Determination of milk concentrations and pharmacokinetics of salicylic acid following acetylsalicylic acid (aspirin) administration in postpartum dairy cows

B. R. Fritz,[1] ● **M. D. Kleinhenz,[2]*** ● **S. R. Montgomery,[1]** ● **G. Magnin,[1] M. S. Martin,[1]†** ● **M. Weeder,[2]**
A. K. Curtis,[1] ● **and J. F. Coetzee[1]** ●
[1]Department of Anatomy & Physiology, College of Veterinary Medicine, Kansas State University, Manhattan 66506
[2]Department of Clinical Sciences, College of Veterinary Medicine, Kansas State University, Manhattan 66506

ABSTRACT

The objectives of this descriptive study were to (1) describe the pharmacokinetics of salicylic acid (SA) in the milk and plasma of postpartum dairy cattle following oral administration of acetylsalicylic acid (ASA; aspirin), (2) to estimate a recommended milk withdrawal period for dairy cattle treated with ASA, and (3) to determine the effect of ASA administration on plasma prostaglandin E_2 metabolite (PGEM) concentrations. Primiparous (n = 3) and multiparous (n = 7) postpartum Holstein dairy cows received 2 oral treatments with ASA at 200 mg/kg of body weight, 24 h apart. Concentrations of SA in plasma and milk from 0 h through 120 h after ASA administration were analyzed using ultra performance liquid chromatography triple quadrupole mass spectrometry and a milk withdrawal period was estimated using the United States Food and Drug Administration Milk Discard App in R. Two withdrawal periods were estimated: (1) a whole-herd treatment scenario with no dilution factor and (2) an individual animal treatment scenario with a bulk tank factor included in analysis. Plasma PGEM concentrations in samples from 0 h to 24 h after ASA administration were determined using a commercially available competitive ELISA. Milk SA concentrations were undetected in all cows by 48 h after the last ASA treatment. Secondary peaks were observed in plasma at 58 and 82 h after the last treatment and in milk at 87 h after the last treatment. In the absence of a tolerance for SA in milk, the estimated milk withdrawal periods were (1) 156 h for the whole-herd treatment scenario and (2) 120 h for the individual animal treatment scenario. Plasma PGEM concentrations were reduced compared with baseline for up to 12 h after ASA administration, with the greatest reduction observed at 2 h. Results from this study suggest that the current milk withhold recommendation for dairy cattle administered ASA may need revision to 120 h (5 d) and that ASA administration may mitigate postpartum inflammation through reduction in prostaglandin production for up to 12 h after treatment. Pharmacokinetic and milk withdrawal data from this study will inform future recommendations for extra-label use of aspirin in postpartum dairy cows. Further research is required to determine the basis for the secondary SA peaks and to elucidate the long-term effects of ASA administration on dairy cow health.

Key words: acetylsalicylic acid, nonsteroidal anti-inflammatory drugs, postpartum, pharmacokinetics

INTRODUCTION

Administration of nonsteroidal anti-inflammatory drugs (**NSAID**) at calving may reduce inflammation in the transition period and the associated negative effect on milk production (Farney et al., 2013a). Previous work has shown that periparturient administration of sodium salicylate (**SS**), acetylsalicylic acid (**ASA**), meloxicam, ketoprofen, or carprofen increases milk yield (Farney et al., 2013b; Stilwell et al., 2014; Carpenter et al., 2016; Kovacevic et al., 2018; Shock et al., 2018; Barragan et al. 2020a). Reported benefits of NSAID administration include decreased SCC, decreased culling rates, increased activity, and increased feeding behavior (Mainau et al., 2014; Stilwell et al., 2014; Shock et al., 2018).

The use of the NSAID, SS, and ASA (aspirin) has also been described in postpartum dairy cows (Farney et al., 2013b; Carpenter et al., 2016; Barragan et al. 2020a). Acetylsalicylic acid and its active metabolite, salicylic acid (**SA**), inhibit cyclooxygenase-1 and -2, reduce tumor necrosis factor-α (TNF-α) mRNA transcription, and prevent nuclear factor kappa B activation

by binding to the protein inhibitor of nuclear factor kappa B kinase subunit beta (Mitchell et al., 1993; Yin et al., 1998; Myers et al., 2010). Acetylsalicylic acid is not currently approved in the United States for use in lactating dairy cattle (Smith et al., 2008). Many authors have reported increased milk yields after treating postpartum, multiparous cows with ASA, similar to the effects seen with other NSAID (Trevisi and Bertoni, 2008; Farney et al., 2013b; Barragan et al., 2020a). A recent study found that cows treated with ASA had a significantly lower incidence of metritis and a tendency toward lower rates of endometritis (Barragan et al., 2021). The authors also reported a tendency in the ASA group to faster conception rates, a lower incidence of pregnancy losses, and increased conception rates following pregnancy loss compared with untreated cattle.

Although the potential benefits of ASA administration are attractive to producers, milk residues need to be investigated. Currently, no published pharmacokinetic (**PK**) data are on SA in milk. Though PK data are available for serum salicylate concentrations (Gingerich et al., 1975; Anderson et al., 1979; Coetzee et al., 2007; Kotschwar et al., 2009; Baldridge et al., 2011; Bergamasco et al., 2011), the treatment regimens used were unlike those described in more recent studies. Some of these earlier studies investigated the use of SS through either a single intravenous administration at 50 mg/kg of BW (Coetzee et al., 2007; Kotschwar et al., 2009; Bergamasco et al., 2011) or oral administration through drinking water for 5 d at doses ranging from 13.6–152 mg/kg of BW (Baldridge et al., 2011). Two studies investigated oral administration of ASA, either at 50 mg/kg of BW for one treatment (Coetzee et al., 2007) or at 100 mg/kg of BW per treatment every 12 h for 5 d (Gingerich et al., 1975). More recently, Barragan et al. (2020a,b, 2021) reported that a short course of ASA treatment—200 mg/kg every 24 h for 2 d—has positive effects on milk production and cow metabolic and reproductive well-being. This revised treatment protocol may be more practical and, thus, more likely to be adopted by researchers and producers for use in postpartum cows. In addition to the updated dosing regimen, the improved quality and sensitivity of analytical equipment make PK data gathered today more accurate. Drug concentration data in plasma and milk are needed to establish the pharmacokinetic profile of SA, to recommend milk withhold times, and to inform future approval decisions and regulations regarding milk withholding periods for lactating dairy cattle. Supplying producers with appropriate, well-founded data on milk residues is critical to maintaining consumer trust and food supply integrity. Minimal pharmacodynamic data are available for dairy cattle following oral administration of ASA. Determining the short- and long-term effects of ASA on dairy cow health would provide producers with information necessary to form optimal postpartum cow health management programs.

