norfentanyl (50). There are several additional minor pathways in the metabolism of fentanyl, primarily amide hydrolysis to despropionyl fentanyl as well as alkyl

hydroxylation to hydroxyfentanyl. Pharmacokinetics of fentanyl metabolites, while readily available in human medical studies, are limited in veterinary medicine. Currently limited to studies reporting norfentanyl concentrations in chickens (51), and primates (52), as well as not detecting measurable quantities of norfentanyl in dogs (53). Fentanyl undergoes hepatic metabolism and is excreted in a renally, and in the case of infusions or other sustained-delivery systems these clearance methods can become saturated. The resulting accumulation results in prolonged drug effects (49) This rapid distribution and short elimination half-life requires fentanyl to be used via a continuous rate infusion or other sustained-delivery system for effective analgesia in most clinical settings.

There are multiple routes of administration for fentanyl. The intravenous formulation is administered via bolus or constant rate infusion. Sustained-release fentanyl transdermal are successfully used for analgesia in cats(54), dogs(55), sheep(56), and goats.(57) A transdermal fentanyl solution was developed for use in dogs,(58) but the safety of this formulation is questionable for ruminants as a study evaluating three different dosages in sheep noted adverse effects at all doses tested.(59) Other formulations of fentanyl exist in human medicine, examples being intranasal fentanyl,(60) effervescent tablets, and fentanyl in a lollipop form,(61) but the utility of these formulations for ruminant practice is unknown at this time.

Fentanyl in veterinary medicine.

In large animals, the pharmacokinetics of IV fentanyl has been described in sheep(62), goats(63), alpacas(64), llamas(65) and horses.(66) In small animals, the IV pharmacokinetics of fentanyl has also been described. Common use in small animals include sustained release approaches in both the dog(55, 67) and the cat.(68, 69) Adverse reactions to fentanyl in non-

ruminant species include an increase in locomotor activity in horses,(70) and respiratory depression with elevated plasma concentrations (>30 ng/mL) in dogs.(71)

Fentanyl usage in ruminant food animal species

Fentanyl usage in the sheep.

In ruminant species, fentanyl use is best described for the sheep, with the initial reports describing fentanyl's use in combination with droperidol in 1964.(72) Studies utilizing sheep as models for human maternal and fetal physiology have identified fentanyl as having parallel pharmacokinetic parameters in both ewes and fetal lambs(73) after intravenous injection. In addition, epidural injections of 50 and 100 μg into the epidural space of the ewe had no adverse effects on the fetus or the ewe.(74) For ovine surgery models, fentanyl patch placement 24-36 hours prior to orthopedic procedures at a dose rate of 2 μg/kg/h has demonstrated analgesic properties.(56)

Effects of fentanyl in the sheep extend beyond analgesia. It has been suggested that fentanyl stimulates the presynaptic dopamine receptors in the corpus striatum, this decreases the synthesis and release of dopamine, and with the resulting inhibition of locomotor activity will lead to immobilization.(75) This has been further explored by Kyles et al. who noted antinociceptive effects when neuroleptics were administered with fentanyl, but not with neuroleptics alone, suggesting that interactions exist between the opioid and dopaminergic systems.(76) In the neonatal lamb, fentanyl administration alone leads to respiratory depression, but when mechanical ventilation is provided to lambs administered fentanyl, cardiac outflow, heart rate, arterial pressure, and organ perfusion is not affected.(77) In lambs, there are several age-related changes in the pharmacokinetics of fentanyl, primarily an increase of volume of

distribution at steady state, most likely due to changes in body composition and clearance.(78) Similarly the extraction ratio for lambs appears to be much less than for adult sheep(79), most likely due to poor hepatic extraction by the neonatal liver. Surgery also appears to have influence on fentanyl clearance, as clearances are slower during than before or after surgery in sheep.(80) Fentanyl use in the sheep blocks cyclic contractions of the reticulum, potentially causing ileus in treated animals.

Fentanyl usage in the goat.

Fentanyl use in the goat is a relatively unexplored therapeutic modality compared to the sheep. Initial studies reported pharmacokinetics of fentanyl via intravenous, intrathecal, and epidural routes, with fentanyl having higher elimination rates from the CSF as well as decreased availability in the CSF.(81) These studies suggests that fentanyl may be less absorbed into the CSF than morphine in the goat than other species. With the use of a transdermal delivery system, maximum plasma concentrations were variable (1.12-16.69 ng/mL; mean: 6.99 ng/mL, SD +/- 6.03 ng/mL) compared to intravenous administration.(63) This suggests that in the goat a transdermal delivery system may not guarantee a stable plasma concentration of fentanyl is achievable. This same study identified a short elimination half-life (1.20 +/- 0.78 hours) for intravenous administration compared to administration via the transdermal route (5.34 ± 5.34 hours). This finding conflicts with a later study that demonstrated consistent fentanyl absorption in goats that underwent orthopedic surgery, with a maximum plasma concentration of 1.84 ng/mL (range: 0.81-3.35 ng/mL), with concentrations maintaining above 0.5 for 40 hours.(57)

In the goat, fentanyl is not limited to use as a stand-alone analgesic agent, as it also possesses the ability to potentiate anesthetics. When combined with isoflurane for inhalational

anesthesia a decreased minimum alveolar concentration was noted with fentanyl protocols than with isoflurane alone (82), with cardiovascular function not being adversely affected. Similarly, when co-administered with alfalaxone, a fentanyl infusion reduces in a dose-dependent manner, the infusion rate of alfalaxone required to prevent motion in response to noxious stimuli.(83) Fentanyl is a component of a Total Intravenous Anesthesia (TIVA) protocol along with propofol. For this purpose, a decreased median dose of propofol was necessary with the fentanyl combination compared to fentanyl and midazolam.(84) Goats treated with the fentanyl-propofol combination also recovered faster from anesthesia. The ability for fentanyl administration to decrease the effective doses of other drugs for anesthesia is a benefit that could minimize adverse anesthetic-related effects for complicated procedures, such as a craniectomy in a goat anesthetized with propofol, lidocaine, midazolam and fentanyl.(85)

Fentanyl use in cattle.

The use of fentanyl in cattle is not well described. An anecdotal recommendation for transdermal patches dosed at 0.05-0.5 μg/kg is described(86), but no PK/PD studies verify this dosing recommendation. An adverse reaction in a post-operative calf administered a fentanyl transdermal has been described.(87) An experimental model to induce respiratory depression via administration of xylazine, fentanyl, and diazepam is also described for calves.(88) Unlike sheep and goats no studies demonstrating the PK/PD for fentanyl exist for cattle.

Adverse effects of fentanyl in ruminant species.

Adverse effects have been reported following the administration of fentanyl to goats. Carroll et al reported a transient increase in rectal temperature after intravenous administration. (63) Behavioral changes have been noted as well in goats administered fentanyl. The changes

range from a variation from recovery following TIVA (84), excessive tail wagging,(82) as well as severe excitatory behavior during recovery from anesthesia.(83) These same adverse effects seem to be more apparent in goats than sheep, as multiple studies mention uneventful recoveries in sheep when fentanyl is used for analgesia or anesthesia,(89, 90) although adverse effects of pacing, head pressing, decreased ruminations, urinary retention and severe sedation have been reported in sheep.(59) These differences in adverse effects could be due to formulation, as the aforementioned studies with no adverse effects explored transdermal patches and intravenous infusions, and the study that noted adverse effects investigated a transdermal solution. Additionally, adverse effects of excessive locomotor activity and vocalization have been associated with the use of a transdermal fentanyl solution in sheep.(59) Similar adverse effects were noted when this transdermal solution was used in camelids (Lakritz, personal communication). An adverse reaction in a post-operative calf administered a fentanyl transdermal patch with morphine is described as agitation, altered mentation, mydriasis, nystagmus, increased locomotor activity, vocalization, myoclonus of the tail, hyperesponsiveness and hyperthermia along with tachycardia and tachypnea has also been described.(87) Additional studies are necessary to determine safe, effective and therapeutic manners to apply the analgesic potential of fentanyl to bovine patients.

Pharmacology of Tulathromycin

Tulathromycin is a member of the macrolide class of antibiotics, which originated with erythromycin. A semisynthetic macrolide, it presents as an equilibrated mixture of an azalide with a 13 member ring in and a 15 member ring.(91) The structure contains three amine groups and these three functional groups classify tulathromycin as the first of a sub-class of macrolides

known as triamilides.(92) While the macrolide antimicrobials can vary structurally, they all maintain similar action, primarily by inhibiting protein synthesis via the reversible binding to 23S ribosomal subunit of bacteria.(93) This mechanism will then serve to inhibit the protein elongation phase by stopping the catalyzation of peptide bonds.(94) Tulathromycin acts as a bacteriostatic antimicrobial when tested against *E. coli* and *Staphylococcus aureus*(93), however this mechanism can be bactericidal when *M. haemolytica*, *A. pleuropneumoniae* and *P. multocida* are challenged at 4 to 8 times the MIC.(91, 95) In the United States tulathromycin is labelled for use in cattle for the treatment and or control of bovine respiratory disease due *to Mannheimia haemolytica*, *Pasteurella multocida*, *Haemophilus somnus* (*Histophilus somni*) and *Mycoplasma bovis*; the treatment of interdigital necrobacillosis due to *Fusobacterium necrophorum*; and treatment of infectious bovine keratoconjunctivitis from *Moraxella bovis*. While the use of tulathromycin is unique to veterinary medicine, the importance of the macrolide antimicrobials is not. In human medicine the World Health Organization has classified macrolide antibiotics on the list of the highest priority, critically important antimicrobials for the preservation of human health.(96)

Tulathromycin is administered via parenteral routes in cattle and swine. No data exist for oral administration in cattle, but for swine the administration of tulathromycin via oral gavage yields a bioavailability of 51.1%.(97) Following subcutaneous administration tulathromycin rapidly achieves systemic absorption and persists in tissues for extended periods of time.(98) It possesses a pKa of 7.4-8.6, as well as hydrophilic properties, which allow for ease of transit from plasma to tissues, as well as the ability to become ion trapped in acidic tissues.(93) Tulathromycin also concentrates in inflammatory cells.(99) Metabolism of tulathromycin is

primarily via hepatic microsomal enzymes. In pigs tulathromycin is excreted by biliary and renal excretion routes (93), and in cattle the major route of excretion is thought to be biliary.(11)

Tulathromycin in sheep.

There is no sheep label for tulathromycin in the United States, so all use of this drug in the US would be considered ELDU. Pharmacokinetics of non-pregnant adult ewes are similar to cattle such that the same dose (2.5 mg/kg) could be suggested to have efficacy as long as the targeted bacteria would have similar inhibitory concentrations to those in cattle.(100) Initial efforts focused on the use of tulathromycin for the treatment of caseous lymphadenitis caused by *Corynebacterium pseudotuberculosis*, due to the high degree of lipid solubility and long-lasting property of the drug.(101) One future potential application of tulathromycin in sheep could be for the treatment of abortion storms caused by *Campylobacter* spp, as research has suggested that an increasing number of ovine isolates are resistant to the traditional treatment with tetracyclines.(102) This use is further supported by fetal ovine PK data demonstrating consistent concentrations between 4 and 288 hours in one study.(103) However, this use would require adherence to AMDUCA, as there is a labelled macrolide antibiotic, tilmicosin, available in the US for ovine use.

Tulathromycin in goats.

Similar to sheep in the US, tulathromycin can be legally used in goats in an extra-label manner when treatment has been deemed clinically ineffective by a labelled drug. The PK of tulathromycin in goats is similar to what has been reported for cattle and swine.(104) When compared to cattle, the pharmacokinetics and tissue elimination of tulathromycin in goats is similar.(104-107) Like sheep, tulathromycin has been identified as a potential therapeutic agent

for infection by *C. pseudotuberculosis*. This is supported by a tissue chamber model of caprine abscesses that found similar concentration of tulathromycin in the chambers as plasma.(108) This therapeutic plan is supported by a randomized clinical trial treating *C. pseudotuberculosis* in client-owned goats.(101) Tulathromycin also appears to be a safe drug for caprine therapy, as administration of ten times the bovine dosage resulted in no gross or microscopic lesions.(109) Tulathromycin's broad spectrum of activity and long-acting character make it an ideal antimicrobial for treatment of respiratory disease in goats. Among caprine respiratory disease isolates, a 100% efficacy was noted against caprine respiratory isolates of *Mannheimia hemolytica*, *Pasteurella multocida*, and *Bibersteinia trehalos*i presented to the Veterinary Diagnostic Laboratory at Iowa State University.(110)

Tulathromycin in cattle.

The majority of tulathromycin use in the US is in cattle. After one subcutaneous administration tulathromycin has high bioavailability, and can persist in lung tissue for extended periods of time.(111) These parameters indicate rapid absorption, with maximum concentrations being reached approximately one hour after administration and a long plasma elimination half-life.(105) When used in cattle, tulathromycin also possesses activity against *Fusobacterium necophorum*, *Porphyromonas levii*, and *Moraxella bovis*. The drug has a significantly higher cure rate when compared to tilmicosin for calves with bovine respiratory disease(112), and higher than either florfenicol or tilmicosin in a separate study.(113) Similar treatment efficacy and a lesser need for retreatment with tulathromycin is seen in dairy heifers treated for preweaning respiratory disease when compared to enrofloxacin.(114) When a similar treatment strategy was utilized on calves at high risk of developing BRD, a significant decrease in

retreatments was also noted for tulathromycin compared to enrofloxacin.(115) This increased retreatment rate was noted for gamithromycin when compared to tulathromycin for treatment of undifferentiated BRDC in a feedlot setting.(116) Resistance to tulathromycin also appears to be a less emergent concern than other antimicrobials, as a lack of significant resistance over multiple-year studies has been observed for *Mannheimia hemolytica* (117, 118), however a 10 year study conducted when tulathromycin entered the US market identified increases in MIC_{90} values for tulathromycin over this time period.(119) Recent work has suggested that tulathromycin is not ideal to use for both BRD control and treatment on the same operation, as this could lead to the development of increased resistance in lower respiratory tract pathogens.(120) This finding presents challenges for clinicians as the treatment of cattle with multiple antimicrobial treatments can lead to increased resistance in BRD pathogens.(121) The emergence of resistance may not only be limited to respiratory pathogens as use of tulathromycin for respiratory concerns results in resistance in enteric populations of *Enterobacter spp*.(122) Not all enteric pathogens follow this trend, notably non-type specific *Escherichia coli* which demonstrates resistance following administration of tetracyclines, but not tulathromycin, to feedlot cattle.(123) Tulathromycin demonstrates efficacy for multiple BRD applications, and the emergence of resistance dictates continued vigilance of this antimicrobial.

Tulathromycin is an effective treatment for other pathogens than just those associated with BRD. *Moraxella bovis*, the causative agent of infectious bovine keratoconjunctivitis is also a label indication for tulathromyin in the US. When prospectively infected with *M. bovis*, treatment with tulathromycin will lead to faster resolution of corneal ulcers, and less frequent isolate recovery when compared to control calves.(124) Tulathromycin is also an effective therapy for *Mycoplasma bovis* in many circumstances.(125) When compared across decades,

tulathromycin is still a prudent choice for the treatment of infections caused by *M. bovis* in cattle, as while MIC_{50} levels increased to 32 μg/mL in the 1990s and then decreased in the 2000s. Tulathromycin also possess activity for infectious pododermatitis in cattle, making it an ideal candidate for individual animal therapy.(126) Contagious bovine pleuropneumonia, caused by *Mycoplasma mycoides* subspecies mycoides (small colony type) demonstrates in vitro sensitivity to tulathromycin, indicating another potential use.(127) Antimicrobial actions may not be the only mechanism of tulathromyin. The immunomodulating effects of promotion of apoptosis of bovine neutrophils, inhibition of proinflammatory signaling, and selective promotion of apoptosis instead of necrosis in bovine macrophages may also confer advantages in respiratory disease.(128) Additional non-antimicrobial activity of tulathromycin may include prokinetic effects, as administration of tulathromycin to calves will increase abomasal emptying rate.(129) This is most likely due to structural similarities to erythromycin, as erythromycin acts on the motilin receptor in a prokinetic fashion.(130) Multiple applications for tulathromycin in cattle exist beyond BRD.

Adverse effects reported to tulathromycin in ruminant food animals.

Limited studies describe adverse effects for tulathromycin when administered to ruminant food animals. Several studies have identified no serious safety concerns or toxicity when overdoses of tulathromycin are administered to goats.(109, 131) The author has noted vocalization when goats are administered tulathromycin in both clinical and research (Smith, in press) settings. When administered to steers by a dart remote delivery system, increased pain, stress, and muscle damage occurs compared to conventional administration(132), although this could be due to the dart administration depositing more drug in an intramuscular, rather than

subcutaneous route. Foals administered tulathromycin tolerated therapy well, with the exception of diarrhea, injection site swellings, and hyperthermia.(133)

Pharmacology of Carbapenem Antimicrobials

The carbapenem class of antimicrobials were initially developed due to the emergence of bacterial resistance to the beta-lactam antimicrobials. Initial research efforts focused upon the inhibition of beta-lactamase enzymes, and a natural compound, olivanic acid was the first beta-lactamase inhibitor identified.(134) Produced by *Streptomyces clavuligerus*, this acid had the early structure of the carbapenems, however due to poor penetration into bacterial cell walls and instability, the olivanic acids were not viable antimicrobials.(134) Two agents originated from this first step investigating olivanic acid: clavulanic acid and theinamycin.(135) Clavulanic acid functions to bind to an active site in the beta-lactamase enzyme and this action deactivates the enzyme. When compared to other beta-lactam antimicrobials, the carbapenem class is more efficacious against bacteria possessing beta-lactamase activity, although spectrum can vary, with meropenem, biapenem, and ertapenem demonstrating some gram-negative organisms, and some other members of the class, such as imipenem and doripenem being effective for gram-positive bacteria.(135) The basis of this improved antimicrobial activity over other beta-lactam antimicrobials is due to two structural moieties: a 1-beta-methyl group that allows for resistance to hydrolysis by renal DHP-1, and resistance to hydrolysis by carbapenemases and beta-lactamases via a trans-hydroxyethyl moiety.(135) This structure is critical as the definition of carbapenem is the 4:5 fused ring lactam of penicillin with a C-2 to C-3 double bond and a substitution of carbon for sulfur at C-1.(134) Additionally the hydroxyethyl side chain is important for the activity of the carbapenems due to the stereochemistry it provides.(136) Carbapenem antimicrobials are organized into three groups. First are the broad-spectrum agents

that have limited efficacy against gram-negative organisms (e.g. ertapenem); second, the agents that have broad spectrum activity and efficacy against gram-negatives (e.g. imipenem, meropenem); and third, those with activity against methicillin-resistant *Staphylococcus aureus* (e.g. Tomopenem(137)).(135) Ertapenem is in the group of carbapenems with activity against gram-positive pathogens and is extensively used in human medicine. Primary indications include complicated infections as well as community-acquired pneumonia.(138) Ertapenem possesses a broad-spectrum of activity for many gram-positive and gram-negative bacteria(139), however it lacks efficacy against *Pseudomonas aeruginosa*. With a longer elimination half-life than most carbapenems (4 hour vs 1 hour)(140), ertapenem can be used in a once-daily dosing regimen.(141) The spectrum and properties of ertapenem support the use of it for community-acquired infections and outpatient intravenous antimicrobial therapy rather than for the treatment of nosocomial infections.(140)

Pharmacokinetics of carbapenems.

The carbapenems have low oral bioavailability, requiring administration by parenteral routes such as intravenous or intramuscular injection, or via local therapy in a regional limb perfusion. Carbapenems rapidly penetrate most body tissues and organs after intravenous administration.(142) Excretion of carbapenems is renal, predominantly via enzymatic breakdown from dehydropeptidase. With imipenem this renal mechanism of excretion can be exploited with the co administration of the cilastatin. Cilastatin has no antimicrobial properties on its own, although it does inhibit a gene in *Aeromonas* bacteria that produces an enzyme that hydrolyzes imipenem.(143) This enzyme is similar to mammalian dehydropeptidase I, and when used in tandem with imipenem, the ability of cilastatin to inhibit the enzyme responsible for destruction

of imipenem prolongs therapeutic concentrations.(144) This mechanism also reduces the risk of tubular necrosis associated with high dosages of imipenem.(144, 145) While the most common adverse effect for this class of drugs is nephrotoxicity, additional adverse effects include oxidative stress leading to spermatogenesis dysfunction(146)and psychosis.(147) Carbapenems are more rapidly bactericidal than the cephalosporins.(148)

Carbapenem usage in veterinary species.

The carbapenems are infrequently used in veterinary practice. Currently there are no carbapenems approved for veterinary medicine, and antimicrobial stewardship recommendations warn against the use of such drugs in veterinary practice.(149, 150) However, in the United States, they can be used extra label in small animals as a "last resort" therapy when antimicrobial resistance precludes using approved veterinary drugs.

The majority of reported use of carbapenems in veterinary species is for imipenem, originally explored as a treatment for multi-drug resistant (MDR) *E. coli* infections in dogs(151) and cats(152), and for the mixed aerobic and anaerobic infections in small animals(153). Imipenem has been used for treatment of MDR nonhemolytic *E. coli* in a dog with septic peritonitis(154), MDR *Pseudomonas aeruginosa* pneumonia in a dog after renal transplantation (155), and for MDR *E. coli* and *Enterobacter* infection in 2 dogs.(156) A case series highlighted the potential utility of imipenem therapy for the treatment of septic peritonitis in dogs and cats, as most of the recovered isolates were susceptible.(157) Imipenem could be utilized for equine therapy, as the intravenous pharmacokinetics would favor q 6 hour administration for most susceptible pathogens when combined with cilastatin.(158) Another equine application of imipenem is regional limb perfusions, as this technique resulted in synovial

fluid concentrations above the MIC of most pathogens for 6 hours in a healthy horse model.(159) Regional limb perfusion with imipenem was used to treat a *Streptococcus* sp*., Staphylococcus* sp*., Escherichia coli*, and *Corynebacterium* sp. mixed infection in a swamp wallaby.(160) The pharmacokinetics of imipenem are described for the sheep, and no adverse renal or hepatic effects were noted.(161) However, due to the human health implications of the emergence of imipenem resistant bacteria, clinicians should exercise judgement prior to using imipenem in a veterinary patient.

