July, 2018

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Received: 15 January 2018 | Revised: 27 May 2018 | Accepted: 6 June 2018

DOI: 10.1111/jvp.12688

ORIGINAL ARTICLE

WILEY Journal of Veterinary Pharmacology and Therapeutics

Comparative plasma and interstitial fluid pharmacokinetics and tissue residues of ceftiofur crystalline-free acid in cattle with induced coliform mastitis

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Funding information
Iowa Healthy Livestock Advisory Council

Abstract

Ceftiofur (CEF) is a third-generation cephalosporin that is the most widely used antimicrobial in the dairy industry. Currently, violative meat residues in cull dairy cattle are commonly associated with CEF. One potential cause for violative residues is altered pharmacokinetics of the drug due to disease, which could increase the time needed for the residue to deplete. The objectives of this study were (a) to determine the absolute bioavailability of CEF crystalline-free acid (CFA) in healthy versus diseased cows; (b) to compare the plasma and interstitial fluid pharmacokinetics and plasma protein binding of CEF between healthy dairy cows and those with disease; and (c) to determine the CEF residue profile in tissues of diseased cows. For this trial, disease was induced through intramammary *Escherichia coli* infusion. Following disease induction and CEF CFA administration, for plasma concentrations, there was not a significant effect of treatment ($p = 0.068$), but the treatment-by-time interaction ($p = 0.005$) was significant. There was a significantly greater concentration of CEF in the plasma of the DIS cows at T2 hr ($p = 0.002$), T8 hr ($p < 0.001$), T12 hr ($p = 0.001$), and T16 hr ($p = 0.002$). For PK parameters in plasma, the slope of the terminal phase of the concentration versus time curve was significantly lower ($p = 0.007$), terminal half-life was significantly longer ($p = 0.014$), and apparent volume of distribution during the elimination phase was significantly higher ($p = 0.028$) diseased group. There was no difference in plasma protein binding of CEF and interstitial fluid pharmacokinetics. None of the cows had kidney CEF residues above the US tolerance level following observation of the drug's withdrawal period, but one cow with a larger apparent volume of distribution and longer terminal half-life had tissue residues slightly below the tolerance. Whereas these findings do not support the hypothesis that severely ill cows need longer withdrawal times, alterations in the terminal half-life suggest that it is theoretically possible.

KEYWORDS

ceftiofur crystalline-free acid, dairy cattle, drug residues, pharmacokinetics

1 | INTRODUCTION

Ceftiofur (CEF) is a third-generation cephalosporin antimicrobial that is the most widely used in the dairy industry due to its broad spectrum of activity and short withdrawal periods for milk and meat (Sawant, Sordillo, & Jayarao, 2005; Schuler, Rice, & Gorden, 2017; Zwald et al., 2004). Broad-spectrum antimicrobials are important for both human and veterinary medicine, leading the World Health Organization to classify third-, fourth-, and fifth-generation cephalosporins among the highest priority critically important antimicrobials for the preservation of human health (WHO, 2017). In addition, due to concerns about the development of antimicrobial resistance to cephalosporins in humans from use in farm animals, extralabel usage of CEF was restricted in major food animal species by the United States Food and Drug Administration (US FDA, 2012).

In calendar year 2013, violative CEF residues became the most frequent residue found in cull dairy cattle at slaughter, surpassing penicillin. Since then, CEF has continued to be the most frequent violative residue in the tissues of cull dairy cattle, as reported by the United States Department of Agriculture (USDA, 2017). The reason for this increase in violative ceftiofur residues is likely multifactorial, including changes to the USDA testing programs that have been implemented over the years (USDA, 2012), producers unintentionally marketing cattle before the meat withdrawals have elapsed due to record keeping errors, producers intentionally marketing cattle before the meat withdrawals have elapsed to avoid losses from animal death, and potentially alterations in drug metabolism in ill animals compared to healthy counterparts. During the drug approval process, sponsoring companies must present the FDA's Center for Veterinary Medicine (CVM) with toxicological and residue depletion studies. Based on these data, the FDA CVM establishes withdrawal periods for meat and milk, if approved for lactating dairy cattle. However, these studies are performed on healthy animals, not animals suffering from infectious diseases. Data examining drug metabolism in sick animals and how this compares with healthy animals are deficient in the veterinary literature. As most veterinary drugs are not intended for use in healthy animals, data on drug metabolism in diseased animals would provide veterinarians with evidence to more accurately prescribe veterinary drugs and to better predict residue depletion in these diseased animals.

Previous research by the authors recently demonstrated that CEF pharmacokinetics (PK) are altered in dairy cows affected with naturally occurring mastitis compared to healthy cattle (Gorden et al., 2016). In that study, plasma terminal half-life ($T_{1/2\ \lambda z}$) of the diseased group was not statistically different from the control group; however, one of the cows in the diseased group had a $T_{1/2\ \lambda z}$ that was nearly twice as long as the mean of the control group (70.9 hr vs. 35.8 hr). In that study, animals were not sacrificed upon completion of the study, so tissue residue concentrations were not determined. However, assuming tissue residue depletion follows plasma PK, this doubling of the $T_{1/2\ \lambda z}$ would indicate that it would take twice as long for the tissues to deplete to the tolerance as would be the case in healthy animals (Riviere, Webb, & Craigmill, 1998). This would necessitate an extension of the withdrawal time by the prescribing veterinarian, even if the drug is used in an on-label manner for dose, duration, and route of administration.

In our previous study (Gorden et al., 2016), the apparent volume of distribution during the elimination phase (V_z/F) and apparent systemic clearance (CL/F) values for CEF were significantly elevated in diseased group. This alteration has also been reported in swine with porcine respiratory and reproductive virus (PRRSv) that were treated with CEF (Day et al., 2015; Sparks et al., 2017; Tantituvanont, Yimprasert, Werawatganone, & Nilubol, 2009). In studies where drugs are not administered via intravenous injection, alterations in bioavailability (F) confound the interpretation of V_z and CL. None of the swine experiments or the previous study included an IV administration component to directly determine F of CEF, but Sparks et al. (2017) estimated a relative bioavailability of 0.8. When relative bioavailability was inputted into V_z/F and CL/F parameters for their study, the differences in these two parameters decreased between the control group and animals challenged with PRRSv (Sparks et al., 2017). Therefore, determination of CEF bioavailability will help determine the underlying cause for changes in V_z and CL.

The objectives of this study were to (a) complete an IV study using CEF sodium to later determine the absolute bioavailability of CEF administered as CEF crystalline-free acid (CFA) sterile suspension via a subcutaneous route in healthy versus diseased animals; (b) compare the plasma and interstitial fluid (ISF) concentrations, plasma protein binding, and plasma and ISF PK of CEF following administration as CEF CFA to healthy dairy cows versus those with disease; and (c) determine the CEF residue profile in kidney, liver, muscle, and fat of diseased cows. In this trial, disease was induced via the administration of *Escherichia coli* via the intramammary (IMM) route. Specifically, we desired to induce the same degree of severity in the disease (DIS) group of animals to mimic the PK profiles of our previous study (Gorden et al., 2016). Our hypothesis was that administration of CEF would result in altered plasma and ISF concentrations, and altered PK in diseased animals compared to healthy animals, necessitating variance in dose regimens and/or withdrawal periods.