The objectives of this study were to (1) describe the pharmacokinetics of SA in the milk and plasma of postpartum dairy cattle following oral administration of aspirin, (2) to establish a recommended milk withdrawal interval (**WDI**) for dairy cattle treated with ASA at 200 mg/kg twice, 24 h apart, and (3) to determine the effect of ASA administration on plasma concentrations of PGE_2 metabolites (**PGEM**) compared with baseline PGEM values.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Kansas State University approved the methods outlined below (protocol number 4432).

Animals, Housing, and Treatments

In this descriptive study, postpartum Holstein cattle (*Bos taurus*; n = 10; 3 primiparous, 7 multiparous) of an average weight of 662 kg (554–859 kg) from the Kansas State University Dairy Teaching and Research Center were enrolled between May 2021 until July 2021, within 3 to 27 h after calving. The Dairy Teaching and Research Center houses approximately 300 cows. A common, straw-bedded pen was used to house cows that were within approximately 30 d of calving. To be eligible for enrollment, cows were required to be free of illness; have a lameness score of ≤2 out of 5 using a visual lameness scoring system (Sprecher et al., 1997); have a dystocia score of 1 out of 5 (Barragan et al., 2020c); and have no history of receiving ASA or other NSAID in the past 30 d. If any cows developed lameness or illness or required other medications during the study period, they were unenrolled. Throughout the enrollment period for each cow, body temperature was measured at least once per day using rectal thermometry and urine ketones were analyzed once per day with ketone reagent strips supplied by the Kansas State Dairy Teaching and Research Center. Farm personnel administered a single bolus of BoviKalc (Boehringer Ingelheim Vetmedica) to cows at the time of calving. Eligible cows were enrolled following the morning milking. Before enrollment, cows were weighed. Following enrollment, cows were moved to an open-sided, freestall barn bedded with sand, along with other postpartum cows. Cows were milked at 0700, 1700, and 2200 h and fed TMR once daily in the morning. The diet was formulated to meet or exceed nutrient requirements for high-producing lactating dairy cattle (NRC, 2001). Cows were provided water ad libitum.

Cattle received oral ASA treatments at enrollment and 24 h later (following the 0700 h milking). A bolus gun was used to administer 3 to 5 boluses of 480 grain (31,104 mg/bolus) ASA (MWI Animal Health) to achieve the target dose of 200 mg/kg of BW. Milk and whole blood were obtained at each time point for SA concentration determination.

Time points for milk collection were before ASA administration (0 h) and 10, 15, 24, 34, 39, 48, 58, 63, 72, 82, 87, 96, 106, 111, and 120 h after ASA administration. Each milk collection time point occurred when cows were scheduled to enter the milking parlor. Cows were milked into floor pails so the entire milking was obtained. Following milking, the milk was well stirred and a milk sample was collected in sample vials (Thermo Fisher Scientific). The milk samples were immediately placed on ice and transported to the laboratory for storage at −20°C until analysis.

Time points for blood collection were before ASA administration and 2, 6, 10, 24, 34, 48, 58, 72, 82, 96, 106, and 120 h after ASA administration. At each collection time point, cows were restrained in a chute or head-lock, and 6 mL of whole blood was obtained by jugular or coccygeal venipuncture using a 1.5 in 18- or 20-gauge needle. Blood samples were collected using sterile 6-mL lithium heparin-coated tubes (Vacuette, Greiner Bio-One). Blood was immediately placed on ice, transported to the laboratory, and centrifuged (4,000 × g for 10 min at 4°C). Plasma was harvested and stored at −80°C until analysis.

Plasma and Milk Salicylic Acid Analysis

Salicylic acid concentrations in milk and plasma samples were determined using ultra performance liquid chromatography triple quadrupole MS (UPLC-MS/MS). Salicylic acid (analytical standard) and salicylic acid-d4 (SA-d4; internal standard, Cerilliant) were purchased from Sigma-Aldrich Inc. Ultrapure water (18.2 MΩ-cm) was obtained from an on-site system (Thermo Fisher Scientific). Optima LC/MS grade acetonitrile, ammonium formate, and ammonium hydroxide, and HPLC grade 85% phosphoric acid were obtained from Thermo Fisher Scientific. The LC/MS and HPLC grade methanol were obtained from Honeywell (Burdick and Jackson). Formic acid (MS standard) was obtained from Waters Corp. Blank filtered lithium heparin bovine plasma (negative control plasma; **NCP**) was obtained from Lampire Biological Laboratories Inc. Negative control milk was obtained from one of the animals enrolled in the study before ASA administration.

Before sample preparation, plasma samples from 2, 6, 10, and 34 h were diluted 500-fold, and samples from 24 and 48 h were diluted 100-fold with NCP; the remaining plasma samples were prepared undiluted. The lipid layers from the milk samples in the collection vials were removed, and 1-mL aliquots from each milk sample were centrifuged at 1,500 × g at 4°C for 10 min. After centrifugation, the remaining lipid layers were removed and the milk samples were vortexed.

Salicylic acid stock solution was prepared at 1,000 µg/mL in methanol and stored at −20°C. Salicylic acid-d4 100 µg/mL stock solution was stored in a glass vial (Waters Corp.) at −20°C. A 100 µg/mL working stock solution of SA was prepared daily by diluting the 1,000 µg/mL stock in 4% phosphoric acid. A 0.050 µg/mL working solution of SA-d4 was prepared daily by diluting the 100 µg/mL stock in 4% phosphoric acid. Working solutions for SA calibration standards (**STC**) and quality controls (**QC**) were prepared fresh daily in NCP or negative control milk. The STC were prepared at 8 concentrations (0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 2.500, and 5.000 µg/mL SA) and QC were prepared at 3 concentrations (0.180, 1.800, and 3.600 µg/mL SA).

For the STC, QC, negative controls, and internal standard controls, 100 µL of NCP or negative control milk was aliquoted. To this, for the STC and QC, 10 µL of the appropriate STC or QC stock and 190 µL of 0.05 µg/mL SA-d4 in 4% phosphoric acid were added. To the internal standard controls, 10 µL of 4% phosphoric acid and 190 µL of 0.05 µg/mL SA-d4 in 4% phosphoric acid were added. To the negative controls, 200 µL of 4% phosphoric acid was added. For the samples, 100 µL of plasma or milk was combined with 10 µL of 4% phosphoric acid and 190 µL of 0.05 µg/mL SA-d4 in 4% phosphoric acid. Samples, STC, QC, negative controls, and internal standard controls were vortexed and then centrifuged at 1,500 × g at 4°C for 10 min.