The use of meropenem has also been reported in veterinary practice, Meropenem differs from imipenem in that it has more gram-negative activity with higher activity against *Pseudomonas aeruginosa* and *Enterobacter* than imipenem(162) and does not require co-administration with cilastatin as it is stable to enzymatic breakdown via dihydropeptidase-1. Because of these characteristics, it is less nephrotoxic than imipenem, with a longer elimination half-life than imipenem.(163) Meropenem was initially investigated for use in dogs(164, 165) and cats(166) as a potential "end of the road" therapy for severe infections that are resistant to common antimicrobials, or for neutropenic patients with severe disease(167), as well as bacteremia or sepsis.(168) Local and regional therapy has been explored with meropenem as it is bactericidal with four-quadrant activity, efficacy at low concentrations, high water solubility, and high stability at high temperatures.(169) When used in regional perfusion, in contrast to imipenem, when meropenem does not achieve therapeutic levels, and would probably be clinically ineffective and promote resistance in once daily dosing fashion.(170) In terms of local therapy, there is more application for meropenem as an antibiotic-impregnated bead, as it appears to be resistant to degradation by the ethylene oxide sterilization process, and can elute (*in vitro*) at levels higher than 4 mcg/mL for a time period of 15-18 days.(169) Additionally, *in*

vitro elution from non-sterilized and sterilized beads was not significantly different after processing via hydrogen peroxide vapor sterilization. While meropenem may have similar utility to imipenem in a clinical setting, the same concerns regarding the emergence of resistant isolates remain.

The effects of clinical states on the PK/PD integration of carbapenems has not been researched in veterinary medicine extensively across multiple studies and species. One study examined the pharmacokinetics of meropenem in beagles receiving intermittent hemodialysis. In that study, patients in renal failure and those undergoing hemodialysis had a reduced half-life meropenem.(168) It is recommended to increase the dose after dialysis to compensate for the residual renal function of these dogs. This suggests higher dosages and dosing frequencies may be necessary for patients undergoing dialysis.(171) Complicated urinary tract infections have also led to lower volumes of distribution, and clearance in human patients.(138) These changes may require altered dosages or dosing strategy. Due the risk that carbapenem resistant infections poses, more research is needed on the effect of clinical states on PK/PD of these antimicrobials for animal models of human disease.

Public health implications of carbapenem usage.

This use of carbapenem in animals can present a significant public health challenge as the treated animal may present a risk for transmission of a resistant organism to a person. This is concerning due to the "big gun" status of the carbapenem antibiotics for the treatment of challenging infections. There is also the potential for carbapenemase-producing bacteria to not be diagnosed by conventional veterinary techniques compared to human laboratories. Since none of these antimicrobials are labelled for animals, most veterinary diagnostic labs are not testing for them and further compounding the problem would be the fact that the use of "generic" human

breakpoints would not necessarily directly translate to bacteria producing carbapenemases in veterinary patients.(150) Despite the infrequent use of carbapenems in veterinary medicine, there are reports of increasing carbapenem resistant bacterial isolates from animals. In France a non-susceptibility rate of 5.7% was found to meropenem and or imipenem in canine and feline veterinary isolates,(172) despite no widespread use of these agents in veterinary species. Increasing rates of resistance in small animal veterinary isolates have also been reported in Spain.(173) There is emerging concern that hospitalized or recently hospitalized companion animals may be a source of carbapenem resistant pathogens for human infections.(174) In food animal species, imipenem resistance has been documented in isolates from pigs in China(175, 176), chickens in Senegal and China (176, 177), dairy cattle in China and India(178, 179). Carbapenem resistance has also been identified in environmental isolates in an Australian equine hospital, indicating the potential for nosocomial risk to veterinary patients and workers.(180) Resistant isolates are not limited to domestic animals, as there are reports of bacteria ranging from wild animals in Italy to Yesso scallops in Korea demonstrating resistance to meropenem and imipenem.(181, 182) Future strategies involving the precision application of the carbapenem antimicrobials as necessary to avoid large scale public health emergencies due to the emergence of resistance to these drugs that are reserved for cases of severe infection.

CHAPTER 2. PHARMACOKINETICS OF FENTANYL CITRATE AND NORFENTANYL IN HOLSTEIN CALVES AND THE EFFECT OF ANALYTICAL PERFORMANCES ON FENTANYL PARAMETER ESTIMATION

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Abstract

This study describes the pharmacokinetics of intravenously administered (i.v.) fentanyl citrate, and its primary metabolite norfentanyl in Holstein calves. Eight calves (58.6 ± 2.2 kg), aged 3–4 weeks, were administered fentanyl citrate at a single dose of 5.0 μg/kg i.v. Blood samples were collected from 0 to 24 hr. Plasma (nor)fentanyl concentrations were determined using liquid chromatography with mass spectrometry and a lower limit of quantification (LLOQ) of 0.03 ng/ml. To explore the effect of analytical performance on fentanyl parameter estimation, the noncompartmental pharmacokinetic analysis was then repeated with a hypothetical LLOQ value of 0.05 ng/ml. Terminal elimination half□life was estimated at 12.7 and 3.6 hr for fentanyl and norfentanyl, respectively. For fentanyl, systemic clearance was estimated at 2.0 L hr−1 kg−1, volume of distribution at steady- state was 24.8 L/kg and extraction ratio was 0.42. At a

hypothetical LLOQ of 0.05 ng/ml fentanyl half‐life, volume of distribution at steady‐state and clearance were, respectively, of 3.0 hr, 8.8 L/kg and 3.4 L kg−1 hr−1. Fentanyl citrate administered i.v. at 5.0 μg/kg can reach levels associated with analgesia in other species. Pharmacokinetic parameters should be interpreted with respect to LLOQ, as lower limits can influence estimated parameters, such as elimination half‐life or systemic clearance and have significant impact on dosage regimen selection in clinical practice.

Key words: Fentanyl, Cattle, Calves, Norfentanyl

Introduction

Analgesia for cattle during production, surgical, and medical procedures is an important tool for promoting animal welfare. While cattle are commonly subjected to potentially painful production procedures and non-routine surgical procedures, practitioners have limited options in terms of pain management as in the US there are currently no drugs labelled for analgesia in cattle.

The synthetic mu receptor opioid agonist fentanyl is commonly used to provide analgesia in veterinary species. Morphine and butorphanol are opioid analgesics that currently are used as an intravenous (IV) bolus in cattle. Morphine, is a primary mu opioid agonist that is used for the treatment of pain in a wide variety of veterinary species. Butorphanol has also been described for use in many veterinary species and is a partial opioid agonist with activity as an agonist for the kappa receptor and weak mu receptor antagonist activity. Butorphanol is thought to have an analgesic value of approximately four to seven times that of morphine.

With a potency that is approximately 100 times more than morphine, and a rapid onset, fentanyl is an ideal clinical analgesic in veterinary medicine. Fentanyl is primarily metabolized

by cytochrome P450 3A enzymes to norfentanyl.(50) There are several additional minor pathways it the metabolism of fentanyl, primarily amide hydrolysis to despropionyl fentanyl as well as alkyl hydroxylation to hydroxyfentanyl.

Among large animal species, the pharmacokinetics (PK) of IV fentanyl has been described in sheep (62), goats (63), alpacas (64), and horses (66). In small animals, the IV pharmacokinetics of fentanyl have also been described. Adverse reactions to fentanyl include an increase in locomotor activity in horses (70), and respiratory depression when too high systemic concentrations are reached (30 ng/mL) in dogs.(71) Pharmacokinetics of fentanyl metabolites, while readily available in human medical studies, are limited in veterinary medicine. Currently limited to studies reporting norfentanyl concentrations in chickens (51), and primates (52), as well as not detecting measurable quantities of norfentanyl in dogs.(53)

While practitioners routinely utilize analgesic drugs in a legal extra-label manner, there are few reports of the pharmacokinetics of fentanyl in ruminant species, and no reports of the use of this analgesic therapy in cattle. Due to the increased analgesic activity of fentanyl compared to morphine and butorphanol it may have clinical uses for bovine analgesia during surgical procedures.

The aim of this study was to describe the pharmacokinetics of fentanyl citrate and its primary metabolite norfentanyl when administered as an IV bolus in calves, as well as to report any adverse reactions. A secondary goal of this study was to examine the impact of the bioanalytical quantification limit of fentanyl with respect to pharmacokinetic parameter estimation.

Materials and Methods

Experimental animals.

This study was completed at the Iowa State University Dairy Farm. Eight female Holstein calves were enrolled in the study. The age of these calves ranged from 23 to 30 days, weighed 58.6 +/- 2.2 kg, and were procured from a single source farm. Approval for the study was secured from the Institution Animal Care and Use Committee (Log # 7-16-8318-B) at Iowa State University. The calves were housed in individual pens since birth, and the study took place in the same individual pens for each calf. The calves were housed in a climate-controlled calf raising facility, and no alterations to feeding or handling schedule was made for this study. During the pre-study time period, all calves were trained to be restrained by a hand placed under the mandible and behind the poll. Criteria for enrollment in this study included a physical assessment by a veterinarian that yielded vital signs within the normal limits for a bovine calf, no previous history of medical illness as well as no history of a previously administered medication. Prior to and during the study all calves were fed a diet that either met or exceeded the NRC requirements for maintenance and growth of bovine calves. Study calves were fed a pasteurized whole milk diet (3 quarts) every eight hours with ad libitum access to a commercial calf starter.

Twenty hours prior to initiation of the study the calves were restrained and 2 IV jugular catheters were aseptically placed. The skin was aseptically prepared utilizing 4 alternating scrubs of chlorhexidine surgical scrub and 70% isopropyl alcohol. Prior to catheter placement the skin at the catheter site was infiltrated with 2% lidocaine. The calf was restrained by study personnel and a 14-gauge catheter was placed in each jugular vein. An injection port was placed and the catheters were sutured to the skin and wrapped for security.

Experimental design and sample collection.

Calves were administered a single 5.0 µg/kg IV bolus of fentanyl citrate (Fentanyl Citrate, Hospira Inc, Lake Forrest, Il) via a catheter inserted in the left jugular vein. Blood collection was achieved through a catheter in the right jugular vein at 2, 5, 10, 30, 45, and 60 minutes, and 1.5, 2, 2.5, 3, 4, 6, 10, 16, and 24 hours after administration. Starting at the 2-hour sampling time point, heart and respiratory rates were measured at each sampling timepoint up to 24 hours.

The 5.0 µg/kg dose was determined as a pilot study investigating a 2.5 µg/kg IV bolus of fentanyl citrate (Fentanyl Citrate, Hospira Inc, Lake Forrest, Il) as reported for sheep (62) failed to achieve a concentration above 1.0 ng/mL and enough data points to accurately analyze pharmacokinetics. Thus the present study was conducted in a new cohort of calves with twice the pilot dosage.

At each sampling timepoint blood was collected from the catheter using a 12-mL syringe and placed into sodium heparin tubes (BD Vacutainer, Franklin Lakes, NJ). The samples were then centrifuged at 1500 G for 10 minutes. The plasma was pipetted off and transferred to cryovials which were then stored at -80 C until analysis.

Sample analysis.

Plasma concentrations of fentanyl, and its metabolite norfentanyl were determined by liquid chromatography-mass spectrometry (LC-MS) after precipitation of proteins by acetonitrile. Briefly, plasma samples were thawed and vortexed, and 200uL aliquots were transferred into a vial with 800uL of internal standard, fentanyl-D5, in acetonitrile with 0.1% formic acid added. Samples were vortexed and then centrifuged at 7500 rpm for 20 minutes. The supernatant was then transferred and the samples were dried down, then reconstituted in 125uL

of 25% acetonitrile in water, vortexed and transferred into an autosampler vial (with glass insert) and then centrifuged for 20 minutes at 2400 rpm and analyzed via LC-MS/MS. The LC-MS system consisted of an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to a Thermo LTQ ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA). The lower limit of quantification (LLOQ) for fentanyl and its metabolite was 0.03 ng/mL for this assay.

Pharmacokinetic analysis.

Pharmacokinetic analysis of total fentanyl and norfentanyl plasma concentrations was completed using a statistical moment (i.e. non-compartmental) approach in commercial software (Phoenix WinNonlin 7.0, Certara, Princeton, NJ, USA). Time versus concentration figures for fentanyl and norfentanyl were produced via a commercial program (GraphPad Prism 7, GraphPad Software, Inc, La Jolla, CA, USA).

Standard PK parameters were generated for individual calves, as follows:

- Maximum (nor)fentanyl concentration, C_0 (fentanyl) or C_{max} (norfentanyl);
- Time of maximum norfentanyl concentration, T_{max};
- Area under (nor)fentanyl concentration-time curve, AUC_{last} and AUC_{inf};
- Area under the moment curve, $AUMC_{inf}$;
- (Nor)fentanyl mean residence time,

  $MRT = AUMC_{inf} / AUC_{inf}$;
- Slope of the elimination phase λ_z, computed by linear regression of the logarithmic concentration vs. time curve during the elimination phase;
- (Nor)fentanyl terminal half-life,

$T_{1/2}$ (λz) = ln (2)/λz;

- Fentanyl systemic clearance, CL = Dose/AUC_{inf};
- Volume of distribution of fentanyl during the elimination phase, V_{area} = Dose/(AUC_{inf} x λz);
- Volume of distribution of fentanyl at steady-state, V_{SS} = CL x MRT

For data analysis, the first value below the LLOQ was inferred to be LLOQ/2, and subsequent data points were excluded from the analysis. A linear/log trapezoidal rule was used to estimate the area under the (nor)fentanyl time-curves. Summary statistics on the individual PK parameters were performed thereafter to derive the geometric mean, median and (min-max) range.

For fentanyl, the extraction ratio (E_{body}) was calculated as reported by Toutain et al (184), with:

E_{Body} = Systemic clearance / Cardiac output [Equation 1]

First calculated for each individual calf, and then combined for a mean value. With the calf cardiac output calculated according to Toutain et al (184) as follows:

Cardiac output = 180 x $BW(kg)^{-0.19}$ [Equation 2]

In a second step and using the same raw source data, an hypothetical analytical LLOQ of 0.05 ng/mL, as reported in the literature in other species (64), was applied and the pharmacokinetic analysis for fentanyl only was repeated using the same workflow as described above.

Results

Animal health.

At enrollment, all study subjects were assessed to be healthy and to have parameters within the normal limits for calves of their respective ages. The injections were well tolerated by all calves, with no adverse effects noted throughout the entire study period. For heart rate, respiratory rate, and temperature no significant elevation or depression from baseline was reported, with the exception of excitement at the timepoints that coincided with the feeding of the calves. Follow up examination 2 weeks and 2 months after the study revealed no abnormalities in behavior or physical assessment.

Pharmacokinetics of fentanyl and its metabolites using a LLOQ of 0.03 ng/mL.

No calf had detectable fentanyl or metabolites in plasma at time zero. The individual time-course of fentanyl and norfentanyl total concentrations in plasma can be found in Figures 1 and 2, respectively. Geometric mean and standard deviations disposition profiles are presented in Table 1 for fentanyl and norfentanyl. Among individuals there appears to be limited variation of time versus concentration data for fentanyl as opposed to norfentanyl. For the LLOQ of 0.03 ng/mL 4.2% (5/120) of the post administration data points had values below the LLOQ. For the theoretical LLOQ of 0.05 ng/mL 21.7% (26/120) of the post administration data points had values below the LLOQ.

Table 1 summarizes the pharmacokinetic parameters for fentanyl and norfentanyl when administered IV. For fentanyl, the systemic clearance was almost 2 L/kg/hr. The average extraction ratio was calculated to be 0.41 ± 0.10. The AUC% extrapolation was estimated to be inferior to 20% (15.4%), while the steady-state volume of distribution (Vss) was 24.8 L/kg. The elimination half-life $T_{1/2}$ (λz) was estimated at approximately 12 hours.

The AUC% extrapolation of 7.2% for norfentanyl was less than that of fentanyl. C_{MAX} and T_{MAX} of norfentanyl were 0.3 ng/mL, and 1.1 hr respectively. The elimination half-life $T_{1/2}$ λz was estimated at 12.7 hours.

Pharmacokinetics of fentanyl and its metabolites using a LLOQ of 0.05 ng/mL.

A comparison of the fentanyl estimated PK parameters with a LLOQ of 0.03 vs. 0.05 ng/mL is provided in Table 2. Despite this relatively small difference in analytical sensitivity (0.02 ng/mL), a noted lack of agreement among parameters was observed. Compared to the quantification limit of 0.03 ng/mL, the clearance of fentanyl was markedly increased (164 % increased) when a hypothetical quantification limit of 0.05 ng/mL was utilized on the study data. In contrast, the estimated volume of distribution markedly decreased (by 68%), and the elimination half-life was 12 hr shorter as compared with the 0.03 ng/mL LLOQ threshold. Interestingly, with the higher quantification limit, the estimated elimination half-life was closer in value to what is reported in the literature for other ruminant species, with a LLOQ ranging from 0.01 (sheep) to 0.1 (goat) ng/mL (Table 3).

Discussion

To the best of our knowledge, this is the first report of the pharmacokinetics of fentanyl in calves. Although the cohort sampling could potentially be a source of bias for this study, it was thought to be minimal as calves had acclimated to the individual pens prior to the study, and the group of individual pens used for the study was from the same block of eight stalls in the temperature, humidity, and ventilation controlled barn. The age and size of the calves utilized for this study was designed to mimic the age of calves presented to the author's hospital for surgical procedures that could potentially benefit from fentanyl analgesia.

In the United States, there is currently no approved formulation of fentanyl citrate for cattle. However, in practice calves routinely undergo orthopedic and other surgical procedures that warrant post-operative analgesia. Several concentrations of fentanyl have been associated with analgesia in various veterinary species. Plasma fentanyl values of 1.07, 0.95, and 0.6 ng/mL or greater have been associated with analgesia in cats (185), dogs (186) and people (187), respectively. In humans, few reports suggest that values as low as 0.2 ng/mL may provide analgesia for individuals that are "opioid naïve" and have not been previously treated with any drugs in the class.(187) The maximum concentration reported in this study (1.5 ng/mL), would be above what is reported to be an analgesic concentration is other veterinary species, although currently the threshold required for analgesia in calves is unknown.

Other studies have evaluated the pharmacokinetics of intravenously administered fentanyl in horses (66), sheep (56, 62), goats (63), and alpacas (64). The mean maximum concentration of 1.5 ug/L reported in our study was less than described by earlier reports in other large animal species when normalized with the input dose (Table 3). The estimated elimination half-life of fentanyl in calves was apparently longer compared with other large animal species, such as sheep (3.1 hours), goats (1.2 hours) and alpacas (1.2 hours).(64) This must be interpreted with caution however, as these values are compared to mature animals in these previous studies, and drug metabolism can be different between young and older animals of the same species. In lambs aged between 3 and 37 days it has been noted that clearance and volume of distribution increase with age.(78) Fentanyl is extracted by the liver via the cytochrome P450 system, and initial activity of this system is low at birth and increases with age.(78) It is uncertain how adult cattle would metabolize this drug, as there would be potential for variation from calves.

It is noteworthy that when the estimated elimination half-life is considered (with a LLOQ of 0.05 ng/mL is applied), the value is much lower (3.0 hr vs 14.9 hr), and this lower value appears to reconcile with other species when a higher quantification limit is applied in calves. However, the HL in sheep was fairly short (3h) despite a very low quantification limit (0.01 ng/mL), therefore, between species differences for fentanyl metabolism are also expected independent of the analytical method.

While the different quantification limits create different pharmacokinetic parameter values, these differences are not trivial. For calculating dosing regimens, clearance is the most important pharmacokinetic parameter.(184) A lower LLOQ can have multiple effects of the pharmacokinetic parameters reported, including clearance. By reducing the number of samples that are below the limit of quantification (BQL), clearance can be overestimated.(188) A higher LLOQ would theoretically result in more sample values BQL, and therefore result in a higher clearance. This finding is supported by the higher average clearance reported for the theoretical 0.05 ng/mL LLOQ for these calves than the average clearance reported for the 0.03 ng/mL LLOQ (3371 vs 2061 mL/hr/kg). Similarly, elimination half-life, important in predicting time to steady-state, as well as drug accumulation, would also be affected by a lower LLOQ. The relationship between elimination half-life and clearance is as follows (189) :

Elimination half-life = (0.693 x Volume of Distribution) / Clearance. [Equation 3]

Therefore, increasing clearance would serve to underestimate the elimination half-life. This is also supported by the theoretical exercise as the elimination half-life was much shorter for the theoretical LLOQ of 0.05 ng/mL vs the theoretical calculation with a LLOQ at 0.03 ng/mL. These differences in calculated parameters could have effects on patients when treated with fentanyl, depending on the pharmacodynamics of the drug. While there is a relative paucity of

the effects of fentanyl in cattle, adverse effects from overdosing have been reported in multiple species.

Volume of distribution at steady state (27.5 L/kg) was also greater than reported values of other ruminant species such as 8.9 L/kg (sheep), 1.5 L/kg (goats), and 1.5 L/kg (alpacas) (64). The estimated systemic clearance (2.1 L/kg/hr) was consistent with other reported clearances in similar large animal species of sheep (3.6 L/kg/hr), goats (2.1 L/kg/hr), and alpacas (1.1 L/kg/hr) .(64)

Extraction data does not appear to be well described for fentanyl in large animal species. The total extraction of the body, reported in this study as E_{body}, can be described as a percentage or ratio of the drug eliminated through one pass of the different organs contributing to clearance .(184) The extraction ratio reported for the calves in this study (0.41 ± 0.10) would be consistent with an extraction percentage of 41.0 ± 10%. This appears to be greater than what has been described in neonatal lambs, as a fentanyl extraction percentage of 16.5 ± 3.0% has been reported.(79) As reported by Toutain et al (184), an extraction value of 0.3 (30%) or higher is indicative of high a clearance of fentanyl in calves.

In adult humans fentanyl is mainly metabolized by cytochrome P450 3A enzymes to norfentanyl.(50) Two other minor metabolites, desproprionyl fentanyl, and hydroxyfentanyl are accomplished by amide hydrolysis and alkyl hydroxylation respectively.(50) The pharmacokinetics of norfentanyl are not widely described in veterinary species, with one recent report identifying parameters in chickens administered fentanyl via a transdermal patch system.(51)

Norfentanyl pharmacokinetics in this study significantly varied from that of the parent compound fentanyl. Notably, the elimination half-life of norfentanyl was estimated at only 3.6

hours vs. 12.7 hours for its parent. Since a metabolite cannot be eliminated faster than it is being formed, the elimination half-life of norfentanyl can either be similar or longer than that of fentanyl, but not shorter. Therefore, the apparent '*shorter*' half-life of norfentanyl is most likely a consequence of the bioanalytical cut-off, such that the reported half-life of 3.6 hours relate to the distribution, rather than the elimination of norfentanyl. This is supported by the similarities in the estimated half-life between fentanyl and norfentanyl as the theoretical LLOQ for the parent increased from 0.03 to 0.05 ng/mL. As no norfentanyl concentrations were measured below 0.05 ng/mL, the PK parameters remain unchanged if re-evaluated with the theoretical LLOQ of 0.05 ng/mL.

At this time the significance of the norfentanyl pharmacokinetic parameters is unknown as a relative paucity of comparative data for this metabolite exists in the veterinary literature. Among human toxicologists it is speculated that the smaller the ratios of blood and urine norfentanyl/fentanyl, the larger the probability of acute fentanyl intake with coexistent fentanyl abstinence, which then predisposes to fentanyl toxicity.(190) Further studies of norfentanyl are necessary to determine the clinical significance of this metabolite in cattle.

