The objective was to determine the effect of milking frequency and dosing interval on pharmacokinetics of cephapirin after intramammary infusion. Six healthy Holstein cows were administered cephapirin (200 mg) into 1 rear mammary gland after each of 2 milkings. Cows were milked twice daily (2×) and dosed at a 12-h interval or 3 times daily (3×) and dosed at an 8- or 16-h interval. A duplicated Latin square design allowed each cow to receive all 3 frequency-dose treatments, with intervening washout periods. Concentrations of cephapirin (CEPH) and desacetylcephapirin (DAC) in milk from the treated glands were determined at each milking after infusion using liquid chromatography-mass spectrometry. Data were fitted using 1- and 2-compartment pharmacokinetic models, as well as a noncompartmental model. Cephapirin was rapidly metabolized to DAC in the mammary gland, with DAC being the predominant agent in milk until 48 h after infusion. Pharmacokinetics of CEPH and DAC were similar for all treatment groups, with a 1-compartment model providing a better fit than a 2-compartment model in most instances. Milking frequency did not affect the length of time that milk CEPH concentration exceeded MIC50 or MIC90 values (the minimum inhibitory antimicrobial concentration needed to inhibit 50 or 90% of microbial activity, respectively) for common mastitis pathogens, except that cows milked 3× and dosed at a 16-h interval maintained inhibitory concentrations approximately 8 h longer than those dosed at an 8-h interval. Time for milk CEPH concentration to reach the FDA tolerance did not differ among treatment groups [mean ± SD; 68 ± 20, 66 ± 22, and 57 ± 18 h after last treatment for cows treated at 12, 16, and 8 h, respectively]. Mean residence time for CEPH in the mammary gland was linearly and negatively associated with the volume of milk produced. Calculated CEPH concentration in composite milk from all 4 mammary glands was below the FDA tolerance in all cows by 96 h after the last infusion, which is the labeled withholding time for the preparation used. Our findings suggest that this CEPH preparation, which is labeled for 2 doses 12 h apart, could be administered at a 16-h interval in herds milking 3×, with no significant effect on inhibitory concentrations in milk or withdrawal time; extended withdrawal times would be prudent for cows with very low milk production. Further investigation is needed to determine if milking frequency affects CEPH pharmacokinetics in cows with clinical mastitis. Key words: milking frequency, cephapirin, pharmacokinetics

INTRODUCTION

Approximately 17% of dairy cows in the United States experience clinical mastitis each year (USDA, 2007). Clinical mastitis episodes are costly and can adversely affect animal well-being. Intramammary (IMM) infusion of antimicrobial agents is a common method of treating clinical mastitis episodes, particularly those caused by streptococci or staphylococci (McDougall et al., 2007). Most IMM antimicrobial preparations for lactating dairy cows in the United States are labeled for 2 or 3 treatments, 12 or 24 h apart. Intramammary antimicrobial preparations are generally intended for use in cows milked twice daily (2×), with milk discard times established accordingly. Although 2× milking is practiced on many farms, 3-times-daily (3×) milking has become popular, especially on large dairies. In a 2002 survey (USDA, 2002), 40% of large herds (≥500 cows) were milked 3×, compared with 20% of medium-sized herds (100 to 499 cows) and 1.7% of small herds (<100 cows). The economic benefits associated with increased production efficiency in herds milked 3× (Smith et al., 2002) may be easier to capture in large herds where personnel can be devoted solely to milking cows. The proportion of herds milking 3× is likely to grow as average herd size continues to increase.
Milking frequency is expected to affect the concentration-time profile of antimicrobial agents in milk after IMM infusion. The additional milking associated with 3× milking should hasten clearance of most antimicrobial agents compared with 2× milking (Knappstein et al., 2003). Also, the 10 to 15% increase in daily milk production associated with 3× milking (Smith et al., 2002) should dilute antimicrobial concentrations in milk and may hasten clearance (Mercer et al., 1970; Whitten, 1999). The effect of 3× milking on antimicrobial concentrations in milk should be investigated, because time above the MIC in milk is a critical determinant of efficacy for most IMM antimicrobial agents (Erskine et al., 2003). Furthermore, antimicrobial preparations labeled for IMM administration every 12 h are used in an extra-label fashion in herds milking 3×; more feasible options are administration of the preparation at 8-h intervals (after 2 consecutive milkings) or 16-h intervals (skipping one milking). Veterinarians need pharmacokinetic data to help them make evidence-based treatment decisions and milk discard recommendations for herds milking 3×.

The objectives of this study were to determine the effect of milking frequency (2× or 3×) and interval between doses (8, 12, or 16 h) on disposition of a commonly-used IMM antimicrobial agent labeled for twice-daily administration in lactating dairy cows in the United States (cephapirin sodium; Cefa Lak, Fort Dodge Animal Health, Fort Dodge, IA). Pharmacokinetic profiles were compared with published MIC values for common mastitis pathogens and with the established tolerance concentration of 0.020 μg/mL for cephalirin in saleable milk in the United States established by the FDA. Pharmacokinetic profiles were compared with the maximum residue limit (MRL) of 0.060 μg/mL for the sum of cephalirin and desacetylcephapirin (DAC) concentrations in saleable milk in Europe.

MATERIALS AND METHODS

The study was conducted between April and May 2006, at the Dairy Research Unit on the University of Illinois at Urbana-Champaign campus. The campus Institutional Animal Care and Use Committee approved all procedures.

