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Determination of Flunixin in Edible Bovine Tissues Using Liquid Chromatography Coupled with Tandem Mass Spectrometry

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An accurate, reliable, and reproducible assay was developed and validated to determine flunixin in bovine liver, kidney, muscle, and fat. The overall recovery and percent coefficient of variation (%CV) of twenty-eight determinations in each tissue for flunixin free acid were 85.9% (5.9% CV) for liver, 94.6% (9.9% CV) for kidney, 87.4% (4.7% CV) for muscle, and 87.6% (4.4% CV) for fat. The theoretical limit of detection was 0.1 μg/kg (ppb, ng/g) for liver and kidney, and 0.2 ppb for muscle and fat. The theoretical limit of quantitation was 0.3, 0.2, 0.6, and 0.4 ppb for liver, kidney, muscle, and fat, respectively. The validated lower limit of quantitation was 1 ppb for edible tissues with the upper limit of 400 ppb for liver and kidney, 100 ppb for fat, and 40 ppb for muscle. Accuracy, precision, linearity, specificity, ruggedness, and storage stability were demonstrated. Briefly, the method involves an initial acid hydrolysis, followed by pH adjustment (~9.5) and partitioning with ethyl acetate. A portion of the ethyl acetate extract was purified by solid-phase extraction using a strong cation exchange cartridge. The eluate was then evaporated to dryness, reconstituted, and analyzed using LC/MS/MS. The validated method is sensitive and specific for flunixin in edible bovine tissue.

KEYWORDS: Flunixin meglumine; LC/MS/MS; validated method

INTRODUCTION

Flunixin (SCH-14714, 2-[[2-Methyl-3-(trifluoromethyl)phenyl]amino]-3-pyridinecarboxylic acid) is approved for use worldwide as a nonsteroidal antiinflammatory agent with analgesic activity for cattle (**Figure 1**). Flunixin is administered intravenously as the meglumine salt. Banamine and Finadyne are the trade names of the formulations approved for use in cattle and horses in the US and for cattle, swine, horses, and dairy cattle in the EU, respectively. Because of use in food producing animal species, methods for regulatory surveillance of flunixin residues in edible tissues of production animals are needed. Flunixin (as free acid) is the marker residue selected by the U. S. FDA and EU-CVMP for monitoring of flunixin residues in cattle tissues. The maximun residue limits (MRLs) for flunixin are liver, 0.125 (FDA) or 0.300 (EMEA) ppm; muscle, 0.010 (EMEA) ppm; kidney, 0.100 (EMEA) ppm; fat, 0.030 (EMEA) ppm.

Previously, our laboratory developed and validated an HPLC-UV determinative and LC/MS/MS confirmatory assay for the determination of flunixin in bovine liver (*1*). The determinative method was subjected to a method trial and the results met the U. S. FDA Center for Veterinary Medicine (USFDA/CVM) performance criteria (*1*). Flunixin and related residues have been investigated previously by others and by our laboratory in milk (*2*). Previously, a determinative and confirmatory method for 5-hydroxyflunixin (flunixin marker residue) in milk at the 1 ppb level was published by our laboratory (*3*). A determinative method for flunixin residues in bovine muscle by HPLC-UV with a limit of quantitation of 15 ppb has been published (*4*). The present method gives improved sensitivity and is applicable to all edible tissues of cattle.

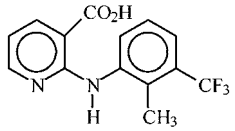

Figure 1. Structure of flunixin (free acid).

MATERIALS AND METHODS

Chemicals, Material, and Solvents. The analytical reference standard of flunixin meglumine salt used in this study was obtained from Schering-Plough Research Institute (SPRI), Union, NJ. Complete characterization (including purity) was provided by SPRI. All solvents used were of HPLC or HR-GC grade. Acetronitrile, formic acid, ethyl acetate, hexanes, and methanol were obtained from EM Science (Gibbstown, NJ). Ammonium hydroxide, hydrochloric acid, phosphoric