2 | MATERIALS AND METHODS

2.1 | Experimental cattle

This study was completed at the Iowa State University Dairy Farm. The lactating herd consists of approximately 400 animals (approximately 90% Holstein and 10% Jersey), with 365-day rolling herd averages per cow of 10,991 kg milk, 404 kg fat, and 342 kg protein. Twenty healthy Holstein cows were utilized in two separate segments to complete the objectives of the trial. Ten cows were assigned to the DIS group, and ten cows of similar age and lactation status were assigned to the control group (CON). In the first segment, all 20 cows received intravenous CEF as CEF sodium to obtain data to later calculate bioavailability of subcutaneously administered CEF as ceftiofur crystalline-free acid (CEF CFA). Segment

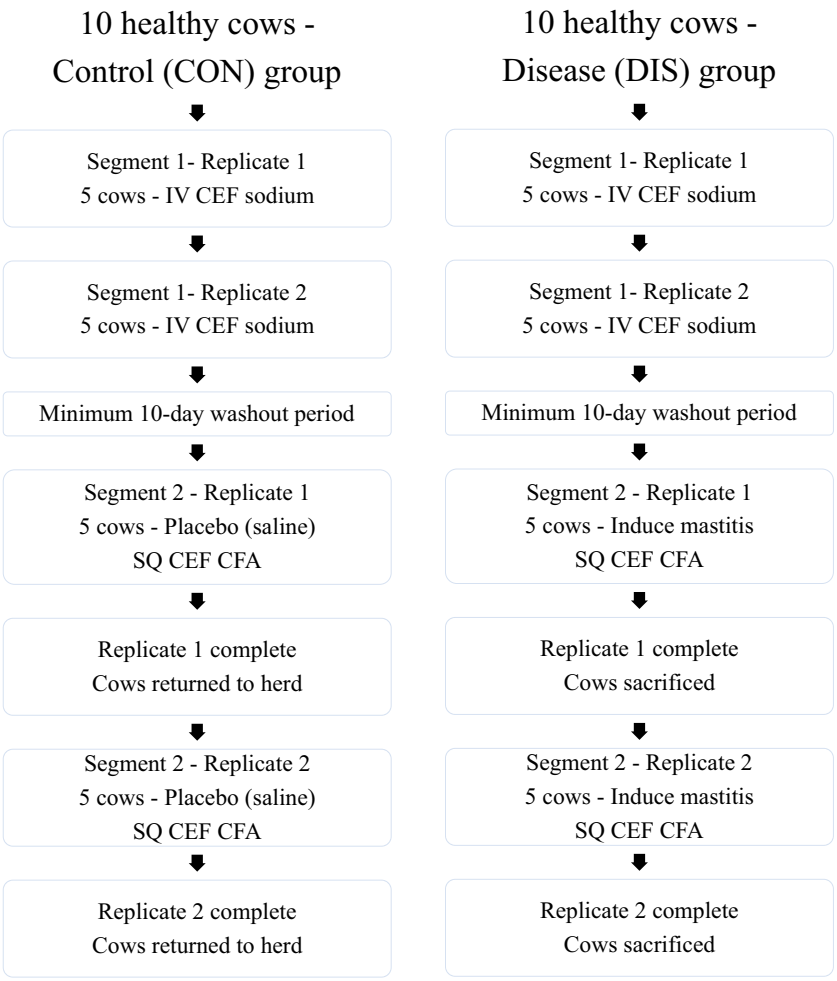

FIGURE 1 Flow diagram showing the chronological flow of the segments. Boxes in the same row occurred at the same time

2 consisted of the IMM challenge to determine CEF concentrations, PK, and residue depletion. Due to the availability of housing, each segment of the trial was done in two consecutive replicates. The trial was carried out as a 1-sequence, 2-treatment, 2-period cross over design, as the cows that would later be challenged with mastitis were to be sacrificed at the end. See Figure 1 for a diagram explaining the chronological flow of the trial. Cows were eligible for the trial if they had not been treated with systemic or IMM CEF within the past 20 days of the first segment and were healthy prior to enrollment. Furthermore, the cows were thirty or more days from their next scheduled dry period.

During each treatment segment, cows were housed in individual box stalls bedded with deep, long-stem straw. Each stall had individual access to feed and water. Cows were milked three times daily (4 a.m., 12 p.m., and 8 p.m.). During the treatment periods, trial personnel milked trial cows per the farm's milking protocol. Between treatments, cows were housed in a free-stall barn bedded with recycled manure solids, which is standard practice for this dairy operation.

Throughout the entire period of this trial, cows were fed a total mixed ration and watered, ad libitum. Ration parameters met or exceeded those recommended by the NRC guidelines (NRC, 2001). Cow housing and management met or exceeded the recommendations listed in the *Guide for Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). Iowa State University's Institutional Animal Use and Care Committee approved the research protocol prior to commencement of trial procedures (protocol number 6-15-8030-B).

2.1.1 | Experimental design—Segment 1—Ceftiofur bioavailability

One day prior to treatment, cows were weighed and moved to their box stall. One intravenous catheter was placed in each jugular vein of all cows to facilitate CEF administration and blood collection. Following restraint in a stanchion, cows were sedated with xylazine at approximately 0.025 mg/kg IV; the skin over the jugular furrow was clipped and aseptically prepped using alternating scrubs of 2% chlorhexidine acetate and 70% isopropyl alcohol. Prior to catheter placement, the area under the skin was infiltrated with 2% lidocaine. Following catheter placement, the catheter was sutured in place using #3 nylon suture. To maintain catheter patency, 3 ml of a heparin saline solution containing 3 USP units of heparin sodium/ml was infused into the catheters every 8 hr until treatments were initiated. Subsequently, catheters were flushed following each blood collection.

On the day of treatment, cows were restrained in a stanchion, where cows received CEF as ceftiofur sodium (Naxcel Sterile Powder; Zoetis Inc., Kalamazoo, MI) at a dose of 2.2 mg/kg via the IV route. Following CEF administration, 3 ml of a heparin saline solution was infused into the catheters to assure complete delivery of the drug. The catheter used for CEF administration was then removed.

At T0 hr prior to CEF administration, two 10-ml blood samples were collected from the jugular catheter into blood tubes containing freeze-dried heparin (Becton, Dickinson and Co, Franklin Lakes, NJ) for plasma harvest. Subsequent blood samples were collected from the jugular catheter into heparinized tubes at 0.05, 0.10, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 16, 24, 32, 40, and 48 hr after drug administration. After blood was collected, samples were immediately placed on ice until plasma could be harvested. Within two hr of collection, blood samples were centrifuged for 20 min at 1,000 *g* at 4°C; then, 5 ml of plasma was harvested and frozen at −70°C until analyzed for drug concentration.

Following the 48-hr time point, IV catheters were removed and the cows were moved back to the free-stall housing for the herd. The cows had a minimum of a 10-day washout period between the two segments of this trial. Both replicates of this segment of the trial were completed prior to initiation of the second segment of the trial.

2.1.2 | Experimental design—Segment 2—Pharmacokinetics and tissue residue determination of CEF CFA in healthy versus diseased cows

One day prior to treatment, cows were moved to box stalls and had one IV catheter inserted as described above. While sedated, all cows had one subcutaneous, in vivo ultrafiltration probe (RUF 3-12, BASi, West Lafayette, IN) placed dorsal-caudal to the scapula to facilitate ISF collection. Briefly, the area was prepped as described for inserting catheters above and the probe was placed by passing a 10-gauge metal introducer needle between two small stab incisions previously made with a #10 scalpel blade into the skin. Following probe placement, the collection tube was stitched in place and connected to a 7-ml red top glass vacuum tube (Becton, Dickinson and Co, Franklin Lakes, NJ) for ISF collection.

