J. vet. Pharmacol. Therap. **32,** 345–352, doi: 10.1111/j.1365-2885.2008.01048.x.

Effect of milk fraction on concentrations of cephapirin and desacetylcephapirin in bovine milk after intramammary infusion of cephapirin sodium

R. M. STOCKLER*
D. E. MORIN*
R. K. LANTZ†
W. L. HURLEY‡ &
P. D. CONSTABLE§

Department of Veterinary Clinical Medicine, University of Illinois at Urbana-Champaign, Urbana, IL; †Rocky Mountain Instrumental Laboratories, Fort Collins, CO; ‡Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL; §Department of Veterinary Clinical Sciences, Purdue University, West Lafayette, IN, USA

Stockler, R. M., Morin, D. E., Lantz, R. K., Hurley, W. L., Constable, P. D. Effect of milk fraction on concentrations of cephapirin and desacetylcephapirin in bovine milk after intramammary infusion of cephapirin sodium. *J. vet. Pharmacol. Therap.* **32**, 345–352.

Clinical mastitis in dairy cows is commonly treated with intramammary (IMM) antimicrobial agents. Pharmacokinetic data are used to design treatment regimens and determine withholding times. In some pharmacokinetic studies, investigators measure antimicrobial concentrations in foremilk, whereas in others, they use bucket milk or do not specify the milk fraction sampled. Our objective was to compare antimicrobial concentrations in foremilk, bucket milk, and strippings after IMM treatment of six healthy Holsteins. One mammary gland/cow was infused with 200 mg of cephapirin (CEPH) after each of the two milkings, using different milking frequencies and treatment intervals in a randomized crossover design. Treated glands were sampled at the first milking following each infusion. Antimicrobial concentrations in milk were measured using HPLC/MS/MS. CEPH concentration was higher in foremilk (geometric mean 44.2 μg/mL) than in bucket milk (15.7 μg/mL) or strippings (18.5 μg/mL), as it was true for desacetylcephapirin (DAC) (59.5, 23.0, and 30.2 μg/mL, respectively). This finding, which was based on milk samples collected at the first milking after IMM infusion, suggests that pharmacokinetic data based on drug concentrations in foremilk may be misleading. Strippings were more representative of bucket milk than foremilk. The relationship between milk fraction and antimicrobial concentration should be investigated for other IMM antimicrobial agents. Meanwhile, it is essential that pharmacokinetic and residue studies report the fraction of milk that was analyzed.

(Paper received 12 August 2008; accepted for publication 25 November 2008)

Ricardo M. Stockler, Mississippi State University College of Veterinary Medicine Department of Pathobiology and Population Medicine, 240 Wise Center Drive, Mississippi State, MS 39762-6100, USA. E-mail: rstockler@cvm.msstate.edu

INTRODUCTION

Treatment of clinical mastitis accounts for a substantial proportion of antimicrobial use on dairy farms (Sawant *et al.*, 2005; Raymond *et al.*, 2006). Antimicrobial agents are often administered by intramammary (IMM) infusion, especially for clinical mastitis episodes caused by streptococci or staphylococci (Hillerton & Berry, 2003; Barkema *et al.*, 2006). For IMM antimicrobial therapy to be successful, the antimicrobial agent must attain and maintain an effective concentration at the site of infection in milk or mammary tissue (Constable & Morin, 2003; Erskine *et al.*, 2003). Following treatment, milk must be withheld from sale until the antimicrobial concentration drops below the allowable tolerance concentration (FDA, 2005). Failure to withhold milk for a sufficient length of time can result in violative residues that adversely affect human health and milk product quality (Saville *et al.*, 2000). Although withholding times for commercially-available IMM antimicrobial products are stated on the product labels, veterinary practitioners must estimate withholding times when antimicrobial agents are used in an extra-label manner, such as when a product labeled for administration every 12 h is used in cows milked every 8 h. Reliable pharmacokinetic data are essential for designing appropriate dosage regimens and determining safe withholding times.

Pharmacokinetic parameters for IMM antimicrobial agents are based on the time course of antimicrobial concentrations in milk after treatment. Therefore, milk samples that reflect milk

antimicrobial concentrations in the gland as a whole should be used as the basis for pharmacokinetic analysis and for guidance when estimating withholding times. Milk fractions differ in nutrient composition, cellular content, enzyme activity, and electrical conductivity (Fernando *et al.*, 1981; Berning *et al.*, 1987; Bansal *et al.*, 2005). For example, fat concentration and somatic cell count (SCC) are substantially higher in samples collected at the end of milking (strippings) than samples collected before milking (foremilk; Vangroenweghe *et al.*, 2002; Sarikaya *et al.*, 2005; Bansal *et al.*, 2005; Sarikaya & Bruckmaier, 2006). In contrast, protein and lactose concentrations are lower in strippings than in foremilk (Urech *et al.*, 1999; Vangroenweghe *et al.*, 2002; Bansal *et al.*, 2005). Such differences might influence the distribution of antimicrobial agents within the gland. Many antimicrobial agents distribute unevenly in milk and mammary tissue as a result of factors such as the type of vehicle, physicochemical properties of the antimicrobial agent, and the presence of inflammation (Ziv, 1980b; Ehinger & Kietzmann, 2000a,b; Gehring & Smith, 2006). Uneven distribution of antimicrobial agents should cause differences in antimicrobial concentrations among milk fractions.