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Table 1. HPLC Solvent Composition and Flow Rate

run time (min)	flow (mL/min)	0.4% formic acid in H_2O (%)	MeOH (%)	0.4% formic acid in CH_3CN (%)	switching valve
0	0.4	55	25	20	waste
8.5	0.4	55	25	20	MS
10.0	0.4	55	25	20	
10.5	0.4	5	50	45	
10.8	0.55	5	50	45	
11.0	0.55	5	50	45	waste
14.8	0.55	5	50	45	
15.0	0.55	55	25	20	
20.0	0.55	55	25	20	
20.2	0.4	55	25	20	

acid, and sodium hydroxide were obtained from J. T. Baker (Phillipsburg, NJ). Benzenesulfonic acid cation exchange (SCX) cartridges (500 mg packing) were purchased from Applied Separations (Allentown, PA). Water was purified on site using a Barnstead NANOPure II Water Purification System (resistivity ≥16.7 MΩ-cm), or obtained from EM Science.

The control bovine tissues from beef cattle were obtained from six different commercial sources. Tissue samples from [14C]flunixin treated beef cattle containing incurred residues were employed in this study. Beef cattle were given Banamine 3.6 mg flunixin per kg (label rate 2.2 mg flunixin/kg) by intravenous administration for 3 days. Tissues containing incurred residues from cattle euthanized at 24- and 48-hour post dose were used for this study.

LC/MS/MS Analysis Conditions. Chromatographic analysis was controlled using Mac-Quan, version 1.5. HPLC was performed with a Waters Alliance 2690 Separations system. All samples were analyzed using a Zorbax Eclipse XDB-C18 column maintained at 40 °C, 2.1-mm i.d. × 150-mm, 5-μm particle (MAC-MOD analytical, Chadds Ford, PA) protected by a Brownlee RP-18 Newguard 3.2-mm i.d. × 15-mm guard column. The injection volume was 60 μL, with the autosampler set at 5 °C. The HPLC mobile phase was 0.4% formic acid in Nanopure water (A), methanol (B), 0.4% formic acid in acetonitrile (C), and A/CH_3CN/B (470/480/50) (D). The samples were analyzed using variable flow rates from 0.4 to 0.55 mL/min using isocratic conditions followed by a solvent wash, as shown in **Table 1**. A six-port injection valve (Valco Instuments, Houston, TX) and actuator control module (Valco Instruments) controlled the flow of solvent and sample to the MS. Column effluent at the peak region (~8.5−11 min) was delivered to the MS source. Prior to and after peak elution, column effluent was diverted to waste, and solvent D was delivered to MS source via a second isocratic pump at 0.3 mL/min. A Perkin-Elmer (Norwalk, CT) Sciex Triple Quadrupole LC/MS/MS Spectrometer, Model API 365 equipped with the Positive Turbo-Ion Spray interface, was used to collect quantitative data. The instrument was operated in the positive-ion mode and optimized to monitor the ionic transition from the precursor ion (*m/z* 297.1) to the product ion (*m/z* 279.1). A product ion scan of *m/z* 297.1 is shown in **Figure 2**. Ion spray voltage was 5500 V; ion source temperature, 400 °C; Nebulizer and Curtain gas flow, 14 and 10 units, respectively. The multiple reaction monitoring (MRM) mode was used with the orifice plate set at 35 V and quad 2 rod offset at −34 V.

Preparation of Standards, Fortification Solutions, and Calibration Curve. Flunixin standard solutions were prepared in methanol at ~500 μg/mL (corrected for purity), and stored at or below −10 °C, at which they are stable for at least 6 months (data not shown). Intermediate stock solutions of 50 μg/mL flunixin were prepared by a 10-fold dilution of the standard solution in MeOH.

Fortification solutions of flunixin were prepared by dilution of the intermediate stock solution with 50% MeOH in H_2O covering a 10−4000 ng/mL range (equivalent to 1−400 ppb flunixin in tissue).

A six-point calibration curve was prepared from 0.125 to 125 ng/mL flunixin (equivalent to 1−500 ppb in tissue). The calibration curve standard solutions were prepared by diluting the intermediate stock solution with a solution of 50% MeOH in H_2O.