2.2 | Intramammary challenge

To induce disease, cows in the DIS group were inoculated with 100–150 colony forming units (cfu) of *E. coli* (strain 487) via the streak canal of a selected mammary gland quarter. To prepare the challenge inoculum, an aliquot of frozen stock culture was streaked onto a trypticase soy agar plate and incubated overnight at 37°C. The following day, two well-isolated colonies were inoculated into trypticase soy broth and incubated overnight in a shaker incubator at 37°C to achieve stationary growth phase. Two-milliliter aliquots of the broth culture were centrifuged in microcentrifuge tubes, and the pellet was washed twice in phosphate-buffered saline (PBS) and then resuspended in PBS to achieve an optical density of 0.35 at 495 nm. Seven 9:1 serial dilutions were then prepared in PBS, and 0.1 ml was plated on trypticase soy agar and incubated overnight. The following day, cfu counts were enumerated. The process was then completed to acquire a challenge inoculum with a desired cfu count of 100–150 diluted in 5 ml of PBS.

Approximately 12 hr prior to CEF administration (T -12 hr), all cows in the DIS group were inoculated with the challenge inoculum in either the right or left front quarter following aseptic preparation of the teat end. Simultaneously, cows in the CON group underwent a placebo challenge by infusing 5 ml of sterile PBS into the right or left front quarter following aseptic preparation of the teat end. The decision on whether to challenge the left or right front quarter was based on position of the dividing gate between stalls. Therefore, the challenged quarter was always opposite from the dividing gate.

Following completion of the IMM challenge, 0.1 ml of challenge inoculum was plated onto trypticase soy agar and incubated overnight to determine *E. coli* challenge dose.

2.3 | Drug administration

At time 0 (T0), all cows received CEF, as CEF CFA (Excede; Zoetis Inc., Kalamazoo, MI), at 6.6 mg CEF equivalents per kg of body weight administered at the base of either ear, following instructions on the package insert. As part of the trial design, rescue therapies (antiinflammatories and fluid support) were included in the trial protocol if needed. No further medications were administered throughout the remainder of the trial.

2.4 | Collection of blood and ISF samples

Prior to CEF administration (T0), two 10-ml blood samples were collected from the jugular catheter into blood tubes containing freeze-dried heparin (Becton, Dickinson and Co, Franklin Lakes, NJ) for plasma harvest. Subsequent blood samples were collected from the jugular catheter into heparinized tubes at 2, 4, 8, 12, 16, 20, 24, 32, 40, and 48 hr and then every 24 hr after drug administration through 312 hr After blood was collected, samples were processed as described above.

Simultaneous with drug administration (T0), a new vacuum tube was attached to the ultrafiltration probe. Interstitial fluid samples were collected at 4, 8, 12, 16, 20, 24, 32, 40, and 48 hr following drug administration and then every 24 hr through 312 hr, by changing the vacuum tube. The tubes were immediately frozen at −70°C until analyzed for drug concentration.

2.5 | Daily observations and infrared thermography

At every milking for the first 5 days following IMM challenge and then daily through the conclusion of the trial, milk was visually evaluated during the milking prep procedure for appearance. In addition, udder consistency was evaluated by palpation of the challenged gland. Using these parameters, cows were assigned a mastitis severity score as previously described by Wenz, Barrington, Garry, Dinsmore, and Callan (2001). In addition, rectal temperatures were

recorded at T0 and then every 8 hr for the first 24 hr and then every 24 hr for the remainder of the trial.

Infrared images of the eye on the side of CEF CFA injection, the ear where the CEF CFA injection was placed, and quarter of the mammary gland that was challenged were obtained using a research quality infrared camera (FLIR SC 660; FLIR Systems, AB, Danderyd, Sweden). Images were obtained prior to IMM challenge (T -12 hr), at T0 prior to CEF CFA injection, at 8, 16, and 24 hr following injection, and then every 24 hr through T168 hr. At each measurement period, three images each (nine total) were collected from the eye, ear, and mammary gland, respectively. Images of the eye and ear were obtained by holding the camera at approximately a 45° angle and 0.5 m from the head. Mammary gland images were collected by placing the camera in a parallel plane lateral to the challenged quarter, approximately 0.5 m from the gland. Camera calibration was performed prior to each measurement period by entering current ambient temperature and relative humidity into the camera's software. Throughout each measurement period, the camera collected changes to ambient temperature and relative humidity and recalibrated automatically.

Analysis of infrared images was completed using research grade software provided by the camera manufacturer (FLIR ExaminIR, North Billerica, MA). For each measurement period, the maximum, minimum, and mean temperature was recorded for each image and a mean value for each the parameters (maximum, minimum, and mean) was determined from the three images for the eye, ear, and mammary gland, respectively.

2.6 | Trial conclusion

At T312 hr, all DIS cows were humanely euthanized with a captive bolt followed by exsanguination. Following euthanasia, kidney, liver, skeletal muscle, fat, and injection site tissues were collected, weighed, and frozen at −70°C until analyzed for drug concentration.

Cows in the CON group were returned to the herd following catheter and subcutaneous ultrafiltration probe removal.

2.7 | Determination of plasma protein binding

Free plasma concentration of CEF was determined on each cow on the T24, T96, and T192 hr plasma samples using a microcentrifugation system (Centrifree Ultrafiltration Device; EMD Millipore Corp., Billerica, MA) to collect plasma ultrafiltrate (UF), as previously described (Gorden et al., 2017). Following collection of plasma UF, samples were immediately frozen at −70°C until analyzed for drug concentration.

2.8 | Plasma, interstitial fluid, and plasma ultrafiltrate ceftiofur concentration analysis

Ceftiofur and its metabolites from plasma were converted to a stable derivative, desfuroylceftiofur acetamide (DCA), and total CEF concentration (as DCA) was then determined using liquid chromatography coupled with mass spectrometry (LC-MS) as previously described (Gorden et al., 2016). Plasma UF and ISF samples were analyzed for free drug concentration in the same manner, except spike and quality control (QC) samples were prepared using blank ISF for ISF sample analysis. The limit of detection (LOD) of the assay was 1 ng/ml, and the limit of quantification (LOQ) was 10 ng/ml. The accuracy and coefficient of variation for the quality control (QC) samples were 98% and 9.4% for the 15 ng/ml QC sample; 105% and 8.7% for the 150 ng/ml QC sample; and 107% and 10.6% for the 1,500 ng/ml QC sample.

2.9 | Screening of kidney samples—Kidney Inhibition Swab (KIS™) test

At tissue harvest, a section of kidney from each cow was bagged and frozen separately. After at least 24 hr of freezer storage, all ten samples were thawed at 4°C and tested for inhibitory residues using the Kidney Inhibition Swab (KIS™) test (Charm Sciences Inc., Lawrence, MA) as described (USDA, 2010). In addition, kidney tissue from a negative control animal was also thawed and tested. Briefly, the cap portion of the swab was used to cut a circular incision into the kidney parenchyma approximately 1–2 cm in depth. The swab was then placed into the incised area and rotated for approximately 30 s to saturate the swab with kidney fluid. This procedure was repeated on up to four swabs in total at one time. The swab was then pierced through the foil and into the clear liquid in the bottom vial of the test, but not perforating the bottom seal. After 2 min, the swab was completed screwed down to pierce the bottom seal and to the point where it was just above the agar in the bottom of the vial. The tube was then tapped firmly five times on the countertop, after which the swab was rotated in the opposite direction and tapped five times again on the countertop. Up to four swabs were then placed into the heating block provided with the test kit and incubated at 64°C for three hr. Following incubation, the agar color was compared to the reference card provided by the manufacturer to determine the test result. The sensitivity of this assay is not reported by the manufacturer but others have reported the lower limit of detection to be 4 ppm (Jones et al., 2014).

