JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Screening and Confirmatory Analyses of Flunixin in Tissues and Bodily Fluids after Intravenous or Intramuscular Administration to Cull Dairy Cows with or without Lipopolysaccharide Challenge

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ABSTRACT: Twenty cull dairy cows (645 ± 83 kg) were treated with 2.2 mg/kg bw flunixin by intravenous (IV) or intramuscular (IM) administration with, or without, exposure to lipopolysaccharide in a two factor balanced design. The usefulness of screening assays to identify violative flunixin levels in a variety of easily accessible ante-mortem fluids in cattle was explored. Two animals with violative flunixin liver residue and/or violative 5-hydroxy flunixin milk residues were correctly identified by a flunixin liver ELISA screen. Oral fluid did not produce anticipated flunixin concentration profiles using ELISA determination. One cow that had liver and milk violative residues, and one cow that had a milk violation at the prescribed withdrawal period were correctly identified by flunixin milk lateral flow analyses. The ratio of urinary flunixin and 5-hydroxy flunixin may be useful for predicting disruption of metabolism caused by disease or other factors potentially leading to violative liver flunixin residues.

KEYWORDS: *flunixin, ELISA, route of administration, concentration, food safety*

INTRODUCTION

Flunixin is a nonsteroidal anti-inflammatory drug approved for use in cattle by the United States Food and Drug Administration (FDA). The indications for flunixin use include treating pyrexia associated with respiratory disease, endotoxemia, and mastitis, and managing inflammation in endotoxemia. The FDA approved route of administration is intravenous (IV), with a preslaughter withdrawal period of 4 days for cattle as well as a minimum milk discard time of 36 h in lactating dairy cattle.[1]

Observance of the appropriate preslaughter withdrawal period is important to ensure tissue and milk residues are below tolerance concentrations in products intended for human consumption [25 and 125 parts per billion (ppb) flunixin free acid in muscle and liver, respectively; 2 ppb of 5-hydroxy flunixin in milk of cattle[2]]. Although the United States has an excellent record of preventing excessive drug residues in meat products, instances of violative residues with flunixin in cattle do occur. The major source of flunixin violations is with market dairy cows, in which about 58% of the total flunixin violations occur.[3] Deyrup et al.[4] have suggested that the probability of a violative flunixin residue increases in cull dairy cows that appear to be lame, that have mastitis or metritis, and/or that have lesions associated with injection.

Under label specifications, flunixin meglumine is administered to cattle as an intravenous injection of 1.1 to 2.2 mg/kg body weight for up to 3 consecutive days. In practice, it is often difficult to administer flunixin meglumine or other drugs by IV infusion to cattle, especially on a repeated basis. As a consequence, flunixin meglumine is commonly administered through extravascular injection.[5] As such, the Food Animal Residue Avoidance Databank has recommended a meat withdrawal period significantly longer than the labeled withdrawal time of 4 days to avoid violative tissue residue in meat when flunixin meglumine is used in an extra-label manner.[6]

The product label for flunixin indicates that "intramuscular administration has resulted in violative residues in the edible tissues of cattle sent to slaughter".[1] In a crossover study comparing the plasma pharmacokinetics of flunixin after intravenous, intramuscular, and subcutaneous dosing,[7] flunixin terminal half-lives were significantly longer for the extravascular routes of administration, which could indicate a longer withdrawal period would be required. Kinetic studies in beef cattle[8] have shown that rates of plasma flunixin depletion did not differ ($P > 0.05$) after subcutaneous or intravenous administration although the differences between Kissell et al.[7]

Received: October 2, 2015
Revised: November 24, 2015
Accepted: December 6, 2015
Published: December 22, 2015

DOI: 10.1021/acs.jafc.5b04793
J. Agric. Food Chem. 2016, 64, 336−345

and Shelver et al.[8] studies may be due to a larger number of animals used by Kissell et al.[7] and differences in animal production class.

Flunixin violations might also occur after flunixin is given to sick animals, which may exhibit prolonged elimination processes. For example, in endotoxemic rabbits, the half-life of flunixin elimination increased, and its clearance decreased, relative to healthy animals.[9] Effects of disease status on flunixin pharmacokinetics have been modeled in cattle, and it was demonstrated that the withdrawal period can be affected by changes in elimination clearance and volume of distribution.[10] Cows with mastitis were shown to have altered flunixin pharmacokinetics requiring prolonged withdrawal.[11]

The USDA FSIS used a flunixin ELISA test kit to screen bovine liver and muscle samples before incorporating the analyte in its UPLC-MS-MS multiresidue screening method.[12,13] In addition, a flunixin ELISA kidney screening method has been established by US-FDA.[14] Immunoassays offer user-friendly, portable formats suitable for running a large number of samples in parallel, while decreasing the stringent sample cleanup requirements often associated with instrumental analysis. Immunoassays also may allow the on-site screening of drug-treated animals for chemical residues. However, immunoassays can be subject to matrix effects and may cross-react with metabolites.

We hypothesized that ante-mortem fluids might reflect tissue flunixin residues and that residue correlations between tissues and ante-mortem fluids might be useful in identifying violative animals prior to slaughter. In this study, flunixin residues from healthy or LPS challenged cows, dosed intramuscularly or intravenously with the maximum flunixin meglumine label dose (2.2 mg/kg bw), were compared. In addition, the maximum label dosing period of 3 consecutive days was employed and cattle were slaughtered using the label withdrawal period of 4 days (96 h). This report describes the determination of flunixin residues in tissues (liver, kidney, and muscle), milk, and body fluids (plasma, oral fluid, and urine) using screening assays which were subsequently verified by either LC-MS (milk) or LC-MS/MS (plasma, tissue, and urine) analysis. The usefulness of using ante-mortem matrices for predicting violative tissue residues was explored.

MATERIALS AND METHODS

Chemicals and Supplies. Banamine (flunixin meglumine; Schering-Plough; Summit, NJ) solution for injection was obtained from Stockman's supply (West Fargo, ND). Flunixin ELISA kits were purchased from Neogen Corporation (Lansing, MI). Lateral flow test strips (sold as Charm flunixin and beta-lactam combo test, LF-FLUBL) were obtained from Charm Sciences, Inc. (Lawrence, MA). Flunixin USP reference standard was obtained from USP (Rockville, MD) and 5-hydroxy flunixin was purchased from Toronto Research Chemicals (Toronto, Canada). Flunixin-d_3 and 5-hydroxy flunixin-d_3 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Lipopolysaccharide (LPS; endotoxin) purified from *E. coli* 0111:B4 by phenol extraction was purchased from Sigma-Aldrich Corporation (St. Louis, MO). All other reagents were obtained from common chemical suppliers.

Animal Treatment and Sample Collection. Details of animal treatment and sample collections were described by Smith et al.[15] The animal protocol was approved by the North Dakota State University IACUC prior to the purchase of experimental animals. Briefly, cull Holstein dairy cows (400 to 500 kg) were purchased from a commercial dairy producer in east-central North Dakota or the North Dakota State University, Dairy Research Unit (Fargo, ND). Animals had *ad libitum* access to water and a corn-based silage ration and were allowed to adapt to the facilities for a minimum of 7 days prior to treatment administration. Control bodily fluids were collected and tested to ensure no prior exposure of flunixin. Cows were randomly selected to receive an intravenous (IV) infusion of 0.2 μg/kg bw of LPS or an infusion of sterile normal saline (NS) for each trial. Each treatment was delivered to a single cow in five replicate trials for a total of 5 cows per treatment. Approximately 2 h after infusion with either LPS or saline, each cow received 2.2 mg/kg bw of flunixin meglumine by IV infusion or IM injection. Flunixin administration was repeated 24 and 48 h after the first flunixin dose. Oral fluid and urine samples were collected prior to dosing and at 2, 4, 8, 12, and 24 h for day one and day two of dosing. On dosing day 3, oral fluid and urine samples were collected at 2, 4, 8, 12, 24, 36, 48, 60, 72, and 96 h post dosing. Oral fluid was collected into 50 mL conical tubes as described by Chiesa et al.[16] Urine was collected via micturition. Milk was collected twice daily, at approximately 12 h intervals. Blood samples were collected on treatment day 1 at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, and 24 h. Subsequent to dosing on day 2, blood was drawn at 0.5, 1, 2, 4, 8, 12, and 24 h. After dosing on treatment day 3, blood was drawn at 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 60, 72, 84, and 96 h. Plasma was harvested by centrifugation. All samples were stored at −20 °C or colder until analyzed.