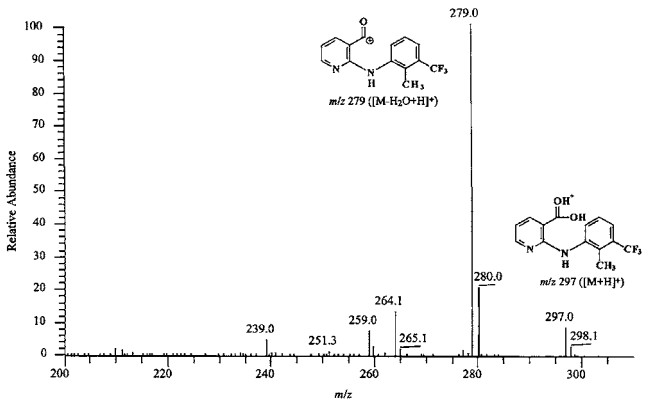

Figure 2. Product ion scan of a flunixin standard.

Preparation of Untreated Control Samples. For each analysis, unfortified tissue samples from a control (untreated animal) served as matrix blanks and were processed and analyzed for interference at the retention time of flunixin.

Preparation of Fortified Samples. Fortified tissues were prepared by adding working standards to control tissues. Control liver and kidney were fortified at flunixin concentrations of 1, 125, 250, and 400 ppb,; muscle at 1, 10, 20, and 40 ppb; and fat at 1, 25, 50, and 100 ppb.

Sample Preparation Procedure. A flow diagram of the procedure is presented in **Figure 3**. A 2 ± 0.1 g tissue sample was mixed with 8 mL of 6 N HCl in a 50-mL round-bottom screw top centrifuge tube and heated at 110 °C in a heating block for 2 h, then cooled to touch. This is a stopping point in the method and samples may be stored at room temperature for up to 3 days. After hydrolysis, to each fat sample 20 mL of hexanes were added, and the mixture was vortexed for 30 s and then centrifuged at approximately 2000 rpm (~700*g*) for 5 min. The hexane layer from each sample was discarded. For the acid hydrolyztate from liver, kidney, and muscle, no hexane extraction was performed. For all samples, the pH of the HCl hydrolysate was adjusted to 9.5 by adding 20% (w/w) NaOH. Ten mL of ethyl acetate was added to each tube. The tube was then capped and vortexed vigorously for 1 min. Each tube was centrifuged for 5 min at 2000 rpm (~700*g*). The ethyl acetate extract was transferred to a graduated 50-mL polypropylene centrifuge tube. The ethyl acetate solvent partitioning was then repeated three more times. The ethyl acetate extracts were combined into the 50-mL polypropylene centrifuge tube, and the final volume was adjusted to 40 mL with ethyl acetate using the graduations on the tube. The tube was capped and mixed thoroughly. The procedure may be stopped at this point, and extract may be stored at room temperature or lower for up to 3 days. A clean 25-mL reservoir was fitted to a preconditioned SCX column cartridge. The SCX cartridge was conditioned with 2 column volumes of water followed by 2 column volumes of 0.1% phosphoric acid in methanol, and approximately 1 mL of the phosphoric acid in methanol was allowed to remain above the column bed. Ten mL of a sample ethyl acetate extract was transferred into the reservoir and mixed with an equal volume of 0.1% phosphoric acid in methanol and then loaded by gravity onto the SCX cartridge. The SCX cartridge was rinsed with 5 mL of ethyl acetate followed by 5 mL of methanol. The sample was eluted with 12 mL of NH_4OH (28−30%)/MeOH (10/90, v/v) into a preconditioned 15-mL disposable glass centrifuge tube. The centrifuge tube was conditioned before use with 2 mL of a 1% formic acid in methanol solution by vortexing for ~30 s, pouring out the residual solution, and allowing the tube to dry. The NH_4OH/MeOH eluate was evaporated to dryness and reconstituted in 2 mL of 50% methanol/water to yield the final extract. A portion of the final extract was transferred to an autosampler vial glass insert for LC/MS/MS analysis. The procedure may be stopped at this point. Samples may be stored at refrigerated (~5 °C) or at lower temperatures for up to 5 months. The analysis was completed via reversed-phase HPLC with MS/MS detection.