Analytes were extracted via solid phase extraction using Oasis MAX 96-well µElution Plates (30µm; Waters Corp.) and a solid phase extraction positive pressure manifold (Positive Pressure-96 Processor, Waters Corp.). The solid phase extraction cartridges were conditioned with 300 µL methanol followed by 300 µL 18.2 MΩ-cm water. After 300 µL sample, control, STC, or QC solution was loaded into the appropriate wells, the cartridge was washed with 300 µL 5% ammonium hydroxide in 18.2 MΩ-cm water followed by 300 µL methanol. Eluate was collected in a clean collection plate (96-well, 2 mL; Waters Corp.) using 50 µL 2% formic acid in acetonitrile-methanol (60:40) and 50 µL 18.2 MΩ-cm water was added to all collection plate wells. The collection plates were covered with pre-slit silicone cap mats (Waters Corp., Milford, MA) and vortexed gently.

Collection plates were loaded onto an ACQUITY H-Class PLUS UPLC system (Waters Corp.). Chromato-

graphic separation was achieved using an ACQUITY UPLC HSS T3 C18 column (100 × 2.1 mm, 1.8 μm; Waters Corp.) kept at 40°C. The UPLC mobile phases consisted of 0.1% formic acid in 5 mM ammonium formate in water (mobile phase A) and 0.1% formic acid in 5 mM ammonium formate in acetonitrile-water (90:10; mobile phase B). A gradient program was used to achieve analyte separation. After sample injection (0 min), a combination of 99% mobile phase A and 1% mobile phase B was linearly changed to a combination of 0% mobile phase A and 100% mobile phase B until 1.49 min. Mobile phase B was linearly reversed to 1% at 2.0 min, and the original mobile phase mixture was held from 2.01 min to 3 min. The flow rate was 0.6 mL/min and the sample injection volume was 2 μL. The LC effluent was diverted to waste for the first 0.7 min and the last 1.0 min of each chromatographic run.

The mass spectrometer was a Xevo TQ-S tandem mass spectrometer (MS/MS) equipped with a Z-spray electrospray ionization interface set in negative ion mode (Waters Corp.). Data were acquired and processed by MassLynx and TargetLynx software, respectively (Waters Corp.). The quantifying transition for SA was m/z 137.0636→92.8904 and the qualifying transition was m/z 137.0636→64.8659. The quantifying transition for SA-d4 was m/z 141.1081→96.9367. The cone voltage and collision energy for the SA quantifying and qualifying transitions were −42 V/−14 V and −42 V/−22 V, respectively. The parameters for SA-d4 were −44 V/−16 V. The dwell time for all compounds was 3 ms.

The concentrations of the eluted standards were 0.0025, 0.005, 0.010, 0.025, 0.050, 0.100, 0.250, and 0.500 μg/mL. The concentrations of the eluted QC were 0.018, 0.180, and 0.360 μg/mL. The limit of detection (**LOD**) was 0.0025 μg/mL and the lower limit of quantification (**LLOQ**) was 0.0043 μg/mL. The standard curve was linear from 0.0025 to 0.500 μg/mL; the correlation coefficient was accepted if it was at least 0.975. The intra-day accuracy and precision were determined by analyzing replicates of SA at 3 different QC levels. Inter-day accuracy and precision calculated by analyzing the 3 levels of QC samples were determined to be 108.38 and 4.29%, respectively.

Plasma Pharmacokinetic Analysis

Pharmacokinetic analysis based on each cow's plasma concentration time curve was performed using PKSolver (Zhang et al., 2010) in Excel (Microsoft Inc.). Using the semi-logarithmic plots of SA, noncompartmental analysis was performed with uniform weighting based on statistical moment theory. Average peak plasma SA concentration (**C_{max}**) and time to peak concentration (**T_{max}**) were determined. The log-linear portion of the terminal section of the log plasma concentration time curve was used to calculate the average terminal elimination rate constant for plasma (**λ_z**) using a linear regression technique. The average terminal half-life of plasma (**$T_{1/2\lambda z}$**) was determined using the equation $T_{1/2\lambda z} = \ln 2/\lambda_z$. The average area under the concentration time curve from 0 to 120 h (AUC_{0-120h}) was calculated using the linear-log trapezoidal method. To account for total drug exposure, the average area under the plasma concentration time curve and area under the first moment curve were extrapolated from the first measurement to infinite time (**$AUC_{0-\infty}$** and **$AUMC_{0-\infty}$**, respectively). All plasma SA concentrations were above LLOQ. Time versus concentration figures were produced using a commercially available software (GraphPad Prism 9.0).

Estimation of Milk Withdrawal Interval

A milk WDI for SA was estimated using the US FDA Milk Discard App in R (version 1.1.0, R Studio) adapting methods from Smith et al. (2020) and Meira et al. (2022). A WDI was calculated for a whole-herd treatment scenario and an individual animal entering the bulk tank. In the whole-herd treatment scenario, the tolerance in the application was set to the LOD of 0.0025 μg/mL, as we observed no established tolerance for SA in milk in the United States. In the second scenario, a tolerance limit of 0.0075 μg/mL was used for the individual animal WDI, where milk enters a commingled bulk tank. This tolerance limit was 3 times the assay LOD and is based on the allowed correction set forth by the FDA (US FDA CVM, 2018) which assumes that no more than one-third of the milk in a bulk tank will come from treated cows. To satisfy linearity and homoscedasticity assumptions, certain time points were excluded from the analysis per the US FDA guidelines (Smith et al., 2020). For cows 1, 4, and 9, time points 10, 15, 24, and 34 h after the second ASA dose were used. For cow 2, time points 10, 15, 24, and 39 h after the second ASA dose were used. For cows 3, 7, and 10, time points 10, 15, and 24 h after the second ASA dose were used. For cows 5, 6, and 8, time points 10, 15, and 87 h after the second ASA dose were used.

The application was set to estimate a milk WDI using the methods described in the US FDA Guidance for Industry #3: General Principles for Evaluation the Human Food Safety of New Animal Drugs Used in Food-Producing Animals (US FDA CVM, 2018). A 99th percentile tolerance limit with a 95% confidence was used. The US FDA Milk Discard App requires

a minimum of 10 animals with samples analyzed in triplicate. To satisfy this requirement, Monte Carlo simulation was performed in Excel using the mean concentrations and the standard deviations from each time point to generate 2 replicate values. Samples with concentrations above the LOD were used in the model, including 2 samples that had concentrations between the LOD and LLOQ. Six samples had concentrations below the LOD; these were not included in the milk withdrawal analysis or when calculating concentration means and standard deviations for Monte Carlo simulation. The concentrations of these 6 samples were recorded as 0 µg/mL.