Further work needs to be completed to investigate the analgesic properties of fentanyl in calves. In addition, more work into alternative dosing formulations, such as continuous rate infusion and transdermal patches needs to be done to evaluate the suitability of these routes for bovine practice. Based on comparison to similar ruminant species it appears that the pharmacokinetic parameters calculated with an LLOQ of 0.05 ng/mL may be more useful for calculating dosing regimens in calves. However, there are species differences in location and distribution of opioid receptors, and as such, differences in responses to opioids have been

described.(191) Future studies should also focus on tissue residue depletion, so that withdrawal guidance could be generated for practitioners.

Limitations

A limitation of this study was the relatively small number of calves used. While eight animals are commonly used in PK studies, it might not account for population variability. Similarly, all of the animals were calves of the approximate same age which may not be reflective of adult cattle. Norfentanyl calculations were limited, as a metabolite, clearance and volumes of distribution cannot be calculated without a priori knowledge on the fractional conversion of fentanyl into norfentanyl. Additional pharmacokinetic studies with norfentanyl per se should consider intravenous injection of the metabolite to derive such parameters.

Conclusions

In conclusion, fentanyl citrate administered intravenously reaches systemic peak concentrations associated with analgesia in other veterinary species. An IV dose of 5.0 µg/kg IV appears to be safely tolerated in calves. Finally, interpretation of pharmacokinetics warrants close investigation of the quantification limits used, as increased or decreased limits of quantification can significantly alter the estimation of pharmacokinetic parameters, which could have important implications for dosing regimen selection in clinical practice.

Author Contributions

JSS was involved in study design and execution, sample collection and analysis, manuscript preparation and submission. JFC was involved in study design and manuscript preparation. IWGF was involved in study design, execution, and manuscript preparation. DJB was involved in study design, method development, sample analysis, and manuscript

preparation. JPM was involved in study design, sample analysis, and manuscript preparation. All authors have read and approved the final manuscript.

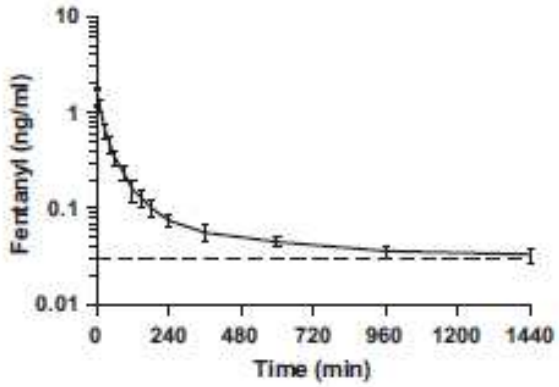

Figure 1. Individual fentanyl pharmacokinetic time-course (log10, mean ± 1SD) following intravenous bolus dosing at 5.0 µg/kg.

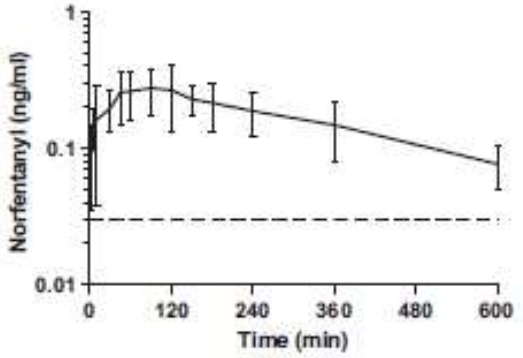

Figure 2. Individual norfentanyl pharmacokinetic time-course (log10, mean ± 1SD) following intravenous bolus dosing at 5.0 µg/kg

Table 1. Pharmacokinetic parameters for fentanyl and norfentanyl in study calves.

Compound	Parameter	Unit	Geomean	Median	Min	Max
	C_0	ng/mL	1.5	1.6	1.0	2.0
	AUC_{last}	ng/mL*hr	2.0	2.1	1.6	2.3
Fentanyl	AUC_{inf}	ng/mL*hr	2.5	2.3	1.8	3.3
	$\%AUC_{extr}$	%	15.4	11.0	7.0	48.1
	$AUMC_{inf}$	$ng/mL*hr^2$	31.1	17.1	16.2	131.1
	MRT	hr	12.4	8.8	7.3	39.3
	CL	mL/hr/kg	1999	2167	1505	2821
	$T_{1/2}$ (λz)	hr	12.7	9.1	7.5	35.1
	V_{ss}	L/kg	24.8	23.3	15.8	58.8
	V_{area}	L/kg	36.7	34.0	23.4	76.1
	Cmax	ng/mL	0.3	0.3	0.2	0.5
	Tmax	hr	1.1	1.5	0.08	2.5
Norfentanyl	AUC_{inf}	ng/mL*hr	1.8	2.2	0.9	2.9
	$\%AUC_{extr}$	%	7.2	7.2	3.6	13.1
	$AUMC_{inf}$	$ng/mL*hr^2$	10.6	13.4	4.6	16.5
	MRT	hr	5.9	6.0	4.8	7.9
	$T_{1/2}$ (λz)	hr	3.6	3.2	2.9	5.4

Table 2. Average (± S.D) fentanyl pharmacokinetic parameters with the study lower limit of quantification (LLOQ) of 0.03 ng/mL compared to a theoretical LLOQ of 0.05 ng/mL.

Parameter	**Unit**	**Calves (Current)**	**Calves (Hypothetical)**
LLOQ	**ng/mL**	**0.03**	**0.05**
AUC_{inf}	ng/mL*hr	2.6 ± 0.6	1.5 ± 0.3
CL	mL/hr/kg	2061 ± 491	3371 ± 813
$T_{1/2}$ (λz)	hr	14.9 ± 9.9	3.0 ± 0.9
Λz	1/hr	0.06 ± 0.03	0.30 ± 0.1
MRT	hr	15.3 ± 11.6	2.7 ± 0.6
V_{ss}	L/kg	27.5 ± 14.7	8.8 ± 1.2
V_{area}	L/kg	39.6 ± 17.1	13.9 ± 3.0

Table 3. Pharmacokinetic parameters of fentanyl in other large animal species. See Table 1 for definition of abbreviated terms.

Parameter	**Unit**	**Calves** (Actual)	**Calves** (Hypothetical)	**Goats** (Carroll, 1999)	**Sheep** (Ahern, 2010)	**Alpacas** (Lovasz, 2017)
LLOQ	ng/mL	0.03	0.05	0.10	0.01	0.05
Dose	µg/kg	5.0	5.0	2.5	2.5	2
$T_{1/2}$ (λz)	hr	14.9	3.0	1.2	3.1	1.2
MRT	hr	15.3	2.7	0.80	-	1.3

CHAPTER 3. ADVERSE REACTIONS TO FENTANYL TRANSDERMAL PATCHES IN CALVES: A PRELIMINARY CLINICAL AND PHARMACOKINETIC STUDY

A paper published in 2018 by the Journal of Veterinary Anesthesia and Analgesia 45:575–580.(192)

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Abstract

Objective: To describe adverse reactions and measure plasma fentanyl concentrations in calves following administration of a fentanyl transdermal patch (FTP).

Study design: Prospective experimental clinical study.

Animals: Six female Holstein calves and one male Angus calf. Four calves were healthy experimental animals and three calves were clinical patients.

Methods: Plasma fentanyl concentrations were measured in blood collected from a jugular vein. FTP (2 μg kg^{-1} $hour^{-1}$) was applied to four calves and FTP (1 μg kg^{-1} $hour^{-1}$) to three calves.

Heart rate, respiratory rate, temperature and ataxia were recorded at the same times as blood collection (0, 2, 4, 6, 12, 24, 36, 48, 60, 72, 84 and 96 hours). Substance P concentrations were determined via radioimmunoassay for two calves.

Results: After application of FTP (2 μg kg^{-1} $hour^{-1}$), two calves developed tachycardia, hyperthermia, excitement and ataxia within 6 hours; no adverse effect was observed in the other two calves. The three calves administered FTP (1 μg kg^{-1} $hour^{-1}$) exhibited tachycardia and excitement, and the FTP were removed at 4 hours. Naloxone was administered to two calves before the adverse clinical signs ceased, while adverse events in the other three calves resolved within 2 hours of FTP removal. Variables returned to previous baseline values by 2–4 hours after FTP removal. Maximum plasma fentanyl concentrations were variable among calves (0.726–6.923 ng mL^{-1}). Substance P concentrations measured in two calves were not consistently depressed during FTP application. Fentanyl concentrations at 4 and 6 hours were significantly associated with the appearance of adverse effects.

Conclusions and clinical relevance: FTP (1–2 μg kg^{-1} $hour^{-1}$) administered to calves may result in adverse behavioral and cardiovascular effects. Patch removal and treatment with an opioid antagonist may resolve these adverse effects. Additional research is needed to determine optimal FTP dosing for cattle.

Introduction

Analgesia for cattle during surgical procedures is important for promoting animal welfare. While cattle are commonly subjected to potentially painful production procedures and nonroutine surgeries, practitioners have limited options for pain management since there are currently no drugs labeled for surgical pain in cattle in the US.(193) Additionally, there are very

few reports of adverse effects of long-acting analgesics, such as fentanyl transdermal patches (FTP), in cattle.

Fentanyl is a μ-opioid agonist that has a short duration of action when administered as a single intravenous bolus. For long-term analgesia, FTP have been developed for humans and have been used in many veterinary species. Adverse effects described in large animal species include sedation in sheep (56), vocalization (bleating) and excitement in goats (63) and increased locomotor responses in horses.(194) The only reported adverse effects of FTP in cattle are mydriasis, nystagmus, increased locomotor activity, vocalization, myoclonus of the tail, hyperesponsiveness and hyperthermia which are described in one case describing administration of a FTP and concurrent morphine epidural in a calf. (87) The effects of FTP as a sole opioid in cattle have not been investigated at this time.

The purpose of this study was to describe the effects of FTP placement on calves, and to generate concentration *versus* time data for FTP use in this species. The hypothesis of this study was that observed effects would be mild and consistent with those reported in other large animal species and to record any differences in adverse effects between healthy animals and animals with clinical disease.

Materials and Methods

This study was approved by the Animal Care and Use Committee of Iowa State University (protocols 6-16-8301, 7-16-8318). Two studies were initiated, one utilizing healthy calves from the university dairy and a second enrolling client-owned animals presented to the Iowa State University Food Animal and Camelid Hospital under informed consent.

Six female Holstein calves aged 3–4 weeks and weighing 52.9 ± 5.2 kg (mean ± standard deviation) and one male Angus calf, 16 weeks old and weighing 171.0 kg were studied. All

calves were individually housed in a climate-controlled facility. A catheter (MILACATH-EU, MILA International Inc., KY, USA)) was placed in a jugular vein for blood collection. FTP (Fentanyl Transdermal System; Mylan Pharmaceuticals Inc., WV, USA) dosing was initially 2 $\mu g\ kg^{-1}\ hour^{-1}$. After adverse reactions were observed in two calves, the dose was reduced to 1 $\mu g\ kg^{-1}\ hour^{-1}$ and rounded to the nearest whole patch (Fig. S1). The hair was clipped and skin prepped with chlorhexidine and alcohol and allowed to dry prior to patch placement. Patches were lightly wrapped as described for sheep.(56)

Heart (HR) and respiratory rates (f_R) were measured by thoracic auscultation and rectal temperature (RT) using a lubricated digital thermometer (VetOne, ID, USA). If possible heart rate was confirmed with a point-of care ECG (AliveCor Inc, CA, USA) as described over the left cardiac apex for calves.(195) These variables and ambulatory status were monitored at a minimum of every other hour for the 96 hour duration of the study. Adverse effects were defined as any deviations from accepted HR and f_R ranges and behavior. Blood samples were collected before (0) and 2, 4, 6, 12, 24, 36, 48, 60, 72, 84 and 96 hours after application. The FTP was to be removed at 72 hours after application. In the event that an adverse effect was noted and deemed deleterious to animal health by the attending veterinarian, the FTP was removed.

Blood (10 mL) was collected from the catheter, after a scavenged sample was discarded, using a syringe and placed into sodium heparin tubes (Vacutainer; Becton Dickinson & Co., NJ, USA). The catheters were flushed with 5 mL of heparinized saline (Heparin Sodium and 0.9% Sodium Chloride Injection; Baxter Healthcare Corp., IL, USA) after each use. Analysis of fentanyl concentrations was performed as described (Smith & Coetzee 2018). The samples were centrifuged at 1500 ***g*** for 10 minutes. Plasma was stored at –80 ºC until analysis of plasma concentrations of fentanyl and two metabolites, norfentanyl and despropionyl fentanyl. Samples

were thawed and vortexed, and 200 µL aliquots were transferred into a vial with 800 µL of internal standard, fentanyl-D5, in acetonitrile with 0.1% formic acid added. Samples were vortexed and then centrifuged at 7500 ***g*** for 20 minutes. The supernatant was transferred and the samples were dried, then reconstituted in 125 µL of 25% acetonitrile in water, vortexed and transferred into an autosampler vial (with glass insert), centrifuged for 20 minutes at 2400 ***g*** and analyzed using high performance liquid chromatography (Agilent 1100; Agilent Technologies, CA, USA) coupled to a Thermo LTQ ion trap mass spectrometer (Thermo Scientific, CA, USA). The lower limit of quantification for fentanyl and the metabolites was 0.03 ng mL^{-1} for this assay. Substance P was measured using radioimmunoassay (Fig. S2) as described by Kleinhenz et al.(196)

Statistical Analysis

Based on the occurrence and severity of adverse events observed in calves, 3 study groups were retrospectively defined: 1) severe adverse reactions with increases in physiologic variables and recumbency (group SA), 2) mild adverse reactions with increases in physiologic variables (group MA) and 3) no adverse reactions (group NA). Plasma fentanyl concentrations at the time of adverse reaction among SA, MA and NA were compared using a Wilcoxon Rank Sum test in R Version 3.3.2 (R Foundation, Austria). p-values < 0.05 were considered statistically significant.

Results

The first two calves were clinical patients. An FTP (2.0 µg kg^{-1} $hour^{-1}$) was placed 12 hours before an arthrotomy. Additional drugs administered were meloxicam (1 mg kg^{-1}; ZyGenerics, India) orally and daily and florfenicol (20 mg kg^{-1}; Nuflor; Merck Animal Health, NJ, USA) intramuscularly (IM) every 48 hours. No adverse events were noted (Table S1). The

maximum plasma fentanyl concentration at 36 hours was 0.859 ng mL^{-1} in calf 1 and 6.92 ng mL^{-1} in calf 2 (Fig. 1). Plasma Substance P concentrations for calves 1 and 2 are reported (Fig. S2). Substance P values were variable amongst these calves. An initial depression was noted, but values for both calves increased while FTPs were applied.

FTP (2 µg kg^{-1} $hour^{-1}$) was administered to calves 3 and 4. Severe adverse events were noted (Table S1). Calf 3 was a 27 day-old healthy calf. At 5 hours after FTP application, the calf developed ataxia progressing to recumbency and excessive vocalization. At 6 hours, HR was 210 beats $minute^{-1}$, f_R 72 breaths $minute^{-1}$ and RT 40.5 ºC. The patch was removed at this time and naloxone (total 0.12 mg; Naloxone HCl; Hospira Inc., IL, USA) was administered intravenously (IV). Clinical signs were normal by 2 hours after patch removal. Plasma fentanyl concentration was 3.29 ng mL^{-1} at the time of patch removal.

Calf 4 was a 120 day-old male calf presented for a nonresolving septic radiocarpal joint of 2 months duration. The calf underwent a radiocarpal joint curettage procedure for ankylosis. The FTP was applied before surgery and was removed 10 hours later when the calf exhibited tachycardia (180 beats $minute^{-1}$) measured by stethoscope and as confirmed with an AliveCor ECG, hyperthermia (40 ºC), pacing and excessive vocalization. Plasma fentanyl concentration at this time was 5.52 ng mL^{-1}. Naloxone was administered IV and physiologic variables and behavior returned to normal within 2 hours.

The FTP dose for calves 5–7 was decreased to 1 µg kg^{-1} $hour^{-1}$. Mild adverse reactions (MA) were observed but did not include recumbency (Table S1). The calves were all healthy calves, 3–4 weeks of age. Tachycardia (174, 180 and 196 beats $minute^{-1}$, respectively) were recorded at 4 hours after patch application, with excitement and increased vocalization, therefore, FTP were removed. At this time, plasma fentanyl concentrations for these calves were

0.73, 1.26 and 0.78 μg kg^{-1} $hour^{-1}$, respectively. No concentrations of fentanyl metabolites were detected in any blood sample.

After adverse effects were noted in the calves 5–7, the study was terminated for animal safety concerns. No long-term effects were observed in the calves that were not attributable to the underlying presentation, and all were healthy ≥ 6 months after the study. At the time of onset of adverse reactions (4–6 hours for all calves), the group plasma fentanyl concentrations were 1.85 ± 0.77, 0.92 ± 0.29 and 0.073 ± 0.038 ng mL^{-1} at 4 hours and 2.27 ± 1.44, 0.41 ± 0.17 and 0.072 ± 0.031 ng mL^{-1} at 6 hours for SA, MA and NA, respectively (Fig. S2). Pooled 4 and 6 hour fentanyl concentrations were significantly different for SA *versus* NA ($p = 0.03$), MA *versus* NA ($p < 0.01$) and SA *versus* MA ($p = 0.02$).

Discussion

Most publications regarding peculiar behaviors associated with fentanyl have been in goats. Dzikiti et al. (2011) found that goats display tail wagging in response to high and low dose fentanyl infusions. Activity increases have been noted in horses and goats.(82, 194) Increased excitatory behaviors post-IV infusion; including, star-gazing, itchiness, restlessness, paddling and bruxism have been noted in goats.(83) By contrast, sheep were moderately sedated after FTP application.(56)

Wide variations were noted in plasma fentanyl concentrations between calves. Factors that could have contributed to this include presence of disease and/or pain. It is not entirely clear as to why some calves had adverse effects and some calves did not. Interestingly, calves exhibiting adverse reactions also had high fentanyl plasma concentrations shortly after patch application, with a pattern resembling a ‘burst’ absorption into the systemic circulation. Conversely, calves with no adverse reactions had a more prolonged absorption of fentanyl in

plasma, typical of extended release formulations. As both calves that exhibited no reactions had concurrent disease, and were administered other medications, it is possible that disease altered fentanyl metabolism, or a potential drug–drug interaction occurred. Disease and disease severity have been shown to alter drug pharmacokinetics in Holstein cattle, an example being the elimination of oxytetracycline based on metritis severity.(197) It has also been suggested that the risk of adverse effects to opioids is inversely related to the clinical disease or pain that the patient is experiencing.(198, 199) This could be partially supported by this project, as two of the three calves with no observed adverse effects had pain from surgery, as well as clinical disease, while all of the calves that were healthy experienced adverse effects. However, calf 4 would not support this, as this was a clinical case. A polymorphism of the G57C fentanyl opioid receptor has been identified in horses that leads to increased locomotor activity when administered fentanyl.(194) It is currently unknown if cattle possess a similar polymorphism.

Limitations of this study include a small sample size, and limited breed representation. The adverse effects noted were not anticipated, so the authors deemed it prudent to decrease the patch dose from 2 $\mu g\ kg^{-1}\ hour^{-1}$ to 1 $\mu g\ kg^{-1}\ hour^{-1}$. After additional effects were observed the decision was made to pause the study until more information could be assessed. Recording of physiological variables was limited, and established at sample collection time points, therefore it is possible that adverse effects could have been present for up to 90 minutes before being recorded and that other cardiopulmonary effects could have gone unnoticed. Additionally more research is needed on the analgesic effects of fentanyl in calves including behavior, appetite, as well as pain biomarkers such as Substance P.

In conclusion, fentanyl administered via FTP resulted in adverse effects in calves manifested by hyperthermia, tachycardia, tachypnea, recumbency, vocalization and increased

locomotor response. These reactions were reversed by naloxone and/or FTP removal. Healthy calves may be more likely to display adverse effects of opioids as opposed to calves with clinical disease, although more research is necessary to investigate this further. Clinicians should exercise prudent judgement when utilizing FTP for calves, considering dosages below 1 $\mu g\ kg^{-1}\ hour^{-1}$, while realizing that the analgesic effects of fentanyl in cattle is currently unknown, and should monitor these calves closely for adverse reactions.

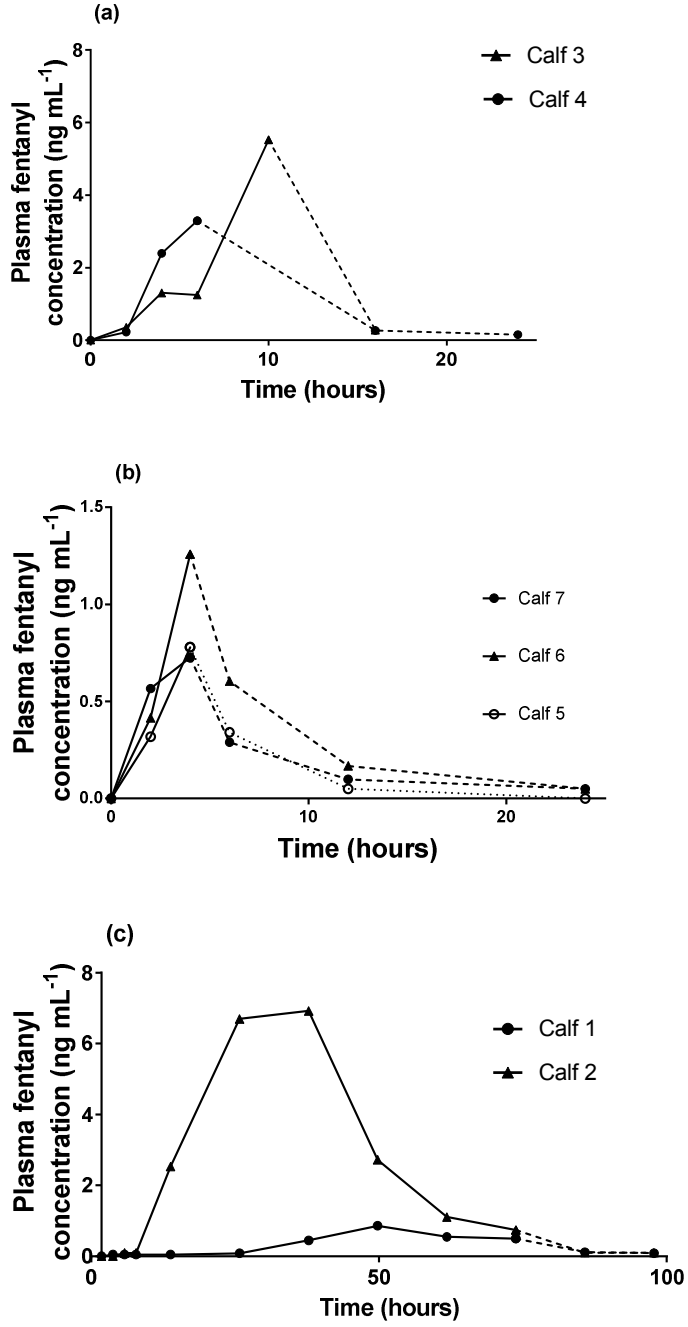

Figure 1. Plasma fentanyl concentrations in seven calves, six Holstein heifers weighing 52.9 ± 5.2 kg and one male Angus weighing 171.0 kg, after application of a fentanyl transdermal patch (FTP). (a) Calves 3 and 4 (FTP 2 μg kg^{-1} $hour^{-1}$) with severe adverse reactions; (b) calves 5–7 (FTP 1 μg kg^{-1} $hour^{-1}$) with mild adverse reactions; and (c) calves 1 and 2 (FTP 2 μg kg^{-1} $hour^{-1}$) with no adverse reactions.

