JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Residue Depletion Study and Withdrawal Period for Flunixin-*N*-methyl Glucamine in Bovine Milk Following Intravenous Administration

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The objective of this study was to establish a withdrawal period for flunixin in milk by quantifying 5-hydroxyflunixin, the marker residue, in bovine milk as a function of time, following intravenous treatment of lactating dairy cows with flunixin-*N*-methyl glucamine (Banamine or Finadyne). Lactating dairy cows were dosed on three consecutive days at 2.2 mg of flunixin free acid/kg of body weight/day. Milk was collected twice daily and assayed using a liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS) procedure. The method was validated at concentrations in the range 0.5−250 ppb. The concentrations for 5-hydroxyflunixin measured 12 h after the last administration of drug ranged from 1.56 to 40.6 ppb for all cows. Milk concentrations for 5-hydroxyflunixin were used to establish withdrawal periods of 36 h using guidelines established by the U.S. Food and Drug Administration/Center for Veterinary Medicine and 24 h using guidelines established by the European Medicinal Evaluation Agency/Committee on Veterinary Medicinal Products.

KEYWORDS: Flunixin meglumine; 5-Hydroxyflunixin; bovine milk; withdrawal period

INTRODUCTION

Banamine injectable solution or Finadyne is a nonsteroidal anti-inflammatory drug and a non-narcotic analgesic. In veterinary medicine, flunixin is formulated as the meglumine [*N*-methylglucamine (NMG)] salt to enhance solubility. In the United States, flunixin is approved for use in beef cattle and nonlactating dairy cows for the control of pyrexia associated with bovine respiratory disease and endotoxemia (*1*). Flunixin is not currently approved for use in lactating dairy cows in the United States. Several studies have been reported on the pharmacokinetics (*2*, *3*) of flunixin in dairy cows and on methods (*4*−*6*) for the analysis of flunixin in milk. There are no reported studies showing the depletion of flunixin in milk as a function of time following treatment of lactating dairy cows with flunixin NMG.

In a total residue depletion study conducted by Feely et al. (*7*), [^{14}C]flunixin meglumine was administered once daily for three consecutive days to eight lactating dairy cows at a dose of 2.2 mg of flunixin free acid/kg of body weight. Milk containing incurred residues of [^{14}C]flunixin was collected and analyzed for parent compound as well as metabolites. 5-Hydroxyflunixin (**Figure 1**) was established as the marker residue for flunixin in bovine milk. The European Medicinal Evaluation Agency, Committee on Veterinary Medicinal Products (EMEA/CVMP), has established a maximum residue limit (MRL) for flunixin (as measured by 5-hydroxyflunixin) in bovine milk as 40 μg/kg (ppb). The U.S. Food and Drug Administration/Center for Veterinary Medicine (FDA/CVM) has established a maximum permitted concentration (R_m) for 5-hydroxyflunixin in bovine milk as 2 ppb.

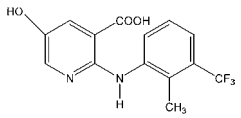

Figure 1. Structure of 5-hydroxyflunixin.

This study was conducted to quantify 5-hydroxyflunixin residues in bovine milk as a function of time and to establish the withdrawal period or milk discard time for flunixin in milk. Lactating dairy cows were treated with 3.65 mg of flunixin-NMG (trade names Banamine in the United States or Finadyne in Europe), equivalent to 2.2 mg of flunixin free acid/kg of body weight/day. The drug was administered once daily for 3 days (~24 h apart). Milk was collected twice daily (~12 h apart)

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10.1021/jf026219+ CCC: $25.00
Published on Web 06/25/2003

from each cow during the 3 days of treatment and for 6 days following the last administration of the drug. The milk was assayed for 5-hydroxyflunixin residues. The limit of quantitation (LOQ) for the method was 0.5 ppb, and the limit of detection (LOD) was 0.18 ppb. The milk concentrations for 5-hydroxyflunixin were used to calculate the withdrawal period.

MATERIALS AND METHODS

Apparatus. The liquid chromatograph consisted of Shimadzu LC-10AT pumps and a Perkin-Elmer series 200 autosampler. The mass spectrometer used was a Sciex API 3000 mass spectrometer controlled by MacQuan version 1.4 software. Separation was achieved using a New Guard RP-18, 15 mm × 3.2 mm, guard column (PE Brown-Lee) connected to an Eclipse XDB-C18 column, 2.1 cm × 15 cm × 5 μm (Zorbax). The solid phase extraction (SPE) cartridges used were SCX Mega Bond Elute cartridges (6 cm^3/g) from Varian.