Prostaglandin E$_2$ Metabolite Analysis

Plasma PGEM concentrations were determined using a commercially available competitive ELISA (Prostaglandin E$_2$ Metabolite ELISA Kit; Cayman Chemical). The protocol supplied by the manufacturer was followed except for the following modifications: (1) during sample purification, samples were centrifuged for 5 min at 3,000 × g and 4°C; (2) 300-µL sample volume and corresponding buffer volumes were used for derivatization; (3) the ethyl acetate extraction step was not performed. The inter-assay CV and intra-assay CV were 9.26 and 18.7%, respectively. The average LLOQ was 1.80 pg/mL. Data were analyzed using a commercially available data analysis tool (MyAssays Desktop). Average PGEM concentrations, average percent changes in PGEM concentrations from the baseline, and the associated standard deviations were calculated using Microsoft Excel. The 80% inhibition concentration of plasma SA was determined using a nonlinear regression technique (GraphPad Prism 9.0).
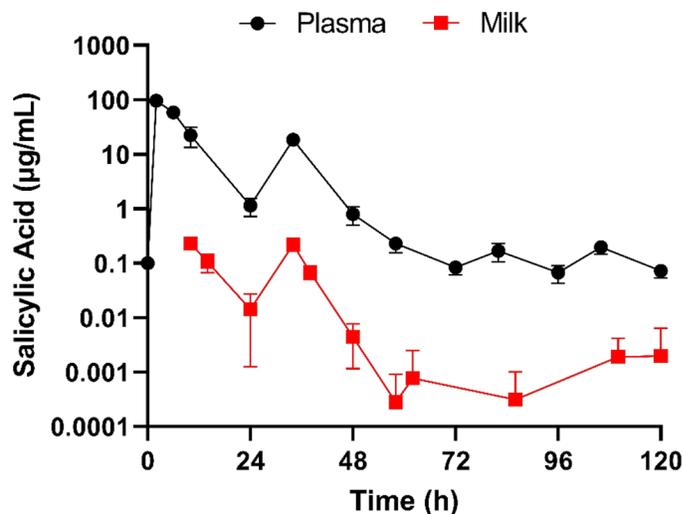

Figure 1. Log-transformed average salicylic acid (SA) concentrations in plasma and milk in 10 postpartum Holstein dairy cattle following oral administration of acetylsalicylic acid at 200 mg/kg of BW. Acetylsalicylic acid was administered after the 0- and 24-h time points. Due to the logarithmic scale, no average concentration icon is shown for time points at which the average concentration was 0 µg/mL. Error bars represent 95% confidence intervals. Error bars were not shown if they were smaller than the average concentration icon or had a value of zero. Data points below the limit of detection were not included. No SA was detected in any milk samples at 0, 72, 82, 96, and 106 h.

RESULTS

No adverse events were noted in any of the cows throughout the study period.

The log-transformed mean values for milk and plasma SA concentrations versus time are shown in Figure 1; plasma SA $T_{1/2\lambda z}$ is reported as the harmonic mean. Pharmacokinetic parameters are summarized in Table 1.

Table 1. Pharmacokinetic (PK) parameters of salicylic acid in plasma and milk in 10 postpartum Holstein dairy cattle following 2 oral administrations of acetylsalicylic acid at 200 mg/kg of BW[1]

Item[2]	Plasma	Milk
C$_{max}$ (µg/mL)	96.637 (50.412–139.577)	0.229 (0.158–0.337)
T$_{max}$ (h)	2.4 (2.0–6.0)	
AUC$_{0–120}$ (h × µg/mL)	977.17 (784.56–1,403.51)	
AUC$_{0-\infty}$ (h × µg/mL)	978.38 (786.16–1,403.99)	
AUC % extrapolated	0.13 (0.03–0.25)	
AUMC$_{0-\infty}$ (µg/mL × h^2)	13,211.85 (10,046.87–17,965.85)	
λ_z (per hour)	0.061 (0.054–0.071)	
T$_{1/2\lambda z}$ (h)	11.49 (9.70–12.79)	

[1]Acetylsalicylic acid was administered at 0 and 24 h; plasma and milk were collected through 120 h for PK analysis.

[2]Pharmacokinetic results are presented as the geometric mean (minimum–maximum), except for half-life, which is presented as the harmonic mean (minimum–maximum); C$_{max}$ = maximum plasma concentration; T$_{max}$ = time to C$_{max}$; AUC$_{0–120}$ = area under the curve for the 120 h after the first treatment; AUC$_{0-\infty}$ = AUC extrapolated to infinite time; AUC % = portion of the AUC$_{0-\infty}$ that is extrapolated after the final concentration measurement; AUMC$_{0-\infty}$ = area under the first moment curve extrapolated to infinite time; λ_z = slope of the terminal phase; and T$_{1/2\lambda z}$ = terminal half-life.
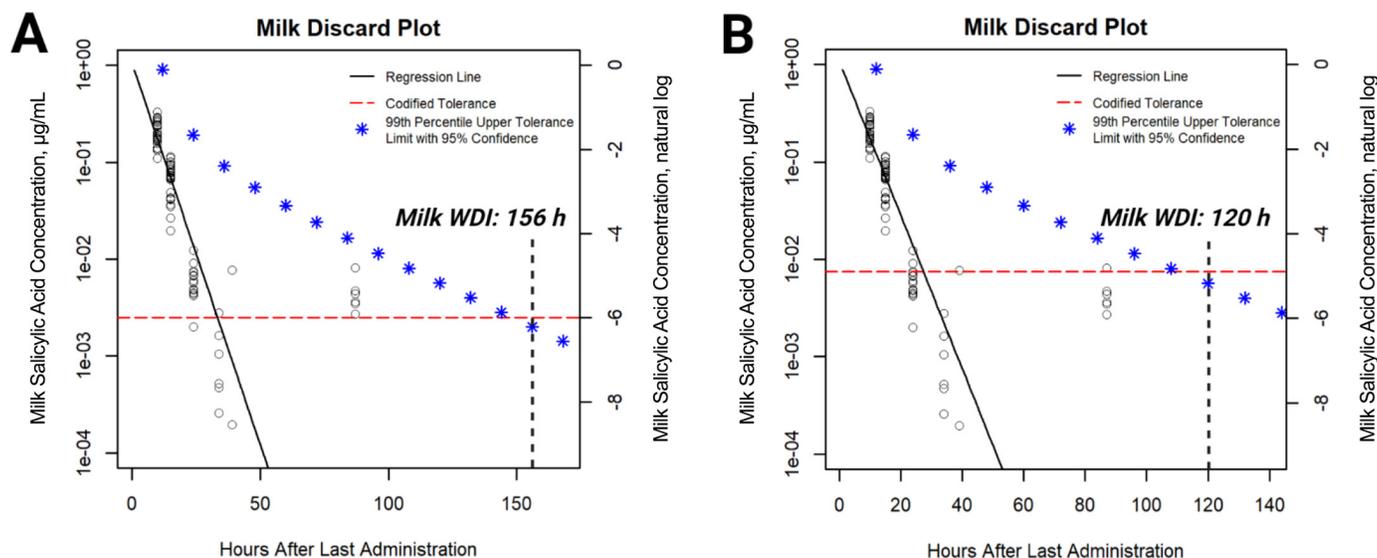
Figure 2. Two milk withdrawal interval (WDI) models for salicylic acid (SA) in postpartum dairy cattle after the last of 2 administrations of acetylsalicylic acid at 200 mg/kg of BW, 24 h apart. Open circles represent actual data points included in the models. Vertical black dotted lines represent the estimated WDI. (A) The first withdrawal calculation was based on a whole-herd treatment scenario where the codified tolerance was equal to the limit of detection of 0.0025 μg/mL for the marker residue SA in milk. The estimated WDI for this scenario was 156 h after the last treatment. (B) The second withdrawal calculation included a 3× correction factor for the limit of detection to account for milk from individually treated cows entering a bulk tank; the codified tolerance was equal to 0.0075 μg/mL SA. The estimated WDI for this scenario was 120 h after the last treatment. Figure created with BioRender.com (version 1.1.0).