Table S1 Responses in seven calves administered a transdermal fentanyl patch (TFP) and peak plasma fentanyl concentrations measured. Note: Physiologic variables are reported at the time of maximum fentanyl concentration. For calves 3-7 this coincided with the time of FTP removal. Bolded physiologic variables correspond with values above normal reference ranges. All calves had initial variables within the accepted physiologic ranges for calves.
HR, heart rate; f_R, respiratory rate; for behavioral observations a + or – indicates that the behavior was or was not noted respectively.

Calf number	TFP dose (μg kg^{-1})	Maximum recorded physiologic variables			Behavior			Peak plasma fentanyl concentration (ng mL^{-1}) and time recorded (hours)
		HR (beats minute^{-1})	f_R (breaths minute^{-1})	Rectal temperature (^oC)	Vocalization	Excitement	Recumbency	
1	2	116	36	38.7	-	-	-	0.859; (48)
2	2	120	30	**39.2**	-	-	-	6.923; (36)
3	2	**210**	**72**	**40.5**	+	+	+	5.522; (10)
4	2	**180**	**78**	**40.6**	+	+	+	3.292; (6)
5	1	**174**	**66**	**39.1**	-	-	-	0.726; (4)
6	1	**180**	**72**	**39.7**	+	+	-	1.258; (4)
7	1	**196**	**72**	**39.8**	+	+	-	0.779; (4)
Accepted normal range		100–140	30–60	38.6–39.4				

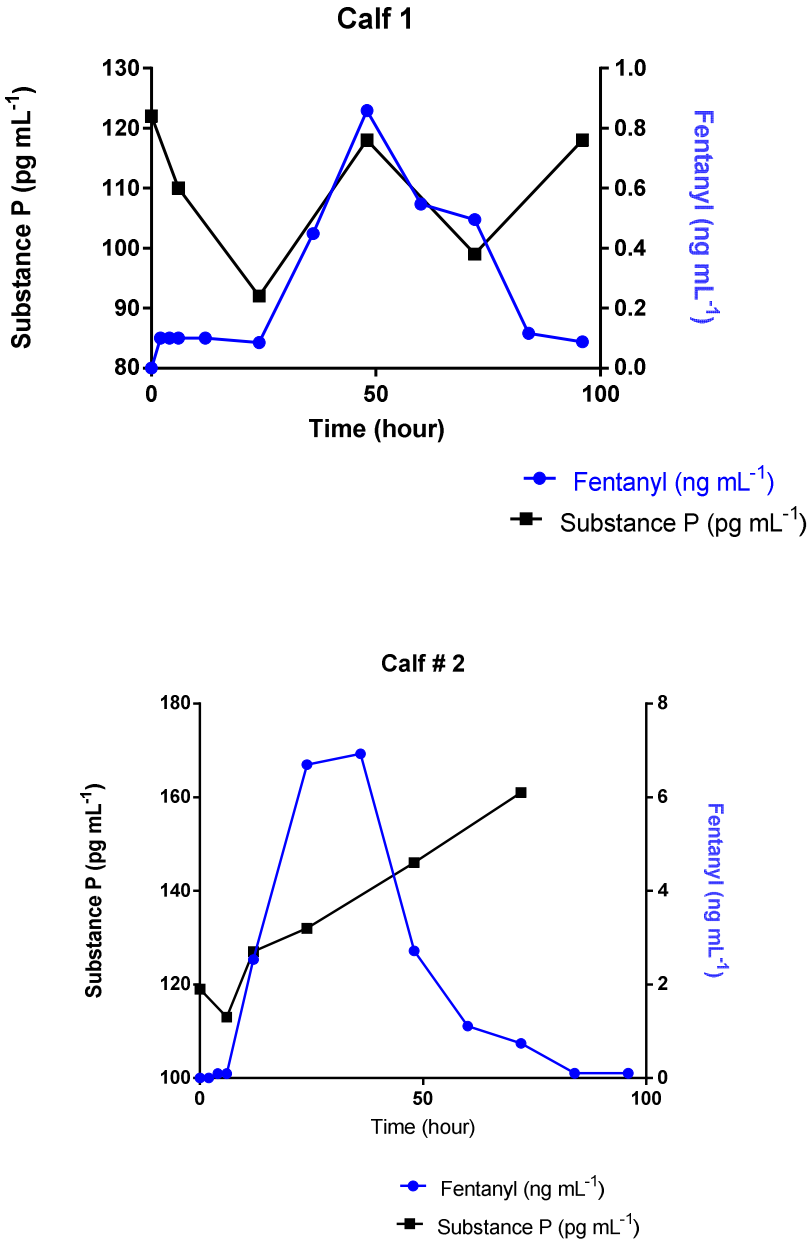

Figure S2. Time *versus* concentration graph of fentanyl and substance P in calf 1 and calf 2.

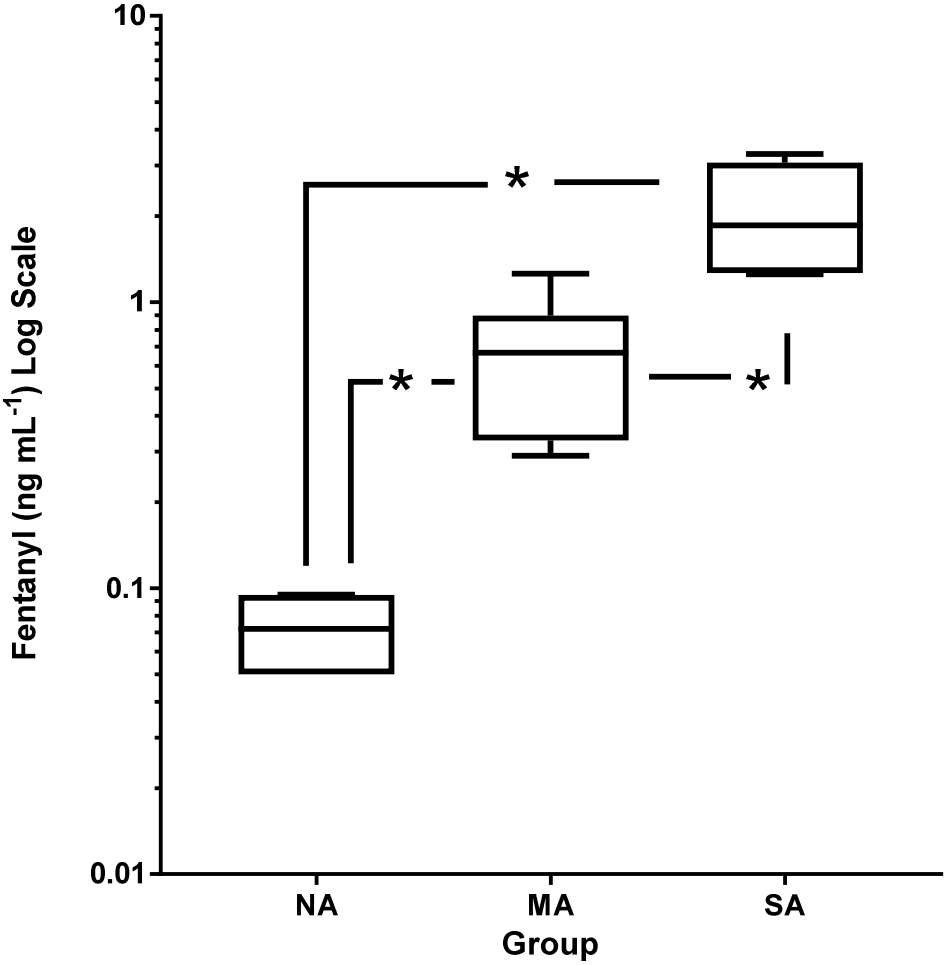

Figure S3. Pooled 4 and 6 hour fentanyl concentrations by group. NA: No Adverse effects noted (calves 1 and 2); MA: Moderate Adverse effects noted (calves 5, 6, and 7); SA: Severe adverse effects noted (calves 3 and 4). Significant difference between groups (SA *versus* NA $p = 0.03$; MA *versus* NA $p < 0.01$; SA *versus* MA ($p = 0.02$).etc) indicated by an asterisk in between plot connecting bar.

CHAPTER 4. EFFECTS OF EXPERIMENTALLY-INDUCED RESPIRATORY DISEASE ON THE PHARMACOKINETICS AND TISSUE RESIDUES OF TULATHROMYCIN IN MEAT GOATS

A paper accepted for publication in 2019 by the Journal of Veterinary Pharmacology and Therapeutics

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Abstract

Tulathromycin is a macrolide antibiotic commonly used for the treatment of respiratory disease in food animal species including goats. Recent research in pigs has suggested that the presence of disease could alter the pharmacokinetics of tulathromycin in animals with respiratory disease. The objectives of this study were (a) compare the plasma pharmacokinetics of tulathromycin in healthy goats as well as goats with an induced respiratory disease; and (b) to compare the tissue residue concentrationss of tulathromycin marker in both groups. For this trial, disease was induced with *Pasteurella multocida*. Following disease induction tulathromycin was administered. Samples of plasma were collected at various time points up to 312 hours post-treatment, when study animals were euthanized and tissue samples were collected. For PK parameters in plasma, V_z (control: 28.7 ± 11.9 mL/kg; experimental: 57.8 ± 26.6 mL/kg), was

significantly higher (P =0.0454) in the experimental group than the control group, and non-significant differences were noted in other parameters. Among time points significantly lower plasma concentrations were noted in the experimental group at 168 hours ($P = 0.023$), 216 hours ($P = 0.036$), 264 hours ($P = 0.0017$), 288 hours ($P = 0.0433$), and 312 hours ($P = 0.0486$). None of the goats had tissue residues above the US bovine limit of 5 µg/g at the end of the study. No differences were observed between muscle, liver, or fat concentrations. A significantly lower concentration ($P = 0.0095$) was noted in the kidneys of experimental goats when compared to the control group. These results suggest that the effect of respiratory disease on the pharmacokinetics and tissue residues appear minimal after experimental *P. multocida* infection, but there is the potential for alterations in diseased vs clinical animals.

Introduction

Tulathromycin is a macrolide antibiotic widley used for respiratory disease in cattle and pigs due to its broad spectrum of antimicrobial activity. The broad spectrum of activity and long-acting formulation also make this an ideal antibiotic for treating respiratory disease in goats, along with its efficacy against caprine respiratory isolates of *Mannheimia hemolytica*, *Pasteurella multocida*, and *Bibersteinia trehalos*i.(110) Due to the importance of broad spectrum antimicrobials for veterinary as well as human medicine, the World Health Organization has classified macrolide antibiotics on the list of the highest priority, critically important antimicrobials for the preservation of human health.(96) This prioritization from the WHO is based on 1) the high number of people affected by diseases for which a macrolide is the sole or one of the few therapies, 2) the high frequency of use in human medicine, and 3) the potential transmission of *Campylobacter* spp from non-human sources.(96)

In the United States tulathromycin can be legally used in goats in an extralabel fashion when approved drugs have been deemed clinically ineffective. In addition to efficacy for the treatment of caprine respiratory disease, tulathromycin has been utilized as therapy for caseous lymphadenitis.(101) When used in cattle, tulathromycin also possesses activity against *Fusobacterium necophorum*, *Porphyromonas levii*, and *Moraxella bovis*. Tulathromycin has demonstrated pharmacokinetics in goats similar to what has been reported for cattle and swine.(104) These parameters indicate rapid absorption, with maximum concentrations being reached approximately one hour after administration and a long plasma elimination half-life.(105) Additionally, tulathromycin has demonstrated similar tissue elimination in goats when compared to cattle.(105)

In other veterinary species the presence of infectious respiratory disease has demonstrated alterations in the pharmacokinetics of tulathromycin. Recently, in pigs infected with *Actinobacillus pleuropneumoniae,* tulathromycin demonstrated both a slower elimination half-life as well as a longer drug persistence when compared to healthy pigs.(10) However, currently no studies demonstrate the effect of respiratory disease on the pharmacokinetics and tissue residue marker concentrations of tulathromycin in goats. The increasing size of the US meat goat herd, as well as the potential for residues in goat products, presents a food safety issue. Withdrawal times are calculated based on healthy animals and the presence of disease may influence pharmacokinetics and tissue residue concentrations. The objective of this study was to determine the pharmacokinetics and tissue residue concentrations of tulathromcyin for goats with experimentally-induced respiratory disease. Our hypothesis was that the presence of disease would result in altered plasma and tissue concentrations of tulathromycin when compared to healthy goats.

Materials and Methods

Experimental animals.

This study was completed at the Iowa State University (ISU) Livestock Infectious Disease Isolation Facility (LIDIF). Twelve healthy 8-10 month old female meat goats (Boer and Boer-cross) weighing 34.7 ± 4.6 kg, were enrolled in the study. Eligible goats had no prior drug administration and no history of respiratory disease. Goats were then randomly assigned by weight into one of two groups: control (N=6) vs. experimental (N=6, details below). Each cohort was group-housed in individual climate controlled rooms at the LIDIF.

During each treatment segment, goats were housed in raised group pens. Each pen had individual access to feed and water. Goats were fed a mixed hay ration and water *ad libitum*. Ration parameters met or exceeded those recommended by the NRC guidelines (NRC, 2001). In addition, animal housing and management met the recommendations listed in the *Guide for Care and Use of Agricultural Animals in Research and Teaching.* (FASS, 2010) The research protocol was approved by the ISU Institutional Animal Use and Care Committee prior to commencement of trial procedures (protocol number-5-17-8517-F).

Experimental design – respiratory challenge.

Three days prior to treatment, the six experimental group goats were administered *P. multocida* strain P1062 (type A3) via intratracheal and intranasal inoculation as described by Smith et al.(200) The isolate was grown from stock culture and standardized as previously reported.(201) Goats were assessed every 12 hours and deemed to be infected when tachypnea (respiratory rate greater than 20% of that recorded at intake), abnormal lung sounds (defined as harsh bronchovesicular sounds, crackles, and wheezes) were noted along with infectious changes

on the leukogram. Following confirmation of infection, the experimental group of goats was treated with tulathromycin.

Drug administration.

For treatment and sample collection, goats were restrained via halter. At time 0 (T0), all goats received tulathromycin (Draxxin®; Zoetis Inc., New York, NY), at 2.5 mg per kg of body weight administered subcutaneously in the left neck as described on the package insert for beef cattle. No further medications were administered throughout the remainder of the experiment.

Collection of blood samples.

Prior to tulathromycin administration (T0), a 10-mL blood sample was collected from the jugular vein via vacutainer (BD Vacutainer; Franklin Lake, NJ) into blood tubes containing freeze-dried heparin (Becton, Dickinson and Co, Franklin Lakes, NJ). Subsequent blood samples were collected from alternating jugular veins into heparinized tubes at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24 hr and then every 24 hr after drug administration through 312 hr. Within 2 h of collection, blood samples were centrifuged for 20 min at 1000 g at 4 °C, then 5 mL of plasma was harvested and frozen at -70°C until analyzed for drug concentration.

Daily observations.

Study goats were observed daily for physical examination parameters relevant to respiratory disease (pyrexia, tachypnea, abnormal respiratory noise, discharge and tachycardia) and were assessed twice daily for general parameters of health such as appetite and responsiveness to stimuli.

Trial conclusion.

To conserve animal resources, two control goats were enrolled in a separate, unrelated study at T312. At T312 hr, all remaining goats were humanely euthanized with a captive bolt as described by Plummer (202) followed by exsanguination. Following euthanasia, kidney, liver, skeletal muscle, and fat were collected, and then frozen at -70 °C until analyzed for common fragment concentration.

Plasma tulathromycin concentration analysis.

Plasma concentrations of tulathromycin were determined using high-pressure liquid chromatography with mass spectrometry detection (LC-MS/MS) after precipitation of plasma proteins with acetonitrile as described previously for cattle(132). LC-MS/MS was performed using an Agilent 1100 Pump, column compartment, and autosampler (Santa Clara, CA, USA) coupled to an ion trap mass spectrometer (LTQ, Thermo Scientific, San Jose, CA, USA).Sequences consisting of plasma blanks, calibration spikes, QC's, and caprine plasma samples were batch processed with a processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA). The processing method automatically identified and integrated each peak in each sample and calculated the calibration curve based on a weighted (1/X) linear fit. Plasma concentrations of tulathromycin in unknown samples were calculated by the Xcalibur software based on the calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. Twelve calibration spikes were prepared in blank caprine plasma covering the concentration range of 1 to 5,000 ng/mL. Calibration curves exhibited a correlation coefficient (R^2) exceeding 0.993 across the concentration range. QC samples at 15, 150, and 1500 ng/mL were within ± 15% of the nominal value with most of the

QC's within ± 5% of the nominal value. The limit of quantitation (LOQ) of the analysis was 1 ng/mL with a limit of detection (LOD) of 0.2 ng mL.

Tissue CP-60,300 concentration analysis.

Tissue concentrations (liver, kidney, muscle, fat) of tulathromycin were determined using high-pressure liquid chromatography with mass spectrometry detection (LC-MS/MS) after acidic hydrolysis of tissue residues of tulathromycin to the common hydrolytic fragment, CP-60,300. LC-MS/MS was performed using an Agilent 1100 Pump, column compartment, and autosampler (Santa Clara, CA, USA) coupled to an ion trap mass spectrometer (LTQ, Thermo Scientific, San Jose, CA, USA). Homogenized tissue samples, tissue spikes, and caprine tissue blanks, 1 gram, were hydrolyzed with 2 N hydrochloric acid (HCl), 4 mL, for 1 hour at 60^{o} C. A second addition of 3.5 mL of HCl to the tissue samples was performed after centrifugation of the tissue digest and removal of the supernatant. The samples were then vortexed and shaken followed by centrifugation. The supernatant from this second extraction was combined with the supernatant from the first digestion and the volume was adjusted to 8 mL For LC-MS/MS analysis the samples and spikes/blanks were diluted 1:20 with a 0.1 M potassium acetate buffer, pH 5.0 in autosampler vials. The buffer contained an internal standard of roxithromycin at a concentration of 50 ng/mL. The vials were then centrifuged at 2,400 rpm prior to analysis.

For LC-MS/MS analysis the injection volume was set to 12.5 µL. The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile at a flow rate of 0.25 mL/min. Separation was achieved with an ACE 3 C18 column, 150 mm x 2.1 mm, 3 µm particles (Mac-Mod Analytical, Chadds Ford, PA, USA) maintained at 40°C. The analysis was performed starting at a solvent composition of 5% B which was increased linearly to 95% B in 8

minutes after injection. The solvent composition was maintained at 95% B for 2 minutes prior to equilibration to 5% B. The flow rate during this time period was 0.325 mL/min. The tulathromycin marker, CP-60,300, and roxithromycin eluted from the ACE 3 C18 column at 5.81 ± 0.05 and 8.29 ± 0.05 minutes, respectively. Full scan positive ion MS of the precursor ions of the analytes was used for residue detection. The doubly charged precursor ion of CP-60,300 (m/z 289.4) and singly charged roxithromycin (m/z 837.3) were used for MS fragmentation in the tulathromycin analysis. The fragment ions of the doubly charged CP-60,300 marker precursor at m/z 289.4 were 158.2, 231.3, and 420.3 m/z. The fragment ions of the roxithromycin precursor ion at m/z of 837.3 were at 522.3, 558.3, and 679.3 m/z.

Each set of tissue samples was run with six calibration spikes (tulathromycin) prepared in the corresponding blank caprine tissue matrix along with tissue blank. The calibration spikes covered a range from 0.2 to 10 ug/g or 0.5-20 ug/g (caprine liver). After a set of tissue samples were run the results were batch processed with a processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA). The processing method automatically identified and integrated each peak in each sample and calculated the calibration curve based on a weighted (1/X) linear fit. Tissue concentrations of the CP-60,300 marker in unknown samples were calculated by the Xcalibur software based on the calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. All calibration curves exhibited a correlation coefficient (R^2) exceeding 0.998 across the concentration range. All of the calibration spikes in each tissue were within ± 7% of the nominal concentration with the majority of the spikes within ± 3%. The limit of quantitation (LOQ) of the analysis was 0.2 µg/g with a limit of detection (LOD) of 0.02 ug/g.

Pharmacokinetic analysis.

Pharmacokinetic analysis of total tulathromycin plasma concentration was completed using a statistical moment (i.e. non-compartmental) approach in commercial software (Phoenix WinNonlin 8.1, Certara, Princeton, NJ, USA). Time versus concentration figures for tulathromycin were produced via a commercial program (GraphPad Prism 8.0, GraphPad Software, Inc, La Jolla, CA, USA).

Standard PK parameters were generated for individual goats, as follows:

- Maximum tulathromycin concentration, C_{max};
- Time of maximum tulathromycin concentration, T_{max};
- Area under tulathromycin concentration-time curve, AUC_{last};
- Area under the moment curve, $AUMC_{inf}$;
- Tulathromycin mean residence time,

  $MRT = AUMC_{inf} / AUC_{inf;}$
- Slope of the elimination phase λz, computed by linear regression of the logarithmic concentration vs. time curve during the elimination phase;
- Tulathromycin terminal half-life,

  $T1/2(\lambda_z) = \ln(2) / \lambda_z$;
- Tulathromycin apparent clearance, $CL/F = Dose / AUC_{inf}$;
- Apparent volume of distribution of tulathromycin during the elimination phase,

  $Vz/F = Dose / (AUC_{inf} \times \lambda_z)$;

For data analysis, the first value below the LLOQ was inferred to be LLOQ/2, and subsequent data points were excluded from the analysis. A linear/log trapezoidal rule was used to estimate the area under the tulathromycin time-curves. Summary statistics on the individual PK parameters were performed thereafter to derive the geometric mean, median and (min-max) range.

Data analysis.

Drug concentrations were compared at each time point using contrasts. Comparison of variables between treatment groups that were single observations (i. e., enrollment variables and PK parameters) were made using a paired t-test when data were normally distributed and with a Wilcoxon signed rank test when distributions were not normally distributed. Comparisons of tissue marker residue (common fragment CP-60,300) concentrations at 312 hours were made using the Wilcoxon signed ranked test. Statistical significance was established when $P < 0.05$.

Statistical Analysis.