Reagents and Solutions. Methanol, ethyl acetate, and acetone were of HPLC grade (Burdick and Jackson), water was of HPLC grade (Optima, Fisher Scientific), and acetonitrile was of reagent grade (Omni solve, E. M. Science). The following acids and base were of reagent grade (J. T. Baker): phosphoric acid, H_3PO_4, 85%; glacial acetic acid, CH_3COOH; hydrochloric acid, HCl, 36.5−38.5%; and ammonium hydroxide, NH_4OH, 28.0−30.0%. Mobile phase A consisted of 0.4% glacial acetic acid prepared in water (v/v). Mobile phase B was made up of 0.2% glacial acetic acid (v/v) in acetonitrile/methanol (35:10).

Standards. 5-Hydroxyflunixin reference standard (**Figure 1**, CAS Registry No. 75369-61-8) was obtained from Schering-Plough Chemical Development (Union, NJ). After correcting for purity, a 500 μg/mL stock standard solution and a 500 μg/mL fortification solution were prepared using methanol. The solutions were stored at −20 °C for 1 month. An intermediate calibration standard solution, 50 μg/mL, was prepared by diluting the stock standard with methanol. Serial dilutions of the 50 μg/mL intermediate standard solution were made using a methanol/water solution (50 + 50, v/v) to give calibration standards at concentrations of 0.25, 0.50, 5.0, 25, 50, and 125 ng/mL. The calibration standards were stable at 4 °C for 1 month.

Test System. Thirty-five lactating Holstein dairy cows weighing 600−900 kg were acclimated for 14 days in a barn. The cows were fed dairy ration and roughage, and fresh water was provided daily. A veterinarian performed physical and fecal examinations on each cow during acclimation. The health history, food intake, and milk productions (second through fifth lactations) for each cow were used to select 27 cows for the study. For the duration of the study, all of the cows were checked daily for general health, behavior, and appearance. Each cow was clinically evaluated twice daily for mastitis. The set of 27 cows had equal numbers of cows with three milk output groups: group I, 9 cows with average milk production >14.4 kg/milking; group II, 9 cows with average milk production <14.4 but >8.7 kg/milking; and group III, 9 cows with average milk production <8.7 kg/milking. Two of the 27 cows (one cow from group I and one cow from group II) were randomly selected as replacement cows to provide 25 cows of varying milk production for the study. No test cows needed to be replaced, so the milk collected from replacement cows was not assayed. Each cow was dosed on three successive days via the jugular vein using a nominal dose of 3.65 mg of flunixin-NMG/kg of body weight/day administered ∼24 h apart. The actual formulation administered was 0.044 mL of Banamine injectable solution or Finadyne/kg of body weight/day, which is equivalent to 2.2 mg of flunixin free acid/kg of body weight/day administered. Banamine injectable solution contains 83 mg of flunixin-NMG salt/mL or 50 mg/mL flunixin free acid. Predose milk was collected in the morning from each cow prior to the administration of the first dose and used as control milk. Milk was collected ∼12 h apart during the 3 days of treatment and for 6 days after the last administration of Banamine injectable solution. Cows with varying milk production were used because milk output can sometimes affect residue levels. Each milking from each cow was separately collected, well mixed, and prepared for subsampling. Subsamples of milk from each milking and from each cow were separately frozen. Frozen subsamples of milk were shipped from the in-life facility (HMS Veterinary Inc.) in boxes containing dry ice to the analytical testing facility (ABC Laboratories Inc.) for analyses.