Routine System Suitability. On each analysis day, a middle range standard was injected at least three times before injection of the standard curve series. The relative standard deviation of the peak area response

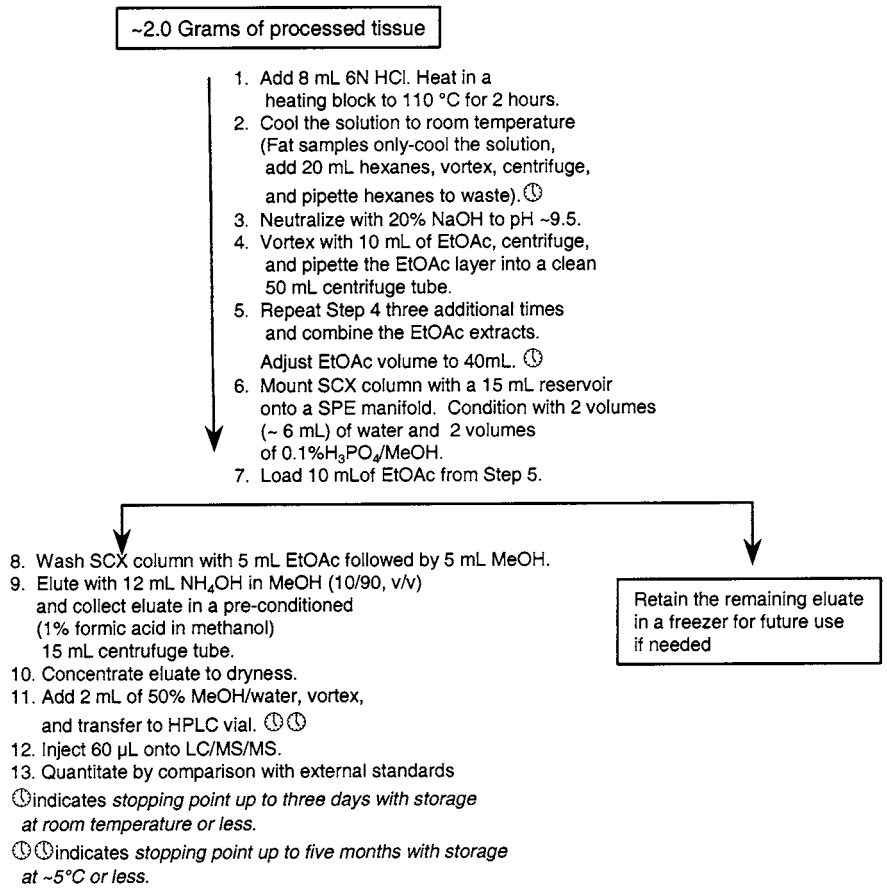

Figure 3. Flow diagram of the procedure to determine flunixin in cattle tissues.

was ≤5%. The retention time for flunixin was 9 min. The within-day retention time for flunixin did not vary more than ±12 s from the mean.

Analysis Sequence. The HPLC system was equilibrated with mobile phase for at least thirty minutes prior to injection. The absolute standard curve series was injected, followed by the processed samples, controls, and fortified samples, and then the absolute standard curve series

Quantitation. Sample concentrations were determined using linear regression standard curves. Peak area was plotted against standard concentration. The resulting sample extract concentration was converted to ppb (ng flunixin/g tissue) by multiplying by the final volume (2 mL) by 4 to take into account that only 1/4 of the ethyl acetate extract was used for the workup. This value was then divided by the sample weight in grams. The result was expressed to the whole number. The formula is expressed in the following equation:

$$\text{Concentration in tissue (ppb)} = \frac{X \times V \times 4}{W}$$

where X = concentration in the final sample extract (ng/mL), V = total volume of the final sample extract = 2 mL, 4 = correction for the subsampling of 1/4 of the ethyl acetate extract, W = total tissue weight (g),

Notes to Analyst and Safety Considerations. Glass centrifuge tubes are used in the procedure. All tubes should be inspected prior to use and cracked or damaged tubes should not be used. Care should be taken with procedures involving 6 N HCl. All manipulations that involve HCl should be performed in properly vented laboratory hoods. Care should be taken to wear proper eye and hand protection at all times in the laboratory. This procedure employs heating of a closed system. Precaution should be taken regarding safe handling of a closed system under high temperature and pressure with corrosive liquid and gas. Use of safety shields and hoods is required. Neutralization of the HCl hydrolysate should be carried out in a fume hood as the process generates heat. Care must be taken to avoid splattering.