At 96 h after the last flunixin administration, cows were slaughtered according to American Veterinary Medicine Association guidelines.[17] Edible tissues (liver, kidney, and skeletal muscle) were collected, diced, pooled, and frozen at −20 °C or less until analyzed.

Instrumental Analyses of Milk, Tissue, and Plasma Samples. Milk flunixin concentrations were measured as described by Kissell et al.[7] Flunixin free acid in skeletal muscle (longissimus), liver, kidney, were determined based on a matrix-matched standard curve with calibration points of 1, 5, 25, 50, 75 ng/g. Details of the tissue samples analyses were described by Smith et al.[15] Plasma sample were analyzed as described by Shelver et al.[8] with a calibration curve of 0.5, 1, 2, 20, 200, 500, 1000, and 2000 ng/mL.

Urine Sample Analyses Using LC-MS/MS. One part of urine with 4 parts of 6N formic acid were mixed and heated at 120 °C for 2 h. The hydrolyzed urine was further subjected to a final dilution of 1:100 in 50% acetonitrile/water (v/v) containing 250 ng/mL each of flunixin-d_3 and 5-hydroxy flunixin-d_3. If flunixin concentration for a given sample was outside the range of the calibration curve, urine aliquots were rediluted at either 1:10 in 50% aqueous acetonitrile or 1:250 using 1:100 control urine as diluent, depending on the results from the original analysis, and reanalyzed.

Quantitative analyses were performed with an ACQUITY UPLC (Waters Corp., Milford, MA) coupled to a Waters triple quadrupole mass spectrometer with a heated electrospray ionization source operated in the positive ionization mode. Data were acquired, processed and quantified using MassLynx 4.1 with TargetLynx systems (Waters Corporation, Milford, MA). Mass spectrometric conditions for flunixin, flunixin-d_3, 5-hydroxy flunixin, or 5-hydroxy flunixin-d_3 were optimized by direct infusion using electrospray ionization in the positive mode to identify the precursor ion, product ions, and the optimum collision energies and cone voltage using AutoTune Wizard with the MassLynx 4.1 software. The column was an ACQUITY UPLC HSS T3 (1.8 μm, 2.1 × 100 mm) maintained at 35 °C. The mobile phase was 0.1% formic acid:acetonitrile (32:68) at 0.4 mL/min. Ions were monitored in the multiple reaction monitoring mode with flunixin m/z 297 → 279, flunixin-d_3 m/z 300 → 282, 5-hydroxy flunixin m/z 313 → 295, and 5-hydroxy flunixin-d_3 m/z 316 → 298 serving as quantified transitions. Flunixin m/z 297 → 264 and m/z 297 → 259, and 5-hydroxy flunixin m/z 313 → 280 and m/z 313 →109 were used as qualifiers. Unknown concentrations were determined by LC-MS/MS with a matrix-matched standard curve with concentrations of 0.5, 1, 2, 20, 200, 1000, 2000 ng/mL using linear calibration with 1/x weighting times the dilution factor. Limits of quantitation (LOQ) were calculated based on standard deviation of the response and the slope[18] and were 0.3 ng/mL for flunixin, 0.4 ng/mL for 5-hydroxy flunixin when the standard curve was made with a 1:100 dilution of urine and 0.3 ng/mL for flunixin and 5-hydroxy flunixin when the standard curve was made with a 1:10 urine curve. Recoveries were

DOI: 10.1021/acs.jafc.5b04793
J. Agric. Food Chem. 2016, 64, 336−345

determined by fortifying flunixin and 5-hydroxy flunixin at 1, 20, and 500 ng/mL into blank urine; percentage recoveries (%RSD) were 98 (8.3), 103 (2.9), 102 (2.2) for flunixin and 95 (6.5), 100 (3.0), 100 (2.3) for 5-hydroxy flunixin at 1, 20, and 500 ng/mL respectively (n = 21, urine 1:100 dilution).

Tissue Sample Analyses Using Plate ELISA. A flunixin rapid screening assay[13] was used to determine flunixin concentrations in liver and skeletal muscle samples. For each sample set, external standard points at 0, 50, and 100 ng/mL were used to establish the inverse relationship between absorbance and concentration. In addition, blank tissue and positive spikes (10 ng/g for muscle and 50 ng/g for liver) were assayed concurrently with the incurred samples. A sample was considered positive when flunixin concentration was ≥10 ng/g for muscle and ≥50 ng/g for liver. Flunixin kidney screenings were conducted according to the FDA-Laboratory Information Bulletin 4246[14] using flunixin fortification levels of 0, 100, 200, and 400 ppb with resulting data fitted with a logit model. The ELISA analyses were independently performed on individual extracts by two analysts on separate days, and the results averaged.

ELISA Procedure for Oral fluid. The flunixin ELISA for analysis of oral fluid utilized a matrix-matched curve diluted 1:5 in 50 mM phosphate buffer, pH 7.4, with calibration points of 0, 0.3, 0.5, 1, 3, 10, 30, 100, and 300 ng/mL. Aliquots of oral fluid samples and controls were diluted 1:5 in 50 mM phosphate buffer. In each well 20 μL of diluted sample, control, or standard were coincubated with 180 μL of enzyme-conjugate solution at room temperature. After 45 min, the plates were washed five times with 350 μL of phosphate buffered saline containing 0.05% Tween 20. Following the plate wash, 150 μL of the single component peroxidase substrate, 3, 3′, 5, 5′-tetramethylbenzidine was added and incubated at room temperature for 30 min. The plates were read at 650 nm (Tecan Ultra 384, Tecan Group Ltd., Austria). Concentrations of flunixin free acid in test samples were computed from the standard curve that was fitted with four parameter logistic equation and adjusted for the dilution factor.

Lateral Flow Immunoassay for Milk, Oral Fluid, and Urine. Lateral flow tests were used for screening raw milk, oral fluid, and urine for the presence of flunixin residues. For each set of analyses, negative control milk and positive controls spiked with 2 ng/mL of 5-hydroxy flunixin were prepared and run concurrently with the incurred samples. Aliquots (300 μL) of milk were pipetted onto the LF-FLUBL (Charm Sciences) test strip and allowed to incubate at 65 °C for 8 min; results were then scored independently by two readers. Inconsistent results between the two scores are reported as "inconclusive" and affected samples were not rerun in order to avoid bias. Urine samples were prediluted 1:10 with raw milk while oral fluid samples were prediluted 1:5 with raw milk before pipetting onto the test strip as described for the milk samples.

RESULTS AND DISCUSSION

Plate ELISA for Kidney, Liver, and Muscle. The correlation between results from the flunixin LC-MS/MS analysis and the flunixin kidney ELISA is shown in Figure 1. With cow 18 included (cow 18 had violative flunixin liver residues), the coefficient of determination between the kidney LC-MS/MS and the ELISA results was 0.98 (Figure 1, panel A); however, with cow 18 excluded, the coefficient of determination between the methods dropped to 0.55 (Figure 1, panel B). Of the 20 cows sampled; 19 had renal flunixin levels less than 50 ng/g, as determined by both ELISA and LC-MS/MS. When cow 18 was excluded (Figure 1B), the slope changed from 1.28 to 1.04, demonstrating the ELISA and LC-MS/MS methods gave similar results, but the coefficient of determination of 0.55 demonstrated considerable scatter. Such results were not surprising, since method FDA LIB-4246[14] was intended to be used as a semiquantitative tool. In addition, immunologically based screening assays can cross-react with metabolites and are frequently subject to matrix interferences

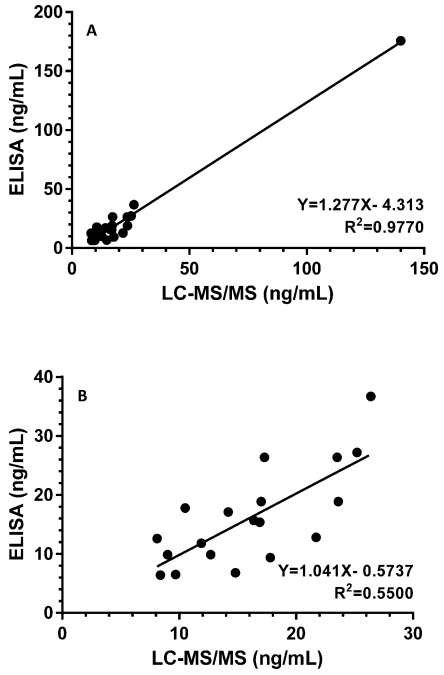

Figure 1. Correlation between kidney flunixin concentrations as determined by ELISA and LC-MS/MS. Panel A includes all animals whereas panel B excludes data derived from cow 18.

that will tend to increase scatter at lower concentrations. The data indicate that, at low concentration, the FDA immunoassay may be expected to return an imprecise estimate of the flunixin residue in kidney; at higher flunixin concentrations, the assay will likely provide a precise estimate of the actual flunixin concentrations in the tissue.