Data distributions for all pharmacokinetic parameters were normality assessed by Shapiro-Wilk tests. Comparisons between the two experimental groups were performed via unpaired t tests for normally distributed parameters and Mann-Whitney tests for nonparametric parameters via a commercial program (GraphPad Prism 8, GraphPad Software, Inc, La Jolla, CA, USA).

Results

Animal health.

At enrollment, all study subjects were assessed to be healthy and to have parameters within the normal limits for goats of their respective ages. The injections were well tolerated by all goats, although three goats from each group vocalized during the injection. For heart rate, respiratory rate and temperature, no significant elevation or depression from baseline was reported amongst the control group. The experimental group had elevations above the normal baseline in rectal temperature and respiratory rate. Hematologically, the experimental group had elevations above caprine normal as well as pre-induction baselines for serum fibrinogen. Five of the six experimental goats had toxic changes present in their neutrophils at the time of treatment. No differences in body weight were noted between the control (34.3 ± 4.1 kg) and experimental (35.1 ± 5.5 kg) groups ($P = 0.77$).

Pharmacokinetics of tulathromycin.

No goat had detectable tulathromycin in plasma at time zero. The mean time□course of tulathromycin total concentrations in plasma can be found in Figure 1. Geometric mean profiles are presented in Table 1 for both groups. Among individuals in each group, there appears to be limited variation of time vs. concentration data noted by moderate variations of AUC_{last} CV% amongst groups (control: 47.3%; experimental: 59.6%). When both groups are compared, there appears to be variation in the initial curve (Figure 2.), however elimination appears to be similar for each group approximated by similar slopes of the terminal phase. Non-significant differences were found on comparison of pharmacokinetic parameters (mean ± SD) between groups of C_{max} (control: 3111.0 ± 2451.4 ng/mL; experimental: 1295.5 ± 630.2 ng/mL; P = 0.17), T_{max} (control:

0.37 ± 0.14 hr; experimental: 0.54 ± 0.25 hr; P = 0.36), CL/F (control: 0.21 ± 0.06 mL/hr/kg; experimental: 0.31 ± 0.11 mL/hr/kg; P = 0.09), T1/2(λ_z) (control: 90.7 ± 24.6 hr; experimental: 125.7 ± 38.6; P = 0.13), AUC_{last} (control: 12630.9 ± 5972.6 hr*ng/mL; experimental: 8873.3 ± 5290.7 hr*ng/mL; P = 0.06), and MRT (control: 85.6 ± 24.6; experimental: 93.0 ± 23.3; P = 0.39). A significant difference was found between groups for V_z (control: 28.7 ± 11.9 mL/kg; experimental: 57.8 ± 26.5 mL/kg; P = 0.045).

Time point comparisons for plasma tulathromycin concentrations are presented in Table 2. With the exception of the +1 hour timepoint, the experimental group displayed an apparent decrease in plasma tulathromycin concentrations when compared to the control group. The initial time point concentrations are much greater for the control group (Table 2) when compared to the experimental group. All time point differences were compared amongst groups. Significant differences were noted in time points at: 168 hours (control: 15.2 ± 3.0 ng/mL; experimental: 7.7 ± 3.4 ng/mL; *P* = 0.02), 216 hours (control: 11.2 ± 3.3 ng/mL; experimental: 7.2 ± 4.4 ng/mL; *P* = 0.03), 264 hours (control: 9.6 ± 2.5 ng/mL; experimental: 4.6 ± 1.4 ng/mL; *P* = 0.0017), 288 hours (control: 6.1 ± 1.4 ng/mL; experimental: 4.1 ± 1.8 ng/mL; *P* = 0.043), and 312 hours (control: 5.5 ± 0.7 ng/mL; experimental: 4.0 ± 1,4 ng/mL; *P* = 0.048).

Tissue residue concentrations of tulathromycin marker.

All tissues contained detectable amounts of CP-60,300. When compared amongst groups no statistically significant concentration differences were found between muscle (control: 0.40 ± 0.045 µg/g; experimental: 0.34 ± 0.045 µg/g; P = 0.21), liver (control: 2.63 ± 0.28 µg/g; experimental: 2.28 ± 0.49 µg/g; P = 0.35), and fat (control: 0.12 ± 0.03 µg/g; experimental: 0.14 ± 0.09 µg/g; P = 0.66). A statistically significant difference was found between kidney tissues of each group (control: 1.56 ± 0.15 µg/g; experimental: 1.20 ± 0.16 µg/g; P = 0.009). Tissue concentration values for each group, of muscle and fat, as well as liver and kidney are presented in Figure 3.

Discussion

To the author's knowledge, this is the first report discussing the pharmacokinetics of tulathromycin in the context of goat respiratory disease. Although the research housing could potentially be a source of bias for this study, it was thought to be minimal as the goats were sourced from the same herd and were group-housed in two separate rooms that had identical temperature, humidity, ventilation, and light control settings. The age, breed, and size of the goats used for this study were designed to mimic young market goats that commonly enter the food chain.

In the United States tulathromycin is not currently labelled for use in goats. However, it may be used in an extralabel manner under a veterinary-client-patient relationship and the AMDUCA guidelines. Currently only two antibiotics, ceftiofur and neomycin are labelled for goats in the United States, so extra-label use is common when treating small ruminants for

respiratory disease if the approved drug is clinically ineffective. Tulathromycin has been shown to be an ideal antimicrobial for the treatment of respiratory disease in goats, based on 100% susceptibility of isolates of *M. haemolytica*, *P. multocida*, and *B. trehalosi* taken from goats with pneumonia.(110) It could be argued that the experimental group in our study exhibited successful treatment with tulathromycin to our isolate of *P. multocida* due to the lack of mortality and resolution of morbidity after treatment in our experimental group.

One recent study has determined altered pharmacokinetics and tissue disposition of tulathromycin with respiratory disease in pigs.(10) In that study differences in the plasma peak concentrations as well increased levels of tulathromycin in the lungs of pigs infected with *Actinobacillus pleuropneumoniae* was observed. A notable difference in the plasma C_{max} was also noted in the goats of our study (control: 3111.1 ± 2451.4 ng/mL; experimental: 1295.5 ± 630.2 ng/mL), but this difference was not statistically significant ($P = 0.11$). Studies in swine with infectious and inflammatory respiratory disease have also noted decreased maximal plasma concentrations in experimental vs control groups.(10, 203)

The pharmacokinetic parameters of tulathromycin reported in our study varied slightly from those previously reported in the literature. Specifically, The C_{max} from our control group (3111.1 ± 2451.4 ng/mL) was significantly higher than the C_{max} (1000 ± 420 ng/mL) reported in healthy goats by Romenet et al(105), despite similar sampling timepoints. In addition, the estimated C_{max} from our study varied significantly from that reported in dairy goats (121.5 ± 19.0 ng/mL), however this study collected plasma samples every 12 hours, so a significant reduction in maximum concentration would be expected.(204) When compared to the 12 hour plasma concentrations of our control goats (225.3 ± 245.6 ng/mL) more similarities were observed. When the 12 hour time point of one outlier goat (721.5 ng/mL) is removed, the 12 hour time

points from our study (126.0 ± 39.3 ng/mL) align very closely to the reported 12 hour timepoint plasma concentrations for dairy goats (121.5 ± 19.0 ng/mL). Variation in the elimination half-life has been noted in infected pigs (10) and this was also noted in our study (control: 90.69 ± 24.63 hr; experimental: 125.75 ± 38.57).

CP-60,300 was utilized for tissue concentration as this is the FDA-approved regulatory method with respect to isoforms of tulathromycin, and the assay utilized has been validated for goat.(107) This technique detects any isoform of tulathromycin and as such, will give the most conservative level of residue concentrations in tissue samples.(105) Muscle, liver, kidney and fat were chosen for analysis as these represent common edible tissues. For cattle in the United States the approved tolerance limit of CP-60,300 is 5 μg/g. Currently for cattle in the European Union the maximum residue limit (MRL) is 4.5 μg/g for liver, 3.0 μg/g for kidney, 0.3 μg/g for muscle and 0.2 μg/g for fat.(205) Among our goats all tissue levels of all groups were below the US bovine tolerance level, and among the EU MRLs only the muscle levels were above the EU Bovine MRL. Since tulathromycin is currently not labelled for goats in the US, the withdrawal interval would be calculated based on the FDA recommendations (206), which would be based on the lower limit of detection, and has been reported by another study to be 34 days for goats with a lower limit of detection of 0.3 ppm.(105) Our equipment was more sensitive than used in that study, with a lower limit of detection of 0.02 ppm.

The goats in our study had higher tissue levels when compared to healthy goats administered a 2.5 mg/kg dose of tulathromycin subcutaneously and tested at 12 days post-injection, despite being collected 24 hours later. Clothier et al. found liver values of 1.18 ± 0.42 μg/g (107) which was lower compared to our goats (2.63 ± 0.28 μg/g; experimental: 2.28 ± 0.49 μg/g). That study also had muscle concentrations measuring < LOD (0.24 μg/g), which would be

lower when compared to the muscle concentrations of the goats in our study (control: 0.40 ± 0.045 μg/g; experimental: 0.34 ± 0.045 μg/g). The levels of tulathromycin in fat found in our goats (control: 0.12 ± 0.03 μg/g; experimental: 0.14 ± 0.09 μg/g) could be similar to what was determined in the Clothier study, as no detectable levels were determined, but the level of detection utilized was 0.14 μg/g, which was close to our results. The kidney concentrations detected in our goats would also be higher than those determined by Clothier et al, at 12 days as they found levels also below the level of detection (0.29 μg/g) and our study goats had levels (control: 1.56 ± 0.15 μg/g; experimental: 1.20 ± 0.16 μg/g; P = 0.0095) higher than those reported. These differences could be due to differences in age, sex, breed and weight of the goats used in both studies as Clothier et al used 5-6 month old, male (castrated and intact), goats of dairy and meat breeds that weighed 13.8-27.4 kg, and our study utilized 8-10 month old, female goats, weighing 34.7 ± 4.6 Kg, of meat breeds. These differences could be due to breed, as differences in tulathromycin clearance has been observed between dairy calves (CL/F 0.33 L/h/kg) (207) and beef calves (CL/F 0.18 L/h/kg).(208)

In other studies tulathromycin has been shown to concentrate in the lung, and swine studies suggest that the lung is the target organ for the drug.(10) In an intranasal challenge model using the *Escherichia coli* lipopolysaccharide in mice, tulathromycin had a 1.7-2.8 times higher exposure in the lungs of mice treated with lipopolysaccharide compared to controls.(209) While plasma levels of macrolide antibiotics have not been shown to correlate with lung concentrations in animals with respiratory disease.(210) It is possible that the significant differences in plasma concentration noted at approximately 168 hours post injection is due to more drug residing in the lungs of the experimental group.

An unexpected finding of our study was the reduced renal concentrations in the experimental group when compared to the controls. In pigs it has been noted that tulathromycin is excreted by biliary and renal excretion (93), and in cattle the major route of excretion is thought to be is biliary excretion (11), these reduced renal concentrations could suggest increased renal excretion by the goat.

Limitations

A limitation of this study was the relatively small number of goats utilized, which could have hampered the statistical power of some of our comparisons. Also, while 4-6 animals are commonly used for PK studies(48), this numbers utilized might not account for all population variability. This limitation is evident by the maximum concentrations observed in mean Cmax values of each group being 2.4 times greater for the control group, but this difference not being statistically significant. This intrinsic high variability of the studied goats, specifically the control goats, with respect to Cmax would prevent the obtaining of a statistically significant result even though there was a 2.4 fold difference in the Cmax of the control and experimental groups. All of the animals were of approximately the same age, which may not be reflective of all meat goat populations. An additional limitation of this study is the reduced number of control goats that had tissue samples collected. While our study focused on edible tissues (liver, muscle, kidney and fat) future studies should consider differences in lung concentration in diseased goats. Since our study evaluated tissue residues at one time point, future studies should consider multiple tissue sampling to determine the effect of respiratory disease on tissue residue disposition in meat goats.

Conclusions

In conclusion, while there appears to be no overall statistically significant differences in the pharmacokinetics of tulathromycin between healthy and diseased goats, we did observe significant differences in tulathromycin plasma concentrations at multiple time points (168-312 hr). Specifically, goats infected with *P. multocida* demonstrated decreased plasma concentrations compared to healthy goats at approximately 168 hours after administration. There do not appear to be significant differences in edible tissue residues, with the exception of decreased kidney concentrations, amongst groups at 13 days post-injection. Similarly, a significant difference was identified in kidney tissue levels, which when considered with the differences in plasma concentration at later time points may suggest a difference in the terminal depletion process. While tulathromycin is currently used in an extralabel manner in goats, the results of this study suggest that experimental *P. multocida* respiratory disease may have the potential to alter pharmacokinetics or tissue residue concentrations of tulathromycin in meat goats. Further studies including larger numbers of animals are warranted to confirm these preliminary observations.

Conflicts of interest.

The authors report no conflict of interest.

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Author contributions.

JSS was involved in study design and execution, sample collection and analysis, manuscript preparation and submission. JPM was involved in study design, sample analysis and manuscript preparation. DJB was involved in study design, method development, sample analysis and manuscript preparation. RWG was involved in study design, sample analysis and manuscript preparation. All authors have read and approved the final manuscript.

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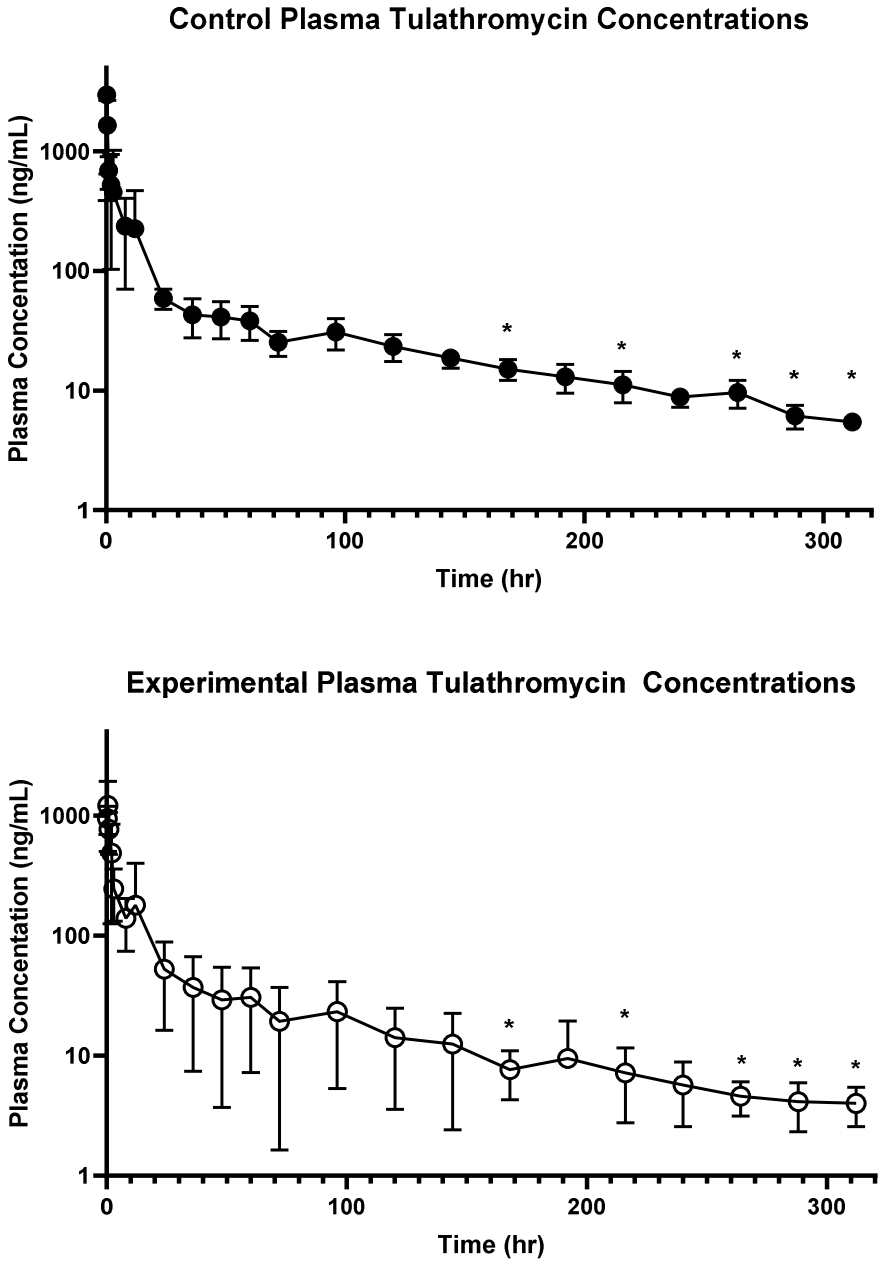

Figure 1. Time vs Concentration data for plasma tulathromycin concentrations between control (top) and experimental (bottom) groups. *Indicates statistically significant differences.

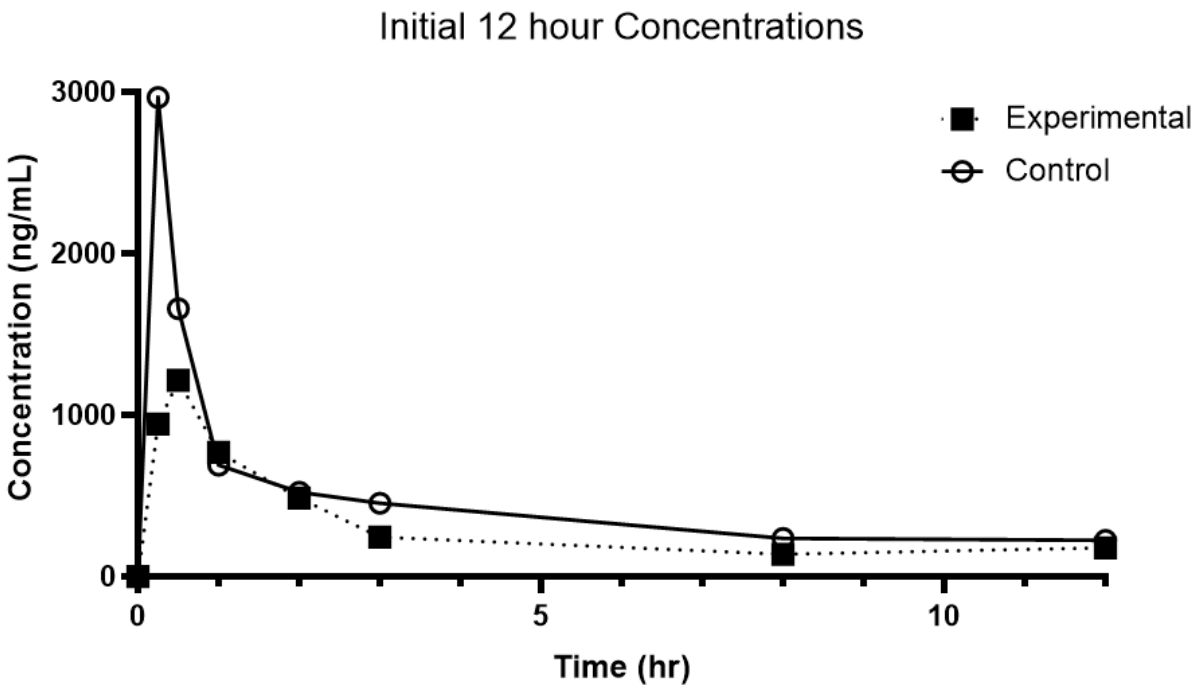

Figure 2. Mean time vs concentration data for plasma tulathromycin concentrations between control (circle) and experimental (square) groups for the first 12 hours of the experiment.

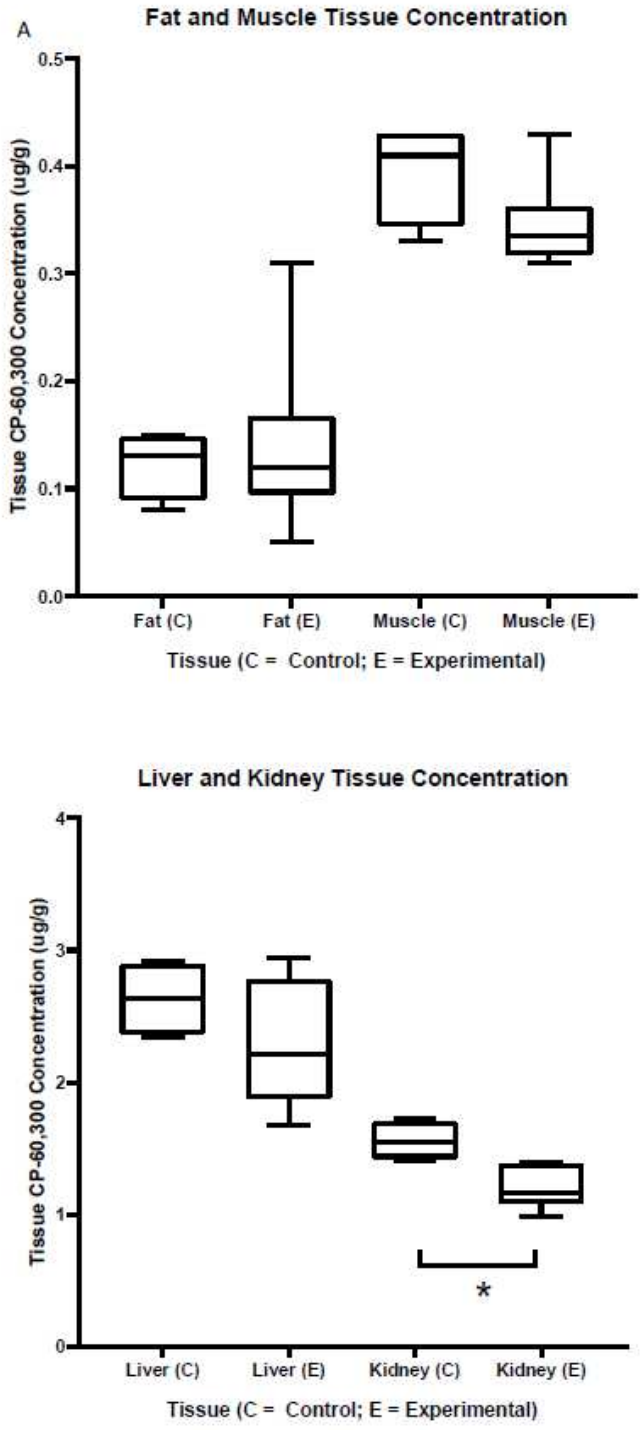

Figure 3. (Upper) Tissue CP□60,300 concentrations for control (C) and experimental (E) groups for fat (left) and muscle (right). (Lower) Tissue CP□60,300 concentrations for control (C) and experimental (E) groups for liver (left) and kidney (right). *Indicates a statistically significant difference of $p < 0.05$

Table 1. Pharmacokinetic parameters for control and experimental goats.