RESULTS AND DISCUSSION

Accuracy. Percentage recoveries generated during the validation of the method as a measure of accuracy are presented in **Table 2**. The method was successfully validated over 3 days for the four bovine tissues as measured by average percentage recoveries ranging from 77.1 to 95.4% for seven replicates of each of four levels for liver, kidney, muscle, and fat. The average percentage recoveries for liver and kidney were 85.9 (N = 28), and 94.6 (N = 28), respectively, over a fortification range of 1−400 ppb. The average percentage recoveries for muscle and fat were 87.4 (N = 28) and 87.6 (N = 28), respectively, over a fortification range of 1−40 ppb for muscle, and 1−100 ppb for fat. Individual recoveries for tissue samples fortified at 1 ppb ranged from 72 to 82.5% for liver, 82.4−112% for kidney, 72.7−83.7% for muscle, and 72.9−81.1% for fat (data not shown). The results of the within day assay (N = 12) were similar to the between day assay results for each tissue, further demonstrating the accuracy of the method (**Table 2**).

Precision. Precision is represented by the coefficient of variation (CV). Percentage CV is expressed as standard deviation divided by the mean expressed as a percentage. The between day precision (N = 7 for each fortification level) for the method for all fortification levels ranged between 4 and 8% CV in liver, between 6 and 18% CV in kidney, and 4 and 5% CV in muscle and fat (**Table 2**). The within day precision (repeatability) for the method for all fortification levels (N = 3 for each level) ranged between 3 and 5% CV in liver, 4 and 27% CV in kidney, <1 and 4% CV in muscle, and between 1 and 4% CV in fat. Between-day assay precision was less than 9% CV for all tissues and fortification levels except the 1 ppb (18% CV) fortification in kidney.

Table 2. Flunixin Bovine Tissue Method Validation Results; Summary of Interday and Intraday Assay Results

between day				within day			
fortification level ppb	average % recovery	% CV	N	fortification level ppb	average % recovery	% CV	N
			Liver				
1	77.2	4.4	7	1	78.5	4.7	3
125	89.2	6.6	7	125	90.2	3.0	3
250	90.2	5.1	7	250	88.1	4.2	3
400	86.8	7.6	7	400	81.7	4.7	3
overall	85.9	5.9	28	overall	84.6	4.2	12
			Kidney				
1	92.7	18.3	7	1	92.4	26.6	3
125	95.4	5.6	7	125	93.5	4.4	3
250	95.2	6.6	7	250	98.3	7.1	3
400	95.2	8.9	7	400	93.7	6.0	3
overall	94.6	9.9	28	overall	94.5	11.0	12
			Muscle				
1	79.3	5.2	7	1	82.0	3.5	3
10	88.9	4.9	7	10	85.1	1.9	3
20	90.0	5.0	7	20	85.1	0.1	3
40	91.5	3.8	7	40	88.4	1.2	3
overall	87.4	4.7	28	overall	85.2	1.7	12
			Fat				
1	77.1	3.5	7	1	77.8	4.2	3
25	89.0	5.3	7	25	85.1	3.5	3
50	90.8	3.6	7	50	89.7	1.4	3
100	93.5	5.0	7	100	90.7	2.5	3
overall	87.6	4.4	28	overall	85.8	2.9	12

Linearity and Range. The relationship between detector response (peak area) and concentration of flunixin was linear and reproducible over the measured concentration range (0.125−125 ng flunixin free acid/mL). Least-squares linear regression weighted $1/x$ analysis of the data for each set of standard injections (two sets of standard injections per trial set), yielded an excellent coefficient of determination (r^2) of >0.99. The effective linear concentration range of the method was from 1 to 400 ppb for liver and kidney, from 1 to 40 ppb for muscle, and from 1 to 100 ppb for fat.

Ruggedness. Ruggedness tests were performed using duplicate fortified liver, kidney, muscle, and fat tissue at two levels. The ruggedness of the determinative method was evaluated by utilizing two cation exchange cartridges from alternate manufacturers and two analytical HPLC columns from the same manufacturer (different lots of packing material). A benzenesulfonic acid SPE cartridge from Varian performed acceptably with average recoveries from duplicate fortifications of 85, 79, 104, and 95% for liver, kidney, muscle, and fat, respectively (data not shown). However, an aliphatic sulfonic acid type from Supleco yielded no detectable (above the LOD) response by LC/MS/MS (data not shown). Thus, benzenesulfonic acid cartridges may likely be used interchangeably, while aliphatic sulfonic acid cation exchange cartridges may yield unacceptable data.