Animals and Eligibility Criteria

Six healthy, multiparous Holstein-Friesian cows were enrolled, 3 with low milk production (<25 kg/d) and 3 with high milk production (≥36 kg/d). Cows were housed in tie stalls on sawdust-covered rubber mats and fed a corn silage-based TMR. Fresh water was available ad libitum. Cows were at least 30 d into lactation to avoid the risk of periparturient disease.

Eligible cows had no history of clinical mastitis during the previous 60 d and a composite SCC <250,000 cells/mL within the previous 30 d. Once these criteria were met, cows were screened for IMI by culturing and measuring SCC in milk from each mammary gland. Milk for culture was collected aseptically and 100 μL was plated, in duplicate, onto 5% sheep blood agar plates. Plates were checked for growth after 24 and 48 h of incubation at 37°C. Infection was diagnosed when both plates corresponding to a given gland contained the same colony type, with at least 1 plate having ≥50 cfu/mL of milk. Colony morphology, hemolysis pattern, Gram staining reaction, catalase reaction, coagulase production, and lactose fermentation on MacConkey agar were used to group isolates as Streptococcus spp., Staphylococcus aureus, CNS, Corynebacterium spp., or coliform bacteria. Somatic cell count was determined electronically (Fossomatic method, Foss Electric, Hilleroed, Denmark). Only cows having bacteriologically-negative milk and SCC <150,000 cells/mL of milk in 1 rear gland (hereafter referred to as the study gland) were used; to reduce the risk of clinical mastitis developing in other glands, cows with major mastitis pathogens (Staph. aureus, Streptococcus spp., coliform bacteria) isolated from any gland were excluded.

Milking Procedures

Cows were moved to a chute in a quiet room for milking. Milking was performed at 0600 and 1800 h for cows milked 2× and at 0600, 1400, and 2200 h for cows milked 3×. A single investigator (RS) was responsible for milking and paid strict attention to milking times and milking order. Pre-milking procedures included spraying the teats with 0.4% chlorhexidine gluconate (Fight Bac, Deep Valley Farm, Brooklyn, CT), allowing 30 s of contact time, and wiping each teat dry with an individual paper towel. Teats were massaged for 30 s while wiping. The milking cluster was attached without removing foremilk.

Just before attaching the cluster; otherwise, the cluster was attached without foremilk. The milk cluster was attached after 30 s of contact time, and wiping each teat dry with an individual paper towel. Teats were massaged for 30 s while wiping. The milking cluster was attached without removing foremilk. The milk cluster was attached without removing foremilk.
ing cluster and quarter milking device were left in place until the mammary glands and teats felt empty and no milk was seen entering the bowl or device for 30 s. Milk production (mL) by the study gland was measured by pouring milk from the quarter milking device into a 4,000-mL graduated cylinder. At the milking following each treatment, 5 mL of milk was hand-stripped from the study gland within 2 min after removing the milking cluster; otherwise, glands were not stripped after milking. The foremilk and stripping samples were used to investigate the effect of milk fraction on antimicrobial concentrations measured in milk; results of that study will be reported elsewhere.

Milk production by the remaining 3 mammary glands (nonstudy glands) was calculated by weighing the milk in the bucket of the milking machine, subtracting the weight of the bucket, and converting weight to mL using the formula: 1 kg = 1,000 mL for milk with 3 to 5% butterfat. Teats were sprayed with chlorhexidine gluconate (Fight Bac) again before returning the cows to their stalls.

Milk and mammary glands were monitored for signs of clinical mastitis throughout the study. At each morning milking, a sample of milk from the quarter milking device was tested for subclinical mastitis using the California Mastitis Test. Milk was cultured if clinical or subclinical mastitis was suspected.

**Study Design**

A duplicated Latin square design enabled each cow to receive 3 experimental treatments and serve as its own control. The treatments were: milking at 12-h intervals, with 200 mg of cephapirin administered into the study gland after 2 (0 and 12 h) consecutive milkings (2x-12 treatment); milking at 8-h intervals, with 200 mg of cephapirin administered into the study gland after the first (0 h) and second (8 h) milkings (3x-8 treatment); and milking at 8-h intervals, with 200 mg of cephapirin administered into the study gland after the first (0 h) and third (16 h) milkings (3x-16 treatment). The order of treatments was determined randomly. The same study gland (right rear or left rear) was used for each cow throughout the study, with the exception of 1 cow that developed mastitis after the second treatment; in that case the opposite rear gland, which met eligibility criteria, was used for the third treatment. Cows were acclimated to the milking regimen (2x or 3x milking) for 48 h before beginning each treatment phase (Figure 1). A 6-d break period was provided between treatment phases. During the first 4 d of the break period, cows were maintained on the previous milking regimen to determine the depletion of cephapirin and DAC from milk. The last 2 d of the break period served as the acclimation period for the next milking regimen. The entire study was conducted over 3 wk.

Cephapirin (200 mg of active cephapirin) as cepha-pirin sodium in a stable peanut oil gel (Cefa-Lak, Fort Dodge Animal Health) was infused into the study gland at the designated times (Figure 1). Infusion occurred within 2 min of removing the quarter milking device after thoroughly scrubbing the teat end with 70% alcohol. The partial insertion method of infusion was used and the infused product gently massaged from the distal teat cistern to the gland cistern after infusion to facilitate dispersion.

**Sample Collection and Analysis**

A well-mixed 20-mL sample of milk was collected from the quarter milking device at each milking time on the treatment day and for 4 d thereafter (Figure 1). These samples, as well as the 5-mL foremilk and stripping samples mentioned above, were frozen at –70°C until analysis.