2.10 | Ceftiofur concentration analysis—tissue samples

Determination of ceftiofur concentrations in kidney tissues was completed using an official method as described by the USDA (2016), with minor alterations to the protocol. The method determines the concentration desfuroylceftiofur cysteine disulfide (DCCD) as a proxy for the marker compound for ceftiofur, desfuroylceftiofur (DFC). Briefly, 0.4-g aliquots of blank bovine kidney for blank, spike, fortified analyst recovery, and QC samples, in addition to 0.4-g aliquots of test kidney samples, were weighed into 15-ml conical bottom, polypropylene tubes. Known concentration of DCCD were added to spike kidney samples to create a calibration curve from 50 to 2,000 ng/g. 5,000 ng of internal standard, DCCD-d3, was then

added to all spike, QC, blank, and test samples but not the fortified recovery analyst tube. All tubes were treated with 3.5 ml of 1% phosphate buffer and shaken on an automated shaker for 10 min at 260 x g. Following shaking, all tubes were centrifuged at 3630 x g for 20 min at room temperature. Solid phase extraction (SPE) cartridges (Strata-X SPE cartridges (60 mg/3 ml); Phenomenex, Torrance, CA, USA) were conditioned with 2 ml of methanol, followed by 2 × 2 ml fractions of ultrapure water. The supernatant following centrifugation was then loaded onto the SPE cartridges and allowed to percolate via gravity. After all the supernatant had percolated through the SPE cartridges, they were washed with 2 ml ultrapure water. Target analytes were eluted with 2 × 1 ml fractions of 50% acetonitrile/ultrapure water (v/v). Following elution, the fortified analyst recovery tube was spiked with sufficient DCCD to create a concentration of 400 ng/g and 5,000 ng of the internal standard, DCCD-d3. The acetonitrile was then evaporated from the samples under a stream of nitrogen at 15 psi and 48°C to a volume of <1 ml. Ultrapure water was added to each sample to bring the total volume to approximately 1 ml and then all samples were vortexed. 150 µl of sample was transferred to labeled autosampler vials equipped with glass inserts for analysis by LC-MS. Autosampler vials were centrifuged for 20 min at 1,000 *g* at room temperature and then loaded onto the autosampler tray.

The LC-MS system consisted of an Agilent 1,100 pump, autosampler, and column compartment (Agilent Technologies, Santa Clara, CA) coupled to an ion trap mass spectrometer (LTQ; Thermo Scientific, San Jose, CA). The injection volume was set to 25 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. The mobile phase began at 5% B with a linear gradient to 95% B at 6.5 min, which was maintained for 1.75 min, followed by reequilibration to 5% B. Separation was achieved with an ACE C18 column (ACE 3 C18, 150 × 2.1 mm, 3 µm particles; Mac-Mod Analytical, Chadds Ford, PA, USA) maintained at 40°C. DCCD was eluted at 3.63 min and the internal standard, DCCD-d3, eluted at 3.61 min.

Sequences consisting of plasma blanks, calibration spikes, quality control samples, fortified analyst recovery, and bovine kidney samples were then batch-processed with an automated processing method developed in the Xcalibur software (Thermo Scientific, San Jose, CA, USA), which identified and integrated each sample peak. The calibration curve was calculated based on a weighted (1/X), linear fit. Tissue concentrations of DCCD in trial samples were calculated based on this calibration curve. Results were then viewed in the Quan Browser portion of the Xcalibur software. The standard curve had a linear range from 50 to 2,000 ng/g, with a correlation coefficient of 0.993. All standards were within ±15% of the nominal value in this range with the exception of the lowest (50 ng/g) standard, which was within +20% of the nominal value. The accuracies for the QC samples were 91% for the 75 ng/g QC, 106% for the 750 ng/g QC, and 110% for the 1,500 ng/g QC. The accuracy of the fortified analyst recovery sample was 94%. The limit of detection and the limit of quantification for this assay was 50 ng/g.

In the United States, the official marker residue for CEF in the bovine is DFC, measured as DCA. In the bovine, the current US tolerance is 0.4 parts per million (400 ng/g), with kidney serving as the target tissue. The method described above has been published as an alternate method to determining DCA in tissues, in which DCCD is measured as a surrogate marker residue for DFC (Feng et al., 2014). To convert measured DCCD concentrations to DFC, the following regression equation was utilized:

$$y = 0.21557 + 1.801 \times x$$

where y = the DFC concentration being calculated and x = the DCCD determined concentration (Feng et al., 2014).

After results from determination of DCCD in kidney tissues were evaluated, other tissue samples were not analyzed.

2.11 | Pharmacokinetic analysis

The total plasma drug and ISF concentration–time profiles from CEF CFA (SQ)-treated cows were analyzed using noncompartmental methods implemented in a commercially available software program (Phoenix® WinNonlin® 7.0; Certara, Inc., Princeton, NJ) to generate the following PK parameters: λ_z (hr^{-1}), slope of the terminal phase; $T_{1/2\ \lambda z}$ (hr), terminal half-life; C_{max} (µg/ml), maximum plasma concentration; T_{max} (hr), time of C_{max}; $AUC_{0-\infty}$ (µg/ml × hr), area under the curve extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; $AUC_{0-24\ hr}$ (µg/ml × hr), area under the curve from T0 to T24 hr; V_z/F (ml/kg), apparent volume of distribution during the elimination phase; CL/F (ml hr^{-1} kg^{-1}), apparent systemic clearance; $MRT_{0-\infty}$ (hr), mean residence time extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; and MAT (hr), mean absorption time using the equation:

$$\text{MRT (SQ)} - \text{MRT (IV)}$$

where MRT(SQ) and MRT(IV) are the mean residence time via the subcutaneous and intravenous route, respectively. AUC and MRT were extrapolated to infinity to account for the total exposure to the drug. The absorption rate constant (k_a) following subcutaneous administration was determined using the equation $\frac{1}{MAT}$. As the minimum interval for ISF collection was 4 hr, no lag time adjustment was made to account for the length of the collection tube for the ultrafiltration tissue probe.