	Parameter	**Unit**	**Geomean**	**Median**	**Min**	**Max**	**CV%**	**Std Dev**
Control	C_{max}	ng/mL	2298.6	2191.6	661.4	6232.9	78.8	2451.4
	T_{max}	hr	0.35	0.375	0.25	0.5	37.8	0.14
	AUC_{last}	hr*ng/mL	11764.2	10257.2	8741.7	24395.8	47.3	5972.6
	MRT_{last}	hr	59.6	61.6	38.7	76.8	28.7	24.6
	CL/F	mL/hr/kg	0.199	0.225	0.101	0.266	28.6	0.06
	$T_{1/2\ (lambda\ z)}$	hr	86.6	98.4	42.9	112.1	27.1	24.6
	V_z	mL/kg	24.99	32.24	6.25	38.85	43.5	11.9
Experimental	C_{max}	ng/mL	1193.3	1157.4	729.7	2493.2	48.6	630.2
	T_{max}	hr	0.05	0.5	0.25	1	46.3	0.25
	AUC_{last}	hr*ng/mL	7947.1	6735.6	4987.2	19298.8	59.6	5290.7
	MRT_{last}	hr	52.4	56.4	41.2	63.0	25.8	23.3
	CL/F	mL/hr/kg	0.287	0.323	0.124	0.460	35.5	0.11
	$T_{1/2\ (lambda\ z)}$	hr	121.3	114.4	85.1	191.2	30.7	38.6
	V_z	mL/kg	50.8	67.2	15.3	88.4	46.0	26.5

Table 2. Mean concentrations and standard deviation per time point for control (left) and experimental (right) groups with *P* value. $P < 0.05$ considered statistically significant (indicated bv *).

Time Point	Control Mean (ng/mL)	Control St. Dev	Time Point	Experimental Mean (ng/mL)	Experimental St. Dev
0.25	2967	2578	944.1	249.2	0.0847
0.5	1659	1011	1214	713.5	0.3991
1	691.7	208.2	768.1	291.8	0.6131
2	523.9	420.5	486.6	360.8	0.8723
3	455.3	566.7	245.3	113.8	0.395
8	237.2	166.7	139.4	65.5	0.2110
12	225.3	245.6	179.3	221.2	0.7406
24	59.2	11.4	52.6	36.4	0.6828
36	43.1	15.4	37.0	29.6	0.6657
48	41.2	14.0	29.1	25.4	0.3353
60	38.3	12.1	30.6	23.4	0.4918
72	25.3	5.9	19.4	17.7	0.4567
96	30.9	9.1	23.4	18.0	0.3813
120	23.4	5.9	14.2	10.6	0.0916
144	18.7	3.3	12.5	10.0	0.1712
168	15.2	3.0	7.7	3.4	0.0023*
192	13.0	3.5	9.5	10.0	0.0649
216	11.2	3.3	7.1	4.4	0.0368*
240	8.8	1.6	5.7	3.2	0.0560
264	9.65	2.5	4.6	1.4	0.0017*
288	6.1	1.4	4.2	1.8	0.0433*
312	5.5	0.7	4	1.4	0.0486*

CHAPTER 5. CO-ADMINISTRATION OF A CARBAPENEM ANTIMICROBIAL AND AN INTERFERON RESPONSE FACTOR 3 ACTIVATOR DOES NOT ALTER ANTIMICROBIAL PHARMACOKINETICS IN AN OVINE MODEL OF URINARY CATHETER ASSOCIATED CYSTITIS

Modified from a paper accepted for publication in *Comparative Medicine*

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Abstract

Sheep are commonly used as animal models for human biomedical research; however, there are currently no descriptions of their use for studying the pharmacokinetics of carbapenem antimicrobials such as ertapenem. Because ertapenem is a critical antimicrobial for human infections, the description of the pharmacokinetics of this drug is of value for research using sheep as models for human diseases, such as urinary tract infections (UTI). There are currently no ovine models for comparative biomedical research of UTI. The objective of this study was to

report the pharmacokinetics of ertapenem in sheep after single and multiple dosing. Additionally the effects of an immunomodulatory drug (Zelnate^{TM}) on the pharmacokinetics of ertapenem in sheep was explored. Eight healthy ewes (weighing 64.4 ± 7.7 kg) were used in an experimental, bacterial cystitis model of human cystitis with *Pseudomonas aeruginosa*. After disease confirmation each ewe was administered 1 gram of ertapenem intravenously once every 24 hr. for 5 administrations. Blood was collected intensively (14 samples) during 24 hr. after the first and last administration. After multiple dose administration the volume of distribution was 84.5 mL/kg, clearance was 116.3 mL/hr/kg, $T1/2_{(\lambda z)}$ was 1.1 hr, and the extraction ratio was 0.02. No significant differences were found between pharmacokinetic parameters or time points between groups treated with the immunostimulant and controls or after the 1st or 5th administration of ertapenem. No accumulation was noted from previous administration. A significant difference in bactiuria was noted in ewes treated with ertapenem and zelnate when compared to controls. Cystitis was confirmed on necropsy in all ewes. Increased resistance to carbapenem antimicrobials was noted from isolates collected at conclusion of the study. Our ovine model can be used to evaluate therapeutic strategies for ertapenem use (varying drug dosing schedules and combinations with other antimicrobials and/or immune modulators) in the context of UTI.

Introduction

Ertapenem is a member of the Carbapenem family and as such has activity against gram-positive and gram-negative aerobic as well as anaerobic bacteria. It demonstrates bactericidal characteristics via the binding to penicillin binding proteins, thereby inhibiting bacterial cell wall synthesis. Due to a high degree of protein binding and stability against renal dehydropeptidase enzymes, ertapenem can be dosed once daily in humans.(211) Similar among carbapenem

antimicrobials, a post anti-biotic effect is noted where bacterial growth is suppressed after concentrations fall below the minimum inhibitory concentration of the organism.(212) These characteristics allow for favorable use of ertapenem for the treatment of complicated urinary tract infections in people,(138) often with excellent efficacy.(141)

Urinary tract infections are a leading cause of nosocomial and resistant bacterial infections in human healthcare, and because of the high rate of resistance in these infections new approaches and models could be beneficial. Complicated urinary tract infections are often associated with immunosuppression, renal disease, renal transplantation, or physical objects such as urinary calculi or indwelling urinary catheters.(213) Animal models of infection are critical for these types of studies; however, traditional models such as mice do not work well for all aspects of urinary tract diseases, most notably due to size and inability to place human urinary catheters. Sample collection could also be impaired in a murine model of cystitis due to indwelling catheterization as a limited volume of urine would be produced, and traditional methods of sample collection, such as cystocentesis, could induce artifact in the form of transient hematuria,(214) which would be less than ideal for a longer-term study. Sheep are currently utilized as research models for many human diseases such as respiratory disease (215), hemophilia (216), and polycystic kidney disease (217). Sheep possess many attributes that are preferable for research models including body size, laboratory disposition, cost, longevity, as well as the ability to be catheterized for long periods allowing for collection of large urinary volumes. The authors do not advocate the treatment of ovine patients with carbapenem antibiotics, but do recognize that due to the attributes that sheep possess they may serve as a model for infectious urinary tract disease for human biomedical research.

A commercially available DNA-based immunostimulant (Zelnate™, Bayer Inc) is approved for use in the aid of bovine respiratory disease in cattle. Available as a cationic lipid/bacterial plasmid DNA liposome, it has been demonstrated to activate the *stimulator of interferon genes* (STING) pathway to activate *interferon response factor* 3 (IRF3). (32) As such, this modulation of the immune response has been demonstrated to decrease both lung lesions and mortality in cattle challenged with bovine respiratory disease. (31) Since this product allows for a non-antimicrobial adjunctive therapy for infectious disease, it may serve as a useful aid for concurrent of infections that rapidly develop antimicrobial resistance, such as urinary tract infections in humans. However, there is currently no information in the literature describing what effect, if any that this immunostimulant may have on the pharmacokinetics of antimicrobials. This effect needs to be explored, as a potential drug interaction may alter antimicrobial pharmacokinetics in a manner that may alter drug efficacy.

While ertapenem is used in human medicine for community-acquired pneumonia as well as mixed and complicated urinary tract infections,(211) there are no reports of use among veterinary species for human biomedical research. Drug efficacy is inherently related to drug exposure for carbapenem antibiotics, as time-dependent exposure exceeding the MIC for 40% and 20% of the dosage interval is necessary to achieve a bactericidal or bacteriostatic effect.(218) Therefore the development of a sheep model for testing of different ertapenem dosing schedules or combination therapies will heavily rely on the comparative pharmacokinetics of ertapenem. As such, one of the primary steps in developing an animal model for human diseases that require ertapenem for treatment is a description of the pharmacokinetics in sheep (*Ovis aries*). The purpose of this study was to define the pharmacokinetics of ertapenem after single and multiple dosing in sheep undergoing

experimental complicated catheter-associated cystitis as well as any pharmacodynamic differences noted from this co-administration. It was hypothesized that the presence of Zelnate would not significantly alter pharmacokinetics, that the ewes would maintain a complicated cystitis, and that resistance in the isolate would be induced by exposure to ertapenem.

Materials and Methods

Experimental animals.

All aspects of this project were reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee (Log # 3-15-7965-O). Eight ewes, (weighing 64.4 ± 7.7 kg), were sourced from a commercial breeder and utilized for this study. Ewes were placed in a climate and humidity controlled room for the entirety of the study, with seventy-two initial hours utilized for acclimation prior to initiation of the study. Animals were randomly assigned by bodyweight to one of 2 groups, an ertapenem only group (n=4), and an ertapenem and immunomodulator (ZelnateTM, Bayer HealthCare, Animal Health, Shawnee Mission, Kansas) group ("ZN"; n=4). Two ewes had infection induced and were not treated with ertapenem to serve as controls. The ewes were housed in individual pens since arrival, and the study took place in the same individual pens for each ewe. Upon acclimated, no alterations to feeding or handling were made for this study. During the pre-study time period, all ewes were trained to be restrained by a halter placed on the head and tied to the wall of the pen. Criteria for enrollment in this study included a normal physical exam that yielded vital signs within the normal limits of an adult ewe, no previous history of medical illness as well as no recent history of a previously administered medication. Prior to and during the study, all ewes were fed a diet that either met or exceeded the National Research Council requirements for maintenance of ewes.

Twenty four hours prior to initiation of the study, ewes were restrained and the skin of the neck was aseptically prepared utilizing four alternating scrubs of chlorhexidine surgical scrub and 70% isopropyl alcohol. Prior to catheter placement, the skin at the catheter site was infiltrated with 2% lidocaine. Two (one in each jugular vein) 14-gauge, 5.5" I.V jugular catheters (MILACATH, MILA International, Inc, Florence, Kentucky) were aseptically placed. After catheter placement an injection port was placed and the catheters were sutured to the skin and wrapped for security.

Urinary tract infection was induced as described by Smith et al.(219) After an acclimation period, an XG Foley catheter was aseptically placed, and ewes were inoculated with *Pseudomonas aeruginosa* strain ATCC 15442. The catheter was clamped for four hours during the inoculation procedure and then left unclamped. Seventy two hours later blood sample collection commenced for the study.

Experimental design and sample collection.

Twenty-four hours prior to collection of samples, ewes in the ZN group received 2.0 mL of the *STING* pathway activator subcutaneously; control ewes received a similar volume of 0.9% saline administered via the same route. At time zero, 1 gram of ertapenem (Invanz®, Merck & Co., INC., New Jersey) was given over a sixty second bolus through the jugular catheter designated for drug administration, with blood sampled through the other jugular catheter with pre-dosing samples collected before drug administration and then at times 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48, 72, and 96 hr after drug administration. Ertapenem was administered at a dose

of one gram every 24 hr. for five doses. At 96 hr., the fifth intravenous dose of ertapenem was given and additional samples were collected at 96, 96.25, 96.5, 96.75, 97, 97.5, 98, 99, 100, 102, 104, 106, 108, and 120 hr. to describe pharmacokinetics at presumed steady state based on human pharmacokinetic data.(211, 220, 221) Complete blood count (CBC) samples were taken the day of and the day after inoculation and submitted to Iowa State's Clinical Pathology lab. After inoculation, daily urine samples were collected for bacterial enumeration of *P. aeruginosa* levels in the urine using standard plate dilution methodology. Urine samples were obtained by clamping the catheter for approximately 10 minutes before collection to allow urine to accumulate. Samples were immediately refrigerated until submission for sample processing.

On day 6 and day 10, 7 urine samples (pre-dose, +1h, +2h, +3h, +6h, +8h, +12h) were collected for measurement of concentration and enumeration of bacterial shedding in the urine. On Day 7, 8, 9, 11, and 12, once-a-day urine samples were taken at 8:00 am. See **Table 1** for a detailed layout of schedule.

At sampling timepoints, blood was collected from the catheter using a 12 mL syringe and placed into sodium heparin tubes. The samples were then centrifuged at 1,500*g* for 10 min. The plasma was pipetted off and transferred to cryovials which were then stored at −80°C until analysis. Urine was collected in 10-12 mL aliquots in falcon tubes and stored at −80°C until analysis.

Sample analysis.

Standards were made in 0.1mM 2-[N-Morpholino]ethanesulfonic acid (MES) buffer containing 0.24M sodium fluoride at pH 6.5, refrigerated, and used within 3 days to ensure the

stability of ertapenem.(222, 223) Frozen samples were thawed in cold water, vortexed, pipetted, and immediately returned to -80ºC freezer to minimize degradation of ertapenem.

Analytical standards for plasma were prepared in 200 µL blank ovine plasma at the following concentrations of ertapenem: 0.25, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 µg/mL. Quality control (QC) samples were prepared in 200 µL blank ovine plasma at the following concentrations of ertapenem: 1.5, 30, and 150 µg/mL. Standards and QCs were kept on ice when not in use. A 25 µg/mL solution of the internal standard, ertapenem-d4, was made in 0.1mM MES buffer containing 0.24M sodium fluoride at pH 6.5 and 10 µL were added to standards, QCs, blank plasma, and samples.

A method based on a previously published method was used for the plasma sample preparation.(224) For the standards, QCs, blank and samples, an aliquot of 200 µL of plasma was transferred into an Eppendorf tube, and 0.25 µg ertapenem-D4 internal standard was added to each tube. A 200 µL portion of chilled 0.1mM MES buffer, containing 0.24M sodium fluoride, was added to each sample, followed by 600 µL of chilled acetonitrile. The samples were mixed using a vortex mixer and then centrifuged for 5 minutes at 2,700*g*. The supernatant was decanted into another tube to which was added 600 µL of chilled dichloromethane. This was followed by mixing using a vortex mixer and centrifuging for 5 minutes at 2,700*g*. A 100 µL portion of the top layer was transferred into LCMS microvials containing glass inserts. The samples were then centrifuged for 20 minutes at 1,500*g* in a chilled centrifuge. Standards and samples were kept on ice during the extraction procedure. Data points with concentrations above the range of the standard curve were diluted with blank plasma and re-extracted to achieve a concentration within the range on the curve.

A method based on a previously published method was used for the urine sample preparation. Standards were prepared daily in 100 µL blank ovine urine at the following concentrations 10, 20, 50, 100, 200, 500, 1000, and 2000 µg/mL. QCs were prepared at 60,400, and 1250 µg/mL. Standards and QCs were kept on ice when not in use. A 10 µL portion of standard, QC or sample was pipetted into an LCMS vial containing an insert and then 10 µL of 0.1 mM MES buffer containing, 0.24 M sodium fluoride, at pH 6.5 was added. The sample was then diluted with 180 µL of water containing 0.1% formic acid and 2ug/mL internal standard, ertapenem-D4. The sample vials were then capped, vortexed and centrifuged for 20 min at 1500 g in a chilled centrifuge. Any samples with an ertapenem concentration greater than the highest standard, 2000 µg/mL, were diluted at ratios of either 1:10 or 1:20 to fit on the curve.

Ovine plasma and urine concentrations of ertapenem were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS). A TSQ Quantum Discovery Max triple quadrupole was coupled to a Surveyor Pump with a chilled Autosampler. The mobile phases consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. The mobile phase began at 15% B with a linear gradient to 70% B at 2.5 minutes, followed by re-equilibration to 15% B. A Kinetex phenyl hexyl column was used (100 mm x 2.1 mm, 2.6 um particles) from Phenomenex (Torrance, CA, USA) with the column temperature set to 35°C. The injection volumes were 10 µL for plasma and 2 µL for urine. The following ions were used for identification: ertapenem (m/z 476) 68, 114, 432 and ertapenem-d4 (m/z 480) 68, 114, 436. The retention time for both compounds was 1.8 minutes. The 432 and 436 ions were used for quantitation of ertapenem and ertapenem-d4, respectively.

Calibration curves were calculated using Quan Browser portion of the Xcalibur software and a quadratic fit. All correlation coefficients (R^2) exceeded that of 0.99. The calibrators were

within a tolerance of 15% of the nominal value except for the lower LOQ, which was <20%. The QCs were within a tolerance of ±15% of the nominal value. The limit of detection (LOD) was 0.1 ug/mL for ovine plasma. The limit of quantitation (LOQ), which was based on the calibration curve was 0.25 ug/mL for ovine plasma.

Pharmacokinetic analysis.

Pharmacokinetic analysis of total ertapenem plasma concentration was completed using a noncompartmental module in commercial software (Phoenix WinNonlin 8.0, Certara, Princeton, NJ, USA). Time vs. concentration figures for ertapenem were produced via a commercial program (GraphPad Prism 8, GraphPad Software, Inc, La Jolla, CA, USA).

Standard PK parameters were generated for individual ewes, as follows:

- Maximum observed ertapenem concentration (µg/mL), C_{max};
- Last observed ertapenem concentration (µg/mL), C_{last};
- Time to maximum concentration (min), T_{max};
- Time to last observed ertapenem concentration (min), T_{last};
- Area under ertapenem concentration–time curve from time zero to infinity ((µg/mL)*hr), AUC_{inf};
- Area under ertapenem concentration–time curve from time zero to last measurement ((µg/mL)*hr), AUC_{last};
- Ertapenem mean residence time (hr),

  MRT = $AUMC_{inf}/AUC_{inf}$; where $AUMC_{inf}$ is the area under the fist-moment curve from time zero to infinity ((µg/mL)*h^2). Area parameters (AUC, AUMC) were calculated using the log-linear trapezoidal rule.

- Ertapenem, elimination half-life (hr)

  $T1/2(\lambda_z) = \ln(2)/\lambda_z$; where λ_z is the slope of the terminal phase of the natural logarithm of concentrations versus time curve.

- Ertapenem systemic clearance (mL/hr/kg), $CL = Dose/AUC_{inf}$;
- Volume of distribution (mL/kg) of ertapenem during the elimination phase,

  $V_{area} = Dose/(AUC_{inf} \times \lambda_z)$; (V_z)

- Volume of distribution (mL/kg) of ertapenem at steady□state,

  $V_{ss} = CL \times MRT$

For ertapenem, the extraction ratio (E_{body}) was calculated as reported by Toutain et al (184), with:

$$E_{Body} = \text{Systemic clearance / Cardiac output} \qquad \text{[Equation 1]}$$

First calculated for each individual ewe, and then combined for a mean value. With the ewe cardiac output described by Tourain et al(184), as follows:

$$\text{Cardiac output} = 180 \times BW(kg)^{-0.19} \qquad \text{[Equation 2]}$$

Bacterial quantification

Urine samples were serially diluted out and plated on blood agar plates to determine CFU/ml. Samples were collected in the morning and refrigerated, and within two hours of collection a standard plating of samples at an order of 10^{-7} was performed. Phosphate-buffered saline (PBS) was used as the dilution fluid and samples were plated on sheep blood agar plates. Count was

recorded approximately 22 hours later, and bacterial counts were calculated using plates that had between 30-300 isolates.

ROSCO Diagnostica Neo-Rapid CARB kit was used to detect the presence of carbapenemases from urine samples taken on the last day of the study, a validated method for *P. aeruginosa* carbapenemase screening. [9] Urine was obtained from Day 13, 10 mL was spun down and the pellet was resuspended in a mixture of 200µL 0.9% NaCl at pH 8.5. 2 mL of Triton X-100 10% sol was diluted in 10 mL of water, and 10 µL was added to the bacterial suspension. Samples were incubated and evaluated at 15 min, 30 min, and 1 hour. Results were interpreted based on color change, as per the manufactures protocol for Kit 98024.

The minimum inhibitory concentration (MIC) of isolates from the last day of Ertapenem (Day 11) was determined by microbroth dilution. *P. aeruginosa* colonies from Day 11 urine samples were streaked on blood agar to grow overnight. A set of 96-well plates were filled with 50 µL of MH broth, except for the final column. Ertapenem, imipenem, and meropenem were mixed to a concentration of 128 µg/ml, and 100 µL of the drug was added to the last well, and then mixed and diluted throughout the rest of the wells. The first well served as a growth control. Colonies from the overnight plates that were grown for 16-24 hours were mixed into MH broth, and the turbidity of suspension was adjusted to achieve 0.5 McFarland turbidity standard (approximately 1 to 2 $x10^8$ CFU/mL). The suspension was then diluted 1000 fold, and 50 µL was added. The top of the plate was sealed, placed in a 37° incubator, and results were read at 24 hours. The MIC was recorded as the concentration of the antibiotic in the first well that did not show evidence of bacterial growth.

Statistical analysis.

The respective data distributions for all pharmacokinetic parameters and group time point concentrations were assessed for normality by means of the Shapiro-Wilk test. Comparisons between the 2 treatment groups were performed with unpaired t tests for parametric statistics and Mann Whitney tests for nonparametric statistics as previously described. (225) A $P < 0.05$ was considered statistically significant.

Necropsy.

At study conclusion, ewes were individually euthanized with an IV barbiturate overdose. Upon necropsy, the bladder and kidneys were grossly evaluated for cystitis and pyelonephritis. The abdomen was opened, and urinary bladder wall samples were taken using sterile instruments for histopathology.

After formalin fixation, bladder samples were examined by a board certified veterinary pathologist and scored on a 0-4 whole point inflammation scale described as follows:

- 0: No or minimal inflammation.
- 1: Mild; few perivascular or interstitial inflammatory cells, rare intra-epithelial inflammatory cells.
- 2: Moderate; moderate numbers of perivascular and/or interstitial inflammatory cells, occasional intra-epithelial inflammatory cells.
- 3: Moderate to severe; moderate numbers of perivascular and/or interstitial inflammatory cells, frequent intra-epithelial inflammatory cells.
- 4: Severe; moderate to large numbers of perivascular and/or interstitial inflammatory cells, frequent intra-epithelial inflammatory cells, mucosal ulceration/loss.

Results

Animal health.

Ertapenem was well-tolerated by all 8 ewes during the study. No changes in appetite, behavior, or stool consistency were noted. No adverse reactions were noted at the catheter sites.

Pharmacokinetics.

No ewe had detectable ertapenem in plasma prior to commencement of the study. The average time course of ertapenem can be found in Figure 1. Geometric mean, median, minimum and maximum profiles after single dose administration are presented in Table 3. Among individuals there appears to be limited variation of time vs. concentration data for ertapenem in plasma. All ewes had no detectable concentrations of ertapenem in plasma after 6 hr. for the single dose study.