Salicylic acid was present in the plasma of all cows at 0 h at an average concentration of 0.1 μg/mL. Following oral administration, plasma SA was present above LLOQ in all cows through 96 h after the last treatment; average SA concentrations were similar to baseline concentrations at 48 h after the last treatment and below baseline concentrations at 72 and 96 h after the last treatment. Secondary SA peaks were observed at 58 and 82 h after the last treatment.

Salicylic acid in the milk of all cows was undetectable at 0 h. Following oral administration, SA was present above the LLOQ in the milk of all cows through 15 h from the last treatment and was undetected by 48 h after the last treatment. A secondary SA peak was observed at 87 h after the last treatment in 3 cows (average concentration across all cows: 0.0019 μg/mL; average concentration for the 3 cows with detectable SA: 0.0063 μg/mL). In one cow, SA was undetected at 34 h after the last treatment but was detected again at low levels at 39 h. The estimated milk WDI using the US FDA application was determined to be 156 h after the last treatment in the whole-herd treatment scenario and 120 h for the second scenario, in which milk from an individually treated cow is diluted in the bulk tank (Figure 2).

Plasma PGEM data are summarized in Table 2; percent changes in PGEM concentrations are shown in Figure 3. The 80% inhibitory concentration (IC_{80}) for plasma SA was determined to be 67 μg/mL.

DISCUSSION

The main findings of the present study were as follows: (1) plasma SA reached an average peak concentra-

Table 2. Plasma prostaglandin E_2 metabolite (PGEM) concentrations in 10 postpartum Holstein dairy cattle following 2 oral administrations of acetylsalicylic acid at 200 mg/kg of BW[1]

	PGEM concentration		Change in PGEM concentration from baseline
Time (h)	Mean ± SD (μ/mL)	Time (h)	Mean ± SD (%)
0	106.11 ± 73.89	0	—
2	35.33 ± 20.37	2	−49.31 ± 54.75
6	57.97 ± 32.32	6	−18.87 ± 84.48
10	84.09 ± 49.99	10	13.03 ± 129.55
24	82.23 ± 56.55	24	12.20 ± 145.86

[1]Acetylsalicylic acid was administered at 0 and 24 h; plasma was collected through 120 h for PGEM analysis.
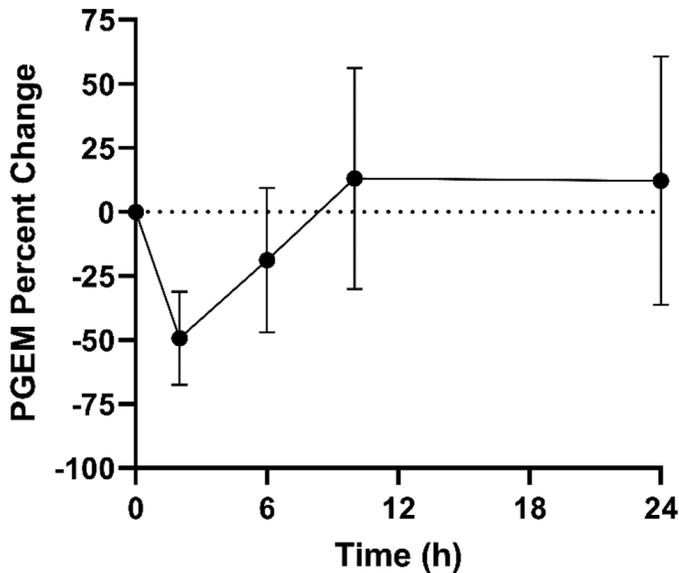
Figure 3. Average percent changes in plasma prostaglandin E_2 metabolite (PGEM) concentrations compared with baseline (0 h) values in 10 postpartum Holstein dairy cattle following the first of 2 oral administrations of acetylsalicylic acid at 200 mg/kg of BW. Error bars represent standard deviations. The dotted line represents no change from baseline.

tion of 96.6 μg/mL at 2.4 h after ASA administration; (2) milk SA reached an average peak concentration of 0.23 μg/mL; (3) milk withdrawal modeling indicated SA levels were below the tolerance limit by 156 h after final ASA treatment, considering whole-herd treatment, and below the tolerance limit by 120 h after the final ASA treatment, considering individually treated animals and a bulk tank factor adjustment; (4) plasma PGEM concentrations were reduced for up to 12 h after ASA administration; (5) the IC_{80} of SA in plasma was 67 μg/mL.

The US Food and Drug Administration, Center for Veterinary Medicine (2015) recommends that 20 dairy animals be used for milk residue studies for establishing WDI for veterinary drugs approved for use in food-producing animals. However, based on the existing literature describing ASA pharmacokinetics in nonlactating dairy cattle, the widespread use of ASA in the dairy industry and in recent research, and the relative safety of ASA (Damian et al., 1997), we elected to enroll 10 cows.

Salicylic acid has been detected in the plasma of cows who have not been treated with ASA, likely due to consumption of forages, which contain salicylates (Gingerich et al., 1975; Anderson et al., 1979). Gingerich et al. (1975) state that salicyluric acid is normally found in the urine and milk of cows. The plasma SA concentrations we observed before ASA administration were lower than previously reported values (Gingerich et al.,

1975), potentially due to differences in diet composition or SA detection methods. Anderson et al. (1979) reported detection of SA before ASA administration, but did not indicate the SA concentration. However, visual comparison of the SA concentration graphs suggests that the baseline SA concentrations detected by Anderson et al. (1979) were lower than those detected by Gingerich et al. (1975). In the study by Gingerich et al. (1975), dietary inclusion of forages with higher salicylate content than the diets in the current study may have been responsible for the higher SA levels detected before ASA administration. Both Gingerich et al. (1975) and Anderson et al. (1979) used a method of extraction and spectrophotometry to quantify plasma salicylate and were able to detect concentrations at approximately 5 μg/mL or greater. Delayed analysis (MacDonald et al., 1965) or the presence of interfering substances in the samples could have altered the values reported by Gingerich et al. (1975), but these possibilities cannot be confirmed. The method employed by Gingerich et al. (1975) and Anderson et al. (1979) was likely much less sensitive than the method implemented in the current study and therefore may not have been able to detect SA at the levels reported in this study. In the present study, ultra performance liquid chromatography triple quadrupole MS (UPLC-MS/MS) was used to quantify plasma SA; the LOD and LLOQ were 0.0025 μg/mL and 0.0043 μg/mL, respectively. Improving assay sensitivity is important for providing more accurate drug pharmacokinetic profiles, which can inform decisions such as drug dosing protocols and withdrawal recommendations.