In some pharmacokinetic and milk residue studies involving IMM antimicrobial agents, investigators have used foremilk samples for drug quantification (e.g., Ziv & Sulman, 1974; Van Eenennaam *et al.*, 1993; Smith *et al.*, 2004). In others, investigators have used the total milk collected during milking (bucket milk; e.g. Moretain & Boisseau, 1989; Whittem, 1999; Moats *et al.*, 2000; Roncada *et al.*, 2000; Knappstein *et al.*, 2003) or specific volumes of milk collected for analysis at designated time points (Wuschko *et al.*, 1998; Bajwa *et al.*, 2007). Some investigators do not report the milk fractions used or simply state that 'quarter milk samples' were collected (e.g., Rollins *et al.*, 1970; Owens & Nickerson, 1990). Given the potential impact of milk fraction on antimicrobial concentrations, the objective of the study reported here was to compare antimicrobial concentrations in three fractions of milk (foremilk, bucket milk, and strippings) 8 or 12 h after administering an IMM antimicrobial agent cephapirin (CEPH) to lactating dairy cows.

MATERIALS AND METHODS

Animals and eligibility criteria

The study was conducted at the Dairy Research Farm of the University of Illinois at Urbana-Champaign. The University's Institutional Animal Care and Use Committee approved all procedures. Six healthy, multiparous Holstein cows were enrolled, three with low milk production (≤ 55 lb/day [25 kg/day]) and three with high milk production (≥ 80 lb/day [36 kg/day]). Cows were at least 30 days into lactation and had no history of clinical mastitis during the previous 60 days. One rear gland (hereafter referred to as the study gland) was selected from each cow. Milk from this gland had to be bacteriologically negative with a SCC < 150 000 cells/mL. When both rear glands were eligible, one gland was selected at random.

Study design

Cows were housed in tie stalls and fed a corn silage-based total mixed ration. Fresh water was available *ad libitum*. The study was conducted in conjunction with an investigation of the impact of milking frequency (twice/day [2×] vs. three times/day [3×]) on pharmacokinetics of CEPH after IMM administration (to be reported elsewhere). A duplicated Latin square design enabled each cow to receive all three experimental treatments. The treatments were: (i) milking at 12-h intervals, with CEPH administered into the study gland after two (0- and 12-h) consecutive milkings (2×-12 treatment); (ii) milking at 8-h intervals, with CEPH administered into the study gland after the first (0-h) and second (8-h) milking (3×-8 treatment); and (iii) milking at 8-h intervals, with CEPH administered into the study gland after the first (0-h) and third (16-h) milking (3×-16 treatment). The order of treatments was determined randomly. CEPH (200 mg; Cefa-Lak®, Fort Dodge Animal Health, Fort Dodge, IA, USA) was infused into the study gland using the partial insertion method (Boddie & Nickerson, 1986). IMM infusion occurred immediately following milking after thoroughly scrubbing the teat end with 70% alcohol. The infused product was gently massaged from the distal teat cistern towards the gland cistern after infusion to facilitate dispersion. The same study gland was used for each cow throughout the study, with the exception of one cow that developed clinical mastitis. Cows were acclimated to the milking regimen (2 or 3×) for 48 h before beginning each treatment phase, and a 4-day washout period was provided between the last CEPH infusion and the next acclimation period.

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. Premilking procedures included spraying the teats with 0.4% chlorhexidine gluconate (Fight Bac®, Deep Valley Farm, Brooklyn, CT, USA), allowing 30 sec of contact time, and wiping each teat and teat end with an individual paper towel. Teats were massaged for 30 sec while wiping. The milking cluster was attached approximately 60 sec after the start of premilking procedures.

Milking was performed using a portable bucket milking machine (Porta-Milker 2-Wheel Base Unit for 1 Unit – Electric Motor 1 hp Motor, Coburn Company, Inc., Whitewater, WI, USA). Milk from the study gland was diverted from the bucket using a portable quarter milking device (Coburn QuarterMilker Model 97QMA with Safety Overflow Valve; Coburn Company, Inc.). The milking 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 sec. Milk production (mL) by the study gland was measured by pouring milk from the quarter milking device into a 4000-mL graduated cylinder.

Sample collection

At the first milking time subsequent to each CEPH infusion (two milking times/cow per treatment phase), three milk samples were collected for drug quantification. These included hand collection of one 5 mL sample of foremilk before applying the quarter milking device (n = 36), one well-mixed 20 mL sample from the quarter milking device (bucket milk; n = 36), and hand collection of one 5 mL sample of strippings collected within 2 min after removing the quarter milking device (n = 36). Because of the high cost associated with HPLC/MS/MS, drug concentrations were measured only at the first milking after each IMM infusion, when the differences among fractions were expected to be the greatest. Two infusions were administered to each cow in each treatment phase, as directed on the label.

The foremilk sample included all of the expressed secretions (no secretions were discarded). The foremilk sample was collected immediately after massaging the teat for 30 sec and wiping the teat dry with an individual paper towel, but within 60 sec after the start of premilking procedures. All milk samples were frozen at −70 °C. The frozen milk samples were shipped on dry ice by overnight mail to Rocky Mountain Instrumental Laboratories, Fort Collins, CO, USA.

Drug testing

Instrumentation

Tandem liquid chromatography mass spectrometry (HPLC/MS/MS) was performed with a SciEx (Applied Biosystems, Foster City, CA, USA) 4000Q triple quadrupole mass spectrometer and ANALYST software (Applied Biosystems; version 1.4.2) coupled to a gradient high performance liquid chromatography (HPLC) system comprised of two Shimadzu (Columbia, MD, USA) LC-10ADvp pumps, SIL-20A autosampler (Shimadzu Scientific Instruments, Columbia, MD, USA) with auxiliary wash pump, DGU-14A vacuum degasser (Shimadzu Scientific Instruments), and SCL-20 controller (Shimadzu Scientific Instruments). Mobile phases were: A, 0.1% formic acid and 0.1 M ammonium formate in water and B, 0.1% formic acid and 0.1 M ammonium formate in acetonitrile. Mobile phases were filtered (Whatman 0.45 μm Nylon; Whatman International, Maidstone, UK) prior to use. The HPLC column was XTerra MS C18, 3.5 μm, 2.1 × 100 mm, PN186000404, and XTerra guard column.