After initial dosing the arithmetic mean ± standard deviation for C_{max} was (232.6 ± 50.8 µg/mL), AUC_{last} was (144.0 ± 24.8 hr*µg/mL), MRT was (0.7 ± 0.08 h), V_{ss} was (78.3 ± 16.1 mL/kg), CL was (109.6 ± 17.9 mL/hr/kg) and $T1/2_{(\lambda z)}$ (1.3 ± 0.6 hr).

Ertapenem concentrations in plasma immediately prior to administration of the 5th dose were below the limit of detection. Geometric mean, median, minimum and maximum profiles for each group after administration of the fifth dose are presented in Table 4, and no detectable concentrations were noted after 6 hr. in the multiple dose study. After multiple dosing the arithmetic mean ± standard deviation for C_{max} was (228.0 ± 52.8 µg/mL), AUC_{last} was (138.7 ±

34.0 hr*μg/mL), MRT was (0.7 ± 0.06 hr), V_{ss} was (84.5 ± 13.1 mL/kg), CL was (116.3 ± 24.4 mL/hr/kg) and $T1/2_{(\lambda z)}$ (1.1 ± 0.6 hr).

No accumulation was noted, as the majority of 8 hr. timepoints, as well as all 10, 12, and 24 hr. timepoints demonstrated ertapenem concentrations below the LOD. The extraction ratio for ertapenem in the ewes was 0.02 ± 0.004. Urine concentrations for the first 12 hours post administration for single and multiple dosing are displayed in table 6.

Statistical analysis.

When parameters were compared amongst the values for the 1st and 5th administration of ertapenem no significant differences were found for $T1/2_{(\lambda z)}$ ($P = 0.44$); C_{max} ($P = 0.86$); AUC_{last} ($P = 0.73$); Vz ($P = 0.65$) CL ($P = 0.88$; AUMC ($P = 0.97$); MRT ($P = 0.47$) and Vss ($P = 0.41$). No significant differences in drug concentrations, or pharmacokinetics were found for ewes when compared for immunomodulator administration (Table 5). No significant differences were noted in urine concentrations when single or multiple dosing was compared, nor were any significant differences noted when the presence or absence of an immunomodulator was compared for single or multiple dosing.

Bacterial analysis.

Following the first treatment of ertapenem, there was a two-fold drop in bacterial shedding within 1.5 hours for the Ertapenem and Ertapenem + Zelnate™ groups. Within 24 hours, the bacterial counts returned to levels that were seen before ertapenem was given. One-way ANOVA results show a significant difference in average bacterial shedding levels between the control group and the Ertapenem + Zelnate™ group (p= 0.0044), however there is no

significance between the Control and Ertapenem group (p=0.1246), and between the Ertapenem and Ertapenem + Zelnate^{TM} group (p= 0.3665). (Figure 3)

Carbapenamase testing was negative for all urine samples collected on the last day. MIC testing for ertapenem, imipenim, and meropenem showed that the majority of isolates taken from Day 11 had in increase in MIC in comparison to the original strain (Figure 4). For ertapenem, all strains had an MIC greater than 64 μg/mL. In imipenem samples, 61% of the isolates tested for resistance showed a 2-fold or more increase in MIC (4 μg/mL) in comparison to the original strain (2 μg/mL). In isolates tested for meropenem resistance, 46% of isolates had a two-fold or more MIC increase (.125 μg/mL) in comparison to the original (.06 μg/ml).

Necropsy and histopathology.

At necropsy, all bladders and kidneys were examined grossly for cystitis and pyelonephritis. There were no cases of pyelonephritis observed. Two ewes in the ertapenem group had enlarged and inflamed bladders, around 7 cm x 7 cm. Within the lumen of one of these, granular mucopurulent material was noted,. The other ewe had a hyperemic bladder with a granular mucosal surface.

All ewes had histopathological evidence of cystitis. The histopathology scores are described in table 7. There was no significant differences of inflammation scoring amongst groups with one way ANOVA (P=0.8956).

Discussion

Before utilizing sheep as a model for human diseases with respect to carbapenem administration, it is important to understand the pharmacokinetics of ertapenem in this species. With the increase of resistance in nosocomial infections in human hospitals requiring carbepenem therapy, additional animal investigations will need to be developed for translational studies. Sheep used in this study displayed ideal characteristics as subjects for a pharmacokinetic study. All were rapidly accustomed to halter restraint by the commencement of the study. All intravenous catheters maintained patency and allowed for ease of sample collection. While ruminant urine pH differs from that of humans, it can be manipulated by dietary factors, such as the addition of ammonium chloride.(226, 227) The body weight of the sheep in this study (65.2 ± 7.7 kg) was similar to the weights of human patients in several human pharmacokinetic studies such as 76.2 ± 9.3 kg (228) and 73 kg.(138) This similarity in bodyweights allow for translational dosing for sheep when ertapenem is dosed at 1 g/patient as humans are frequently dosed. However, this similarity in total body mass does not account for differences in anatomy, such as the relatively larger size of the ruminant gastrointestinal system. The sheep in our model were able to maintain infection throughout the duration of the study, and histopathology immune scoring confirmed infection at study's end.

Our analysis revealed a short half-life similar to what is described in human patients. The pharmacokinetic parameters and concentration at individual timepoints also demonstrated no differences after one or five administrations of ertapenem in sheep. This is of importance as at the 1 gram/patient dose sheep appear to have linear pharmacokinetics, again similar to humans where the pharmacokinetics of ertapenem are not dose-proportional. When ertapenem is administered to people it can be given as a 30 minute or 5 minute infusion, typically once

daily.(228) The C_{max} of our study in sheep (arithmetic mean ± SD: 232.6 ± 50.8) resembles the 5 min bolus in humans (195.9 ± 34.0). (228)

V_{ss} after adjustment for bodyweight for our ewes was 5.5 ± 0.14 L, which is descriptively similar to what is reported for human outpatients with complicated urinary tract infections (4.85 ± 1.8 L)(138), but less than what is reported for healthy female human volunteers (7.5 ± 0.9 L).(211) The elimination half-life in our ewes was also less than reported in either of those human studies. While some of the pharmacokinetic parameters of ertapenem in sheep appeared lower than what is reported in human patients, it is important to note that specific disease status can alter ertapenem pharmacokinetics, as sepsis can increase V_{ss} and UTIs can decrease this parameter. For example, in patients with severe sepsis treated with ertapenem, lower C_{max} and AUC as well as larger V_{ss} were observed when compared to healthy human volunteers. (220) A decreased V_{ss} has also been noted in human outpatients with complicated UTI when compared to healthy volunteers. (138) Age may also present an effect on the PK of ertapenem as in humans elderly people have higher AUC values when compared to younger individuals. (229) The extraction ratio of ertapenem in our study sheep would be classified as low according to Toutain et al.(184), as it is less than $E = 0.05$. This is in the range of extraction by glomerular filtration (i.e. 2% of cardiac output) and in agreement with previous descriptions in humans. (230) While our results suggest some species-specific differences in the pharmacokinetics of ertapenem in sheep and humans, it is important to note that analytical method sensitivity can have a profound impact on pharmacokinetics, as recently illustrated in the comparative pharmacokinetics of fentanyl in large animal species.(183) The limit of quantification of our assay was 0.25 μg/mL, and in the human literature LOQs of 0.125 μg/mL have been reported. (211) As noted for fentanyl concentrations in large animal species, when comparing pharmacokinetic parameters it

is important to consider analytical sensitivity as a lower limit of quantification can lead to the reporting of a longer elimination half-life. As such, it is possible that our analytical limits would yield decreased half-life and other parameters due to the higher LOQs.

Adverse effects with ertapenem administration in people are primarily of concern for the nervous system. Several human patient undergoing peritoneal dialysis have developed seizures after the administration of ertapenem.(231, 232) Seizures have also been reported in elderly patients receiving ertapenem. (233, 234) Among humans, stroke, low hemoglobin, and a low platelet count were identified as risk factors for seizures when administered ertapenem.(235) Less commonly reported adverse effects, such as thrombocytopenia have also been reported in human patients.(236). While safety was not a primary goal of this study, none of the sheep administered ertapenem had observed seizure activity or neurologic disease, and none displayed hematologic abnormalities associated with ertapenem in people.

Of note, organisms susceptible to ertapenem are typically inhibited by *in vitro* concentrations of $\leq$ 4 μg/mL.(230) The time above MIC required for bacteriostasis for ertapenem in people is approximately 30% of the dosing interval. (237) Based on the shorter elimination half-life of ertapenem in sheep when compared to human patients (1hr vs. 3.5hr), future studies utilizing sheep models of infection may need to employ an increased dosage used for this study to maintain plasma concentrations above 4 μg/mL for thirty percent of the day with once daily-dosing. It should be noted that most strains of *P. aeruginosa* are resistant to ertapenem, so the infection of the sheep in this model was to insure ongoing infection throughout the study period to investigate the development of resistance.

P. aeruginosa is one of the leading causes of nosocomial infections, with many cases being severe and life threating due to the emergence of antibiotic resistance.(238, 239)

Resistance was developed by the isolates over the course of this study, however the presence of carbapenemases was not determined with our rapid test. The results of this study demonstrate that in an *in vivo* model of CAUTI, *P. aeruginosa* resistance to meropenem and imipenem emerged after just six days of treatment with ertapenem. This is the first direct evidence using an *in vivo* model of a selective pressure for carbapenem antimicrobial resistance associated with ertapenem use. Ertapenem use *in vitro* selects for isolates with broad-spectrum resistance to β-lactams, displaying increased MIC for ertapenem and meropenem, as well as selection for isolates with resistance to carbapenem and non-carbapenem β-lactams.(240) However, based on evidence of the emergence of *P. aeruginosa* cross-resistance during ertapenem treatment *in vitro*, Livermore *et al.* speculated that this resistance would persist only briefly *in vivo.*(240) From our *in vivo* study, the isolates taken the day after and two days after ertapenem treatment, still showed increased MICs. A possible next step would be to test isolates from the last day, 72 hours after treatment to look for the persistence of resistance. Drawing *in vivo* conclusions based on *in vitro* evidence is a difficult presumption. There are different selective pressures in an *in vivo* environment in comparison to *in vitro*, and the emergence of resistance mechanisms in some isolates could additionally increase the competitive fitness of that strain.[12] Even if this resistance is transient, additional consideration should be given to the environment in which these isolates are found. For example, healthcare workers attending to multiple patients with CAUTIs and have the potential to serve as vectors for the spread of infectious pathogens, such as resistant *P. aeruginosa* between patients.(241)

A limitation of our study was the relatively small sample size, however for veterinary pharmacokinetic studies a sample size of 4 to 6 animals is typically adequate to describe the pharmacokinetics of a test drug.(242) Additional studies are required to explore the

pharmacodynamics of ertapenem in sheep, as well as the potential synergistic effects of ertapenem and immunomodulators on bacteriuresis and resistance development.

Conclusions

In conclusion, our study established the pharmacokinetics of ertapenem in sheep used as a model for human biomedical research. No drug accumulation was reported after 5 days of dosing, which is consistent with the short elimination half-life of this antimicrobial in sheep. Likewise, the absence of notable difference between ertapenem CL after single and multiple dosing is indicative of first order elimination in sheep. Ewes maintained infection throughout the study, and at study conclusion resistance was noted in the remaining isolates. This ovine model can be used to evaluate pharmacokinetics for therapeutic strategies for ertapenem use (varying drug dosing schedules and combinations with other antimicrobials and/or immune modulators) in the context of UTI.

Acknowledgements

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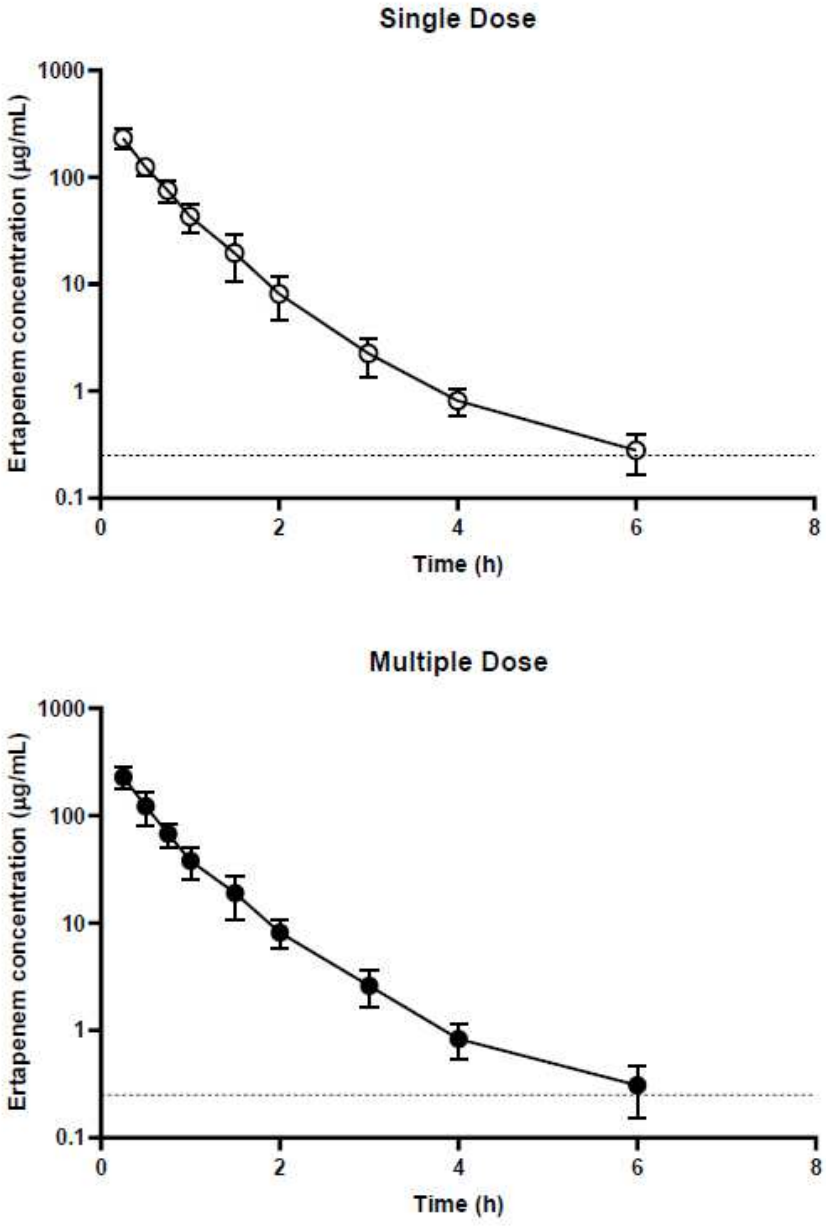

Figure 1: Mean (error bars, ± 1 SD) plasma concentration after initial intravenous administration (above); and multiple dose administration (below). The dashed line illustrates the limit of quantification for the assay (0.25 µg/ml).

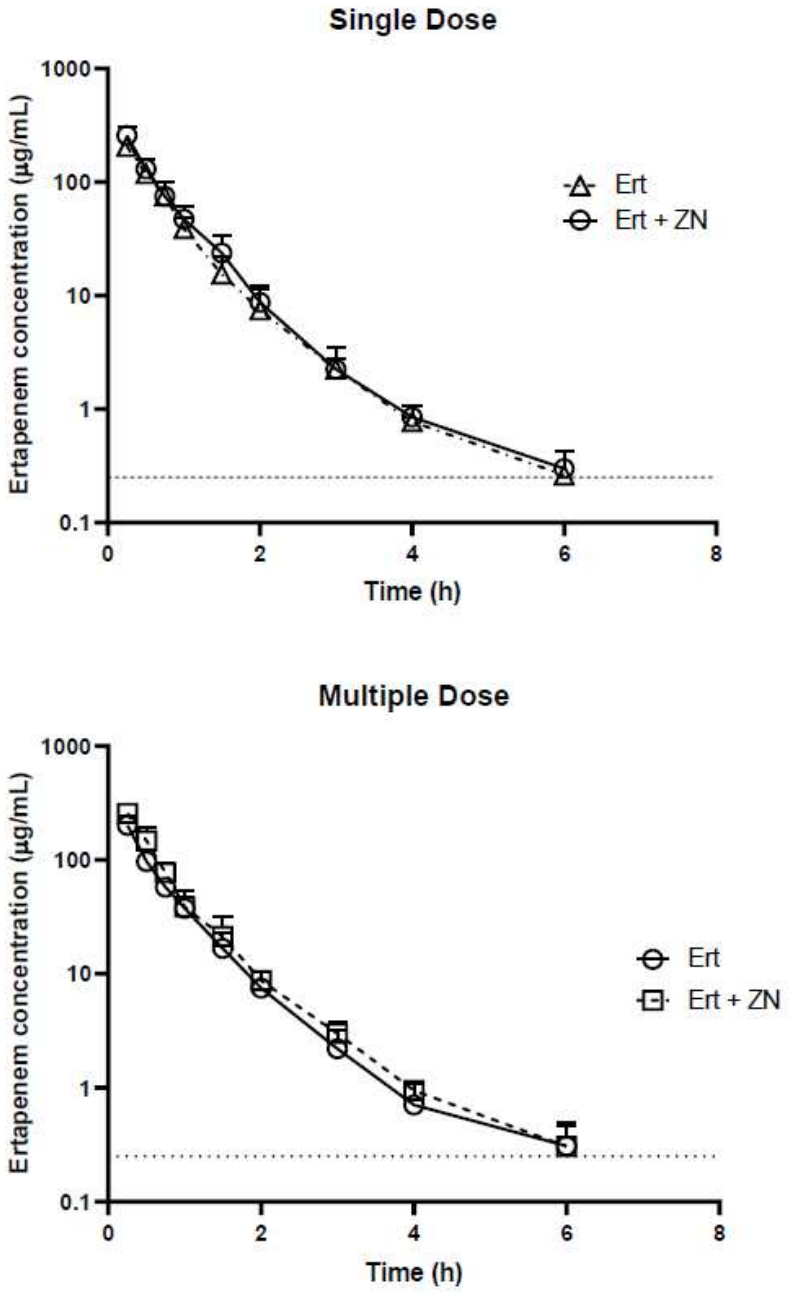

Figure 2: Mean (error bars, ± 1 SD) plasma concentration after initial (above) and multiple (below) intravenous administration of ertapenem in groups treated without immunomodulator (Ert) and with immunomodulator (Ert + ZN). The dashed line illustrates the limit of quantification for the assay (0.25 µg/ml).

Table 1. Experimental schedule by day for all ewes by group.

		Day 0 Acclimation	Day 1 Catheterization	Day 2	Day 3 Inoculation	Day 4	Day 5	Day 6 PKSD	Day 7 SPPK (trough)	Day 8 SPPK (trough)	Day 9 SPPK (trough)	Day 10 PKSS	Day 11 Wash-out	Day 12 Wash-out	Day 13 Wash-out + Euthanasia	Total	Grand Total/Group	
Group 1 (n=2)	**Control**				1 urine sample	1 urine sample	1 urine sample	5 urine samples	1 urine sample	1 urine sample	1 urine sample	5 urine samples	1 urine sample	1 urine sample	1 urine sample	19 urine samples	38 urine samples	Urine inoculum
					1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	11 urine samples	22 urine samples	UpH + Gravity
					1 plasma sample	1 plasma sample										2 plasma samples	4 plasma samples	CBC
Group 2 (n=4)	**Ertapenem**							*Ertapenem qd*	*Ertapenem qd*	*Ertapenem qd*	*Ertapenem qd*	*Ertapenem qd*						
					1 urine sample	1 urine sample	1 urine sample	7 urine samples	1 urine sample	1 urine sample	1 urine sample	7 urine samples	1 urine sample	1 urine sample	1 urine sample	23 urine samples	92 urine samples	Urine inoculum
					1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	11 urine samples	44 urine samples	UpH + Gravity
								U.PK: 7 samples P.PK: 13 samples	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	U.PK: 7 samples P.PK: 13 samples	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	20 urine samples 32 plasma samples	80 urine samples 128 plasma samples	Urine Ertapenem PK Plasma Ertapenem PK
					1 plasma sample	1 plasma sample										2 plasma samples	4 plasma samples	CBC
					Just before inoculation													
Group 3 (n=4)	**Ertapenem + Zelnate**							*Ertapenem qd*	*Ertapenem qd*	*Ertapenem qd*	*Ertapenem qd*	*Ertapenem qd*						
					Zelnate													
					1 urine sample	1 urine sample	1 urine sample	7 urine samples	1 urine sample	1 urine sample	1 urine sample	7 urine samples	1 urine sample	1 urine sample	1 urine sample	23 urine samples	92 urine samples	Urine inoculum
					1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	1 urine sample	11 urine samples	44 urine samples	UpH + Gravity
								U.PK: 7 samples P.PK: 13 samples	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	U.PK: 7 samples P.PK: 13 samples	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	U.PK: 1 sample P.PK: 1 sample	20 urine samples 32 plasma samples	80 urine samples 128 plasma samples	Urine Ertapenem PK Plasma Ertapenem PK
					1 plasma sample	1 plasma sample										2 plasma samples	4 plasma samples	CBC
					Just before inoculation													
															Summary/# of samples for the entire study		**222 urine samples**	Urine inoculum
																	110 urine samples	UpH + Gravity
																	160 urine samples	Urine Ertapenem PK
																	256 plasma samples	Plasma Ertapenem PK
																	20 plasma samples	CBC

Table 2. Complete blood count (CBC) results for total white blood cell count (WBC) as well as neutrophil counts from day before (Day 2) and the day of (Day 3) inoculation and Zelnate™ treatment. Control group included Ewe 1 and 2, Ertapenem (Ewe 3-6) and Ertapenem and Zelnate™ (Ewe 7-10).

WBC									
Ewe 1	Ewe 2	Ewe 3	Ewe 4	Ewe 5	Ewe 6	Ewe 7	Ewe 8	Ewe 9	Ewe 10
6.45	5.36	5.46	5.56	7.26	7.64	4.58	7.5	4.56	7.36
5.71	5.9	5.37	5.28	7.09	8.72	4.83	6.23	3.87	7.11
Neutrophils									
3.81	2.84	3.28	1.89	3.27	2.98	1.65	3	1	3.24
2.91	2.83	2.15	2.06	3.12	3.14	2.8	1.87	1.16	3.56

Table 3: Pharmacokinetic parameters of ertapenem in sheep after single (ERT SD) dosing.

Compound	Parameter	Unit	Geometric mean	Median	Min	Max
ERT SD	C_{max}	µg/mL	228.1	216.5	182	324
ERT SD	$T_{1/2}$ (λz)	hr	1.09	1.13	0.65	2.7
ERT SD	AUC_{last}	(µg/mL)*hr	142.0	146.5	112.3	176.7
ERT SD	CL	mL/hr/kg	108.3	109.1	131.4	81.2
ERT SD	AUMC	(µg/mL)*hr^2	97.3	97.1	73.4	137.9
ERT SD	MRT	hr	0.69	0.70	0.55	0.78
ERT SD	V_{SS}	mL/kg	76.8	78.1	52.7	113.4

Table 4: Pharmacokinetic parameters of ertapenem in sheep after multiple (ERT MD) dosing.