To determine the apparent bioavailability (F) in cows treated with CEF CFA, the $AUC_{0-\infty}$ was determined from plasma samples collected following IV ceftiofur sodium administration. Bioavailability was then determined using the equation, assuming no change in clearance between the two routes of administration (Toutain & Bousquet-Mélou, 2004):

$$F(\%) = 100 * \frac{\text{AUC (SQ)} * \text{D (IV)}}{\text{AUC (IV)} * D\,(SQ)}$$

where AUC(SQ) = $AUC_{0-\infty}$ determined for CEF CFA via the subcutaneous route; D(IV) = dose of ceftiofur sodium administered via the IV route; AUC(IV) = $AUC_{0-\infty}$ determined for ceftiofur sodium via the

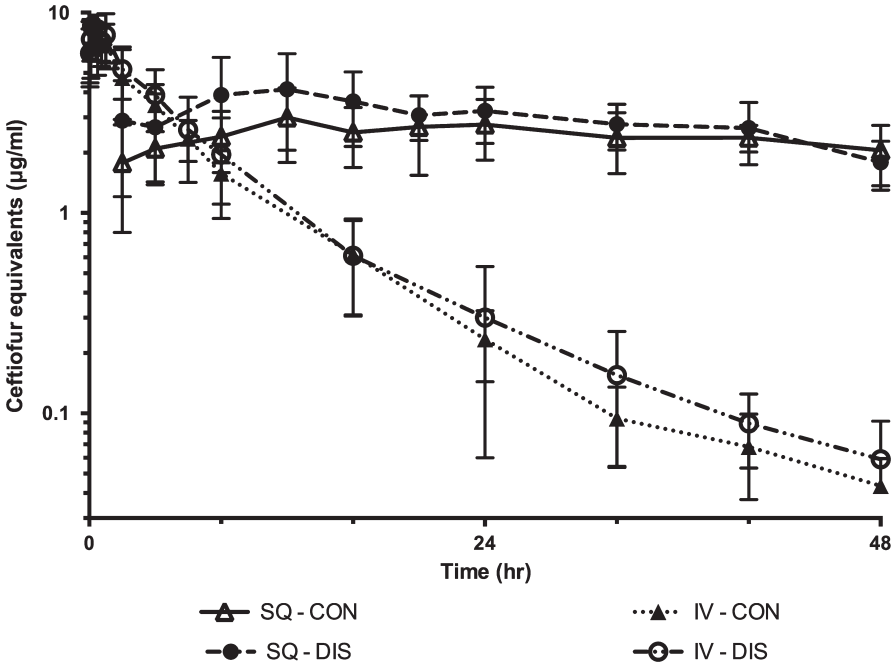

FIGURE 2 Semilogarithmic transformations of mean plasma ceftiofur equivalent concentrations (±*SD*) following a single intravenous ceftiofur sodium versus a single subcutaneous administration of ceftiofur crystalline-free acid. Cows in the CON group represented healthy control animals, whereas cows in the DIS group underwent an intramammary disease challenge prior to SQ ceftiofur crystalline-free acid administration. The concentration at which the *x*-axis intersects the *y*-axis represents the level of quantification for the analytical assay

IV route; D(SQ) = dose of CEF CFA administered via the subcutaneous route.

2.12 | Data analysis

Statistical analysis was performed using a commercially available software program (SAS 9.4, SAS Institute, Cary, NC). All data are expressed as arithmetic mean ± *SE* and geometric mean. Comparison of variables between treatment groups that were single observations (e.g., enrollment variables and PK parameters) was made using a paired *t* test unless the values were not normally distributed (ISF C_{max} and $AUC_{0-\infty}$). For these parameters, means were compared using the Wilcoxon two-sample rank-sum test. Drug concentrations in plasma and ISF, protein binding, rectal temperatures, and IRT values for the DIS and CON groups were analyzed via the GLIMMIX procedure using repeated measures, with the animal being the subject of repeated measures. Fixed effects were treatment (DIS or CON), time, and the interaction between treatment and time. Replicate was included as a random effect. Statistical significance was established when $p < 0.05$.

3 | RESULTS

At enrollment, there was no statistical difference for any of the animal enrollment variables between the treatment groups. Between the first and second segments of this trial, two CON cows were removed due to illness that was treated with systemic CEF therapy. Therefore, only the eight remaining cows were used in the calculation of F of CEF CFA in the CON group. The two animals were replaced for the completion of the second segment of the trial; therefore, all other parameters have 10 animals per treatment.

Following IMM challenge, *E. coli* concentration in the challenge inoculum was determined to be 105 cfu for replicate 1 and 184 cfu for replicate 2. Following challenge, all cows in the DIS group developed clinical mastitis within 12 hr (by T0 hr). As a result, four of ten cows developed clinical mastitis classified as moderate and the remaining were classified as severe. However, none of the cows developed clinical signs necessitating rescue therapy.

No cow had detectable CEF in plasma or ISF at the beginning of either segment (time 0). Figure 2 shows plasma CEF concentrations during the first 48 hr following IV and SQ administration. Following IV administration of CEF sodium, all cows had measurable CEF in their plasma throughout the 48-hr monitoring period. In addition, when CEF CFA was administered, it demonstrated flip-flop kinetics.

Plasma and ISF concentrations of CEF for cows in Segment 2 are displayed in Figure 3. Following CEF CFA administration, CEF was detected in all subsequent plasma samples throughout the entire study period. For plasma concentrations, there was not a significant effect of treatment ($p = 0.068$) but the treatment-by-time interaction ($p = 0.005$) was significant. There was a significantly greater concentration of CEF in the plasma of the DIS cows at T2 hr ($p = 0.002$), T8 hr ($p < 0.001$), T12 hr ($p = 0.001$), and T16 hr ($p = 0.002$). There were no other time points that were significantly different between the two groups for the remainder of the trial.

For the ISF samples, only eight cows had quantifiable CEF concentrations in their ISF 4 hr after therapy, but by 8 hr, all cows with functional ultrafiltration probes had quantifiable CEF concentrations in their ISF. One cow had a malfunctioning ultrafiltration probe for the first 16 hr after CEF CFA administration. Two cows did not have quantifiable CEF in ISF past 48 hr and seven cows had quantifiable CEF through 192 hr. There was a significant effect for time ($p < 0.001$) but treatment and the treatment-by-time interaction were not significant.

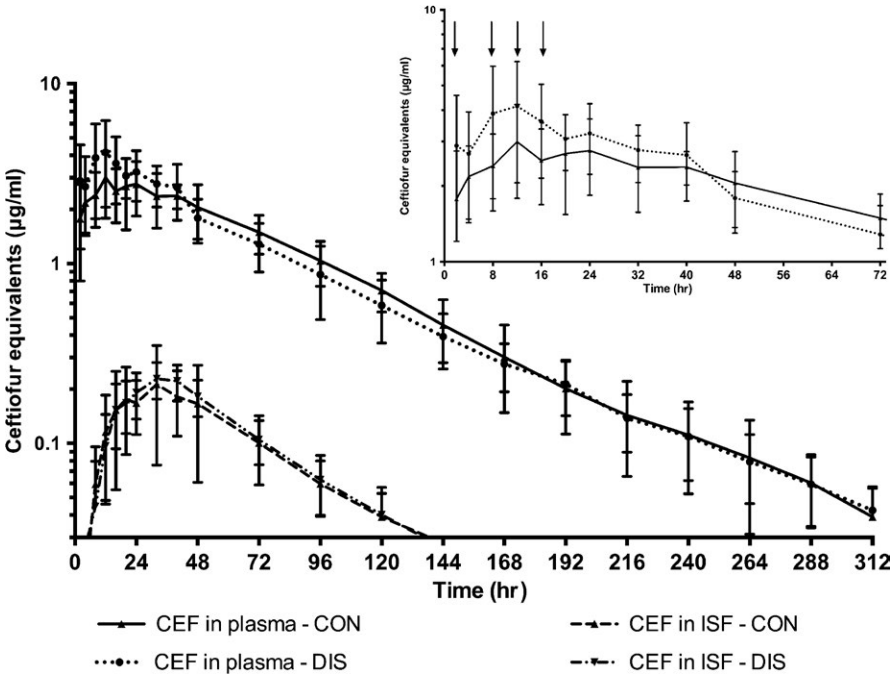

FIGURE 3 Semilogarithmic transformations of mean plasma and interstitial fluid ceftiofur equivalent concentrations (±*SD*) for ten healthy cows (CON) versus ten cows with induced coliform mastitis (DIS) following a single subcutaneous administration of ceftiofur crystalline-free acid. The concentration at which the *x*-axis intersects the *y*-axis represents the level of quantification for the analytical assay. The insert represents the same data, except only for plasma during the first 72 hr. The arrows indicate the time points where there are significant differences between the mean plasma concentrations