The secondary peaks observed in milk and plasma may be due to environmental contamination by urine or feces containing SA, consumption of salicylate-containing forages, or both (Gingerich et al., 1975; Anderson et al., 1979; Bates et al., 2020). Considering that the secondary peaks in plasma were consistently observed at the evening milkings—after the morning feeding time—they are perhaps more likely due to forage consumption than environmental contamination and subsequent drug transfer. Even though drug transfer has been reported for other NSAID in swine and horses (Popot et al., 2007; Hairgrove et al., 2019; Bates et al., 2020), acetylsalicylic acid resulting in detectable plasma or milk SA concentrations in dairy cattle has no drug transfer data. Anderson et al. (1979) did report urine salicylate concentrations in cattle for 84 h following ASA administration, so it is possible that urinary contamination of the environment resulted in the observed rebound SA concentrations. This would help explain the relatively high rebound milk SA levels at 96 h after the last treatment. The smaller secondary peaks we observed in milk at 39 and 87 h after the last treat-

Table 3. Comparison of the plasma pharmacokinetic parameters of salicylate between the present study and 6 previous studies

Study	Item[1]			
	C_{max} (µg/mL)	T_{max} (h)	$AUC_{0-\infty}$ (h x µg/mL)	$T_{1/2 \: \lambda z}$ (h)
Current study[2]	96.637 (50.412–139.577)	2.4 (2.0–6.0)	978.38 (786.16–1,403.99)	11.49 (9.70–12.79)
Gingerich et al., 1975[3]	63	2–4	N/A	3.7 ± 0.44
Anderson et al., 1979[4]	117	2–8	N/A	5.8
Coetzee et al., 2007[5]	200–230	N/A	219.30 ± 18.23	0.63 ± 0.04
Coetzee et al., 2007[6]	≤10	N/A	N/A	N/A
Kotschwar et al., 2009[7]	226.9 ± 4.9	N/A	201.65 ± 8.50	0.62 ± 0.02
Baldridge et al., 2011[8]	61.134 ± 10.312	41.7	82.05 ± 14.27	N/A
Bergamasco et al., 2011[9]	198.720 ± 8.220	N/A	192.73 ± 21.22	0.68 ± 0.08

[1]C_{max} = maximum plasma concentration; T_{max} = time to C_{max}; $AUC_{0-\infty}$ = area under the curve extrapolated to infinite time; $T_{1/2 \: \lambda z}$ = terminal half-life; N/A = not applicable.

[2]Aspirin (acetylsalicylic acid) administered at 200 mg/kg of BW at 0 and 24 h. Pharmacokinetic results are presented as the geometric mean (minimum–maximum), except for half-life, which is presented as the harmonic mean (minimum–maximum).

[3]C_{max} value is based on results from the multiple dose oral aspirin experiment (100 mg/kg of BW every 12 h for 5 d). This value was visually estimated from Figure 5 in Gingerich et al. (1975). The T_{max} and $T_{1/2}$ values are based on results from the single-dose oral aspirin experiment (100 mg/kg of BW). Standard deviation is listed with $T_{1/2}$ value. Salicylate concentrations determined using an extraction and spectrophotometry protocol.

[4]Sodium salicylate (SS) administered orally at 100 mg/kg of BW at 0, 12, 25, and 36 h. C_{max} value was visually estimated from Figure 1 in Anderson et al. (1979). Salicylate concentrations determined using an extraction and spectrophotometry protocol.

[5]SS administered intravenously at 50 mg/kg of BW immediately before castration. Results are presented as mean ± SE, except C_{max}, which was estimated visually from Figure 1 in Coetzee et al. (2007). Salicylate concentrations determined using fluorescence polarization immunoassay.

[6]Aspirin (acetylsalicylic acid) administered orally at 50 mg/kg of BW immediately before castration. Results were extracted from the discussion.

[7]SS administered intravenously at 50 mg/kg of BW 4 min after lameness induction. Results are presented as mean ± SE. Salicylate concentrations determined using fluorescence polarization immunoassay.

[8]SS administered for 5 d surrounding the time of castration and dehorning via free-choice consumption of water containing SS (SS concentrations in water: 2.5–5 mg/mL). Results are presented as mean ± SEM (SEM not reported for T_{max}). Salicylate concentrations determined using fluorescence polarization immunoassay.

[9]SS administered intravenously at 50 mg/kg of BW immediately before castration. Results are presented as mean ± SEM. Salicylate concentrations determined using fluorescence polarization immunoassay.

ment are more easily explained by forage consumption. Future studies could be designed to determine the exact reason for these late secondary SA peaks. This information could guide the actions of regulatory officials if low SA residues are detected in milk samples of cows reportedly meeting ASA withdrawal recommendations.

A comparison of plasma salicylate PK profiles in 7 publications is presented in Table 3. These previous studies investigated the PK profiles of SS following a variety of treatment regimens and used different quantification methods than in the present study. The 2 earliest publications (Gingerich et al., 1975; Anderson et al., 1979) used extraction and spectrophotometry to determine salicylate concentrations, whereas some later studies have used fluorescence polarization immunoassay (Coetzee et al., 2007; Kotschwar et al., 2009; Baldridge et al., 2011; Bergamasco et al., 2011). These methods of determining SA concentrations have higher LOD than the method described in this study; the results reported by Gingerich et al. (1975) suggest that the extraction protocol had a sensitivity around 5 µg/mL, which was also the LOD of the fluorescence polarization immunoassay used in the later studies. The low LOD of the method used in the present study improves the resolution and utility of the PK data. In the previ-

ous studies describing the PK profile of salicylate, the drug administered was often different (SS versus SA), doses administered were smaller (50 or 100 mg/kg of BW), or the doses were administered via a different route (e.g., intravenous, free-choice through the water) than in the present study. The more recent studies investigating the effects of aspirin (Farney et al., 2013a,b; Carpenter et al., 2016; Barragan et al., 2020a,b,c, 2021) have not described PK profiles of SA.