All solvents were purchased from EMD Science (Gibbstown, NJ, USA), and formic acid and ammonium formate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Solid phase extraction 96-well plates were polymeric Oasis HLB (Waters Associates, Milford, MA, USA) PN 186000128. CEPH standard was purchased from The US Pharmacopeia (Rockville, MD, USA), desacetylcephapirin (DAC) was a gift from Fort Dodge Laboratories (Fort Dodge, IA, USA) and from Alan Lightfield, USDA, Eastern Regional Research Center, Wyndmoor, PA, USA) and amoxicillin was purchased from Sigma Chemical Co. (St Louis, MO, USA). Known antibiotic negative milk was purchased from the University of Illinois Dairy Research Farm.

Preparation of Stock solutions and standards

The primary CEPH stock was prepared at 2.0 mg/mL, the DAC stock at 650 μg/mL, and the amoxicillin stock at 1.0 mg/mL, in 50% aqueous acetonitrile. Working stocks were prepared in acetonitrile, and final working standards were prepared in drug-free milk. The internal standard stock solution was prepared in 50% aqueous acetonitrile to be 1 mg/mL, and then diluted each day for use by mixing 250 μL stock with 25 mL water (10 ng/μL). Standard concentrations were nominally 2–20 000 ng/mL for CEPH and 10–13 000 ng/mL for DAC. Control samples were prepared at nominal concentrations of 100, 400, and 15 000 ng/mL CEPH and 65, 260, and 9750 ng/mL DAC. Samples, standards, and controls were aliquoted and frozen at −80 °C until used.

Extraction

The SPE matrix was first activated by addition of 500 μL methanol, followed by two aliquots of 500 μL water. Then, 100 μL of defatted milk (sample, standard or control) was added, followed by 25 μL of the internal standard. Solutions were allowed to flow through under gravity, and each well was rinsed with water three times. The matrix was then dried under vacuum for 15 min. Drugs were eluted from the plate by the addition of 50% aqueous acetonitrile (250 μL three times). The acetonitrile was then removed under vacuum centrifugation (SpeedVac, Savant Instruments, Farmingdale, NY, USA), and extracts were reconstituted in 100 μL mobile phase A (below) and transferred to a small volume autosampler vial. Then, 20 μL of each concentrated eluate was injected onto the LC/MS system twice.

HPLC/MS/MS

A linear gradient of 5% B to 90% B over 12 min was used with a flow rate of 325 μL/min, where mobile phase A was prepared by the addition of 500 μL formic acid and 100 mg ammonium formate to 1 L water, and mobile phase B was prepared by the addition of 500 μL formic acid and 100 mg ammonium formate to 1 L acetonitrile. A typical chromatogram is shown in Fig. 1. Two tandem mass spectrometry (MS/MS) transitions (stated in m/z) were used for CEPH (424.0/292.0 and 424.0/152.0), one for DAC (382.0/124.2) and one for the internal standard, amoxicillin, (366.2/208.1). Two sets of standards and controls were used for each 96-well plate, calibration curves were generated for each standard set, and approximately 10% of samples were extracted and analyzed in duplicate. When results were above the calibration curve, samples were diluted before being reanalyzed.

Calibration curve and assay validation

Calculations were performed using the ANALYST software. Samples yielding results above 10 μg/mL were reassayed after dilution, even if results were within the calibration curve. Correlation coefficients were recorded for each run and always exceeded 0.99. Within day precision was 5.2% and between day precision 8.5%. Accuracy was ±4% to 6% using spiked milk samples. The limit of detection (LOD) for both CEPH and DAC was 0.0005 μg/mL, and the limit of quantification (LOQ) for