TABLE 1 Plasma pharmacokinetic parameters for ceftiofur for ten cows with induced coliform mastitis (DIS) compared to ten healthy cows (CON) following a single subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid at the base of the ear. Results are presented as arithmetic mean ± *SE*[a]

Parameter	Disease (DIS)	Control (CON)
λ_z (hr^{-1})	0.012 ± 0.0007 (0.012)	0.015 ± 0.0006 (0.015)[b]
$T_{1/2\,\lambda z}$ (hr)	58.52 ± 4.0 (57.35)	45.87 ± 2.08 (45.47)[b]
C_{max} (µg/ml)	4.74 ± 0.58 (4.43)	3.47 ± 0.35 (3.33)[c]
T_{max} (hr)	15.0 ± 2.62 (12.4)	15.4 ± 2.39 (12.8)
AUC_{0-24} (µg/ml × hr)	78.01 ± 9.0 (73.29)	57.82 ± 5.66 (55.44)[c]
$AUC_{0-\infty}$ (µg/ml × hr)	263.3 ± 13.3 (260.1)	253.03 ± 11.91 (251.2)
V_z/F (L/kg)	2.135 ± 0.134 (2.098)	1.745 ± 0.090 (1.723)[b]
CL/F (ml hr^{-1} kg^{-1})	25.69 ± 1.42 (25.36)	26.45 ± 1.03 (26.27)
$MRT_{0-\infty}$ (hr)	66.87 ± 4.23 (65.68)	72.14 ± 4.57 (70.87)
F (%)	167.9 ± 20.8 (158.1)	164.8 ± 15.5 (160.1)

Notes. λ_z (1/hr), slope of the terminal phase; $T_{1/2\,\lambda z}$ (hr), terminal half-life; C_{max} (µg/ml), maximum plasma concentration; T_{max} (hr), time of C_{max}; $AUC_{0-24\,hr}$ (µg/ml × hr), area under the curve from T0 to T24 hr; $AUC_{0-\infty}$ (µg/ml × hr), area under the curve extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; V_z/F (ml/kg), volume of distribution per fraction of the dose absorbed; and CL/F (ml hr^{-1} kg^{-1}), clearance per fraction of the dose absorbed; $MRT_{0-\infty}$ (hr), mean residence time extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; and *F*, apparent bioavailability.

[a]Values in parentheses are the geometric mean; [b]Means within the columns differ ($p < 0.05$); [c]Means within the columns differ ($p < 0.10$).

Mean protein binding (±1 *SE*) of CEF at T24 hr was 91.1% ± 0.93 for the CON group and 93.0% ± 0.93 for the DIS group. At T96 hr, mean protein binding was 92.5% ± 0.93 and 90.6% ± 0.93 for the CON and DIS groups, respectively, whereas at T192 hr, the bound fraction was 93.8% ± 1.3 and 94.3% ± 1.03, respectively. There were no significant differences in protein binding between any of the groups.

Plasma pharmacokinetic parameters following CEF CFA administration are shown in Table 1. Between the two groups, λ_z was significantly lower ($p = 0.007$), $T_{1/2\,\lambda z}$ was significantly longer ($p = 0.014$), and V_z/F was significantly higher ($p = 0.028$) in the DIS group. The mean (and range) value for $T_{1/2\,\lambda z}$ was 58.52 (44.82–80.37) hr for the DIS group and 45.87 (39.05–58.01) hr for the CON group. For V_z/F, the mean (and range) of value for the CON group was 1.745 (1.230–2.146) L/kg whereas for the DIS group was 2.135 (1.574–2.910) L/kg.

Comparisons of λ_z following IV ($\lambda_{z\,(IV)}$) and SQ ($\lambda_{z\,(SQ)}$) administration and calculated k_a are presented in Table 2. The k_a /$\lambda_{z\,(IV)}$ ratio was 0.16 for the CON group and 0.25 for the DIS group, indicating the CEF CL following subcutaneous CEF CFA administration was much lower than following IV administration. In addition, the mean

TABLE 2 Comparison of the elimination rate constant (λ_z) of CEF determined via the intravenous (IV) and subcutaneous (SQ) route, and the absorption rate constant (k_a) of CE determined following extravascular administration of CEF CFA

Cow	IV elimination rate constant ($\lambda_{z\ (IV)}$)	SQ elimination rate constant ($\lambda_{z\ (SQ)}$)	Absorption rate constant (k_a)	Ratio $k_a/\lambda_{z\ (IV)}$
Control group (CON)				
9156	0.085	0.018	0.019	0.22
9244	0.055	0.015	0.015	0.27
9325	0.113	0.017	0.015	0.14
9353	0.114	0.017	0.016	0.14
9480	0.110	0.012	0.014	0.12
9657	0.110	0.014	0.013	0.12
9725	0.109	0.014	0.011	0.10
9760	0.130	0.017	0.017	0.13
Mean (CON)	0.100	0.016	0.015	0.16[a]
Diseased group (DIS)				
8972	0.081	0.010	0.018	0.22
9146	0.089	0.013	0.017	0.19
9206	0.045	0.009	0.018	0.39
9233	0.067	0.015	0.020	0.30
9389	0.082	0.014	0.020	0.24
9456	0.056	0.015	0.013	0.23
9709	0.103	0.009	0.027	0.26
9728	0.062	0.015	0.016	0.27
9776	0.077	0.011	0.018	0.24
9779	0.065	0.012	0.012	0.18
Mean (DIS)	0.070	0.012	0.018	0.25[a]

Note. [a]Means between the treatment groups differ ($p < 0.05$).

difference between the $k_a/\lambda_{z\ (IV)}$ ratios was significant ($p = 0.003$), indicating CL in the DIS group was impacted by disease.

The mean apparent bioavailability in the DIS group was 167.9%, whereas the mean in the CON group was 164.8%. These were not statistically different. There were no other statistically significant differences between any of the PK parameters.

Interstitial fluid PK parameters are displayed in Table 3. There were insufficient samples from three cows (2 CON and 1 DIS) to determine all the PK variables, so their data were excluded. There were no statistically significant differences between any of the ISF PK parameters between the two groups.

All kidney KIS tests were negative. Kidney DCCD concentrations in are presented in Table 4. Only two cows had DCCD concentrations in kidney tissue above the LOQ for the assay. When converted to DFC, both cows had concentrations below the US tolerance for CEF in bovine kidney tissues. As none of the kidney tissues had violative kidney residues, the remaining tissues were not analyzed.