Extraction. Milk samples were assayed in 25 sets (1 set per animal) each consisting of duplicate quality control (QC) samples (at four concentrations of 0.5, 10, 50, and 150 ppb), 2 matrix blanks (composite milk from nontreated cows), and 54 milk samples containing incurred residues of 5-hydroxyflunixin from the same cow (3 replicates per time point × 18 time points). To prepare an extract, 2 g of milk was weighed into a 15-mL polypropylene tube. HCl (0.1 N, 1.5 mL) was added to the milk. The milk sample was mixed using a vortex mixer for ∼3 min. Six milliliters of acetone/ethyl acetate solution (1:1, v/v) was added to the milk sample. The contents of the tube were mixed for ∼30 s at high speed using a vortex mixer. The tube was centrifuged at 2500*g* for ∼3 min. The supernatant was removed and placed into a 50-mL polypropylene centrifuge tube. Six milliliters of acetone/ethyl acetate solution (1:1, v/v) was added to the precipitate in the 15-mL polypropylene tube. The contents of the tube were mixed and centrifuged, and the supernatant was combined with the previous supernatant. The extraction with acetone/ethyl acetate solution (1:1, v/v) followed by centrifugation and separation of the supernatant was repeated two more times for a total of four extractions. The volume of the supernatant (in the 50-mL polypropylene tube) was adjusted to 30 mL using acetone/ethyl acetate solution (1:1, v/v). An aliquot of the extract, 7.5 mL, was placed into a 15-mL glass centrifuge tube and completely dried using a slow stream of nitrogen and a water bath set at a temperature of 50 °C. The residue was dissolved in 10 mL of H_3PO_4/methanol (0.1:99.9, v/v) and the contents of the centrifuge tube were mixed for ∼30 s using a vortex mixer. An SCX SPE cartridge was conditioned with 12 mL of water followed by 12 mL of H_3PO_4/methanol (0.1:99.9, v/v). The flow control valve on the SPE manifold was closed, allowing ∼0.5 mL of H_3PO_4/methanol (0.1:99.9, v/v) to remain above the cartridge bed. The extract dissolved in H_3PO_4/methanol (0.1:99.9, v/v) was added to the SCX cartridge and allowed to flow using gravity. The flow rate was adjusted to 1−2 drops per second using the flow control valve. The centrifuge tube, which contained the extract, was rinsed with 10 mL of H_3PO_4/methanol (0.1:99.9, v/v), and the rinse was used to wash the SCX cartridge after the sample had been loaded. Once the H_3PO_4/methanol (0.1:99.9, v/v) wash solution drained to the surface of the column bed, a vacuum of 5 in. of Hg was applied for 5 s to completely drain the SCX cartridge. The analyte was eluted from the SCX cartridge using 5 mL of NH_4OH/methanol (10:90, v/v). A vacuum of 5 in. of Hg was applied for ∼5 s to completely drain the SCX cartridge. The extract was evaporated to dryness using a gentle stream of nitrogen and a water bath at a temperature of 50 °C. The residue was reconstituted in 1 mL of methanol/water (50:50, v/v). The extract was transferred to a microcentrifuge tube and centrifuged at 9800*g* for ∼5 min. The supernate was removed and analyzed by LC-MS/MS. The extract is stable for 3 days at temperatures in the range 2−10 °C.

Analysis. The LC-MS/MS system consisted of Shimadzu pumps, a Perkin-Elmer autosampler, and the Sciex API 3000 mass spectrometer. The electrospray interface was operated in the positive ionization mode. The source variables were optimized to monitor the ionic transition from the precursor ion (*m*/*z* 313.1) to the product ion (*m*/*z* 295.1) using multiple reaction monitoring acquisition mode. The following instrument variables were used: collision energy, 29 eV; turbo-ion spray gas flow, 7000−8000 mL/min; nebulizer gas flow, 12 units; and ion source temperature, 400 °C. The injection volume was 20 μL, and separation was achieved using a Zorbax Eclipse XDB-C18 column (2.1 cm × 15 cm × 5 μm) preceded by a New Guard RP-18 guard column (3.2 mm × 15 mm). The column was maintained at a temperature of 40 °C, and the autosampler temperature was 4 °C. Gradient elution was used for the separation. Initially, the gradient was held for 3 min at 50% mobile phase A (0.4% CH_3COOH in H_2O) and 50% mobile phase B [0.2% CH_3COOH in acetonitrile/methanol (35:10)]. The gradient was then stepped to 10% mobile phase A and 90% mobile phase B in 0.1 min. These conditions were maintained for 0.4 min. The gradient conditions were stepped to 50% mobile phase A and 50% mobile phase B in 0.1 min. The latter conditions were maintained for 2.4 min. The LC system was equilibrated using the initial gradient conditions for at least 30 min prior to the daily analyses of three

Table 1. Postdose Milk Concentrations (Parts per Billion) for 5-Hydroxyflunixin, Group I Cows