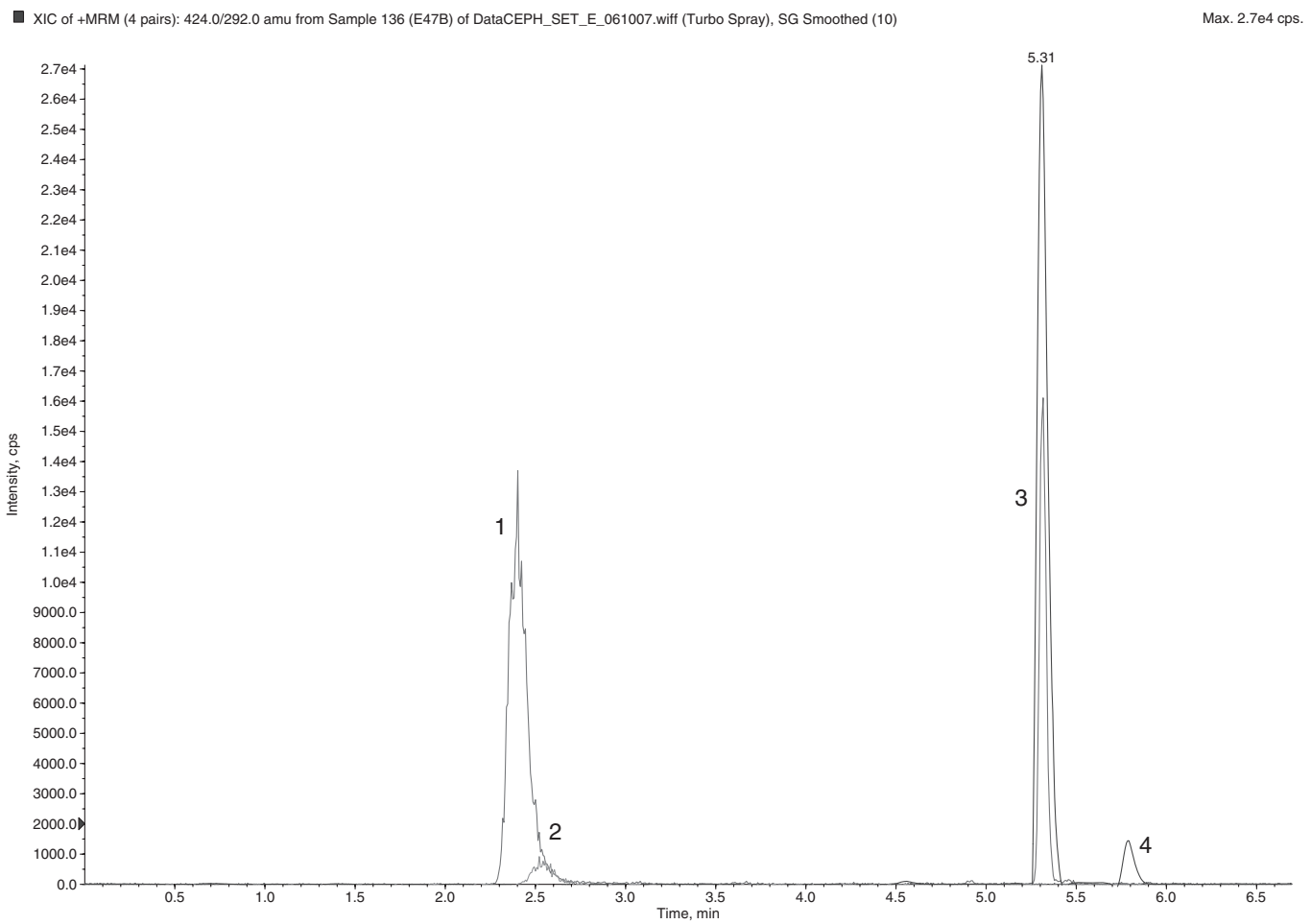

Fig. 1. Typical chromatogram for a Holstein Friesian cow treated with 200 mg of cephapirin as cephapirin sodium. 1 desacetyl-cephapirin (382.0/124.2), 2 amoxicillin internal standard (366.2/208.1), 3 cephapirin (424.0/152.0 and 424.0/292.0), 4 unidentified, possibly related, contaminant with only one transition in common with cephapirin (424.0/292.0).

each was 0.002 μg/mL, using signal to noise ratio of 6 for each compound.

Data analysis

Data are presented as geometric mean and 95% confidence interval, and $P < 0.05$ was considered significant. Concentrations of CEPH and DAC in milk samples were summed to estimate total active cephapirin equivalent antimicrobial concentrations ([TA]) in those samples by multiplying the [DAC] by the molecular weight ratio of CEPH (423.5 g) to DAC (381.5 g). Concentrations of CEPH, DAC, and TA, as well as the coefficient of variation for the two measurements of each milk fraction and frequency combination for each cow, were log transformed to achieve a normal distribution. Mixed models analysis of variance (PROC MIXED, SAS Inc., Cary, NC, USA) was used to compare the main effects of milk fraction (three levels; foremilk, bucket milk, strippings), milking frequency (three levels; 2×-12, 3×-8, 3×-16), milk production (two levels; high, low), and the interaction between milk fraction and milking frequency on CEPH, DAC, and TA concentrations, with cow as a random factor.

RESULTS

Cows were in second to seventh lactation and had been lactating for 190–529 days at the time of enrolment. Milk yield of the low producing cows ranged from 46 to 53 lb/day (21–24 kg/day) and for the high producing cows from 89 to 92 lb/day (40–42 kg/day). Four left rear and two right rear glands were enrolled, with SCC in milk from those glands ranging from 12 000 to 118 000 cells/mL.

Study glands remained free of clinical and subclinical mastitis, with one exception. One cow developed clinical mastitis in the study gland during the washout period between the second and third treatment phase. Mastitis developed after samples for drug testing had been collected and was expected to have no impact on study results. The opposite rear gland, which met all enrolment criteria, was used in the third treatment phase.

Table 1. Effect of milk fraction and milking frequency on concentrations of CEPH, DAC, and the combination of CEPH and DAC (TA) measured in milk samples (n = 36) of six lactating dairy cows after intramammary infusion of CEPH sodium using three different treatment protocols

	CEPH (μg/mL)	DAC (μg/mL)	TA (μg/mL)
Milk fraction*			
Foremilk	44.2[a] (3.9–508.0)	59.5[a] (4.9–727.6)	113.0[a] (9.9–1,291.2)
Bucket milk	15.7[b] (5.4–45.5)	23.0[b] (8.2–64.7)	43.1[b] (17.8–104.1)
Stripping	18.5[b] (2.3–147.2)	30.2[b] (3.5–263.0)	54.3[b] (7.0–421.5)
Milking frequency†			
2×-12	12.5[c] (1.3–118.1)	18.7[c] (1.9–180.1)	34.1[c] (3.7–311.2)
3×-8	35.1[d] (6.1–203.0)	52.3[d] (8.3–330.2)	97.5[d] (18.3–519.6)
3×-16	29.3[d] (4.7–180.7)	42.4[d] (7.4–241.3)	79.4[d] (15.0–421.1)

Values are geometric mean with 95% confidence interval in parentheses. *Samples were collected only at the first milking after each IMM infusion. †2×-12: cows milked every 12 h (2×) and treated at 0 and 12 h; 3×-8: cows milked every 8 h (3×) and treated at 0 and 8 h; 3×-16: cows milked every 8 h (3×) and treated at 0 and 16 h. CEPH, cephapirin; DAC, desacetylcephapirin; TA, total active antimicrobial agent. Milk fraction and milking frequency values with different superscripts within columns are significantly different ($P < 0.05$).