There was no effect of treatment or time-by-treatment interaction on rectal temperature; however, time had a significant effect ($p < 0.001$). Specifically, T0 hr ($p < 0.001$) and T8 hr ($p = 0.031$) were significantly elevated, whereas T288 ($p = 0.004$) and T312 hr

TABLE 3 Comparative interstitial fluid pharmacokinetic parameters (arithmetic ± *SE*[a]) for ceftiofur in DIS and CON cows ($n = 10$) following a single injection of 6.6 mg/kg of ceftiofur crystalline-free acid

Parameter	Disease (DIS)	Control (CON)
λ_z (hr^{-1})	0.018 ± 0.0012 (0.018)	0.020 ± 0.0012 (0.020)
$T_{1/2\ \lambda z}$ (hr)	39.75 ± 3.03 (39.02)	35.06 ± 2.16 (34.54)
C_{max} (μg/ml)	0.24 ± 0.047 (0.21)	0.24 ± 0.015 (0.24)
T_{max} (hr)	33.2 ± 2.53 (32.3)	35.2 ± 1.77 (34.7)
AUC_{0-24} (μg/ml × hr)	2.75 ± 0.49 (2.48)	2.30 ± 0.27 (2.15)
$AUC_{0-\infty}$ (μg/ml × hr)	15.72 ± 1.56 (15.26)	16.17 ± 1.33 (15.74)
$MRT_{0-\infty}$ (hr)	69.84 ± 3.51 (69.24)	65.54 ± 2.51 (65.16)

Notes. λ_z (1/hr), slope of the terminal phase; $T_{1/2\ \lambda z}$ (hr), terminal half-life; C_{max} (μg/ml), maximum ISF concentration; T_{max} (hr), time of C_{max}; $AUC_{0-24\ hr}$ (μg/ml × hr), area under the curve from T0 to T24 hr; $AUC_{0-\infty}$ (μg/ml × hr), area under the curve extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$; CL/F (ml hr^{-1} kg^{-1}), clearance per fraction of the dose absorbed; and $MRT_{0-\infty}$ (hr), mean residence time extrapolated to infinity using the equation $\frac{C_{last}}{\lambda_z}$.

[a]Values in parentheses are the geometric mean.

TABLE 4 Kidney concentration of desfuroylceftiofur cysteine disulfide (DCCD) and calculated desfuroylceftiofur (DFC) in DIS cows (n = 10) following a single SQ injection of 6.6 mg/kg of ceftiofur crystalline-free acid. The limit of quantification for the assay was 0.05 μg/g

Cow	Measured DCCD concentration (μg/g)	Calculated DFC concentration[a] (μg/g)
8972	<LOQ	–
9146	<LOQ	–
9206	0.094	0.38
9233	<LOQ	–
9389	0.057	0.32
9456	<LOQ	–
9709	<LOQ	–
9728	<LOQ	–
9776	<LOQ	–
9779	<LOQ	–

Notes. <LOQ, below the limit of quantification of the assay.
[a]Desfuroylceftiofur (DFC) concentrations were calculated from DCCD values.

(p < 0.001) were lower than the mean temperature. Thermography was evaluated at the base of the ear at the injection site, on the ipsilateral eye, and on the challenged quarter of mammary gland. For ear images, there were no significant treatment, time, or treatment-by-time interactions between the two groups, except the time variable for maximum (p = 0.016) and minimum (p = 0.012) temperature. For eye images, the time variable for maximum, minimum, and average temperatures were significant (p < 0.001). There was no effect for treatment or the treatment-by-time interaction for eye images. There were also significant time differences for maximum (p = 0.033), minimum (p < 0.001), and average (p = 0.009) mammary gland temperatures, but not treatment or treatment-by-time interaction.

4 | DISCUSSION

In this trial, no cow required rescue therapy as a consequence of their illness. Compared to previous work (Gorden et al., 2016), the cows in the current trial did not get as clinically ill and the duration of illness was shorter. In addition, all cows continued lactating as compared to the previous work where five of eight cows developed agalactia as a result of their illness.

Plasma C_{max} concentrations of CEF following administration of CEF CFA were approximately equal to those published for dairy cattle in the package insert and lower than those for beef cattle from the package insert (Zoetis Inc., 2013). C_{max} values were also lower for a beef cattle study published by others (Washburn et al., 2005). Time to maximum concentration was approximately 4 hr shorter in this work compared to the package insert, the $AUC_{0-\infty}$ was slightly lower in this work, and the $T_{1/2}$ in the CON group was approximately equal to the package insert (Zoetis Inc., 2013).

Initially, the cows in the DIS group had a numerically higher CEF plasma concentration, which persisted through 40 hr posttreatment. This phenomenon was also present in our previous work for approximately 10 hr after the first dose of CEF hydrochloride (Gorden et al., 2016). Whereas C_{max} in this trial was not determined to be significantly higher in the DIS group, there was a tendency for a higher C_{max} (p = 0.081). It is plausible that the febrile response associated with the clinical mastitis in the DIS group could have resulted in more blood flow to the injection site as previously described by Groothuis, van Miert, Ziv, and Nouws (1978), Groothuis, Werdler, van Miert, and van Duin (1980), resulting in numerically higher plasma drug concentrations early in the course of disease.

In addition, the cows in this trial had saw toothlike CEF plasma concentrations that continued until approximately 32 hr after treatment. This was also noted in the previous work on individual cows, but was not apparent on the mean concentration graph (Gorden et al., 2016). Desfuroylceftiofur is reported to have a lower initial volume of distribution than CEF (Whittem, Freeman, Hanlon, & Parton, 1995), which will likely account for the up and down pattern of plasma concentrations as CEF is absorbed from the injection site. In addition, altered hepatic metabolism of parent CEF to the DFC metabolite in the DIS group could have contributed in differences in plasma concentration over time between groups. In Segment 1 of this trial, plasma CEF concentrations following IV administration increased from T0.05 to T0.5 hr (data not shown). This phenomenon has previously been described and is apparently the result the lower initial volume of distribution of DFC compared to its parent compound (Whittem et al., 1995).

Data from the IV CEF study were used to calculate the absolute bioavailability of CEF administered as CEF CFA. In both groups, F was determined to be approximately 160%, which is a reason for concern as bioavailability values >100% are theoretically implausible in cases of linear clearance (Toutain & Bousquet-Mélou, 2004). There are three potential issues at hand that could contribute to this phenomenon. First, this trial was conducted as a 1-sequence, 2-treatment, 2-period crossover design, as compared to the recommended 2-sequence crossover approach when completing bioavailability trials. This potentially introduces the risk of having a period effect and/or a carryover effect from the first period to the second on the exposure estimates. Another explanation for the elevated bioavailability value is due to changes in CL associated with extravascular administration. And finally, CEF was injected on the same side of the head as was the jugular vein used for blood collection, so we cannot exclude direct absorption of drug from the injection site into the jugular.

Visual inspection of CEF time courses following IV and SQ dosing (Figure 2) together with the estimated differences in the slope of the terminal phase (Table 2) support the hypothesis of flip-flop kinetics for CEF after extravascular administration. Under these circumstances, values for $\lambda_{z\,(IV)}$ and k_a (termed $k_{a,fl}$ in the remainder of the manuscript) should be approximately equal if CL is unaffected. However, in this trial the value for $k_{a,fl}$ was only 16% and 25% of the value for $\lambda_{z\,(IV)}$ in the CON and DIS groups, respectively. As $k_{a,fl}$

is substantially lower than $\lambda_{z\ (IV)}$, CL following subcutaneous administration of CEF CFA is lower compared with IV dosing. This results in increased exposure of drug following SQ administration and an elevated AUC(SQ), which resulted in a bioavailability value that was much greater than 100%. Interestingly enough, in an equine trial using a parallel design versus IV CEF sodium, the absolute bioavailability of CEF CFA has been reported to be 100%, with a 90% confidence interval ranging from 92.4% to 109% (Collard et al., 2011), supporting our findings in dairy cattle.