The flunixin screening ELISA procedure from FSIS[12] for liver and muscle flunixin ELISA worked well as a qualitative screening tool with fortified liver and muscle samples repeatedly passing the method's quality assurance plan (data not shown). In addition, the flunixin ELISA, when applied to liver samples, identified 2 cows having flunixin residues exceeding the 50 ng/g positive control liver spike. One of the cows, cow 18, had violative liver flunixin levels (178 ng/g) that were confirmed by LC-MS/MS analysis.[15] The ELISA assay of the liver from cow 1 (in the IV-NS group), which had violative milk 5-hydroxy flunixin residues, indicated a liver flunixin concentration above the 50 ng/g liver positive control threshold, but the LC-MS/MS result (31 ng/g) indicated that the ELISA returned a false positive, possibly due to the presence of cross-reactive metabolites.

Analysis of muscle by flunixin ELISA indicated that none of the 20 cows had muscle concentrations which exceeded the flunixin positive muscle spike of 10 ng/g. The LC-MS/MS analyses of skeletal muscle confirmed the ELISA results.

Taken together, results from the plate ELISA tissue analyses indicate that with simple sample treatment, ELISA results can be used as a rapid screen with cutoff values to predict violative liver residues or to provide semiquantitative results for kidney residues. Within the constraints of the limited number of animals used in this study, comparison of the flunixin liver and muscle ELISA results with those reported using the LC-MS/MS measurements[15] confirmed the ELISA's usefulness in screening out the nonviolative tissue residues. The liver flunixin ELISA correctly identified an animal that had violative residues, but also produced one false positive (5%). The results confirm

DOI: 10.1021/acs.jafc.5b04793
J. Agric. Food Chem. 2016, 64, 336−345

that the liver flunixin ELISA is suitable as a screening method that when judiciously used could reduce the number of samples submitted for labor intensive confirmatory analyses.

Flunixin Oral Fluid Plate ELISA. Analyses of flunixin in oral fluids using ELISA clearly demonstrated that oral fluid did not reflect the expected changes in flunixin concentrations associated with dosing and drug depletion (Figure 2). Given

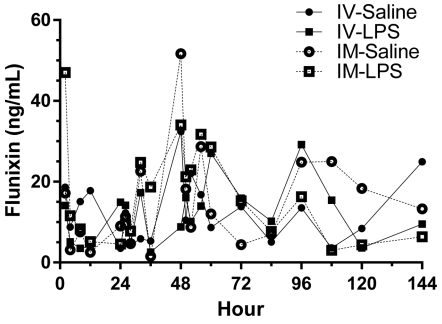

Figure 2. ELISA determination of flunixin concentrations in oral fluids of cows dosed with flunixin meglumine by IV or IM administration, with or without prior LPS infusion (5 cows per treatment). Oral fluids were diluted with 1:5 before measurement by ELISA. Oral fluid flunixin concentrations less than or greater than the calibration dynamic range were omitted from the calculation of mean. Flunixin was dosed at 0, 24, and 48 h.

the seemingly random pattern of flunixin measured in oral fluids over the study period, it is not surprising that there was little to no relationship between flunixin concentrations in oral fluids and tissues at slaughter (i.e., at 96-h withdrawal; data not shown). Although oral fluids have been used to monitor drug exposures, and have been used as a matrix for calculating bioavailability or for determining pharmacokinetic parameters of drugs, particularly in humans,[19] results of this study were not encouraging in those respects. Even though oral fluids are an attractive ante-mortem matrix for drug monitoring because of the ease in which they can be collected, in cattle, the oral fluid output is large and variable and not all drugs are partitioned to salivary gland in a predictable manner. Thus, we must conclude that oral fluids are a poor choice for the ante-mortem monitoring of flunixin residues in cattle. A similar conclusion was drawn by Chiesa et al.[16] with respect to sulfadimethoxine residues, even though their data indicated a fairly consistent pattern between kidney and oral fluid residues.

Lateral Flow Analysis for Milk, Oral Fluid, and Urine. Lateral flow immunoassays provide opportunities for the rapid (often within 10 min) and economical on-site evaluation of chemical residues. Both flunixin and 5-hydroxy flunixin cross-reacted with the antibody employed in the test strip (data not shown). Our results confirm those of Douglas et al.,[20] who reported a test strip sensitivity of 1.9 ng/mL for 5-hydroxy flunixin. However, because of the antibody cross-reactivity, it is possible that either the presence of 5-hydroxy flunixin or parent flunixin or a combination of both could produce a positive result.

Table 1 presents lateral flow immunoassay and quantitative LC-MS results of milk samples collected at withdrawal periods of 36 and 48 h. At the minimum milk-discard time of 36 h, 6 cows had 5-hydroxy flunixin levels exceeding the 2 ng/mL tolerance. With the exception of cow 1, flunixin and 5-hydroxy flunixin levels were lower or unchanged at the 48 h withdrawal in comparison to the 36 h withdrawal. The increase in 5-hydroxyflunixin concentration in milk from cow 1 as the withdrawal increased was probably due to the very low milk yield at the 48 h withdrawal (0.6 kg vs 1.6 kg at 36 h withdrawal)[15]. Because the antibody reacts with 5-hydroxy flunixin and flunixin, their sum from the LC-MS analysis (Table 1) was used to predict the accuracy of lateral flow tests. At the 36-h withdrawal period, a total of 11 cows had summed concentrations of 5-hydroxy flunixin and flunixin greater than 2 ng/mL, as determined by LC-MS analysis. Correspondingly, lateral flow immunoassay of the same milk samples resulted in 12 positives with 7 true positives and 5 false positives. In addition, three false negatives and four ambiguous (conflicts between observers) results occurred. After a 48-h withdrawal, lateral flow immunoassays indicated that cows 1 and 18 had violative milk residues (true positives), but the assays also returned three false positives and two inconclusive results. Collectively, the milk lateral flow assay produced false positives and false negatives but when the sum of flunixin and 5-hydroxy flunixin concentrations were greater than 5 ng/mL positive

Table 1. Comparison of Milk Lateral Flow Results with Those Obtained from LC-MS Analyses of Flunixin and 5-Hydroxy Flunixin (ng/mL)

	Intravenous										Intramuscular									
	Saline					+LPS					Saline					+LPS				
ID	1	6	12	13	19	3	8	10	15	17	2	5	11	14	20	4	7	9	16	18
36-h withdrawal																				
5OHF[a,b]	**19.1**	**2.0**	0.6	0.4	0.7	0.4	1.0	0.5	0.4	**2.8**	0.9	**2.4**	**4.7**	0.9	1.8	1.8	1.3	1.4	0.9	**8.3**
Flunixin	1.9	0.3	0.2	–	0.3	0.6	1.1	0.1	0.3	0.7	0.5	0.2	0.8	0.4	0.6	1.0	1.7	1.3	0.6	10.3
Sum[c]	**21**	**2.3**	0.8	0.4	1.0	1.0	***2.1***	0.6	0.8	**3.5**	1.4	**2.6**	**5.5**	1.3	***2.4***	***2.8***	***3.0***	**2.7**	1.5	**18.6**
Lateral[d]	+	x	x	–	+	+	–	+	+	–	+	+	+	x	+	+	–	+	x	+
48-h withdrawal																				
5OHF	**34.4**	0.5	0.3	0.1	0.1	0.4	0.2	0.2	0.1	0.6	0.4	0.2	1.4	0.3	0.8	0.4	0.4	0.7	0.3	**2.5**
Flunixin	4.1	–	0.1	–	–	1.0	0.1	–	0.1	0.2	0.5	–	0.4	0.1	0.3	0.5	0.5	0.9	0.2	4.6
Sum	**38.5**	0.5	0.4	0.1	0.1	1.4	0.3	0.2	0.2	0.8	0.9	0.2	1.8	0.4	1.1	0.9	0.9	1.6	0.5	**7.1**
Lateral[a]	+	–	x	–	–	+	–	+	–	–	+	–	–	–	x	+	–	–	–	+

[a]Tolerance is 2 ppb for 5-hydroxy flunixin (5 OHF) in milk. [b]Bold face indicates violative residues. [c]Bold face indicates samples that had 5OHF + Flunixin concentrations >2 ppb in addition to those that had 5OHF > 2 ppb. [d]LFA, lateral flow assay. + , –, and X indicated unanimous positive, unanimous negative, and dissimilar scores, respectively, returned by two independent scorers. The lateral flow sensitivity for 5-hydroxy flunixin in milk was 1.9 ppb, as reported by Douglas et al. (2012).