An analytical column from a different lot from the same manufacturer was used with no significant impact on the analytical results. The average recoveries for duplicate fortifications determined on the original and (alternate) column were 84 (90), 93 (92), 87 (91), and 88% (85%) for liver, kidney, muscle, and fat, respectively (data not shown). The average retention time (R_t) of flunixin by the second analytical column ($R_t \sim 9$ min) did not differ significantly from the standard column ($R_t \sim 9.1$ min) used in the determinative method validation.

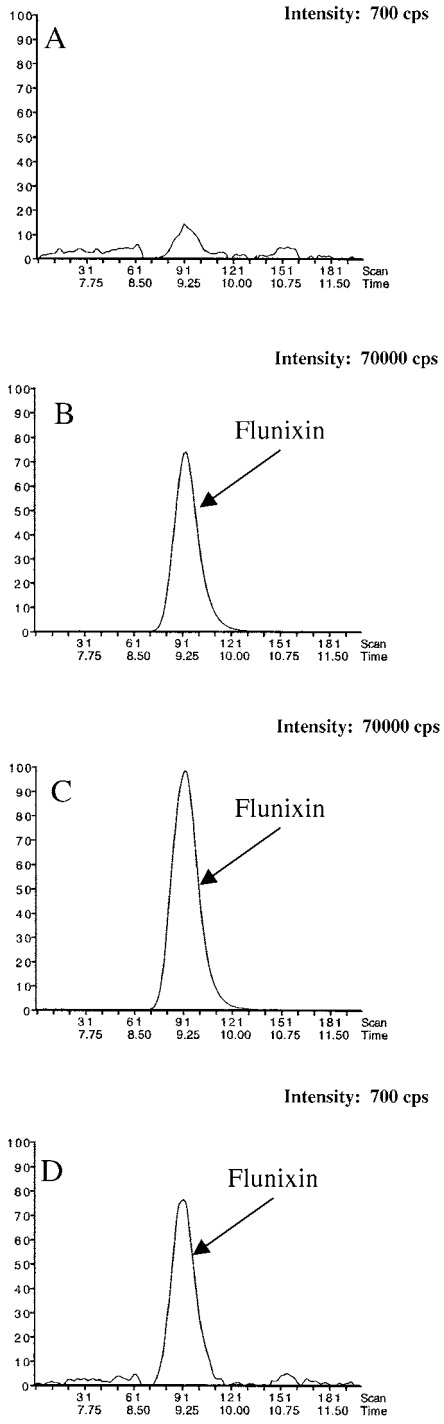

Figure 4. Representative LC/MS/MS chromatograms of (A) control liver, (B) fortified liver at 125 ppb, (C) incurred residue liver at 168 ppb, (D) calibration standard at 0.125 ng/mL (equivalent to 1 ppb).

Selectivity. Chromatograms of control samples were free from interference. **Figure 4** presents typical chromatograms obtained from the method.

Specificity. Specificity of the method was confirmed by assessing the potential for interference in the LC/MS/MS assay by 16 drugs potentially used in cattle and 3 flunixin metabolites. The drugs included bacitracin zinc, chlortetracycline hydrochloride, fenbendazole, lasalocid sodium, levamisole hydrochloride, monensin sodium, oxytetracycline dihydrate, penicillin G sodium, sulfamethazine sodium, tylosin tartrate, ceftiofur sodium, flavomycin (bambermycins), laidlomycin propionate potassium salt, tilmicosin, eprinomectin, and cephalonium. The

flunixin metabolites included 5-hydroxy flunixin, 2′-hydroxymethyl flunixin, and 4′-hydroxy flunixin. Compounds evaluated were prepared at a concentration of approximately 1 μg/mL and tested at a concentration equivalent to ~100 ppb. The compounds were tested in groups of 3−4 compounds by addition of the solutions to 6N HCl followed by extraction with ethyl acetate. A subsample of each ethyl acetate extract was dried and reconstituted with 50% methanol/water and evaluated by LC/MS/MS as for the determinative method. None of the drugs or flunixin metabolites yielded a response above the LOD by LC/MS/MS under the conditions used confirming the specificity of the method (data not shown).