Maximum CEF concentrations in ISF were lower and time to reach maximum concentration was longer in the current trial compared to previous work (Gorden et al., 2017). In addition, CEF was detected in ISF for approximately 120 hr in the current study compared to 60 hr in the previous work. Given that CEF CFA exhibits flip-flop kinetics, this is not surprising. Foster, Jacob, Warren, and Papich (2015) have also reported ISF PK parameters using similar tissue probes utilized in the current study. In their work, they reported higher ISF CEF C_{max}, a more rapid T_{max}, but nearly identical AUC compared to the current work in a study utilizing healthy 6-month-old Holstein steers administered CEF sodium at 2.2 mg/kg. These C_{max} and T_{max} values were similar to our previous work, where ISF CEF concentration following CEF hydrochloride administration was determined (Gorden et al., 2017). Again, differences in kinetics of absorption likely account for these differences. Washburn et al. (2005) also reported lower C_{max} and a longer T_{max} using fluid collected from uninfected tissue cages following CEF CFA administration in trials using feedlot animals, compared to earlier work by the same research group when CEF sodium was administered via the IV route (Clarke et al., 1996). However, the C_{max} and AUC values reported by Washburn et al. (2005) are significantly higher than those reported in our current work or that reported by Foster et al. (2015). Washburn et al. (2005) also observed even higher CEF C_{max} and AUC values in tissue cages infected with *Mannheimia haemolytica* compared to uninfected cages. Tissue cage data should be interpreted carefully, as these create an artificial fluid filled space that allows protein to escape the vasculature and enter the tissue cage (Davis, Salmon, & Papich, 2005). As CEF is reported to be 50%–90% protein bound (Brown, Jaglan, & Banting, 1991), a major portion of the drug represented in the Washburn et al. (2005) would be protein bound and not biologically active. Clarke et al. (1996) state that bound fractions of CEF will dissociate quickly in chemically reduced environments found in areas of inflammation. However, it would seem prudent to utilize tissue probe data to interpret biological function of CEF in ISF.

The fact that none of the Kidney Inhibition Swab (KIS™) tests were positive on the DIS animals is not surprising given the fact that all the kidney tissues were below the tolerance. In addition, the reported sensitivity of this assay is 4 ppm, which is 10-fold higher than the tolerance for CEF (Jones et al., 2014), making it a questionable choice for screening cull dairy cattle for CEF residues. This is thought-provoking as cull dairy cows have the highest incidence of violative residues among adult cattle classes (USDA, 2017) and CEF is the most commonly used antimicrobial in the US dairy industry (Sawant et al., 2005; Schuler et al., 2017; Zwald et al., 2004). Taken together, it is highly likely that animals with violative residues for CEF are not being submitted for confirmatory testing due to the limited sensitivity of this screening test for CEF.

During the drug approval process, sponsoring companies must present the FDA CVM with toxicological and residue depletion studies. Based on these data, the FDA CVM establishes withdrawal periods for meat and milk, if approved for lactating dairy cattle. However, these studies are performed on healthy animals, not animals suffering from infectious diseases. In this study, we were able to determine CEF residue levels in kidney tissues following CEF CFA treatment in animals that experienced induced coliform clinical mastitis. Of the ten animals that were challenged, only two had kidney residues for DCCD above the LOQ for the assay. To convert measured DCCD concentrations to DFC, a regression equation was utilized (Feng et al., 2014). As a result, one cow was determined to have a DFC residue level of 0.32 μg/g (cow #9389) and another of 0.38 μg/g (cow #9206). These are both below the established tolerance for DFC in kidney tissue of 0.4 μg/g (US FDA, 2006a). When looking at the individual PK parameters for cow #9206, her plasma CEF $T_{1/2\ \lambda z}$ (80.3 hr) was the highest of all the DIS cows. This is nearly two times as long as the CON average. In addition, this cow was the one who suffered the most severe clinical disease based on clinical appearance, rectal temperature, and daily feed refusal. In previous work (Gorden et al., 2016), a much wider range for V_z, *CL*, and subsequently $T_{1/2\ \lambda z}$ was observed in severely ill animals. If tissue residue depletion follows plasma PK, this doubling of the $T_{1/2\ \lambda z}$ would indicate that it would take twice as long for the tissues to deplete to the tolerance as would be the case in healthy animals provided that a monoexponential slope was observed (Riviere et al., 1998). To account for variation in tissue depletion among animals, the FDA utilizes a process based on the statistical tolerance limit procedure (De Gryze, Langhans, & Vandebroek, 2007). In applying this procedure to the determination of withdrawal periods for a drug, FDA selects the 99th percentile tolerance limit with a 95% confidence. This should mean that 99% or more of tissue samples are at or below the tissue tolerance in the target tissue when the withdrawal period has elapsed (US FDA, 2006b).

The primary weakness of this trial was not creating the level of illness in DIS group animals as was seen in the previous trial (Gorden et al., 2016). In that trial, five of eight trial cows developed severe disease and agalactia, despite aggressive supportive therapy. In this work, no cows required rescue therapy. In addition, the attempts to objectively characterize febrile responses using rectal temperature and IRT measurements to separate out treatment effects between the DIS and CON animals proved to be unsuccessful. In both replicates of Segment 2, high ambient temperatures during the first 48 hr of each challenge period likely confounded our ability to assess differences in rectal and IRT temperatures. Future research should focus on identifying clinical parameters that are associated with altered PK parameters, which would allow producers to implement longer withdrawal periods in order to minimize the risk of marketing an animal with a violative residue.

It should be noted that under the FDA's cephalosporin prohibition (US FDA, 2012), the use of CEF sodium via the IV route and CEF CFA for the treatment of healthy animals is technically illegal. However, prior to initiation of the trial, conversations were undertaken with a representative from the FDA CVM who recommended a prolonged slaughter withdrawal period should be implemented. Therefore, a 30-day slaughter withdrawal was implemented.

In conclusion, the results of this trial support previous work that cows suffering clinical disease associated with mastitis may have altered CEF volume of distribution and terminal half-life. It did not however support the hypothesis that severely ill cows need longer withdrawal times following CEF therapy. However, substantially larger V_z/F and longer $T_{1/2\,\lambda z}$ on some cows suggest that this may be possible in a clinical disease in a large population. Future population modeling work needs to identify parameters in cattle that should be monitored in order to implement longer withdrawal periods on cows potentially at risk for maintaining violative residues past their withdrawal period (Fink et al., 2013; Mochel et al., 2013).

ACKNOWLEDGMENTS

The authors would like to thank Rochelle Warner, Mike Burchard, Cassie Rice, Kristen Raymond, Caitlyn Dierks, Nick Van Engen, Holly Brown, Logan Domeyer, Isaac Jepsen, Kris Haymen, Aislinn Pomfert, Micaela Mertens, and Curtis Korthanke for their assistance with animal care, sample collection, and sample processing. This project was funded by Iowa State University's College of Veterinary Medicine.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

PJG, JFC, JAY, MDK, and RWG designed the study; PJG, JAY, MDK, TAB, and JSS carried out animal work; PJG, RWG, LWW, and SMR carried out laboratory analysis; PJG, JPM, MZ, and PKS carried out data analysis; PJG, JPM, MDK, and JFC wrote the manuscript; and all authors have read and approved the final version of the manuscript.

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How to cite this article: Gorden PJ, Ydstie JA, Kleinhenz MD, et al. Comparative plasma and interstitial fluid pharmacokinetics and tissue residues of ceftiofur crystalline-free acid in cattle with induced coliform mastitis. *J vet Pharmacol Therap*. 2018;00:1–13. https://doi.org/10.1111/jvp.12688