DOI: 10.1021/acs.jafc.5b04793
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Table 2. Comparison of Oral Fluid and Urine Lateral Flow Results to Those Obtained from LC-MS/MS or ELISA of Flunixin Residues (ng/mL) at Withdrawal Periods 72 and 96 h

	Intravenous										Intramuscular									
	Saline					+LPS					Saline					+LPS				
ID	1	6	12	13	19	3	8	10	15	17	2	5	11	14	20	4	7	9	16	18
Oral fluid 72-h withdrawal																				
ELISA[a]	12.2	<min[e]	20.1	0.2	1.2	0.3	0.9	13.5	1.1	2.4	5.5	<min[e]	65.6	2.1	0.1	1.0	2.5	9.4	4.6	165.2
Lateral[b]	−	−	+	−	+	−	−	X[f]	−	−	−	−	X[f]	+	X[f]	−	−	X[f]	+	+
Oral fluid 96-h withdrawal																				
ELISA[a]	66.7	<min[e]	26.1	3.6	1.3	13.6	<min[e]	13.3	<min[e]	1.7	1.6	1.9	44.8	4.7	<min[e]	<min[e]	5.4	16.1	2.1	2.0
Lateral[b]	−	−	+	−	−	−	−	+	−	+	−	−	+	+	−	−	X[f]	X[f]	+	−
Urine 72-h withdrawal																				
LC-MS[c]	381	9	197	8	26	35	14	24	10	29	301	14	416	75	204	106	187	354	84	7516
Lateral[d]	+	+	+	+	+	+	X[f]	+	+	+	+	−	+	+	+	+	+	+	+	+
Urine 96-h withdrawal																				
LC-MS[c]	207	9	117	5	10	19	11	14	7	11	130	17	103	46	61	38	19	168	18	1911
Lateral[d]	+	X[f]	+	+	+	+	+	X[f]	+	+	+	−	+	+	+	+	−	+	+	+

[a]Flunixin oral fluid concentrations were determined by ELISA using a matrix matched calibration curve fitted with a four parameter logistic equation. [b]Oral fluid was diluted with milk 1:5 followed by lateral flow immunoassay. Lateral flow results from scores that nearly completely eliminate the flunixin line (faint line) and that were scored independently by two scores. [c]Flunixin urine concentrations were determined by UPLC-MSMS using a matrix matched calibration curve with flunixin-d_3 as the internal standard and fitted with a linear equation. [d]Urine was diluted with milk 1:10 followed by lateral flow immunoassay. Lateral flow results from scores that nearly completely eliminate the flunixin line (faint line) and that were scored independently by two scores. [e]Concentration was lower than the lowest calibration curve. [f]Indicated no consensus between 2 scorers.

DOI: 10.1021/acs.jafc.5b04793
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Table 3. Flunixin and 5-Hydroxy-flunixin Concentrations (ng/mL) in Urine after Administration of Saline or of LPS to Cows via Intravenous or Intramuscular Routes