The frozen milk samples were shipped on dry ice by overnight mail to Rocky Mountain Instrumental Laboratories (Fort Collins, CO). Tandem liquid chro-
matography mass spectrometry was used to quantify cephapirin (CEPH) and its active metabolite DAC, using methods described elsewhere (Stockler et al., 2009). Briefly, liquid chromatography mass spectrometry was performed with a SciEx (Applied Biosystems, Foster City, CA) 4000Q triple quadrupole mass spectrometer, Waters X Terra C18 HPLC column (2.1 mm × 10 cm), methanol-buffer (ammonium formate-formic acid) gradient, and amoxicillin internal standard. Two sets of standards and controls were included in each analysis, and calibration curves were generated for each standard set. Cephapirin standard was obtained from the United States Pharmacopeia (Rockville, MD), and the DAC standard was a gift from Fort Dodge Laboratories (Fort Dodge, IA) and from Alan Lightfield, USDA, Eastern Regional Research Center (Wyndmoor, PA). Standard concentrations were from 0.002 to 20 μg/mL for CEPH and 0.01 to 13 μg/mL for DAC. Control samples were prepared at concentrations of 0.1, 0.4, and 15 μg of CEPH/mL and 0.065, 0.26, and 9.75 μg of DAC/mL. Calculations were performed using Analyst software (version 1.4.2, Applied Biosystems Inc., Foster City, CA). Correlation coefficients were recorded for each analysis and always exceeded 0.99. Within-day precision was 5.2% and between-day precision was 8.5%. Accuracy was ±4 to 6% using milk samples with added standards. The limit of detection for both CEPH and DAC was 0.0005 μg/mL and the limit of quantification for each was 0.002 μg/mL using a signal to noise ratio of 6 for each compound.

Pharmacokinetic Modeling

The concentrations of CEPH ([CEPH]) and DAC ([DAC]) in milk samples were summed to estimate total active CEPH equivalent (TACEPH) antimicrobial concentrations ([TACEPH]) in those samples by multiplying the [DAC] by the molecular weight ratio of CEPH (423.5 g) to DAC (381.5 g). The mass of TACEPH recovered in milk from the treated mammary gland at each milking was calculated by multiplying the [TACEPH] in milk from that gland by the volume of milk removed by the quarter milking device; when foremilk and stripping samples were collected from the treated gland, the masses of TACEPH in those samples were added to the mass removed by the quarter milking device. The total amount of TACEPH recovered in milk from the study gland was determined from the cumulative mass data and expressed as a percentage of the infused dose (2 × 200 mg of cephapirin).

The ratio of CEPH to DAC in milk from the study gland was calculated at each collection time after the first infusion, after correcting for differences in molecular weight as described previously. The maximum concentrations (Cmax) of CEPH and DAC, and the maximum value for the sum of [CEPH] and [DAC] in milk samples after infusion were determined. The time required for [CEPH] in milk from the treated gland to initially drop below the FDA tolerance concentration of 0.020 μg/mL after the last IMM infusion was determined, as was the time required for the sum of [CEPH] and [DAC] to initially drop below the maximum residue limit of 0.060 μg/mL for saleable milk in Europe. The time above a range of MIC50 and MIC90 values (the MIC needed to inhibit 50 or 90% of microbial activity, respectively) for

Streptococcus agalactiae, Streptococcus uberis, Staph. aureus, and Escherichia coli was calculated by determining the duration after first infusion that [CEPH] exceeded 16, 1, 0.5, 0.25, 0.125, and 0.06 μg/mL. The concentrations of CEPH and DAC were estimated in composite milk (milk from all 4 glands) to explore the effects of milking frequency on withdrawal time at the cow level, assuming only 1 gland was infused twice with cephapirin.

Changes in concentrations of CEPH, DAC, and TA-
CEPH in milk from the study gland after the last IMM infusion of each treatment phase were analyzed using commercially available software (WinNonLin, Pharsight Corp., Cary, NC). Because reports of pharmacokinetic modeling of IMM cephapirin do not appear to be available, 1-compartment and 2-compartment models were fit to the milk concentration data. Noncompartmental analysis was performed. Time points at which the concentrations of CEPH and DAC were below the limit of quantification were not included in the pharmacokinetic modeling.

An open 1-compartment model with bolus input (WinNonLin model 1) was applied as: y = A × e−k10 × t,
where y was the concentration, t (time) was the time of milk collection in hours after the last infusion, A was a constant, and k10 the elimination rate constant. An open 2-compartment model with bolus input (WinNonLin model 7) was applied as: y = A × e−α × t + B × e−β × t, with A, α, B, and β being hybrid constants. The rate constant for elimination from the central compartment (k10) was calculated from the hybrid constants of the 2-compartment model using standard equations. Various weighting methods were investigated and the best weighting method was selected by inspecting plots of residuals versus time and of concentration-predicted versus concentration-observed. The choice of compartmental model was based on Akaike’s information criterion and the coefficient of variation for the estimated traits. Clearance of CEPH, DAC, and TACEPH from the milk, and the half time for elimination (t1/2) were calculated from the final compartmental model using standard equations.

A noncompartmental model was fitted using an i.v. bolus model (WinNonLin Model 201) that applied a
linear trapezoid method from the time of the last infusion to the last sample time and was extrapolated to infinity as the ratio of last measured milk concentration and the terminal slope of the milk concentration versus time curve. Mean residence time was calculated from the noncompartmental analysis using standard equations.