The greater average C_{max} reported here compared with that reported by Gingerich et al. (1975) may be appropriate, considering the higher target dose used in this study. However, the multiple dose aspirin experiment conducted by Gingerich et al. (1975) had minimal blood sampling times, so the peak concentration values presented in the figures may not be an accurate representation of the true plasma salicylate C_{max}. In a study conducted by Anderson et al. (1979), 3 nonlactating female Holstein cows were administered SA orally at 100 mg/kg of BW at 0, 12, 25, and 36 h; the plasma salicylate C_{max} value was similar to the values reported in this study. Considering the similarity between treatment regimens in the study conducted by Gingerich et al. (1975) and Anderson et al. (1979), it is likely that blood collection in the study by Gingerich et al.

(1975) was not properly timed to observe the plasma salicylate peaks, and a lower average C_{max} was thus observed. Baldridge et al. (2011) reported a lower mean C_{max} value than that reported here, which is most likely due to differences in dosage route: free-choice SA was administered in water at a concentration between 2.5 and 5 mg/mL, with doses ranging from 13.62 to 151.99 mg/kg. The higher C_{max} values reported by Coetzee et al. (2007; intravenous SS group), Kotschwar et al. (2009), and Bergamasco et al. (2011) are reasonable, considering that SS was administered intravenously. In Holstein calves administered oral aspirin at 50 mg/kg of BW immediately before castration, plasma salicylate levels did not exceed 10 µg/mL (Coetzee et al., 2007). Overall, the differences in C_{max} among the different studies likely reflect the variation in dosing and the timing of sample collection. Although AUC is a better indicator of drug exposure than C_{max}, it was less frequently reported. Future research should focus on reporting AUC values to more accurately compare drug exposure differences among studies.

The T_{max} reported in this study is similar to the values reported by Gingerich et al. (1975) and Anderson et al. (1979); any numerical differences are likely due to random variation or different sampling times. The T_{max} reported by Baldridge et al. (2011) is significantly longer, which may be explained by the dosage route (free-choice through the water versus via oral bolus in the present study). Because calves were self-medicating, the timing of peak water intake may have artificially increased T_{max}.

A summary by the Center for Veterinary Medical Products (CVMP, 1999) reported an absorption half-life of 2.9 h for cattle administered oral aspirin (20 to 100 mg/kg of BW); in the same report, following intravenous administration of ASA DL-lysine to cattle at 90 mg/kg of BW, the $T_{1/2\lambda z}$ was found to be 36.5 min. Comparing the values in Table 3, the $T_{1/2\lambda z}$ reported in the studies evaluating oral administration of ASA or SS are considerably longer than the $T_{1/2\lambda z}$ of salicylate following intravenous administration of SS. The relatively slow absorption of aspirin from the rumen—the absorption half-life is approximately 3 h (Gingerich et al., 1975; Anderson et al., 1979; CVMP, 1999)—is one potential reason for the prolonged $T_{1/2\lambda z}$ in these studies. Even though a flip-flop phenomenon is possible (the rate of absorption, rather than the rate of elimination, is the limiting step in final drug elimination; Toutain and Bousquet-Mélou, 2004b), this does not fully explain the greatly extended $T_{1/2\lambda z}$ we observed in the present study. We did observe a much higher $T_{1/2\lambda z}$ in the present study than was reported by Gingerich et al. (1975) or Anderson et al. (1979). Because the dose used in the present study was double the dose used in the

earlier studies, it is possible that a metabolism pathway was saturated and we thus observed a longer $T_{1/2\lambda z}$. (Salicylate and SA are primarily metabolized through glucuronidation pathways in the liver; CVMP, 1999.) However, the authors feel two other explanations for the extended $T_{1/2\lambda z}$ are more robust. First, improved assay sensitivity may have allowed detection of a secondary terminal phase in the present study. Toutain and Bousquet-Mélou (2004b) have shown that terminal half-life is heavily influenced by assay sensitivity; as assay sensitivity improves, terminal half-life will appear to increase due to detection of late terminal phases. Second, decrease in drug clearance due to altered postpartum physiology may have increased $T_{1/2\lambda z}$. In a comparison of the pharmacokinetic profiles of meloxicam in postpartum and mid-lactation cows, Gorden et al. (2018) reported that C_{max}, T_{max}, and mean residence time were increased and clearance was decreased in postpartum cows; the reduction in clearance resulted in an increase in drug exposure and relative bioavailability. In addition, the authors detected higher levels of meloxicam in milk in postpartum cows compared with mid-lactation cows at all time points. However, they did not detect any difference in $T_{1/2\lambda z}$. Furthermore, Warner et al. (2020) reported similar findings in a study evaluating the pharmacokinetics of both intravenous and oral meloxicam in postpartum versus mid-lactation cows. In this study, the authors also reported a significantly longer $T_{1/2\lambda z}$ in the postpartum cows. The authors postulated that these clearance differences could have been due to changes in plasma protein binding or reduced liver enzyme levels in postpartum cows. Considering this evidence for change in NSAID clearance in postpartum cows, it is likely that the postpartum cows used in this study had decreased clearance of SA compared with cows involved in previous studies evaluating PK profiles after oral ASA administration. Because we did not administer ASA intravenously, we were unable to quantify clearance or bioavailability in this study (Toutain and Bousquet-Mélou, 2004a). The bioavailability of oral ASA in dairy cattle has been reported to be 70% (Gingerich et al., 1975). The present study was also not designed to measure changes in plasma protein, protein binding, or liver enzymes. Further research investigating changes in plasma protein-drug interactions and metabolic enzyme expression in the liver could determine the differences in NSAID pharmacokinetics in postpartum dairy cows compared with cows at other lactation stages. Such research would be useful in optimizing NSAID treatment protocols for cows in different production stages. In summary, increases in assay sensitivity and postpartum decreases in drug clearance in the present study are likely responsible for the longer $T_{1/2\lambda z}$ observed. Future research is needed to compare

clearance and bioavailability of SA in postpartum and mid-lactation cows following oral ASA administration.

No data are currently available on SA in the milk of postpartum dairy cattle, so the data reported here are novel. Postpartum cows were specifically selected as the study population based on recent efforts by Barragan et al. (2020a,b,c, 2021) that describe positive benefits of ASA given at 200 mg/kg in the postpartum period. The use of ASA for an anti-inflammatory indication following parturition would constitute an extra-label drug use (**ELDU**). Under the guidance of the Animal Medicinal Drug Use Clarification Act, the prescribing veterinarian would be responsible for determining an appropriate WDI (US FDA, 1996). We determined the C_{max} of SA in milk to be 0.23 µg/mL. Our findings suggest that a milk withhold period of 120 h (5 d) would be most appropriate for cattle in the immediate postpartum period to meet US FDA tolerance guidelines for unapproved drugs. Our description of the PK and milk residue profiles of SA in lactating dairy cows will help producers make informed treatment decisions and ensure food supply safety. Though our data can inform withdrawal decisions, the methods employed in this study are not practical for field use due to their time-intensive nature. To facilitate SA residue detection in milk, future research should focus on developing a rapid assay. Although aspirin is considered to be of low regulatory concern, a 24-h milk WDI is currently recommended based on potential risk for individuals with Reye's syndrome (Damian et al., 1997). However, the risk of Reye's syndrome is correlated with exposure to high doses of sodium acetylsalicylate, which are unlikely to be obtained through consumption of residues in milk (CVMP, 1999).