		postdose milking no. and time for group I cows[a]					
cow	replicate	1, 12 h	2, 24 h	3, 36 h	4, 48 h	5, 60 h	6, 72 h
1599	1	17.1	1.47	BQL	BQL	BQL	ND
	2	17.6	1.47	BQL	BQL	BQL	ND
	3	11.6	1.25	BQL	BQL	BQL	ND
1725	1	10.0	BQL	BQL	BQL	BQL	ND
	2	8.93	BQL	BQL	BQL	BQL	ND
	3	10.6	BQL	BQL	BQL	BQL	ND
3215	1	25.7	3.38	BQL	BQL	BQL	ND
	2	26.2	3.70	BQL	BQL	BQL	ND
	3	25.5	3.37	BQL	BQL	BQL	ND
9457	1	28.7	14.1	2.64	1.29	0.595	BQL
	2	26.0	9.61	2.76	1.38	0.617	BQL
	3	1.56	10.5	2.79	1.52	0.623	BQL
12497	1	15.7	0.973	BQL	BQL	BQL	ND
	2	15.2	0.894	BQL	BQL	BQL	ND
	3	15.6	1.05	BQL	BQL	BQL	ND
13619	1	30.4	3.55	0.830	BQL	BQL	BQL
	2	22.5	4.23	0.825	BQL	BQL	BQL
	3	25.1	3.98	0.841	BQL	BQL	BQL
13942	1	15.5	5.23	BQL	BQL	BQL	ND
	2	21.0	7.53	BQL	BQL	BQL	ND
	3	28.0	5.90	BQL	BQL	BQL	ND
14218	1	23.1	1.41	BQL	BQL	BQL	ND
	2	21.9	1.41	BQL	BQL	BQL	ND
	3	20.7	1.29	BQL	BQL	BQL	ND

[a] Milk concentrations for 5-hydroxyflunixin for all cows for time points between 72 and 156 h either were below the limit of detection or were not detected (ND). BQL means below the quantifiable limit (0.5 ppb).

Table 2. Postdose Milk Concentrations (Parts per Billion) for 5-Hydroxyflunixin, Group II Cows

		postdose milking no. and time for group II cows[a]					
cow	replicate	1, 12 h	2, 24 h	3, 36 h	4, 48 h	5, 60 h	6, 72 h
176	1	18.3	3.06	BQL	BQL	BQL	ND
	2	17.5	2.57	BQL	BQL	BQL	ND
	3	19.3	2.66	BQL	BQL	BQL	ND
1996	1	17.2	1.57	BQL	BQL	BQL	ND
	2	19.3	1.34	BQL	BQL	BQL	ND
	3	18.2	1.55	BQL	BQL	BQL	ND
10795	1	26.3	3.63	0.616	BQL	BQL	ND
	2	24.8	3.04	0.776	BQL	BQL	ND
	3	28.8	3.90	0.790	BQL	BQL	ND
11825	1	16.8	1.25	BQL	BQL	BQL	ND
	2	16.5	0.837	BQL	BQL	BQL	ND
	3	16.9	0.875	BQL	BQL	BQL	ND
12002	1	30.5	5.14	0.602	BQL	BQL	ND
	2	31.7	5.22	0.525	BQL	BQL	ND
	3	32.3	5.27	0.620	BQL	BQL	ND
12145	1	12.1	1.28	BQL	BQL	BQL	ND
	2	12.8	2.71	BQL	BQL	BQL	ND
	3	13.2	3.32	BQL	BQL	BQL	ND
14072	1	36.7	4.46	1.04	BQL	BQL	BQL
	2	40.6	4.39	1.02	BQL	BQL	BQL
	3	40.3	4.09	1.00	BQL	BQL	BQL
20660	1	8.37	1.59	BQL	BQL	BQL	ND
	2	8.16	1.56	BQL	BQL	BQL	ND
	3	8.08	1.66	BQL	BQL	BQL	ND

[a] Milk concentrations for 5-hydroxyflunixin for all cows for time points between 72 and 156 h either were below the limit of detection or were not detected (ND). BQL means below the quantifiable limit (0.5 ppb).

Table 3. Postdose Milk Concentrations (Parts per Billion) for 5-Hydroxyflunixin, Group III Cows

		postdose milking no. and time for group III cows[a]					
cow	replicate	1, 12 h	2, 24 h	3, 36 h	4, 48 h	5, 60 h	6, 72 h
1963	1	32.5	2.64	BQL	BQL	BQL	ND
	2	31.3	2.28	BQL	BQL	BQL	ND
	3	35.2	3.46	BQL	BQL	BQL	ND
9430	1	13.7	5.12	1.07	BQL	BQL	ND
	2	12.8	6.19	0.961	BQL	BQL	BQL
	3	13.4	3.62	1.05	BQL	BQL	ND
9710	1	40.2	1.81	0.629	BQL	BQL	ND
	2	38.1	0.702	0.630	BQL	BQL	ND
	3	24.1	1.03	0.660	BQL	BQL	ND
10220	1	10.1	6.05	0.797	BQL	BQL	ND
	2	8.62	5.60	1.05	BQL	BQL	ND
	3	11.0	4.05	1.04	BQL	BQL	ND
11541	1	27.2	1.59	BQL	BQL	BQL	BQL
	2	22.2	2.76	BQL	BQL	BQL	BQL
	3	27.5	1.82	BQL	BQL	BQL	BQL
11907	1	22.0	6.48	0.621	BQL	BQL	ND
	2	22.9	6.11	0.476	BQL	BQL	ND
	3	25.3	6.23	0.570	BQL	BQL	ND
12347	1	21.8	3.51	0.561	BQL	BQL	ND
	2	21.4	3.79	0.512	BQL	BQL	ND
	3	20.0	2.61	0.538	BQL	BQL	ND
20174	1	9.44	1.36	BQL	BQL	BQL	ND
	2	9.59	1.29	BQL	BQL	BQL	ND
	3	9.37	1.29	BQL	BQL	BQL	ND
20601	1	26.2	3.31	BQL	BQL	BQL	ND
	2	24.7	3.59	BQL	BQL	BQL	BQL
	3	25.4	2.48	BQL	BQL	BQL	BQL