**Statistical Analysis**

Data are presented as mean ± SD or median and range, and \( P < 0.05 \) was considered significant. Mixed models ANOVA (PROC MIXED of SAS, SAS Institute Inc., Cary NC) was used to compare the main effects of milking frequency (3 levels: 2×-12, 3×-8, 3×-16), milk production (2 levels: high, low), and the interaction between milking frequency and milk production on pharmacokinetic values, withdrawal times, percentage recovery, and time above specific concentrations for CEPH, DAC, and TACEPH, with cow as a random factor.

**RESULTS**

Cows were in their second to seventh lactation and 190 to 529 DIM at the time of enrolment. Composite milk SCC determined within 30 d before the start ranged from 17,000 to 107,000 cells/mL. Milk yield for the low-producing cows ranged from 21 to 24 kg/d and for the high-producing cows from 40 to 42 kg/d at the time of enrollment. The mean measured milk production from the study gland and calculated total milk production per day were similar for all 3 groups (Table 1). Three-times-daily milking resulted in no significant increase in measured daily milk production from the study gland (\( P = 0.099; 2×, 6.7 ± 3.6 \) L; 3×, 7.3 ± 3.9 L) or in calculated total milk production (\( P = 0.19; 2×, 24.5 ± 9.7 \) L; 3×, 26.3 ± 11.6 L).

Four left rear and 2 right rear glands were enrolled, with SCC in milk from those glands ranging from 12,000 to 118,000 cells/mL. The order of treatments was 2×-12, 3×-8, 3×-16 (2 cows), 3×-8, 3×-16, 2×-12 (2 cows), and 3×-16, 2×-12, 3×-8 (2 cows). All cows remained systemically healthy. Nevertheless, 1 low-producing cow developed clinical mastitis in the study (left rear) gland. This occurred on d 3 of the break period between the second and third treatment phases. Signs included enlargement of the gland, discoloration of the milk, garget (flakes) in the milk, and a drop in milk yield from the gland of approximately 50%. *Corynebacterium* spp. were isolated from the milk. The affected gland was infused with amoxicillin trihydrate
(Amoximast, Schering-Plough Animal Health, Union, NJ) after each of 3 consecutive milkings and clinical signs resolved after 5 d. The opposite (right rear) gland remained healthy. Because milk from that gland was bacteriologically negative and had an SCC of 40,000/mL, the right rear gland was used for the final treatment phase.

The best weighting method was unweighted for 1-compartment models and the reciprocal of concentration for 2-compartment models. For CEPH, 16 of the 18 analyses were best modeled as a 1-compartment model, and 2 analyses were best modeled as a 2-compartment model (both in the 3×-8 group; Figure 2). The coefficient of variation for k10 in the 18 fitted compartment models was 4.2 ± 4.0%. For DAC, 16 of the 18 analyses were best modeled as a 1-compartment model, and 2 analyses were best modeled as a 2-compartment model (1 in the 3×-8 group and 3×-16 group; Figure 3). The coefficient of variation for k10 in the 18 fitted compartment models was 4.3 ± 2.8%. The ratio of CEPH to DAC indicated that metabolism to DAC occurred within 8 h of administration, with DAC being the predominant antimicrobial agent until 48 h, after which time CEPH was the predominant antimicrobial agent (Figure 4). For TACEPH, 8 of the 18 analyses were modeled as a 1-compartment model, 7 analyses were modeled as a 2-compartment model, and 3 analyses could not be fitted with a 1 or 2-compartment model (Figure 5). The coefficient of variation for k10 in the 11 fitted models was 4.3 ± 2.8%. Noncompartmental models were fitted for all 18 analyses.

Table 1 summarizes results of the analyses for k10, clearance, $t_{1/2}$, and the mean residence time of CEPH, DAC, and TACEPH in milk after the second infusion of CEPH when cows were milked at 8-, 12-, or 16-h intervals. There was no effect of milking frequency on any of the pharmacokinetic values for CEPH, DAC, or TACEPH. Mean residence time (MRT) was negatively and linearly related to volume of milk produced by the study gland (Figure 6; $y = 4.8 - 5.2x$; $R^2 = 0.22; P = 0.049$), with high values for MRT associated with low milk volumes.

Table 2 summarizes the continuous time that [CEPH] or the sum of [CEPH] and [DAC] in milk from the study gland exceeded specific MIC after the first IMM infusion. Neither milking frequency nor milk production level affected the time above specific MIC, except for 16 μg/mL. The time above an MIC of 16 μg/mL was significantly shorter for CEPH and the sum of CEPH and DAC for cows in the 3×-8 group than for cows in the 2×-12 group ($P = 0.0003$) or 3×-16 group ($P < 0.0001$).

The time after the last IMM infusion required for [CEPH] in milk from the study gland to initially fall below the FDA tolerance concentration for [CEPH] in saleable milk (0.020 μg/ml) and the time after the last IMM infusion for the sum of [CEPH] and [DAC] in milk from the study gland to initially fall below the European MRL for these agents in saleable milk (0.060 μg/ml) were similar for all 3 treatment groups (Table 2, Figure 2, and Figure 5).

Antimicrobial concentration in composite milk from all 4 glands was calculated from the antimicrobial concentration in milk from the study gland, the weight of the milk produced by the study gland, and the total weight of the milk produced by all 4 glands (Table 2). The calculated time after the last IMM infusion of CEPH for [CEPH] in composite milk to fall below the FDA tolerance concentration for saleable milk was less than the labeled withdrawal time of 96 h for cows in all 3 treatment groups.