	Flunixin (ng/mL)[a]								5-hydroxy-flunixin (ng/mL)[a]							
	IV-Saline		IV-LPS		IM-Saline		IM-LPS		IV-Saline		IV-LPS		IM-Saline		IM-LPS	
Time (h)	Mean ± SD	n[b]	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
T2	$1.0 \times 10^5 \pm 7.7 \times 10^4$	5	$5.3 \times 10^4 \pm 3.8 \times 10^4$	5	$1.1 \times 10^5 \pm 4.8 \times 10^4$	5	$5.1 \times 10^4 \pm 2.7 \times 10^4$	5	$1.1 \times 10^4 \pm 7.6 \times 10^3$	5	$4.3 \times 10^3 \pm 3.1 \times 10^3$	5	$1.1 \times 10^4 \pm 4.9 \times 10^3$	5	$2.7 \times 10^3 \pm 1.5 \times 10^3$	5
T4	$1.3 \times 10^5 \pm 1.3 \times 10^5$	4[c]	$2.0 \times 10^4 \pm 2.1 \times 10^4$	5	$1.3 \times 10^5 \pm 5.8 \times 10^4$	5	$1.3 \times 10^4 \pm 7.8 \times 10^3$	5	$1.0 \times 10^4 \pm 6.3 \times 10^3$	4	$1.7 \times 10^3 \pm 1.4 \times 10^3$	5	$1.3 \times 10^4 \pm 5.4 \times 10^3$	5	$8.2 \times 10^2 \pm 5.3 \times 10^2$	5
T8	$2.5 \times 10^4 \pm 1.8 \times 10^4$	5	$7.4 \times 10^3 \pm 5.8 \times 10^3$	5	$4.2 \times 10^4 \pm 3.1 \times 10^4$	5	$9.7 \times 10^3 \pm 4.4 \times 10^3$	5	$3.7 \times 10^3 \pm 2.7 \times 10^3$	5	$8.8 \times 10^2 \pm 7.1 \times 10^2$	5	$5.4 \times 10^3 \pm 4.3 \times 10^3$	5	$6.8 \times 10^2 \pm 4.5 \times 10^2$	5
T12	$9.6 \times 10^3 \pm 5.6 \times 10^3$	5	$6.0 \times 10^3 \pm 3.6 \times 10^3$	5	$1.3 \times 10^4 \pm 7.0 \times 10^3$	5	$1.2 \times 10^4 \pm 6.5 \times 10^3$	5	$1.6 \times 10^3 \pm 1.2 \times 10^3$	5	$7.2 \times 10^2 \pm 5.0 \times 10^2$	5	$2.0 \times 10^3 \pm 1.2 \times 10^3$	5	$8.8 \times 10^2 \pm 5.1 \times 10^2$	5
T24	$1.4 \times 10^3 \pm 5.5 \times 10^2$	5	$2.8 \times 10^3 \pm 1.9 \times 10^3$	5	$3.0 \times 10^3 \pm 2.5 \times 10^3$	5	$9.8 \times 10^3 \pm 3.8 \times 10^3$	5	$2.2 \times 10^2 \pm 8.5 \times 10^1$	5	$2.9 \times 10^2 \pm 1.7 \times 10^2$	5	$5.1 \times 10^2 \pm 4.4 \times 10^2$	5	$7.0 \times 10^2 \pm 1.6 \times 10^2$	5
T26	$2.0 \times 10^5 \pm 9.3 \times 10^4$	5	$1.9 \times 10^5 \pm 1.5 \times 10^5$	5	$1.6 \times 10^5 \pm 8.1 \times 10^4$	5	$1.4 \times 10^5 \pm 6.6 \times 10^4$	5	$2.3 \times 10^4 \pm 1.2 \times 10^4$	5	$1.5 \times 10^4 \pm 8.6 \times 10^3$	5	$2.1 \times 10^4 \pm 1.2 \times 10^4$	5	$7.8 \times 10^3 \pm 4.7 \times 10^3$	5
T28	$1.2 \times 10^5 \pm 4.0 \times 10^4$	5	$1.1 \times 10^5 \pm 5.9 \times 10^4$	5	$1.2 \times 10^5 \pm 6.4 \times 10^4$	5	$1.9 \times 10^5 \pm 4.6 \times 10^4$	5	$1.3 \times 10^4 \pm 4.5 \times 10^3$	5	$7.3 \times 10^3 \pm 2.6 \times 10^3$	5	$1.6 \times 10^4 \pm 7.6 \times 10^3$	5	$8.1 \times 10^3 \pm 1.9 \times 10^3$	5
T32	$4.8 \times 10^4 \pm 2.3 \times 10^4$	4[c]	$4.4 \times 10^4 \pm 2.3 \times 10^4$	5	$6.2 \times 10^4 \pm 4.2 \times 10^4$	5	$9.3 \times 10^4 \pm 5.7 \times 10^4$	5	$6.1 \times 10^3 \pm 3.5 \times 10^3$	4	$3.6 \times 10^3 \pm 2.3 \times 10^3$	5	$8.4 \times 10^3 \pm 5.7 \times 10^3$	5	$3.3 \times 10^3 \pm 9.2 \times 10^2$	5
T36	$1.9 \times 10^4 \pm 1.2 \times 10^4$	5	$2.5 \times 10^4 \pm 2.6 \times 10^4$	5	$1.9 \times 10^4 \pm 1.5 \times 10^4$	5	$5.4 \times 10^4 \pm 4.4 \times 10^4$	5	$2.5 \times 10^3 \pm 1.8 \times 10^3$	5	$2.0 \times 10^3 \pm 2.1 \times 10^3$	5	$3.2 \times 10^3 \pm 2.5 \times 10^3$	5	$2.4 \times 10^3 \pm 6.5 \times 10^2$	5
T48/W0	$2.4 \times 10^3 \pm 1.7 \times 10^3$	4[c]	$5.9 \times 10^3 \pm 5.5 \times 10^3$	5	$5.5 \times 10^3 \pm 3.7 \times 10^3$	5	$1.7 \times 10^4 \pm 1.1 \times 10^4$	5	$4.3 \times 10^2 \pm 4.1 \times 10^2$	4	$7.1 \times 10^2 \pm 6.9 \times 10^2$	5	$8.6 \times 10^2 \pm 6.3 \times 10^2$	5	$1.2 \times 10^3 \pm 4.6 \times 10^2$	5
W2	$2.0 \times 10^5 \pm 1.3 \times 10^5$	5	$1.9 \times 10^5 \pm 9.1 \times 10^4$	5	$1.6 \times 10^5 \pm 5.2 \times 10^4$	5	$1.5 \times 10^5 \pm 3.7 \times 10^4$	5	$2.2 \times 10^4 \pm 1.4 \times 10^4$	5	$2.2 \times 10^4 \pm 6.6 \times 10^3$	5	$1.9 \times 10^4 \pm 4.2 \times 10^3$	5	$9.6 \times 10^3 \pm 3.2 \times 10^3$	5
W4	$1.4 \times 10^5 \pm 7.6 \times 10^4$	5	$9.9 \times 10^4 \pm 5.7 \times 10^4$	5	$1.0 \times 10^5 \pm 6.1 \times 10^4$	5	$1.7 \times 10^5 \pm 6.1 \times 10^4$	5	$1.3 \times 10^4 \pm 7.9 \times 10^3$	5	$9.1 \times 10^3 \pm 4.6 \times 10^3$	5	$1.3 \times 10^4 \pm 6.5 \times 10^3$	5	$9.4 \times 10^3 \pm 2.4 \times 10^3$	5
W8	$3.9 \times 10^4 \pm 2.4 \times 10^4$	5	$3.6 \times 10^4 \pm 2.1 \times 10^4$	5	$4.0 \times 10^4 \pm 3.7 \times 10^4$	5	$7.6 \times 10^4 \pm 6.0 \times 10^4$	5	$4.1 \times 10^3 \pm 1.8 \times 10^3$	5	$4.7 \times 10^3 \pm 4.2 \times 10^3$	5	$5.6 \times 10^3 \pm 5.2 \times 10^3$	5	$4.3 \times 10^3 \pm 1.9 \times 10^3$	5
W12	$1.2 \times 10^4 \pm 7.0 \times 10^3$	5	$1.6 \times 10^4 \pm 1.5 \times 10^4$	5	$1.7 \times 10^4 \pm 8.3 \times 10^3$	5	$4.4 \times 10^4 \pm 4.2 \times 10^4$	5	$1.2 \times 10^3 \pm 4.2 \times 10^2$	5	$1.8 \times 10^3 \pm 1.3 \times 10^3$	5	$2.4 \times 10^3 \pm 1.0 \times 10^3$	5	$2.5 \times 10^3 \pm 5.0 \times 10^2$	5
W24	$2.3 \times 10^3 \pm 1.8 \times 10^3$	5	$2.0 \times 10^3 \pm 1.3 \times 10^3$	5	$3.4 \times 10^3 \pm 1.2 \times 10^3$	5	$2.1 \times 10^4 \pm 3.4 \times 10^4$	5	$2.6 \times 10^2 \pm 1.5 \times 10^2$	5	$2.8 \times 10^2 \pm 2.2 \times 10^2$	5	$4.9 \times 10^2 \pm 2.0 \times 10^2$	5	$9.7 \times 10^2 \pm 9.7 \times 10^2$	5
W36	$3.9 \times 10^2 \pm 5.3 \times 10^2$	5	$1.9 \times 10^2 \pm 1.6 \times 10^2$	5	$8.7 \times 10^2 \pm 6.4 \times 10^2$	5	$7.1 \times 10^3 \pm 1.4 \times 10^4$	5	$3.4 \times 10^1 \pm 2.6 \times 10^1$	5	$3.4 \times 10^1 \pm 3.5 \times 10^1$	5	$1.3 \times 10^2 \pm 8.9 \times 10^1$	5	$3.2 \times 10^2 \pm 5.7 \times 10^2$	5
W48	$2.7 \times 10^2 \pm 3.8 \times 10^2$	5	$5.1 \times 10^1 \pm 3.7 \times 10^1$	5	$5.2 \times 10^2 \pm 4.3 \times 10^2$	5	$3.7 \times 10^3 \pm 7.3 \times 10^3$	5	$1.9 \times 10^1 \pm 1.5 \times 10^1$	5	$1.0 \times 10^1 \pm 7.7 \times 10^0$	4	$6.7 \times 10^1 \pm 6.2 \times 10^1$	5	$1.3 \times 10^2 \pm 1.9 \times 10^2$	5
W60	$1.5 \times 10^2 \pm 1.8 \times 10^2$	5	$1.9 \times 10^1 \pm 8.8 \times 10^0$	5	$3.4 \times 10^2 \pm 3.2 \times 10^2$	5	$3.1 \times 10^3 \pm 6.5 \times 10^3$	5	$1.8 \times 10^1 \pm 2.7 \times 10^0$	3	$4.0 \times 10^0 \pm 8.6 \times 10^{-1}$	4	$4.6 \times 10^1 \pm 4.6 \times 10^1$	5	$9.9 \times 10^1 \pm 1.7 \times 10^2$	5
W72	$1.2 \times 10^2 \pm 1.5 \times 10^2$	5	$2.2 \times 10^1 \pm 1.0 \times 10^1$	5	$2.0 \times 10^2 \pm 1.6 \times 10^2$	5	$1.6 \times 10^3 \pm 3.3 \times 10^3$	5	$1.8 \times 10^1 \pm 1.0 \times 10^1$	3	$4.6 \times 10^0 \pm 2.3 \times 10^0$	4	$3.4 \times 10^1 \pm 3.9 \times 10^1$	5	$5.1 \times 10^1 \pm 7.9 \times 10^1$	5
W96	$7.0 \times 10^1 \pm 8.1 \times 10^1$	5	$1.2 \times 10^1 \pm 4.4 \times 10^0$	5	$7.1 \times 10^1 \pm 4.5 \times 10^1$	5	$4.3 \times 10^2 \pm 8.3 \times 10^2$	5	1.3×10^1	2			$1.4 \times 10^1 \pm 1.1 \times 10^1$	5	$2.5 \times 10^1 \pm 2.8 \times 10^1$	3

[a]LC-MS/MS values represented the mean of two independent sample measurements on two different days. [b]Number of samples that have flunixin or 5-hydroxy flunixin concentrations that are >LOQ and were used for calculation. Limits of quantitation (LOQ) were 0.3 ng/mL for flunixin, 0.4 ng/mL for 5-hydroxy flunixin at 1:100 dilution, and 0.3 ng/mL for flunixin, and for 5-hydroxy flunixin at 1:10 dilution. [c]No samples were collected from one of the cows for the treatment times 4, 32, or 48 h.