Compound	Parameter	Unit	Geometric mean	Median	Min	Max
ERT MD	C_{max}	µg/mL	222.3	217.5	136	324
ERT MD	$T_{1/2}$ (λz)	hr	1.01	0.86	0.56	2.16
ERT MD	AUC_{last}	(µg/mL)*hr	134.6	136.3	77.8	183.8
ERT MD	CL	mL/hr/kg	114.3	112.4	92.5	170.9
ERT MD	AUMC	(µg/mL)*hr^2	42.9	46.9	47.2	138.7
ERT MD	MRT	hr	0.71	0.72	0.61	0.79
ERT MD	V_{SS}	mlL/kg	77.2	72.7	67.1	105.6

Table 5. Geometric means of the pharmacokinetics observed in groups treated with ertapenem alone and ertapenem with concurrent immunomodulator (ZelNate) and the statistical comparisons between each group.

Group	Parameter	Unit	Ertapenem (Geometric mean)	Ertapenem +ZelNate (Geometric mean)	*P* value
ERT SD	C_{max}	µg/mL	203.9	255.1	0.1537
ERT SD	$T_{1/2}$ (λz)	Hr	1.1	1.0	0.6537
ERT SD	AUC_{last}	(µg/mL)*hr	129.6	155.51	0.1878
ERT SD	CL	mL/hr/kg	112.5	104.2	0.4761
ERT SD	AUMC	(µg/mL)*hr^2	89.6	105.7	0.4353
ERT SD	MRT	Hr	0.69	0.68	0.9108
ERT SD	V_{SS}	ML/kg	80.5	73.2	0.5816
ERT MD	C_{max}	µg/mL	196.6	251.4	0.1157
ERT MD	$T_{1/2}$ (λz)	Hr	0.92	1.11	0.3429
ERT MD	AUC_{last}	(µg/mL)*hr	117.6	154.0	0.2000
ERT MD	CL	mL/hr/kg	124.0	105.4	0.2675
ERT MD	AUMC	(µg/mL)*hr^2	83.7	108.6	0.2679
ERT MD	MRT	Hr	0.71	0.71	0.8372
ERT MD	V_{SS}	ML/kg	91.2	76.7	0.1056

Table 6. Urine ertapenem concentrations (μg/mL) by time and dose. SD after single dosing; MD after multiple dosing.

Time (hr)	Dosing	Mean	Median	Min	Max
1	SD	8473.375	3886.5	2024	28612
2	SD	836.875	591	100	1929
3	SD	238.5	265	28	417
6	SD	66.875	36.5	5	267
8	SD	12.4	5	5	42
12	SD	23	21	0	32
1	MD	16083.5	14812.5	1213	32441
2	MD	1973	1465	397	5631
3	MD	493.25	482.5	99	940
6	MD	194.25	127.5	43	539
8	MD	61.875	49.5	17	155
12	MD	34.625	16.5	5	93

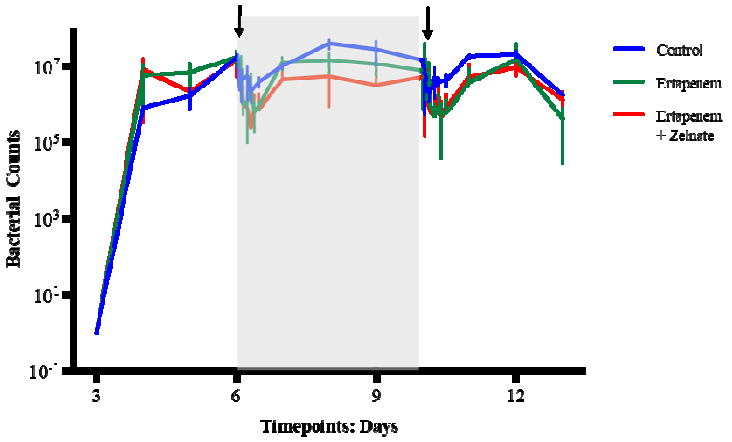

Figure 3. Relative number of bacterial shedding in the urine. Statistical significance found between the Control and the Ertapenem and Zelnate™ group (p=0.004). Arrows signify PK days in which 7 urine samples were taken: Pre-dose, +1h, +2h, +3h, +6h, +8h, +12h. Days in which ertapenem was administered is highlighted in gray (Day 6-10).

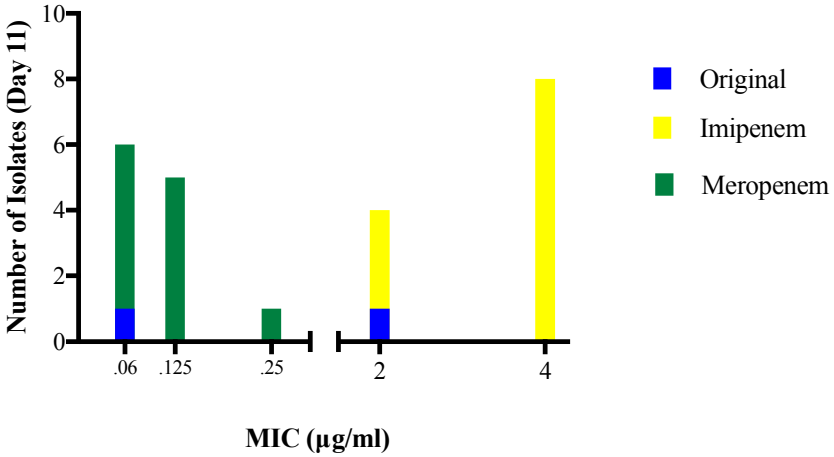

Figure 4. MICs of 13 isolates taken from the day after the final ertapenem treatment (Day 11). Isolates grown in meropenem are green bars, with MICs of .06 µg/mL (n=7*), .125 µg/mL (n=5), and .25 µg/mL (n=1). Isolates grown in imipenem are yellow bars, with MICs of 2 µg/mL (n=4*), and 4 µg/mL (n=8). Original strain MICs (*) are blue bars, meropenem and imipenem MICs being .06, and 2 µg/mL, respectively.

Table 7. Inflammation scores, presence of ulceration, and histopathology descriptions of bladder wall samples from the study ewes

ID	Score	Ulceration	Description and Comments
1	3	-	Multifocally, there is moderate hyperplasia of transitional cells manifested by thickening of normally oriented urothelium, and solid down-growths of nests of urothelium, which are both contiguous and non-contiguous with overlying epithelium. Diffusely, the lamina propria is moderately expanded by a well-vascularized and lightly eosinophilic, often fibrillar collagenous stroma with scattered fibroblasts, and contains moderate numbers of scattered polymorphonuclear cells with brightly eosinophilic cytoplasm (eosinophils) with fewer plasma cells and lymphocytes. Multifocally, lymphocytes and plasma cells form nodular/follicular aggregates. Capillaries are often lined by hypertrophied endothelium. Neutrophils or eosinophils are frequently present within the urothelium along with rare lymphocytes and occasional necrotic cells. Multifocal transitional cells are vacuolated. There are low numbers of lymphocytes and plasma cells around few blood vessels within the tunica muscularis. The interstitium of the tunica musuclaris is multifocally expanded by pale eosinophilic homogeneous fluid.
2	2	-	Changes are similar to Sheep #1 but less severe and with fewer eosinophils. Diffusely, there is mild hyperplasia of the urothelium characterized by thickening of normally oriented urothelium. Low to moderate numbers of scattered plasma cells and lymphocytes, rarely forming nodular perivascular aggregates, are present within the submucosa, which is multifocally mildly to moderately expanded by similar loose fibrovascular tissue. There are infrequent intra-epithelial polymorphonuclear cells with eosinophilic cytoplasm (neutrophils or eosinophils). There are multifocal areas of urothelium loss interpreted as artifactual with subjacent crush artifact. Similar low numbers of lymphocytes and plasma cells around few blood vessels within the tunica muscularis.
3	1	-	Multifocally, the submucosa is expanded by similar loose pale fibrovascular tissue. Infrequently, there are perivascular and scattered low numbers of lymphocytes and plasma cells, and rare polymorphonuclear cells with eosinophilic cytoplasm of variable intensity (eosinophils or neutrophils). There are occasional eosinophils and lymphocytes present within the urothelium, which is multifocally vacuolated. There are low numbers of lymphocytes and plasma cells around rare blood vessels within the tunica muscularis. The interstitium of the tunica musuclaris is multifocally expanded by pale eosinophilic homogeneous fluid.
4	4	+	Most severe of group. The urothelium is multifocally ulcerated. The submucosa is expanded by loose pale fibrovascular tissue containing moderate to large numbers of neutrophils (concentrated in areas of ulceration) and multifocal hemorrhage. Neutrophils are frequently present within the urothelium. There is also multifocal hemorrhage within the urothelium with multifocal vacuolated epithelial cells and occasional foci of thickened urothelium with mitotic figures (hyperplasia). The tunica muscularis is edematous with scattered moderate numbers of neutrophils. The serosa is also diffusely mildly expanded by edema with scattered neutrophils, few plasma cells and macrophages. Mesothelial cells are hypertrophied.
5	2	-	The submucosa is multifocally edematous and contains low to moderate numbers of polymorphonuclear cells with variably intense eosinophilic cytoplasm (neutrophils or eosinophils), often present at the epithelial-propria/submucosa junction or within the urothelium, and low numbers of plasma cells and lymphocytes, which multifocally form variably-sized nodular aggregates. The interstitium of the tunica musuclaris is multifocally expanded by pale eosinophilic homogeneous fluid.
6	1	-	The submucosa is focally edematous and contains low to moderate numbers of polymorphonuclear cells with variably intense eosinophilic cytoplasm (neutrophils), primarily within the edematous region, and low numbers of perivascular lymphocytes and plasma cells. Neutrophils are occasionally present within the urothelium. Basal layers of the urothelium are also moderately vacuolated within this region. The interstitium of the tunica musuclaris is multifocally expanded by pale eosinophilic homogeneous fluid. The serosa is mildly edematous with scattered low numbers of macrophages, neutrophils.
7	0	-	Looks most normal of all samples. Compact propria-submucosa. Rare subepithelial edema/vacuolation (minimal to mild) with rare neutrophils and rare intra-epithelial lymphocytes. The interstitium of the tunica musuclaris is multifocally expanded by pale eosinophilic homogeneous fluid. At one end of the section, the serosa is moderately expanded by similar pale eosinophilic fluid.
8	3	-	Diffusely, the submucosa is expanded by similar loose pale fibrovascular tissue. There are moderate numbers of scattered lymphocytes, plasma cells and polymorphonuclear cells with eosinophilic cytoplasm (neutrophils or eosinophils). Polymorphonuclear cells are most commonly subepithelial and present within the urothelium. Occasional lymphocytes are also present within the urothelium along with occasional necrotic cells. There are multifocal areas of mild hemorrhage within the superficial propria-submucosa, with mild superficial vacuolation (edema). Lymphocytes rarely form nodular aggregates. The urothelium is diffusely mildly thickened with occasional mitoses. There is multifocal sloughing of surface urothelial cells creating a slightly ragged surface appearance. Blood vessels of the propria-submucosa are often lined by hypertrophied endothelium. The interstitium of the tunica musuclaris is multifocally expanded by pale eosinophilic homogeneous fluid. The serosa is mildly edematous with scattered low numbers of macrophages, lymphocytes and rare mild hemorrhage.
9	2	-	Changes are similar to Sheep #2. There are low to moderate numbers of lymphocytes and plasma cells within the propria-submucosa, occasionally forming small nodular aggregates. There are low numbers of scattered polymorphonuclear cells often with granulated cytoplasm (eosinophils). Intra-epithelial inflammatory cells are rare. The interstitium of the tunica musuclaris is multifocally expanded by pale eosinophilic homogeneous fluid.
10	3	-	Changes are similar to Sheep #8. There are moderate numbers of PMNs (neutrophils) present within the urothelium, occasional lymphocytes, and occasional necrotic cells. There is multifocal sloughing of surface urothelial cells creating a slightly ragged surface appearance.

wes

CHAPTER 6. SUMMARY AND FUTURE DIRECTIONS

Summary

Determination of pharmacokinetic and pharmacodynamic (PK/PD) relationships in veterinary medicine is based on examination of relatively small populations that often are free of disease or clinical deviations from normal. This can counter the goal of such studies, as clinically healthy animals are seldom treated therapeutically, especially within the ruminant food animal species where drug residue avoidance is a major component of human food safety. PK/PD data obtained from healthy animals may not directly translate to sick animals, and presents a critical knowledge gap to the field of ruminant food animal clinical pharmacology.

The objective for this dissertation was to explore the effects of clinical states on the PK/PD of various drugs in ruminant food animal species. Our central hypothesis was that the physiologic differences brought upon by different clinical states such as stress, pain, respiratory disease, and drug co-administration would cause differences in the PK/PD of various drugs in ruminants. To test this hypothesis, we assessed the following: 1) the effect of clinical pain on the pharmacokinetics and pharmacodynamics of fentanyl in clinical patient calves and healthy calves; 2) the effect of *Pasteurella multocida* pneumonia on the pharmacokinetics and tissue residue levels of tulathroymycin in meat goats; and 3) the effect of co-administration of an immunomodulatory agent on the pharmacokinetics and pharmacodynamics of ertapenem in sheep with catheter-associated *Pseudomonas aeruginosa* cystitis.

First, we determined the intravenous pharmacokinetics of fentanyl citrate in healthy calves.(183) We initially observed a longer terminal elimination half-life of 12.7 and 3.6 hours for fentanyl and norfentanyl, respectively. These elimination half-lives were markedly higher than what has been reported for other ruminant species, and similar relationships were noted amongst other pharmacokinetic parameters. However, upon closer examination we discovered that our analytical lower level of quantification (LLOQ) of 0.03 ng/mL was lower than reported in the comparative literature. Upon re-analyzing our data with a theoretical LLOQ of 0.05 ng/mL, parameters much closer to what has been reported comparatively in other ruminant species were achieved. While this study characterized the pharmacokinetics of fentanyl for Holstein calves, it also demonstrated the analytical sensitivity of pharmacokinetic parameter estimation.

Additionally, we determined the PK/PD of fentanyl transdermal patches (FTPs) in clinically healthy and clinical calves to investigate the differences that may be brought upon by altered clinical states such as disease and pain.(192) While this study was limited due to the adverse effects noted and the resultant IACUC modifications, we demonstrated that among calves with concurrent acute disease no adverse reactions occurred, whereas among calves with no disease or chronic disease adverse effects such as tachycardia, hyperthermia, excitement, and ataxia occurred. Maximum plasma concentration was variable amongst calves, and concentrations at 4 and 6 hours post patch application were significantly associated with the occurrence of adverse effects. This preliminary work supports other clinical observations about the effect of acute pain on the presence or absence of adverse effects with opioids in veterinary patients, but also warrants further investigation for dose optimization for calves. Clinical states

such as acute disease or pain appear to minimize the occurrence of adverse effects from the application of FTPs in calves.

Second, we investigated the effect of *Pasteurella multocida* respiratory disease on the pharmacokinetics and pharmacodynamics of tulathromycin in meat goats. Goats with respiratory disease had lower plasma concentrations of tulathromycin from 168 hours to 312 hours and had a higher volume of distribution when compared to controls (50.8 ± 26.5 mL/kg vs 24.99 ± 11.9 mL/kg). While no differences were noted at 312 hours in terms of tissue CP 60,300 concentration in muscle, liver or fat, a significantly ($P = 0.0095$) lower concentration was noted in the kidney tissue of experimental goats. Thus, this work demonstrated that respiratory disease has the potential ability to alter plasma concentrations, pharmacokinetics, as well as tissue concentration of tulathromycin in meat goats.

Finally, we investigated the effect on PK/PD of administration of ertapenem with and without an immunomodulator in a model of catheter-induced cystitis in sheep to explore this drug-drug interaction. An ovine model of human catheter-associated cystitis due to *Pseudomonas aeruginosa* was developed, and colonization of the bladder was confirmed via culture, urinalysis, and histopathology of the bladder wall. No significant differences in ertapenem pharmacokinetics were noted in either the group receiving ertapenem or ertapenem with the concurrent administration of an immunomodulator. When bacterial counts were quantified, a significant ($P = 0.004$) decrease was noted between the group administered ertapenem with an immunomodulator as compared to the control group. In addition, MIC testing for ertapenem, imipenim, and meropenem demonstrated that isolates had increased MICs when compared to the initial strain. The results generated by this study may lead to the use of non-antimicrobial adjunctive therapies to decrease bacterial numbers in infections of high consequence.

Collectively, these results provide new insights into the effects of clinical states on PK/PD in ruminant food animal species. These findings demonstrate that it is vital to study drugs in the face of clinical states to determine differences on PK/PD compared to administration of the drug in a normal animal.

Future Directions

The models utilized in this work provide a basis for further studies into the effects of clinical states on PK/PD in ruminant food animals. Based on the work presented here several areas merit additional research. The pharmacokinetics of fentanyl transdermal patches in calves need to be further elucidated, utilizing our study's recommendation of a dose below 1.0 ug/kg(192), and potentially using the anecdotally reported dose of 0.05 - 0.5 μg/kg(86) to determine a safe therapeutic application for fentanyl transdermal patches in this species. Additional pharmacodynamic work will be necessary to determine the effect of sustained fentanyl concentrations as an analgesic in calves. Further study will also be necessary to determine the effect of naturally occurring *P. multocida* pneumonia on the pharmacokinetics and tissue residue concentrations of tulathromycin and the marker residue in meat goats and other ruminants, as this could vary from the results seen with experimental infection. Finally, the ovine model of catheter-associated *P. aeruginosa*, while providing exciting results with the effect of the immunomodulator, will need to be further assessed by additional trials with increased subject numbers and drug combinations to determine its full comparative utility to human medicine.

Due to the paucity of clinical descriptions of pharmacokinetics and pharmacodynamics in ruminant food animal species it is common for practitioners to use information from one ruminant species to formulate a treatment plan for another species. An example of this being the

use of pantoprazole for increasing gastric pH. The PK/PD data for this treatment is based on alpacas(243), and clinically this treatment has been extrapolated to other ruminant food animals, such as the goat.(43) However, currently clinical judgments of this type have to be based on caution, as pharmacodynamics are not always constant across ruminant species. For example, the use of fenbendazole and NSAIDs in camelids(244) as a successful treatment for infections of *P. tenuis* has been successfully extrapolated to meat goats(42, 245) without adverse effects. In contrast, the use of ketamine, xylazine, and butorphanol has been demonstrated to be a safe injectable anesthetic in cattle(246), yet this combination has been shown to cause profound aspiration when used in a similar ruminant, the yak.(247) This potential for adverse reactions was also noted by our work, as an ovine dose for fentanyl transdermal patches yielded adverse effects in calves.(192) Our data supports that more species-specific research is necessary to completely examine the effect of clinical states on PK/PD in ruminant food animal species.

The presence or absence of disease, pain, or other clinical condition is not the only factor that warrants further investigation. Recently studies in people, epigenetics and quasi-epigenetics have been recognized as additional considerations in therapeutic drug use, as these unintentional effects can act upstream to conventional drug mechanisms, as these “off target” effects could potentially be beneficial or adverse.(248) Examples of these epigenetic and quasi-epigenetic phenomenon include: the cardiac protective effect of the chemotherapeutic 5-Azacytidine,(249) the neuroprotective effect of beta-lactam antimicrobials,(250, 251) and the anti-neoplastic properties of the NSAID class of drugs.(252) While these off-target effects have been described for human patients, more work is needed to determine these effects in ruminant species.

Additional physiologic factors have been demonstrated to lead to differences in PK/PD in veterinary medicine. Gender, age, and body composition have all been demonstrates to impact

drug pharmacokinetics in cattle.(253) Conversely the effects of renal and hepatic impairment on drug pharmacokinetics have not yet been well-described in cattle, although inflammation, infection, pregnancy and lactation's effects on drug pharmacokinetics has been described for cattle.(1) Chronobiology and circadian rhythm have been explored for companion animals(254), but research is lacking among cattle(253) and other ruminants. Numerous other factors specific to clinical patients will also need to be accounted for in future work when considering the effect of clinical states on PK/PD.

Considerations need to be taken regarding the methods used to determine PK parameters, as calculation can add additional complexity to interpretation. In addition to the effect of analytical performance demonstrated by our fentanyl study, more work needs to investigate the clinical effects of non-compartmental parameter estimation. For example, our ertapenem study revealed a mean residence time (MRT) that was less than the elimination half-life ($T_{1/2}$) for the drug. Mathematically this is not possible as demonstrated by the following:

- MRT = 1 / ke; where ke represents the elimination constant.
- $T_{1/2}$ = 0.693 / ke; therefore:
- $T_{1/2}$ = 0.693 x MRT

Our results from the ertapenem study would conflict this. Similarly our tulathromycin study found a statistically significant difference in the Volume of Distribution (V) between groups, but no difference in clearance (CL). This is also counterintuitive as the mathematical relationship of these parameters is:

- CL = V x ke; where ke is the previously defined elimination constant.

While V and CL are independent parameters; an increase in V could lead to an increase in $T_{1/2}$ unless the CL changed to the same extent as V ($_{/2}$ = LN(2) x V/CL). These findings are not

uncommon in non-compartmental pharmacokinetic literature as an MRT < $T_{1/2}$ has been reported for other rapidly metabolized intravenously administered drugs such as flunixin(255) and fentanyl(183) calculated through non-compartmental methods. The reasoning for these discrepancies is probably due to the selection of the slope of the terminal phase of noncompartmental software programs. However, the clinical effects of this parameter calculation, if any, are unknown at this time.

Future work needs to focus on the impact that clinical disease takes on ruminant food animal patients as well as models to predict these interactions. Clinical studies utilizing patients in veterinary referral centers, potentially with sparse sampling or naïve pooling schedules would be potential tools for this next step. These techniques allow for minimal sample collection, but larger populations of diseased patients to draw results.

Additionally, application of modelling techniques will be crucial to allow for further extrapolation of the effect of clinical states on PK/PD in ruminant food animals, potentially for the prediction of tissue residues. Nonlinear mixed effects (NLME) modelling may very well be a tool for this purpose, as though while different from the method used by the United States Food and Drug Administration, this modelling technique could serve as a way to identify components of clinical disease that lead to increased risk of tissue residue.(256) NLME models allow for the flexibility of analyzing dense or sparse data, while accounting for many covariates that could serve as valuable clinical characteristics of food animal patients.

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APPENDIX. IOWA STATE UNIVERSITY INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE STUDY REFERENCE NUMBERS

Chapter 2: 7-16-8318-B

Chapter 3: 7-16-8318-B; 6-16-8301-B

Chapter 4: 5-17-8517-F

Chapter 5: 3-15-7965-O