The percentage of infused drug recovered in milk from the study gland (percentage recovered) was similar for all 3 treatment groups, ranging from a mean of 59% for the 2×-12 group to 73% for the 3×-16 group (Table 2). Milk production level tended ($P = 0.056$) to affect percentage recovered, with percentage recovered being 86 ± 16% for high-producing cows and 46 ± 21% for low-producing cows. The percentage recovered was related in a curvilinear manner to volume of milk produced by the study gland (Figure 7; $y = −9.9 + 0.47x - 0.00057x^2; R^2 = 0.78; P < 0.0001$), with low quarter volumes being associated with low percentage recoveries.

**DISCUSSION**

We believe that this is the first study to report the effect of milking frequency on pharmacokinetics of an antimicrobial agent administered by IMM infusion to lactating dairy cows, and the first to determine the effect of dosing interval on the time course of antimicrobial concentrations in milk for cows milked 3×. We are aware of only a few published studies on IMM antimicrobial treatment in which milk concentrations of antimicrobial agents were pharmacokinetically modeled. Ziv and Sulman (1974) fitted a 2-compartment model to milk concentrations of polymixin B, colistin, neomycin, spiramycin, and several tetracyclines after administering these agents as aqueous solutions or suspensions in distilled water. They fitted a 1-compartment model to 26 other antimicrobial agents administered as aqueous formulations in saleable milk (0.020 μg/ml) and the time after the last IMM infusion for the sum of [CEPH] and [DAC] in milk from the study gland to initially fall below the European MRL for these agents in saleable milk (0.060 μg/ml) were similar for all 3 treatment groups (Table 2, Figure 2, and Figure 5).
and ceftiofur, respectively, whereas Bajwa et al. (2007) used noncompartmental modeling to study the pharmacokinetics of erythromycin. None of the previous studies investigated the effect of milking frequency or dosing interval on pharmacokinetics. Because reports of pharmacokinetic modeling for IMM CEPH did not appear to be available, 1-compartment and 2-compartment models were attempted with our data, as well as a noncompartmental analysis. We acknowledge that the compartmental modeling assumptions of instantaneous and homogeneous mixing and constant distribution volume were violated following IMM administration and that the identification of 1- or 2-compartments had minimal physiologic relevance. Nonetheless, we believe that compartmental modeling can be helpful in predicting appropriate withdrawal times following IMM infusion.

A 1-compartment model provided a better fit than a 2-compartment model in most instances. Sixteen of the 18 CEPH analyses, and 16 of the 18 DAC analyses were fitted with a 1-compartment model, compared with 2 CEPH analyses and 2 DAC analyses with a 2-compartment model; analyses that did not fit a 1-compartment model were not consistently associated with a particular treatment group, supporting Ziv and Sulman (1974) who found that a 1-compartment model provided a better fit for cephalosporin antimicrobial agents (cephaloridine sodium, cephaloglycin monohydrate, cephalexin monohydrate) than a 2-compartment model when those agents were infused IMM as aqueous solutions.

It is not clear to what extent the number of compartments for pharmacokinetic modeling is influenced by physicochemical properties of the antimicrobial agent (e.g., lipid solubility, pKa, extent of binding to milk

Figure 2. Cephapirin concentrations in milk from the treated gland of 6 lactating Holstein-Friesian cows administered 2 infusions of cephapirin (200 mg) as cephapirin sodium at an 8-h (group B), 12-h (group A), or 16-h (group C) interval into 1 rear mammary gland. Time = 0 h is the time of the last dose. Group A cows (2×-12 group) were milked every 12 h, whereas cows in groups B and C (3×-8 and 3×-16 groups, respectively) were milked every 8 h. Data are expressed as mean ± standard deviation. The horizontal dashed line is the tolerance concentration (0.020 μg/mL) for cephapirin in saleable milk set by the Food and Drug Administration in the United States. The horizontal solid line indicates the intersection at 0 h. Six cows contributed data to each time point except for the following: group A, 48 to 72 h (n = 5), 84 to 96 h (n = 4); group B, 56 h (n = 5), 104 h (n = 4); group C, 96 h (n = 5).
proteins or udder tissue) or the vehicle. A 1-compartment model assumed instantaneous distribution of the antimicrobial agent within a single compartment and a constant rate of elimination. A 2-compartment model may reflect extensive binding of the antimicrobial agent to udder tissue (Ziv and Sulman, 1974), with slow release and elimination of bound drug following the initial rapid elimination of unbound agent. The infrequent sampling intervals (8 or 12 h) used here might have influenced model fit; ideally sampling should be conducted at least as frequently as the elimination t_{1/2} of the drug in milk (Riviere, 1999) which was approximately 2.7 h for CEPH and 3.0 h for DAC reported here. Still, more frequent sampling would have required repeated removal of foremilk, which has a different CEPH concentration than bucket milk (Stockler et al., 2009). Concentrations of CEPH and DAC in foremilk after IMM cephapirin infusion were significantly (more than 2-fold) higher than concentrations in bucket milk or strippings, reflecting uneven distribution within the mammary gland. Therefore, collecting foremilk at frequent intervals after CEPH infusion might have removed a substantial proportion of the infused dose; this would have reduced external validity of our results, because milk is typically removed only at milking time (8- or 12-h intervals) on US dairy farms.

Noncompartmental models were fitted to all analyses, thereby allowing investigation of differences in MRT among treatment groups. Noncompartmental modeling eliminated some of the assumptions associated with compartmental modeling but assumed instantaneous mixing and a constant volume for the sampling space (Veng-Pedersen, 2001). The latter assumptions were not satisfied in the lactating dairy cow because drug distribution is heterogeneous (Stockler et al., 2009) and the sampling space varied because of ongoing milk production and milk removal at each milking. Model assumptions are not completely satisfied when applying...
compartmental or noncompartmental pharmacokinetic analysis to concentration-time data obtained after the administration of drugs by IMM infusion to lactating dairy cows. New approaches to pharmacokinetic modeling are required to more accurately characterize the concentration-time relationship after IMM treatment.