DOI:10.1021/acs.jafc.5b04793
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Table 4. Flunixin and 5-Hydroxy-flunixin Concentrations (ng/mL) in Plasma after Administration of Saline or of LPS to Cows via Intravenous or Intramuscular Routes

	Flunixin (ng/mL)								5 hydroxy Flunixin (ng/mL)							
	IV-Saline		IV-LPS		IM-Saline		IM-LPS		IV-Saline		IV-LPS		IM-Saline		IM-LPS	
Time (h)	Mean ± SD	n[a]	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
T2	$1.2 \times 10^3 \pm 1.7 \times 10^3$	5	$5.3 \times 10^2 \pm 2.6 \times 10^2$	5	$2.5 \times 10^3 \pm 1.4 \times 10^3$	5	$1.4 \times 10^3 \pm 7.0 \times 10^2$	5	$1.5 \times 10^2 \pm 2.1 \times 10^2$	5	$6.3 \times 10^1 \pm 3.1 \times 10^1$	5	$1.5 \times 10^2 \pm 4.6 \times 10^1$	5	$8.6 \times 10^1 \pm 5.2 \times 10^1$	5
T4	$6.0 \times 10^2 \pm 3.1 \times 10^2$	5	$5.0 \times 10^2 \pm 3.8 \times 10^2$	5	$7.8 \times 10^2 \pm 2.8 \times 10^2$	5	$7.0 \times 10^2 \pm 3.4 \times 10^2$	5	$1.1 \times 10^2 \pm 7.4 \times 10^1$	5	$6.3 \times 10^1 \pm 3.2 \times 10^1$	5	$8.6 \times 10^1 \pm 3.5 \times 10^1$	5	$6.8 \times 10^1 \pm 8.0 \times 10^1$	5
T8	$2.5 \times 10^2 \pm 1.7 \times 10^2$	5	$3.2 \times 10^2 \pm 2.5 \times 10^2$	5	$3.1 \times 10^2 \pm 1.3 \times 10^2$	5	$4.2 \times 10^2 \pm 1.8 \times 10^2$	5	$4.3 \times 10^1 \pm 3.2 \times 10^1$	5	$3.4 \times 10^1 \pm 1.7 \times 10^1$	5	$4.3 \times 10^1 \pm 2.7 \times 10^1$	5	$4.1 \times 10^1 \pm 3.0 \times 10^1$	5
T12	$1.2 \times 10^2 \pm 7.5 \times 10^1$	5	$1.9 \times 10^2 \pm 1.8 \times 10^2$	5	$1.9 \times 10^2 \pm 1.1 \times 10^2$	5	$2.2 \times 10^2 \pm 5.2 \times 10^1$	5	$2.0 \times 10^1 \pm 1.2 \times 10^1$	5	$1.5 \times 10^1 \pm 9.0 \times 10^0$	5	$1.9 \times 10^1 \pm 6.2 \times 10^0$	5	$1.8 \times 10^1 \pm 9.9 \times 10^0$	5
T24	$1.5 \times 10^1 \pm 1.3 \times 10^1$	5	$5.2 \times 10^1 \pm 6.2 \times 10^1$	5	$2.5 \times 10^1 \pm 2.0 \times 10^1$	5	$8.8 \times 10^1 \pm 5.1 \times 10^1$	5	$2.6 \times 10^0 \pm 2.7 \times 10^0$	5	$3.0 \times 10^0 \pm 3.0 \times 10^0$	5	$2.4 \times 10^0 \pm 1.8 \times 10^0$	5	$4.2 \times 10^0 \pm 1.6 \times 10^0$	5
T26	$5.9 \times 10^2 \pm 3.2 \times 10^2$	5	$7.7 \times 10^2 \pm 4.0 \times 10^2$	5	$1.3 \times 10^3 \pm 4.5 \times 10^2$	5	$1.7 \times 10^3 \pm 9.4 \times 10^2$	5	$7.8 \times 10^1 \pm 3.7 \times 10^1$	5	$4.9 \times 10^1 \pm 1.8 \times 10^1$	5	$1.1 \times 10^2 \pm 3.8 \times 10^1$	5	$5.7 \times 10^1 \pm 2.7 \times 10^1$	5
T28	$6.6 \times 10^2 \pm 1.8 \times 10^2$	5	$6.4 \times 10^2 \pm 4.9 \times 10^2$	5	$7.1 \times 10^2 \pm 3.2 \times 10^2$	5	$9.5 \times 10^2 \pm 4.6 \times 10^2$	5	$1.1 \times 10^2 \pm 4.3 \times 10^1$	5	$5.1 \times 10^1 \pm 2.4 \times 10^1$	5	$8.7 \times 10^1 \pm 2.3 \times 10^1$	5	$4.0 \times 10^1 \pm 8.0 \times 10^0$	5
T32	$2.4 \times 10^2 \pm 1.1 \times 10^2$	5	$3.0 \times 10^2 \pm 1.9 \times 10^2$	5	$3.2 \times 10^2 \pm 1.3 \times 10^2$	5	$3.8 \times 10^2 \pm 1.4 \times 10^2$	5	$3.6 \times 10^1 \pm 1.3 \times 10^1$	5	$2.6 \times 10^1 \pm 1.3 \times 10^1$	5	$3.8 \times 10^1 \pm 1.3 \times 10^1$	5	$2.0 \times 10^1 \pm 6.6 \times 10^0$	5
T36	$1.0 \times 10^2 \pm 4.9 \times 10^1$	5	$1.8 \times 10^2 \pm 1.6 \times 10^2$	5	$1.8 \times 10^2 \pm 6.0 \times 10^1$	5	$3.0 \times 10^2 \pm 2.3 \times 10^2$	5	$1.8 \times 10^1 \pm 7.6 \times 10^0$	5	$1.8 \times 10^1 \pm 1.6 \times 10^1$	5	$1.8 \times 10^1 \pm 5.4 \times 10^0$	5	$1.3 \times 10^1 \pm 4.9 \times 10^0$	5
T48/W0	$2.1 \times 10^1 \pm 1.5 \times 10^1$	5	$4.5 \times 10^1 \pm 5.2 \times 10^1$	5	$7.1 \times 10^1 \pm 9.5 \times 10^1$	5	$1.0 \times 10^2 \pm 1.1 \times 10^2$	5	$5.1 \times 10^0 \pm 3.6 \times 10^0$	5	$5.8 \times 10^0 \pm 7.2 \times 10^0$	5	$7.3 \times 10^0 \pm 9.2 \times 10^0$	5	$5.4 \times 10^0 \pm 3.4 \times 10^0$	5
W2	$5.5 \times 10^2 \pm 4.0 \times 10^2$	5	$5.0 \times 10^2 \pm 3.3 \times 10^2$	5	$1.1 \times 10^3 \pm 3.7 \times 10^2$	5	$1.2 \times 10^3 \pm 5.6 \times 10^2$	5	$7.8 \times 10^1 \pm 5.5 \times 10^1$	5	$5.2 \times 10^1 \pm 4.1 \times 10^1$	5	$1.0 \times 10^2 \pm 2.4 \times 10^1$	5	$5.2 \times 10^1 \pm 1.2 \times 10^1$	5
W4	$5.7 \times 10^2 \pm 2.6 \times 10^2$	5	$5.7 \times 10^2 \pm 3.8 \times 10^2$	5	$6.8 \times 10^2 \pm 1.5 \times 10^2$	5	$6.5 \times 10^2 \pm 1.5 \times 10^2$	5	$1.2 \times 10^2 \pm 8.7 \times 10^1$	5	$9.1 \times 10^1 \pm 9.6 \times 10^1$	5	$8.0 \times 10^1 \pm 2.1 \times 10^1$	5	$4.7 \times 10^1 \pm 1.9 \times 10^1$	5
W8	$2.7 \times 10^2 \pm 1.3 \times 10^2$	5	$2.5 \times 10^2 \pm 1.6 \times 10^2$	5	$3.3 \times 10^2 \pm 9.2 \times 10^1$	5	$3.8 \times 10^2 \pm 2.1 \times 10^2$	5	$5.0 \times 10^1 \pm 4.0 \times 10^1$	5	$4.0 \times 10^1 \pm 4.6 \times 10^1$	5	$4.0 \times 10^1 \pm 1.1 \times 10^1$	5	$2.2 \times 10^1 \pm 8.2 \times 10^0$	5
W12	$1.0 \times 10^2 \pm 3.1 \times 10^1$	5	$1.2 \times 10^2 \pm 1.2 \times 10^2$	5	$1.5 \times 10^2 \pm 3.6 \times 10^1$	5	$2.1 \times 10^2 \pm 7.9 \times 10^1$	5	$2.1 \times 10^1 \pm 1.1 \times 10^1$	5	$1.4 \times 10^1 \pm 8.2 \times 10^0$	5	$1.8 \times 10^1 \pm 4.0 \times 10^0$	5	$1.4 \times 10^1 \pm 5.7 \times 10^0$	5
W24	$1.6 \times 10^1 \pm 1.4 \times 10^1$	5	$1.9 \times 10^1 \pm 2.3 \times 10^1$	5	$4.5 \times 10^1 \pm 4.2 \times 10^1$	5	$7.6 \times 10^1 \pm 8.6 \times 10^1$	5	$7.9 \times 10^0 \pm 1.1 \times 10^1$	4	$2.7 \times 10^0 \pm 2.0 \times 10^0$	3	$4.6 \times 10^0 \pm 4.3 \times 10^0$	5	$4.1 \times 10^0 \pm 3.7 \times 10^0$	5
W36	$4.9 \times 10^0 \pm 4.4 \times 10^0$	3[b]	$3.7 \times 10^0 \pm 5.7 \times 10^0$	5	$1.1 \times 10^1 \pm 7.3 \times 10^0$	5	$2.7 \times 10^1 \pm 3.6 \times 10^1$	5	$2.1 \times 10^0 \pm 1.7 \times 10^0$	3	1.9×10^0	1	2.1×10^0	2	1.7×10^0	2
W48	$2.8 \times 10^0 \pm 3.8 \times 10^0$	3[b]	$1.7 \times 10^0 \pm 1.8 \times 10^0$	3	$1.1 \times 10^1 \pm 8.9 \times 10^0$	4	$2.1 \times 10^1 \pm 3.8 \times 10^1$	5	2.7×10^0	1	1.6×10^0	1	2.0×10^0	1	3.1×10^0	1
W60	5.9×10^0	2	1.4×10^0	1	$5.2 \times 10^0 \pm 3.7 \times 10^0$	4	$2.1 \times 10^1 \pm 4.2 \times 10^1$	5	1.2×10^0	1					2.4×10^0	1
W72	4.1×10^0	2	5.0×10^{-1}	1	$4.1 \times 10^0 \pm 4.4 \times 10^0$	4	$1.1 \times 10^1 \pm 2.0 \times 10^1$	4	2.4×10^0	1			1.8×10^0	1	8.6×10^1	1
W96	1.4×10^0	2	5.7×10^{-1}	1	$1.3 \times 10^0 \pm 9.4 \times 10^{-1}$	4	1.0×10^1	2								