Theoretical Limits of Detection (LOD) and Quantitation (LOQ). Duplicate extractions of each tissue type from six control samples and duplicate injections per each sample extract were made ($n \geq 20$, for each tissue type). The baselines at the retention time of flunixin (ca., 9.1 min) were manually integrated and quantified against the average response of the lowest concentration flunixin calibration standard. The average concentration ($\bar{X}$) found for each control tissue type was 0.06 ppb (SD = 0.02 ppb) for liver, 0.04 ppb (SD = 0.02 ppb) for kidney, 0.06 ppb (SD = 0.05 ppb) for muscle, and 0.07 ppb (SD = 0.03 ppb) for fat. The theoretical limit of detection (LOD) is defined as "LOD = $(\bar{X}) + 3 \times$ SD" and was 0.1, 0.1, 0.2, and 0.2 ppb for liver, kidney, muscle, and fat, respectively. The theoretical limit of quantitation (LOQ) is defined as "LOQ = $(\bar{X}) + 10 \times$ SD" and was 0.3, 0.2, 0.6, and 0.4 ppb for liver, kidney, muscle, and fat, respectively. The lowest level used for validation of the method was 1 ppb, and this level is the LOQ for the method.

Assay Timing and Repeats. The method takes approximately 12 h to prepare 30 samples for LC/MS/MS analysis. A typical set for this method would consist of 27 samples, a control, a fortified control at low and high levels, and a set of 6 standards injected before and after the samples. The rate-limiting step is the sample hydrolysis step and depends on the capacity of the heating block. If repeat analysis is necessary, another subsample of the ethyl acetate extract could be subjected to the remaining method steps.

Stability. Storage stability analyses conducted with stored control-fortified and incurred tissue samples at ~ −20 °C indicated that flunixin was stable for a minimum of one year in liver and at least 2 months in muscle, kidney, and fat (data not shown). Storage stability analyses conducted with stored calibration standard solutions indicated that they were stable for a minimum of one month (data not shown).

In-Process Extract Stability. One control extract from each tissue type and duplicate samples of extracts from tissue fortified with flunixin at 125 and 400 ppb (liver/kidney), 10 and 40 ppb (muscle), and at 25 and 100 ppb (fat) levels of flunixin free acid were prepared for LC/MS/MS analyses. The final extracts were analyzed on the day of extraction, after at least 24 h at room temperature, and after at least 6 days at ~ −20 °C. Average recoveries for 125 and 400 ppb fortified liver (and kidney) samples stored for 24 h at room temperature were 88 (91) and 91% (88%), respectively. Average recoveries for muscle stored at room-temperature fortified at 10 and 40 ppb were 88, and 91%, respectively. Average recoveries for fat stored at room-temperature fortified at 25 and 100 ppb were 94, and 95% respectively. Similar recoveries were also found after frozen storage. Results demonstrated that the final extracts were stable over the 24 h and 6 day interval under the specified conditions. In addition, the ethyl acetate extracts were also stored for at least 5 days at ~ −20 °C and then analyzed. Results demonstrated the stability of the storage samples (data not shown).

Freeze/Thaw Stability. The stability of flunixin in all four bovine tissues was evaluated after three freeze/thaw cycles. Each cycle consisted of thawing the fortified samples at room temperature and returning to freezer (~ −20 °C) for approximately 24 h. The results demonstrated the stability of the samples. Average recoveries for 125 and 400 ppb fortified liver (and kidney) samples were 86.3 (91.3) and 88.5% (92.5%), respectively. Average recoveries for muscle fortified at 10 and 40 ppb were 97.5, and 98.2%, respectively. Average recoveries for fat fortified at 25 and 100 ppb were 90.5, and 95.6%, respectively.

The method reported herein is a rapid and sensitive procedure for the determination of low levels of flunixin in edible bovine tissues. This method has a validated LOQ of 1 ppb for all tissues, and a LOD of 0.1, 0.1, 0.2, and 0.2 ppb for liver, kidney, muscle, and fat, respectively. The procedure was also shown to possess a sufficient level of ruggedness such that chromatographic materials from different manufacturers would not result in a significant difference in determined values. Potential interference from several other animal drugs and flunixin metabolites were investigated and showed no interference. Overall fortification recoveries for liver (1−400 ppb), kidney (1−400 ppb), muscle (1−40 ppb), and fat (1−100 ppb) tissues were 85.9, 94.6, 87.4, and 87.6%, respectively.

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