The efficacy of β-lactam antimicrobial agents, such as CEPH, depends upon the length of time the infectious agent is exposed to concentrations above the MIC (Erskine et al., 2003). Failure to maintain concentrations above the MIC for a sufficient time may result in treatment failure. Here, the effect of milking frequency and dosing interval on time that milk [CEPH] met or exceeded typical MIC\textsubscript{50} or MIC\textsubscript{90} thresholds for common mastitis pathogens is discussed. MIC\textsubscript{50} values of 0.06 μg/mL were reported for Strep. agalactiae (Guérin-Faublée et al., 2003) and Staph. aureus (Owens et al., 1993), and MIC\textsubscript{50} or MIC\textsubscript{90} values of 0.25 μg/mL for Strep. agalactiae (Guérin-Faublée et al., 2003), Strep. uberis (Owens et al., 1997), and Staph. aureus (Owens and Watts, 1987). Staphylococcus aureus had an MIC\textsubscript{90} value of 0.5 μg/mL (Guérin-Faublée et al., 2003), whereas MIC\textsubscript{50} values from 1.0 μg/mL to 16 μg/mL were reported for E. coli (Guérin-Faublée et al., 2003). Cows here maintained milk [CEPH] above all of these MIC thresholds for at least 24 h after the first dose, and above the lowest threshold (0.06 μg/mL) for at least 54 h after the first dose, regardless of milking frequency or dosing interval.

Cows milked 3× and dosed at a 16-h interval maintained [CEPH] ≥ 16 μg/mL significantly longer than cows milked 3× and dosed at an 8-h interval. Cows milked 3× and dosed at a 16-h interval maintained [CEPH] above all selected MIC thresholds 8 h longer than cows milked 3× and dosed at an 8-h interval, which
reflected the difference in dosing interval. Therefore, to achieve a longer time above MIC, cows milked 3× should be infused with CEPH at 16-h intervals rather than at 8-h intervals. A 24-h dosing interval should be investigated in future studies, because all MIC thresholds were exceeded for at least 24 h after the last IMM infusion of CEPH in these cows. The relationship between MIC results and treatment efficacy has not been sufficiently investigated in cows with mastitis.

Approximately 60 to 70% of the infused CEPH dose was recovered in milk as CEPH or DAC, and percentage recovery was not affected by milking frequency. The high recovery rate indicated limited systemic absorption, as would be expected with a hydrophilic antimicrobial agent that has low lipid solubility, and is predominantly ionized in milk. Cephapirin is highly water-soluble, has low lipid solubility, and is a weak organic acid with pKa 2.67 and 4.49, making it almost completely ionized at normal milk pH (Gehring and Smith, 2006). Mean recovery rates of 54, 43, and 52% were reported for another cephalosporin antimicrobial agent, cefquinome, after IMM administration to healthy cows milked 3×, 2×, and 1.5×, respectively (Knappstein et al., 2003).

We observed that low-producing cows had less CEPH and DAC recovered in milk compared with high-producing cows. This may have been caused by higher [CEPH] (less dilution) in milk of low-producing cows, which may have created a higher concentration gradient between milk and blood that favored passive systemic absorption. The vehicle can markedly influence percentage recovery of an antimicrobial agent in milk after IMM infusion; for example, mean recovery rates for procaine penicillin G associated with 3 different IMM infusion products were 16, 42, and 71% (Moretain and Boisseau, 1989). The vehicle for CEPH here was a stable peanut oil gel. Mean recovery rate for cephalixin in a vehicle

Figure 5. Sum of cephapirin and desacetylcephapirin concentrations in milk from the treated gland of 6 lactating Holstein-Friesian cows administered 2 infusions of cephapirin (200 mg) as cephapirin sodium at an 8-h (group B), 12-h (group A), or 16-h (group C) interval into 1 rear mammary gland. Time = 0 h is the time of the last dose. Group A cows (2×-12 group) were milked every 12 h, whereas cows in groups B and C (3×-8 and 3×-16 groups, respectively) were milked every 8 h. Data are expressed as mean ± standard deviation. The horizontal dashed line is the maximum residue limit in Europe (0.060 μg/mL) for the sum of cephapirin and desacetylcephapirin concentrations in saleable milk. The horizontal solid line indicates the intersection at 0 h. Six cows contributed data to each time point except for the following: group A, 60 to 72 h (n = 5), 84 h (n = 4), 96 h (n = 5); group B, 56 h (n = 5), 104 h (n = 4); group C, 96 h (n = 5).
of glyceryl trihydroxystearate in peanut oil was only 3.5% (Moretain and Boisseau, 1989), which highlights the inappropriateness of extrapolating findings for 1 cephalosporin-containing product to another.

Concentration of CEPH in milk from the treated mammary gland dropped below the FDA tolerance of 0.020 μg/mL before the end of the withdrawal time for the infused product (96 h) in all treatment groups. Concentrations below the tolerance first occurred at 57 to 68 h after treatment, but there was marked variability among cows. Cephalospirin concentrations above tolerance were subsequently observed in some cows, with 5 of 18 cows having milk [CEPH] above the tolerance at 96 h; a similar effect was observed with [TACEPH] with respect to the MRL. This apparent rebound effect most likely resulted from the release of bound CEPH from mammary tissue. The fluctuations should not pose a risk for violative residues; however, because calculated [CEPH] in composite milk from all 4 mammary glands was below the tolerance at 96 h (the labeled withdrawal time) in all cases. The calculated [TACEPH] was below the MRL in 16 of 18 cases. Our study did not meet FDA criteria for determining withdrawal times in milk (20 animals tested in triplicate; http://www.fda.gov/cvm/Guidance/1732.htm), so veterinarians prescribing IMM CEPH in cows milked 3× (extra-label use) must still use judgment in establishing conservative withdrawal times.