[a]The number of samples including flunixin or 5-hydroxy flunixin concentrations that are > LOQ was used for calculation. The limits of quantitation were 0.5 ng/mL for flunixin and 0.9 ng/mL for 5-hydroxy flunixin. [b]Cow 12 36-h withdrawal and 48-h withdrawal samples were excluded from calculation due to misplaced samples.

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DOI: 10.1021/acs.jafc.5b04793

results were correctly identified (i.e., milk violations at both 36-h and 48-h withdrawal).

Although urine could not be analyzed directly with the lateral flow immunoassay, simple dilution with raw milk (1:10) produced satisfactory results. Table 2 shows a comparison of the qualitative urine lateral flow assay tests results with quantitative results obtained by LC-MS/MS analyses. At the 72-h withdrawal period, lateral flow immunoassays returned 18 positives (of 20 cows) with one cow inconclusive and another negative. At the 96-h withdrawal period, 16 cows tested positive, tests on two cows were inconclusive, and two cows tested negative. For both time points, cows that had violative milk residues and/or violative liver residues [as assessed by LC-MS (milk) or LC-MS/MS (liver)] had urine that tested positive by lateral flow immunoassay. In all cases, urinary flunixin concentrations greater than 24 ng/mL (2.4 ng/mL after dilution for analysis) tested positive by lateral flow immunoassay. When urinary flunixin concentrations were below the limit of the lateral flow assay sensitivity, it would be difficult to discern a positive or negative result, consistent with the fact that the two inconclusive results (at 96-h withdrawal) had urinary flunixin concentrations of 9 to 14 ng/mL (0.9−1.4 ng/mL after dilution).

Despite the relatively simple urine dilution procedure in comparison to the pretreatment steps employed by Jones et al.[21] prior to analysis by lateral flow immunoassay, the performance of the screening test was comparable. The milk lateral flow assay, adapted for urine by Jones et al.[21] had a limit of detection of 30 ppb. With the adapted test, they correctly identified 3 of 12 heifers having 96-h postdosing urinary flunixin concentrations 30−70 ppb, as quantified by HPLC (limit of detection 20 ng/mL). However, urine from 4 of 12 animals collected 96-h post dosing also tested positive by the lateral flow immunoassay; these same animals had non-detectable flunixin concentrations as determined by HLPC indicating that false positives occurred.

Similar to urine, oral fluid required sample treatment before lateral flow immunoassay could be performed. After a 1:5 dilution with milk, oral fluid lateral flow immunoassay performed as expected with the flunixin test line progressively fading as the fortified flunixin concentration increased (data not shown). Table 2 demonstrates that there were only 5 positives returned by lateral flow analysis at the 96-h withdrawal period. None of the 96-h withdrawal positives were from cows that had milk and/or liver violative flunixin/5-hydroxy flunixin residues. What's more, the cow that had oral fluid ELISA result of 67 ng/mL tested negative with the lateral flow immunoassay. Such erratic results strongly suggest that the lateral flow immunoassay would not be useful with oral fluids for a flunixin screen.

Urinary Flunixin Concentration as Determined by LC-MS/MS. Total flunixin concentration in urine was determined after acid hydrolysis of conjugates (Table 3). In addition, urinary 5-hydroxy flunixin was measured in parallel (Table 3). Flunixin concentrations greater than 100,000 ng/mL were measured in urine collected 2 h after dosing in 30% of the cows on day 1 (max 240,000 ng/mL), 70% of cows on day 2 (max 430,000 ng/mL), and 90% of cows on day 3 (max 390,000 ng/mL). Urine flunixin levels increased sharply shortly after dosing and decreased in a biphasic fashion.

At the 96-h withdrawal period, flunixin was quantified (146 ± 420 ng/mL) in all urine samples: IV flunixin-saline 70 ± 90 ng/mL; IV flunixin-LPS 12 ± 4 ng/mL; IM flunixin-saline 71 ± 45 ng/mL; and IM flunixin-LPS 431 ± 830 ng/mL. Across all groups, urinary flunixin concentrations at the 96-h withdrawal ranged from 5 to 1,911 ng/mL with a median concentration of 19 ng/mL. The highest urinary concentration at 96-h withdrawal (1,911 ng/mL) was from an animal (cow 18) in the IM flunixin-LPS treatment group; this cow also had violative milk and liver residues. An additional cow (cow 1) that had violative milk residues (in the IV-saline group) had a urinary flunixin concentration of 207 ng/mL at the 96-h withdrawal period.

Urinary 5-hydroxy flunixin was quantifiable in urine of all cows at the 48-h withdrawal period and was present in 96-h withdrawal urine in 10 of the 20 cows. Unlike milk, in which 5-hydroxy flunixin is secreted in greater quantities than parent flunixin (with fluninx/5-hydroxy flunixin concentration ratios being less than 1[22]), urinary flunixin levels were 6 to 38-fold greater than urinary 5-hydroxy flunixin concentrations. The median, flunixin/5-hydroxy flunixin ratio was ∼9-fold in urine (Table 3).