The concentration of CEPH was affected by milk fraction (P = 0.0007 for the main effect of fraction; Table 1) and milking frequency (P = 0.023 for the main effect of frequency), but not by the interaction of milk fraction and milking frequency (P = 0.17) or by milk production (P = 0.96; data to be reported elsewhere). The concentration of DAC was also affected by milk fraction (P = 0.0022 for the main effect of fraction) and milking frequency (P = 0.017 for the main effect of frequency), but not by the interaction of milk fraction and milking frequency (P = 0.22) or by milk production (P = 0.71; data to be reported elsewhere). Similarly, the concentration of TA was affected by milk fraction (P = 0.0011 for the main effect of fraction) and milking frequency (P = 0.017 for the main effect of frequency), but not by the interaction of milk fraction and milking frequency (P = 0.20) or by milk production (P = 0.80; data to be reported elsewhere).

Concentrations of CEPH were much higher in foremilk than in bucket milk (P = 0.0003) and strippings (P = 0.0011), but concentrations in bucket milk and strippings were similar (P = 0.43). Similarly, concentrations of DAC were higher in foremilk than in bucket milk (P = 0.0008) or strippings (P = 0.0051), with concentrations in bucket milk and strippings being similar (P = 0.29). Therefore, the same relationships were observed for TA concentrations in foremilk, bucket milk, and strippings.

Antimicrobial concentrations were lower (P = 0.047; Table 1) in milk samples collected from cows in the 2×-12 treatment group than in either of the 3× treatment groups, where samples were collected 8 h after infusion instead of 12 h. Concentrations were similar for cows in the 3×-8 and 3×-16 treatment groups ($P > 0.25$).

Milk fraction tended (P = 0.054) to have an effect on the within-cow variability of CEPH concentrations for the milk samples collected following each infusion in each treatment phase, based on coefficients of variation of 34%, 14%, and 27% for foremilk, bucket milk, and stripping samples, respectively. The main effect of milk fraction was not significant for DAC (P = 0.60), but tended to be significant for TA (P = 0.092). The range of the 95% confidence interval for antimicrobial concentrations was always smaller for bucket milk than strippings or foremilk (Table 1). These results suggested that precision may be best with bucket milk samples. Frequency had no effect on the coefficient of variation ($P > 0.20$).

DISCUSSION

Milk fraction had a substantial impact on the concentrations of CEPH, DAC, and TA measured in milk, 8 or 12 h after IMM infusion of CEPH sodium. In particular, antimicrobial concentrations in foremilk were more than twice as high and more variable than those in bucket milk or strippings. Because foremilk was not representative of conditions throughout the gland, pharmacokinetic parameters and withholding times derived on the basis of antimicrobial concentrations in foremilk may be misleading.

Results of the study reported here must be interpreted in light of the definitions of milk fractions. The terms used to describe milk fractions can be confusing. In general, foremilk refers to the first secretions (number of streams or mL) removed from the teat at milking time. Foremilk represents milk that was produced or released soon after the previous milking and stored in the teat cistern and lower gland cistern between milkings. Some investigators use 'strict foremilk' to refer to the very first stream(s) of milk and 'foremilk' to refer to a subsequent sample, whereas others subdivide foremilk into even smaller fractions, with each fraction representing as little as one stream of milk to as much as 15–60 mL of milk (Fernando & Spahr, 1983; Bansal *et al.*, 2005; Sarikaya *et al.*, 2006). Cisternal milk refers to the entire volume of milk that is stored in the cisterns and large ducts between milkings (Bruckmaier *et al.*, 1994a). Although foremilk represents a portion of cisternal milk, some studies define them separately based on when they are collected (Bruckmaier & Blum, 1996; Vangroenweghe *et al.*, 2002; Sarikaya *et al.*, 2006). There is little information on the fraction of cisternal milk stored in the teat, but in one study, the teat cisterns of 25 cows contained approximately 3 mL of milk (Sarikaya *et al.*, 2006). Teat size varies among cows, which impacts the amount of milk that can be stored. In our study, we defined foremilk to be the first 5 mL of secretion collected from

the teat after udder preparation. As such, we presume that the foremilk samples consisted predominantly of teat cistern milk.

Alveolar milk represents milk that is stored in the small ducts and alveoli between milkings and released only after milk ejection occurs (Bruckmaier *et al.*, 1994b; Sarikaya *et al.*, 2005, 2006; Sarikaya & Bruckmaier, 2006). Approximately, 75–90% of the total milk volume in dairy cows is alveolar milk, compared with 10–25% cisternal milk (Bruckmaier *et al.*, 1994a; Pfeilsticker *et al.*, 1996). However, the cisternal fraction varies among cows, increases with lactation number and decreases with lactation length (Pfeilsticker *et al.*, 1996). Strippings refers to secretions that are manually removed (stripped) from the teats after milking, although the volume collected varies among studies. Residual milk is also collected after milking, but only after oxytocin is administered to eject secretions remaining in the alveoli (Urech *et al.*, 1999; Vangroenweghe *et al.*, 2002). Both strippings and residual milk are part of alveolar milk. In our study, strippings refers to the first 5 mL of milk collected from the teat after removing the milking cluster.