We are aware of only 1 group of investigators that has studied the impact of milking frequency on excretion of IMM antimicrobial agents. Knappstein et al. (2003) milked healthy German Holstein cows at intervals of 8 h (3×), 14 and 10 h (2×), or 16 h (1.5×). All 4 glands of each cow were infused with 75 mg of cefquinome 3 times within 24 h or with 3 g of procaine-benzylpenicillin G 3 times within 48 h. Cefquinome-treated cows milked 3× had shorter withdrawal times (71.8 ± 6.5 h) than those milked 2× (111.6 ± 4.8 h) or 1.5× (99.9 ± 5.6 h), but individual cow variability had a greater effect on withdrawal time than did milking frequency. Penicillin-treated cows milked 3× (64.7 ± 4.6 h) had a shorter withdrawal time than those milked 1.5× (96.3 ± 4.5 h), and milking frequency was the most significant determinant of withdrawal time for penicillin.

Although milking frequency did not affect time for [CEPH] to drop below tolerance concentration (i.e., withdrawal time), withdrawal time may be affected by milk production. Low-producing cows had longer MRT despite lower recovery of the infused drug in milk, supporting findings for other IMM antimicrobial agents in cows (Whittem, 1999; Knappstein et al., 2003). Therefore, it may be prudent to routinely extend the time that milk is withheld from sale when cows with very low milk production are treated with IMM antimicrobial agents.

The crossover design employed here allowed each cow to serve as its own control and reduced the total number of animals required. This was important because inter-cow variability in IMM antimicrobial disposition can be high (Knappstein et al., 2003). Nonetheless, because our objective was to determine the effect of milking frequency on CEPH pharmacokinetics in milk, the study design had to assure that cows responded appropriately to changes in milking frequency. Previous research suggested that a 2-d acclimation period would be sufficient to cause milk production to respond (Dewhurst and Knight, 1994). Indeed, we observed a nonsignificant 8.7% increase in milk yield from the treated gland in cows milked 3× compared with 2×, and a nonsignificant 7.3% increase in composite milk from all 4 glands. This change was less than the 10 to 15% we anticipated based on field studies (Smith et al., 2002), but similar to the volume increase identified in a review of 19 previously published studies conducted by Erdman and Varner (1995).

High-performance liquid chromatography with tandem mass spectrometry was a highly sensitive, reliable method for quantifying CEPH and DAC in milk, as has been observed by others (Heller et al., 2000; Holstege et al., 2002). Some pharmacokinetic and residue studies
Table 2. Summary of time metrics related to cephapirin concentration or the sum of cephapirin and desacetylcephapirin concentrations