When estimating milk WDI, we included all data above the assay LOD. This decision was based on recent publications describing the issues with defining LLOQ and excluding data that fall below that level (Jelliffe et al., 2015; Woodward and Whittem, 2019). In general, assay LLOQ is determined based on an arbitrary threshold for sample variation (CV%; often set at >20%); when measurements lie below this threshold, they are often discarded form the analysis (Jelliffe et al., 2015). However, CV% increases as measurements approach zero, so the establishment of an LLOQ based on this information is erroneous and may exclude valuable data (Jusko, 2012; Jelliffe et al., 2015). Woodward and Whittem (2019) proposed 3 options for handling data below LLOQ: (1) discard data below LLOQ, (2) censor data using maximum likelihood estimates, and (3) use data below LLOQ without adjustment. Keizer et al. (2015) reported improved model performance and precision and decreased bias in population PK analyses when using all data above LOD compared with other

methods of censoring data below LLOQ. This effect was more pronounced when the proportion of data below LLOQ was larger. In the present study, only 2 milk samples were below LLOQ and above LOD. Compared with a model that excluded data below LLOQ, our final model that included all data above LOD resulted in a longer bulk tank factor WDI (120 vs. 108 h) and a shorter whole-herd WDI (156 vs. 168 h). Two other possible combinations of time points were able to be modeled, but these models had a poorer fit and were thus not selected over the final model.

Prostaglandin E_2, or PGEM, concentrations in postpartum dairy cattle have been minimally reported in the literature. Farney et al. (2013a) reported an elevation in the total concentration of plasma eicosanoids after cessation of oral treatment with sodium salicylate in drinking water, but they did not provide reports on all individual eicosanoids evaluated. prostaglandin E_2 as a primary mediatory of inflammation and pain (Myers et al., 2010), is of particular interest in pharmacodynamic analysis. We observed reduction in PGEM concentrations at plasma SA levels of approximately 700 and 425 μM at 2 and 6 h, respectively. Myers et al. (2010) reported that 300 μM of aspirin resulted in reduction in prostaglandin E_2 (Myers et al., 2010). The discrepancy between our results and those of Myers et al. (2010) can be explained by the weaker inhibition of cyclooxygenase by SS compared with ASA (Mitchell et al., 1993). However, considering the short half-life of ASA in plasma (Gingerich et al., 1975), the active metabolite SA may play a larger role than ASA in inhibiting prostaglandin E_2 production following oral administration of ASA. We observed large standard deviations in both PGEM concentrations and change in PGEM concentrations following ASA administration. Thus, although we did report at least 6 h of prostaglandin inhibition, this result may not be truly representative of the response of postpartum dairy cattle. Wischral et al. (2001) reported that, compared with cows without retained fetal membranes, those with retained fetal membranes had significantly higher PGEM concentrations at 24, 48, 72, and 120 h before and 12 h after calving and lower PGEM concentrations 1 h after calving. Even though none of the cows in this study had retained fetal membranes, it is possible that other, undetected health events could have similarly altered PGEM concentrations, resulting in the large standard deviations observed. It is also unclear whether the transient reduction in prostaglandin production reported here is biologically significant. Future research should focus on establishing reference ranges for PGEM concentrations in postpartum dairy cattle and determining the long-term effects of ASA on postpartum inflammation and whole-lactation production and health. Research such as that conducted by

Vailati Riboni et al. (2015) analyzing adipose and liver gene expression could help elucidate long-term effects of ASA administration and help determine whether the transient inhibition of prostaglandin production reported here is clinically meaningful.

Literature suggests that IC_{80} is more closely correlated with analgesia than half-maximal inhibitory concentration (IC_{50}; Huntjens et al., 2005); thus, we chose to calculate IC_{80}. The IC_{80} value of 67 μg/mL determined in the present study is more than twice the 30 μg/mL therapeutic level previously reported (Gingerich et al., 1975). Gingerich et al. (1975) stated that they selected the 30 μg/mL therapeutic level based on therapeutic minimum levels in humans, the presumed difficulty of achieving greater levels in cattle, and the alleviation of pain observed in 2 of the animals enrolled in the study. In the previous study (Gingerich et al., 1975), no lactating cows were enrolled and no pharmacodynamic evaluation was performed. Lactating and nonlactating dairy cattle likely exhibit different NSAID PK profiles (Gorden et al., 2018; Warner et al., 2020) and the therapeutic level of SA in lactating dairy cattle may differ from that in other cattle. Our analysis of PGEM concentrations and calculation of IC_{80} is a more objective pharmacodynamic measure than lameness evaluation, which was used by Gingerich et al. (1975). Hence, the therapeutic level we report here may be more accurate and reliable than that previously reported. More comprehensive analyses of the pharmacodynamic effects of NSAID in postpartum dairy cattle are needed to refine treatment recommendations for managing postpartum inflammation.

CONCLUSIONS

The results of the present study suggest that the current 24-h milk withdrawal recommendation for cattle treated with ASA may require revision to 120 h after ASA treatment. Furthermore, these data suggest that ASA administration transiently reduces prostaglandin production for up to 12 h. Given that aspirin is not approved for use in lactating dairy cattle in the United States, producers must consult with a veterinarian and demonstrate a valid veterinarian-client-patient relationship before initiating use of ASA in postpartum dairy cattle. Furthermore, because aspirin is not approved by the FDA, ELDU requires adherence with Animal Medicinal Drug Use Clarification Act, which stipulates that ELDU is permitted only if the well-being of the animal is threatened, and that ELDU for production purposes is strictly prohibited. Further research should focus on determining the etiology of the secondary SA peaks following ASA administration and expounding on the pharmacodynamics of ASA and its long-term

effects on dairy cow health. Evaluating the differences in NSAID pharmacokinetics between postpartum cows and cows at other production stages and determining reference values for PGEM in postpartum dairy cattle may allow for more robust research and refinement of current NSAID treatment protocols.

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ORCIDS

B. R. Fritz ● https://orcid.org/0000-0003-2828-4001

M. D. Kleinhenz ● https://orcid.org/0000-0001-9453-3657

S. R. Montgomery ● https://orcid.org/0000-0002-8194-397X

M. S. Martin ● https://orcid.org/0000-0002-4746-5011

A. K. Curtis ● https://orcid.org/0000-0002-3087-2841

J. F. Coetzee ● https://orcid.org/0000-0003-1802-3991