Bucket milk is the total volume of milk collected via the milking machine after removing the foremilk. The terms bulk milk, main milk, and primary milk are used synonymously with bucket milk. In our study, bucket milk was the total volume of milk collected by the quarter milking device after removing the foremilk.

Some investigators collect foremilk before manipulating the teats, to avoid milk ejection, which may cause mixing of alveolar and cisternal milk (Bruckmaier & Blum, 1996; Sarikaya *et al.*, 2006). Others fully clean and dry the teats before collecting the foremilk (Ziv & Sulman, 1974; Urech *et al.*, 1999; Vangroenweghe *et al.*, 2002). We collected foremilk within 60 sec after beginning udder preparation. Because milk collected in the first 50 sec after stimulating the teats is suggested to be free of alveolar milk (Bruckmaier & Hilger, 2001; Sarikaya *et al.*, 2006), we believe any contamination of our 5 mL foremilk samples with alveolar milk was negligible.

The differences we observed in antimicrobial concentrations among milk fractions are probably the result of uneven distribution of drug in the mammary gland and dilution of drug in the alveolar regions of the gland by newly produced milk. Distribution of antimicrobial agents within the mammary gland depends on factors such as the nature of the vehicle and the physicochemical properties of the antimicrobial agent (Ziv, 1980a,b). Edema or inflammation of the gland and blockage of ducts by necrotic or inflammatory debris can also impact distribution (Ziv, 1980b; Owens & Nickerson, 1990). Ziv (1980b) used autoradiography to document differences in IMM distribution among various classes and formulations of antimicrobial agents and as a result of mastitis. More recently, Ehinger and Kietzmann (2000a), using an isolated perfused udder model, demonstrated a reduction in benzylpenicillin concentration in mammary tissue with an increase in vertical distance from the teat. Similar findings were observed for cefquinome after IMM infusion (Ehinger *et al.*, 2006). As IMM antimicrobials are delivered ventrally and directly into the teat cistern, it is logical that concentrations in the foremilk and low portions of the gland would be higher than those in the strippings and dorsal portions of the gland due to gravitational effects and proximity to the site of infusion. The isolated perfused udder model also demonstrated an effect of type and particle size of vehicle on the distribution of IMM antimicrobial agents (Ehinger & Kietzmann, 2000a,b). Differences in milk composition among milk fractions, such as higher protein concentrations in foremilk than strippings and higher fat concentrations in strippings than foremilk, might impact antimicrobial distribution in the mammary gland, as antimicrobial agents vary in protein binding capacity and lipophobicity (Ziv, 1980a,b; Gehring & Smith, 2006).

Our findings indicate that for CEPH and its active metabolite DAC, foremilk is not an ideal sample to use in pharmacokinetic studies. Bucket milk is the best because it reflects the conditions within the gland as a whole, is similar to milk that enters the bulk tank, and had the lowest coefficient of variation for milk antimicrobial concentration following treatment. Bucket milk is easy to collect by conventional milking machine when the study design involves infusion of all four mammary glands. However, when only a single gland is infused, as is more typical of practices in the field, a special quarter milking machine or milking device is required to collect bucket milk from one gland. If this is not feasible, results of the study reported here suggest that strippings are a more appropriate sample for pharmacokinetic studies than foremilk, at least for CEPH and healthy cows.

Cephapirin is a weak organic acid (p*K*a, 2.67 and 4.49) with low lipid solubility, which means it is poorly absorbed from the milk into the systemic circulation (Ziv, 1980b; Gehring & Smith, 2006). CEPH sodium, which is highly soluble in water (1.03 g/L) and insoluble in most organic solvents, is formulated in a stable peanut oil gel for IMM infusion (Freedom of Information Summary, 2003). The total infusion volume is 10 mL. Once infused into the mammary gland, CEPH is partially metabolized. The predominant metabolite, DAC, can equal or exceed CEPH concentration in milk, as in the study reported here. Moreover, DAC is biologically active, which highlights the need to measure both CEPH and DAC in milk (Moats *et al.*, 2000). CEPH has been analyzed in biological samples using a variety of technologies, but LC/MS is especially sensitive and specific (Heller *et al.*, 2000 and Mastovska & Lightfield, 2006). The HPLC/MS/MS method we developed, which uses two transitions for CEPH and one for DAC along with the gradient HPLC system, allowed clear identification of the two compounds and differentiation from possible interfering substances. This method also allowed for excellent sensitivity with small sample volumes and a matrix that can be difficult. The LOD (0.0005 μg/mL) and LOQ (0.002 μg/mL) for CEPH were well below the US tolerance concentration for CEPH in milk (0.02 μg/mL). The relationship between milk fraction and antimicrobial concentration should be investigated for other drugs with different physicochemical properties and for cows with mastitis to determine if findings of the study reported here can be generalized.