<table>
<thead>
<tr>
<th>Treatment interval</th>
<th>Milking interval</th>
<th>12 h</th>
<th>8 h</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephapirin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time &gt;16 μg/mL after first infusion (h)</td>
<td></td>
<td>24 (12 to 24)</td>
<td>16 (16 to 24)</td>
<td>24 (24 to 24)</td>
</tr>
<tr>
<td>Time &gt;1 μg/mL after first infusion (h)</td>
<td></td>
<td>30 (24 to 36)</td>
<td>24 (24 to 32)</td>
<td>32 (24 to 40)</td>
</tr>
<tr>
<td>Time &gt;0.5 μg/mL after first infusion (h)</td>
<td></td>
<td>36 (24 to 36)</td>
<td>32 (24 to 72)</td>
<td>40 (24 to 80)</td>
</tr>
<tr>
<td>Time &gt;0.25 μg/mL after first infusion (h)</td>
<td></td>
<td>36 (24 to 48)</td>
<td>36 (32 to 72)</td>
<td>44 (32 to 80)</td>
</tr>
<tr>
<td>Time &gt;0.125 μg/mL after first infusion (h)</td>
<td></td>
<td>36 (36 to &gt;108)</td>
<td>40 (32 to &gt;112)</td>
<td>48 (32 to 80)</td>
</tr>
<tr>
<td>Time &gt;0.060 μg/mL after first infusion (h)</td>
<td></td>
<td>54 (36 to &gt;108)</td>
<td>60 (32 to &gt;112)</td>
<td>72 (56 to 96)</td>
</tr>
<tr>
<td>Cephapirin and desacetylcephapirin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time &gt;16 μg/mL after first infusion (h)</td>
<td></td>
<td>24 (24 to 24)</td>
<td>16 (16 to 24)</td>
<td>24 (24 to 32)</td>
</tr>
<tr>
<td>Time &gt;1 μg/mL after first infusion (h)</td>
<td></td>
<td>36 (24 to 36)</td>
<td>36 (32 to 72)</td>
<td>36 (24 to 40)</td>
</tr>
<tr>
<td>Time &gt;0.5 μg/mL after first infusion (h)</td>
<td></td>
<td>36 (24 to 48)</td>
<td>40 (32 to &gt;112)</td>
<td>48 (32 to 80)</td>
</tr>
<tr>
<td>Time &gt;0.25 μg/mL after first infusion (h)</td>
<td></td>
<td>42 (36 to &gt;108)</td>
<td>44 (32 to &gt;112)</td>
<td>52 (32 to 80)</td>
</tr>
<tr>
<td>Time &gt;0.125 μg/mL after first infusion (h)</td>
<td></td>
<td>54 (36 to &gt;108)</td>
<td>56 (40 to &gt;112)</td>
<td>56 (40 to 96)</td>
</tr>
<tr>
<td>Time &gt;0.060 μg/mL after first infusion (h)</td>
<td></td>
<td>54 (48 to &gt;108)</td>
<td>68 (40 to &gt;112)</td>
<td>76 (64 to &gt;112)</td>
</tr>
<tr>
<td>Percentage of infused dose recovered</td>
<td></td>
<td>59 ± 32</td>
<td>66 ± 22</td>
<td>73 ± 30</td>
</tr>
<tr>
<td>Withdrawal times for milk from the treated gland</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to initially fall below the tolerance concentration for cephapirin of 0.020 μg/mL after the last infusion (h)</td>
<td></td>
<td>68 ± 20</td>
<td>66 ± 22</td>
<td>57 ± 18</td>
</tr>
<tr>
<td>Proportion of cows with concentrations above the tolerance concentration of 0.020 μg/mL at 96 h after the last infusion</td>
<td></td>
<td>2/6</td>
<td>1/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Time to initially fall below the maximum residue limit for the sum of cephapirin and desacetylcephapirin concentrations of 0.060 μg/mL after the last infusion (h)</td>
<td></td>
<td>58 ± 21</td>
<td>63 ± 23</td>
<td>65 ± 17</td>
</tr>
<tr>
<td>Proportion of cows with concentrations above the maximum residue limit for the sum of cephapirin and desacetylcephapirin concentrations of 0.060 μg/mL at 96 h after the last infusion</td>
<td></td>
<td>1/6</td>
<td>1/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Withdrawal times for composite milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calculated time to initially fall below the tolerance concentration for cephapirin of 0.020 μg/mL after the last infusion (h)</td>
<td></td>
<td>36 (36 to 60)</td>
<td>40 (32 to 48)</td>
<td>36 (24 to 48)</td>
</tr>
<tr>
<td>Proportion of cows with calculated composite concentrations above the tolerance concentration of 0.020 μg/mL at 96 h after the last infusion</td>
<td></td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Calculated time to initially fall below the maximum residue limit for the sum of cephapirin and desacetylcephapirin concentrations of 0.060 μg/mL after the last infusion (h)</td>
<td></td>
<td>36 (36 to 60)</td>
<td>40 (32 to 64)</td>
<td>36 (24 to 48)</td>
</tr>
<tr>
<td>Proportion of cows with calculated composite concentrations above the maximum residue limit for the sum of cephapirin and desacetylcephapirin concentrations of 0.060 μg/mL at 96 h after the last infusion</td>
<td></td>
<td>1/6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

1Length of time after the first infusion that cephapirin concentration, or the sum of cephapirin and desacetylcephapirin concentrations, exceeded specific minimum inhibitory concentrations in milk from the treated gland in 6 lactating Holstein-Friesian cows milked at 12- or 8-h intervals and receiving 2 intramammary infusions of cephapirin (200 mg) at 8-, 12-, or 16-h intervals. Withdrawal times for milk from the treated gland, and calculated withdrawal times for composite milk from all 4 glands, after the last infusion. Data are expressed as mean ± SD or median with range in parentheses.

2Values available for only 12 analyses.
use microbial inhibition tests as less expensive alternatives for detecting antimicrobial agents in milk (Moretain and Boisseau, 1989; Bajwa et al., 2007). However, nonspecific antimicrobial inhibitors in milk can affect microbial inhibition results (Andrew, 2001), and microbial inhibition tests cannot precisely distinguish CEPH and DAC. Both CEPH and DAC have antimicrobial activity, and [DAC] in milk can equal or exceed [CEPH] after IMM infusion of CEPH (Moats et al., 2000). Therefore, it is important to quantify both CEPH and DAC in pharmacokinetic studies. Metabolism of CEPH to DAC occurred within 8 h of cephapirin administration, with DAC being the predominant antimicrobial agent in milk until 48 h after infusion, after which time CEPH was predominant. Although CEPH produces other metabolites in milk besides DAC (Heller et al., 2000), they are present in negligible concentrations in milk and probably irrelevant to efficacy.

CONCLUSIONS

The pharmacokinetics of CEPH and DAC after infusing 200 mg of CEPH into a single mammary gland following each of 2 milkings were similar for cows milked 2× and cows milked 3×. Cephapirin was rapidly metabolized to DAC in the milk, with DAC being the predominant antimicrobial agent for the first 48 h after infusion. Approximately 60 to 70% of the infused dose was recovered in milk as CEPH or DAC. Compared with 2× milking, milking cows 3× did not significantly reduce time above published MIC50 or MIC90 values for common mastitis pathogens or time above the FDA tolerance concentration for cephapirin in milk. In all cows, calculated [CEPH] for composite milk from all 4 mammary glands was below tolerance by 96 h after the last infusion, which is the labeled withholding time for the infused product. A dosing interval of 16 h prolonged the duration of inhibitory concentrations for common mastitis pathogens by approximately 8 h compared with a dosing interval of 8 h in cows milked 3×. Cows with low milk production appeared to absorb more of the infused CEPH dose than cows with high milk production and had a longer MRT. Healthy cows were used, so results should not be directly extrapolated to cows with mastitis, which can alter mammary gland permeability, reduce milk yield, or cause blockage of ducts or changes in milk pH or composition that influence drug distribution and absorption.

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REFERENCES