Comparison between Plasma and Urinary Flunixin Residues. Flunixin and 5-hydroxy flunixin levels in plasma are provided in Table 4. Nine cows had quantifiable plasma flunixin at 96-h withdrawal after the final flunixin dose; none of the cows had quantifiable 5-hydroxy flunixin at the 96-h withdrawal collection (Table 4). At 96-h withdrawal, the cow having the highest plasma flunixin concentration (19.9 ng/mL) was the cow having both violative liver (178 ng/g) and milk 5-hydroxy flunixin residues (8.3 ng/mL; cow 18). The cow having a milk residue violation (19.1 ng/mL 5-hydroxy flunixin; cow 1), but no liver violation, had the third highest plasma flunixin concentration (1.8 ng/mL). The relationship between plasma and urinary flunixin concentrations across all time points indicates that urinary flunixin concentrations were approximately 70-fold higher than those measured in plasma at any given time point (Figure 3). However, across all cows, the

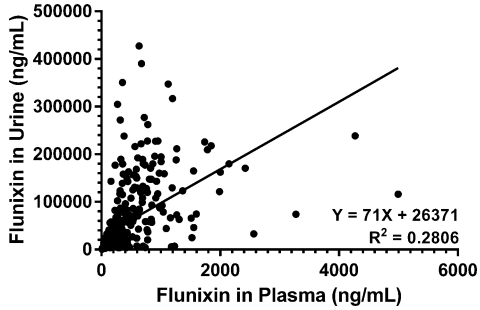

Figure 3. Correlation between urinary and plasma flunixin concentrations. Note the difference in scales of the X and Y axes.

coefficient of determination between urine and plasma flunixin concentrations was poor (R^2 = 0.3). Plasma drug concentrations are most often used for pharmacokinetic modeling and prediction of elimination patterns because of consistency and physiological relevance. The relation with urine drug concentrations varies with urine production and the kinetics of the elimination of the drug in urine. Because of this complexity, considerable animal to animal variation is clearly demonstrated in our data. An advantage of using urine as an antemortem matrix relative to plasma would be the higher flunixin concentrations in comparison to plasma in addition to the noninvasive collection of urine.

Comparison of Flunixin/5-Hydroxy Flunixin Ratios among Milk, Plasma, and Urine. LPS can alter drug

DOI: 10.1021/acs.jafc.5b04793
J. Agric. Food Chem. 2016, 64, 336−345

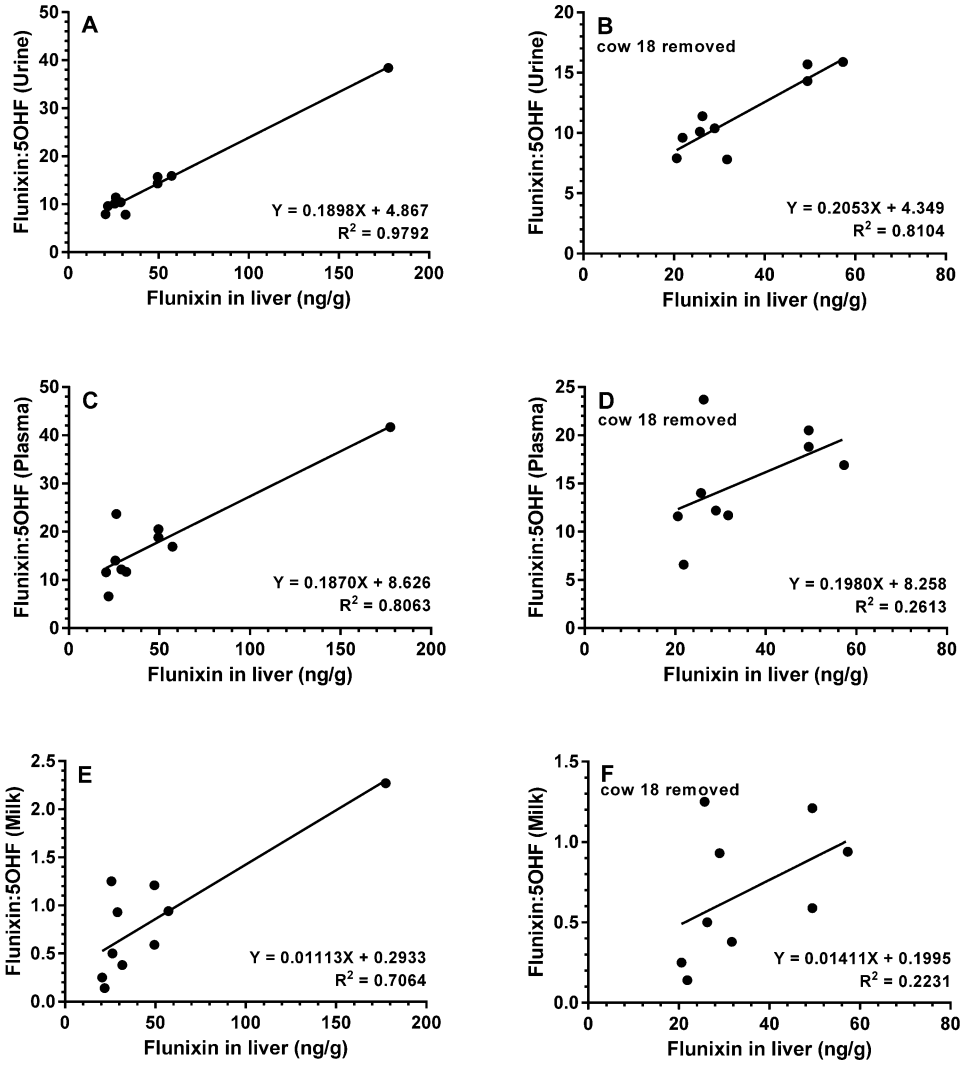

Figure 4. Correlations between ratios of flunixin:5-hydroxy flunixin and flunixin liver residues from cows challenged with LPS for urine (top panel), plasma (middle panel), and milk (lower panel). The correlations shown in panels B, D, and F have cow 18 removed. Flunixin liver concentrations were obtained from the ARS analysis of liver rather than the FSIS analysis (Smith et al. 2015,[15] supplementary Table 4).

metabolism pathways through cytochrome P_{450} inhibition[23−25] and consequently could alter the relationship between parent and metabolite. Flunixin and 5-hydroxy flunixin concentration ratios within the individual animals were found to be relatively constant (~2-fold variation) among all collection time points in urine (data not shown), remarkable because of the independence of the various processes involved. Between animals more variation was observed. The flunixin:5-hydroxy flunixin ratios in urine, milk, and plasma (computed across all collection time points) were correlated with flunixin liver concentration at slaughter. The correlations between flunixin liver residue and flunixin:5-hydroxy flunixin ratios in urine, milk, and plasma were quite low (R^2 < 0.1, data not shown) in cows that were not exposed to LPS. In contrast, urine, milk, and plasma correlations (R^2) of flunixin:5 hydroxy flunixin ratios with liver residues in LPS-treated cows were 0.98, 0.71, and 0.81 respectively (Figure 4A, 4C, and 4E). When cow 18 was removed from the analysis (because of its very high liver flunixin concentration), only the urinary flunixin:5-hydroxy flunixin ratio correlated strongly with liver residue level (R^2 = 0.81, Figure 4B) while coefficient of determination for plasma and milk with liver were 0.26 and 0.22, respectively (Figure 4D and 4F). The regression equation implies an increase of flunixin liver residue with an increase in the proportion of flunixin relative to 5-hydroxy flunixin, despite the independence of hepatic and renal function. Based on these correlations, urinary flunixin to 5-hydroxy flunixin ratios might serve to indicate metabolic problems related to disease or other factors that might contribute to potential violative levels in liver.

Taken together, the FSIS or FDA flunixin ELISA screening methods worked well to identify violative tissue residues in incurred samples. Flunixin lateral flow assays provided a feasible on-site test for the presence of flunixin residues in both milk and urine. With LC-MS/MS analysis, flunixin and 5-hydroxy flunixin ratios might be indicative of endotoxin exposure as well as potential violative residues.

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Funding

Support for the Food Animal Residue Avoidance Databank program (FARAD) at KSU, UC−Davis, and NCSU was provided by USDA-NIFA.

DOI: 10.1021/acs.jafc.5b04793
J. Agric. Food Chem. 2016, 64, 336−345

Notes

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors wish to thank Missy Berry, Jim Brooks, Grant Herges, Jason Holthusen, Amy McGarvey, Jim Yeatts, and Scott Wetzlich for skillful technical assistance. The animal care, slaughter, and sampling assistance provided by Dee Ellig, Austen Germolus, J. Michael Giddings, Justin Gilbertson, Kelsey Heiberg, Erin Loeb, Sara Lupton, Malinda Scherf, and Terry Skunberg is gratefully acknowledged as are the efforts of student employees of the North Dakota State University Meats Laboratory and Animal Physiology and Nutrition Center. Clint Mitchell's helpful discussion is greatly appreciated.

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DOI: 10.1021/acs.jafc.5b04793
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