Producers and veterinarians must be able to assess accurately the residue status of tanker trucks, bulk tanks, and individual lactating cows on the farm (Cullor, 1995). Although

antimicrobial residue test kits were not designed for use with individual cows, producers typically use residue kits on composite milk collected from all four mammary glands of a treated cow or on foremilk or strippings from the treated gland (Cullor, 1993). Although our results suggest that milk fraction may influence residue test results in cows treated with CEPH sodium, we cannot draw this conclusion because we only sampled cows 8 or 12 h after treatment, not 96 h after treatment as performed in the field. This was a pilot study to examine the differences in antimicrobial concentrations among milk fractions at the first milking after IMM infusion. The impact of milk fraction might be less at subsequent milking times. The higher antimicrobial concentrations we observed in cows milked 3× compared with those milked 2× can be attributed to the time between the treatment and milk sampling (8 h for cows milked 3× and 12 h for cows milked 2×). The antimicrobial agents had more time to be absorbed in cows milked 2× and would have been diluted by the additional accumulating milk.

CONCLUSIONS

Antimicrobial concentrations measured in milk at the first milking after IMM infusion of antimicrobial agents can be influenced by the fraction of milk sampled. For CEPH, antimicrobial concentrations in foremilk were more than twice as high as those in bucket milk or strippings, reflecting uneven distribution within the mammary gland. Therefore, it is imperative that authors of pharmacokinetic and residue studies provide specific details about how the milk samples were collected. The relationship between milk fraction and antimicrobial concentrations should be investigated for other classes and formulations of antimicrobial agents, and at later sampling times so that appropriate samples can be used for pharmacokinetic studies and residue testing.

ACKNOWLEDGMENTS

This study was funded, in part, by Illinois Department of Agriculture Cattle Research Funds and through a cooperative agreement with Fort Dodge Animal Health, Fort Dodge, IA.

REFERENCES

Bajwa, N., Bansal, B., Srivastava, A. & Ranjan, R. (2007) Pharmacokinetic profile of erythromycin after intramammary administration in lactating dairy cows with specific mastitis. *Veterinary Research Communications*, **31,** 603–610.

Bansal, B., Hamann, J., Grabowskit, N. & Singh, K. (2005) Variation in the composition of selected milk fraction samples from healthy and mastitic quarters, and its significance for mastitis diagnosis. *Journal of Dairy Research*, **72,** 144–152.

Barkema, H., Schukken, Y. & Zadoks, R. (2006) Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *Journal of Dairy Science*, **89,** 1877–1895.

Berning, L., Paape, M., Miller, R. & LeDane, R. (1987) Variation in *N*-acetyl-beta-*D*-glucosaminidase activity and somatic cell count among various milk fractions. *Journal of Dairy Science*, **70,** 1054–1060.

Boddie, R. & Nickerson, S. (1986) Dry cow therapy: effects of method of drug administration on occurrence of intramammary infection. *Journal of Dairy Science*, **69,** 253–257.

Bruckmaier, R. & Blum, J. (1996) Simultaneous recording of oxytocin release, milk ejection and milk flow during milking of dairy cows with and without prestimulation. *Journal of Dairy Research*, **63,** 201–208.

Bruckmaier, R. & Hilger, M. (2001) Milk ejection in dairy cows at different degrees of udder filling. *Journal of Dairy Research*, **68,** 369–376.

Bruckmaier, R.M., Rothenanger, E. & Blum, J.W. (1994a) Measurement of mammary gland cistern size and determination of the cisternal milk fraction in dairy cows. *Milchwissenschaft*, **49,** 543–546.

Bruckmaier, R., Schams, D. & Blum, J. (1994b) Continuously elevated concentrations of oxytocin during milking are necessary for complete milk removal in dairy cows. *Journal of Dairy Research*, **61,** 323–334.

Constable, P. & Morin, D. (2003) Treatment of clinical mastitis using antimicrobial susceptibility profiles for treatment decisions. *Veterinary Clinics of North America: Food Animal Practice*, **19,** 139–155.

Cullor, J.S. (1993) Antibiotic residue tests for mammary gland secretions. Veterinary Clinics of North America. *Food Animal Practice*, **9,** 609–620.

Cullor, J.S. (1995) Milk antibiotic residue tests and veterinary practice. *The Compendium*, **17,** 863–871.

Ehinger, A. & Kietzmann, M. (2000a) Tissue distribution of benzylpenicillin after intramammary administration in the isolated perfused bovine udder. *Journal of Veterinary and Pharmacology Therapeutics*, **23,** 303–310.

Ehinger, A. & Kietzmann, M. (2000b) Tissue distribution of oxacillin and ampicillin in the isolated perfused bovine udder. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine*, **47,** 157–168.

Ehinger, A., Schmidt, H. & Kietzmann, M. (2006) Tissue distribution of cefquinome after intramammary and ''systemic'' administration in the isolated perfused bovine udder. *Veterinary Journal*, **172,** 147–153.

Erskine, R., Wagner, S. & DeGraves, F. (2003) Mastitis therapy and pharmacology. *Veterinary Clinics of North America: Food Animal Practice*, **19,** 109–138.

FDA (2005) http://www.cfsan.fda.gov/~ear/pmo03toc.html. Accessed January 2008.

Fernando, R. & Spahr, S. (1983) Effects of milking interval on selected milk constituents from normal and infected quarters. *Journal of Dairy Science*, **66,** 1155–1161.

Fernando, R., Rindsig, R. & Spahr, S. (1981) Effect of length of milking interval and fat content on milk conductivity and its use for detecting mastitis. *Journal of Dairy Science*, **64,** 678–682.

Freedom of Information Summary (2003) *Cephalosporins Veterinary-Systemic* http://www.usp.org/pdf/EN/veterinary/cephalosporins.pdf. Accessed January 2008.

Gehring, R. & Smith, G. (2006) An overview of factors affecting the disposition of intramammary preparations used to treat bovine mastitis. *Journal of Veterinary and Pharmacology Therapeutics*, **29,** 237–241.

Heller, D., Kaplan, D., Rummel, N. & von Bredow, J. (2000) Identification of cephapirin metabolites and degradants in bovine milk by electrospray ionization – ion trap tandem mass spectrometry. *Journal of Agriculture and Food Chemistry*, **48,** 6030–6035.

Hillerton, J. & Berry, E. (2003) The management and treatment of environmental streptococcal mastitis. *Veterinary Clinics of North America: Food Animal Practice*, **19,** 157–169.

Knappstein, K., Suhren, G. & Walte, H. (2003) Influence of milking frequency on withdrawal period after application of beta-lactam antibiotic-based drugs. *Analytica Chimica Acta*, **483,** 241–249.

Mastovska, K. & Lightfield, A. (2006) *Personal Communications USDA ERRC*. Eastern Regional Research Center, Wyndmoor, PA.

Moats, W., Anderson, K., Rushing, J. & Buckley, S. (2000) Conversion of cephapirin to deacetylcephapirin in milk and tissues of treated animals. *Journal of Agriculture and Food Chemistry*, **48,** 498–502.

Moretain, J. & Boisseau, J. (1989) Excretion of penicillins and cephalexin in bovine milk following intramammary administration. *Food Additive and Contaminants*, **6,** 79–90.

Owens, W. & Nickerson, S. (1990) Treatment of *Staphylococcus aureus* mastitis with penicillin and novobiocin: antibiotic concentrations and bacteriologic status in milk and mammary tissue. *Journal of Dairy Science*, **73,** 115–124.

Pfeilsticker, H., Bruckmaier, R. & Blum, J. (1996) Cisternal milk in the dairy cow during lactation and after preceding teat stimulation. *Journal of Dairy Research*, **63,** 509–515.

Raymond, M., Wohrle, R. & Call, D. (2006) Assessment and promotion of judicious antibiotic use on dairy farms in Washington State. *Journal of Dairy Science*, **89,** 3228–3240.

Rollins, L., Mercer, H., Carter, G. & Kramer, J. (1970) Absorption, distribution, and excretion of penicillin and dihydrostreptomycin in dairy cows following intramammary infusion. *Journal of Dairy Science*, **53,** 1407–1414.

Roncada, P., Tomasi, L., Stracciari, G., Ermini, L. & Strocchia, A. (2000) Milk depletion of dicloxacillin residues in cows and sheep following intramammary administration. *Journal of Veterinary and Pharmacology Therapeutics*, **23,** 237–241.

Sarikaya, H. & Bruckmaier, R. (2006) Importance of the sampled milk fraction for the prediction of total quarter somatic cell count. *Journal of Dairy Science*, **89,** 4246–4250.

Sarikaya, H., Werner-Misof, C., Atzkern, M. & Bruckmaier, R. (2005) Distribution of leucocyte populations, and milk composition, in milk fractions of healthy quarters in dairy cows. *Journal of Dairy Research*, **72,** 486–492.

Sarikaya, H., Schlamberger, G., Meyer, H. & Bruckmaier, R. (2006) Leukocyte populations and mRNA expression of inflammatory factors in quarter milk fractions at different somatic cell score levels in dairy cows. *Journal of Dairy Science*, **89,** 2479–2486.

Saville, W., Wittum, T. & Smith, K. (2000) Association between measures of milk quality and risk of violative antimicrobial residues in grade-A raw milk. *Journal of the American Veterinary Medical Association*, **217,** 541–545.

Sawant, A., Sordillo, L. & Jayarao, B. (2005) A survey on antibiotic usage in dairy herds in Pennsylvania. *Journal of Dairy Science*, **88,** 2991–2999.

Smith, G., Gehring, R., Riviere, J., Yeatts, J. & Baynes, R. (2004) Elimination kinetics of ceftiofur hydrochloride after intramammary administration in lactating dairy cows. *Journal of the American Veterinary Medical Association*, **224,** 1827–1830.

Urech, E., Puhan, Z. & Schällibaum, M. (1999) Changes in milk protein fraction as affected by subclinical mastitis. *Journal of Dairy Science*, **82,** 2402–2411.

Van Eenennaam, A., Cullor, J., Perani, L., Gardner, I., Smith, W., Dellinger, J., Guterbock, W. & Jensen, L. (1993) Evaluation of milk antibiotic residue screening tests in cattle with naturally occurring clinical mastitis. *Journal of Dairy Science*, **76,** 3041–3053.

Vangroenweghe, F., Dosogne, H. & Burvenich, C. (2002) Composition and milk cell characteristics in quarter milk fractions of dairy cows with low cell count. *Veterinary Journal*, **164,** 254–260.

Whittem, T. (1999) Pharmacokinetics and milk discard times of pirlimycin after intramammary infusion: a population approach. *Journal of Veterinary and Pharmacology Therapeutics*, **22,** 41–51.

Wuschko, S., Kunter, U., Schwabe, K.-H. & Kalvelage, H. (1998) Erythromycin in the udder – results of pharmacokinetic study on bioavailability. *Der praktische Tierarzt*, **79,** 1157–1159.

Ziv, G. (1980a) Practical pharmacokinetic aspects of mastitis therapy–1: parenteral treatment. *Veterinary Medicine, Small Animal Clinician*, **75,** 277–290.

Ziv, G. (1980b) Practical pharmacokinetic aspects of mastitis therapy–3: intramammary treatment. *Veterinary Medicine, Small Animal Clinician*, **75,** 657–670.

Ziv, G. & Sulman, F. (1974) Absorption of antibiotics by the bovine udder. *Journal of Dairy Science*, **58,** 1637–1644.