[a] Milk concentrations for 5-hydroxyflunixin for all cows for time points between 72 and 156 h either were below the limit of detection or were not detected (ND). BQL means below the quantifiable limit (0.5 ppb).

replicates of the system validation standard (50 ng/mL) followed by the analyses of calibration standards. Milk extracts were analyzed in a set consisting of duplicate injections of six calibration standards, duplicate QC milk extracts at four different concentrations, 2 control milk extracts, and 54 milk extracts containing incurred residues of 5-hydroxyflunixin. QC milk extracts were interspersed throughout the analysis set. Calibration standards analyzed at the end of the sequence were used to monitor instrument performance. The analysis set was considered to be acceptable if the following criteria were met: the instrument response for two-thirds of the second set of calibration standards was ±15% of the response for the calibration standards analyzed at the start of the sequence, the coefficient of determination (r^2) for the calibration curve was ≥0.98, and the percent recoveries for six of eight QC samples assayed were 70−110% for 5-hydroxyflunixin milk concentrations ≤100 ppb and 80−110% for concentrations >100 ppb.

Calculations. The peak areas and the calibration standard concentrations for 5-hydroxyflunixin were used to construct calibration curves by applying least-squares fit linear regression. The concentration for 5-hydroxyflunixin in milk (ng/g or ppb) was calculated using the following equation: $(X \times V \times 4)/W$, where X is the concentration for 5-hydroxyflunixin in the final sample extract (ng/g) derived from the calibration curve, V is the volume of the final sample extract (1 mL), 4 is the correction factor for subsampling (one-fourth of the acetone/ethyl acetate extract), and W is the weight (g) of milk extracted.

RESULTS AND DISCUSSION

Incurred milk was collected from 25 cows, which were divided into three groups based on milk output during the acclimation phase of the study. Three replicate milk extracts

Table 4. Average Postdose Milk Concentrations[a] (Parts per Billion) for 5-Hydroxyflunixin

postdose milking time (h)	group I ($n = 8$), av milk production >14.4 kg per milking	group II ($n = 8$), av milk production >8.7 and <14.4 kg per milking	group III ($n = 9$), av milk production <8.7 kg per milking
12	19.4	21.4	21.7
24	4.1	2.8	3.4
36	1.8	0.8	0.7
48	1.4	ND	ND
60	0.6	ND	ND
72	ND	ND	ND

[a] Milk concentrations of 5-hydroxyflunixin for all cows for time points between 72 and 156 h either were below the limit of detection or were not detected (ND).

from each time point (from each cow) were separately extracted, analyzed, and reported. The standard curves for 5-hydroxyflunixin were linear in the concentration range 0.25−125 ng/mL (0.5−250 ppb equiv in milk). The coefficients of determination for calibration curves were routinely ≥0.98 for the concentration range 0.5−250 ppb. Analyses of predose (control) milk collected from the 25 cows prior to dosing did not show any peaks in the chromatographic window for 5-hydroxyflunixin that could interfere with the assay. The QC samples ($n = 50$ per concentration) assayed with incurred milk gave the following average percent recoveries (and coefficients of variations): 77.2% (24.6%) at 0.5 ppb; 83.9% (15.5%) at 10 ppb; 90.0% (14.2%) at 50 ppb; and 88.8% (16.5%) at 150 ppb. Representative chromatograms for 5-hydroxyflunixin, and milk containing incurred residues of 5-hydroxyflunixin are presented in **Figures 2**−**5**.

The measured concentrations for 5-hydroxyflunixin in milk for the three groups of cows are presented in **Tables 1**−**3**, and the average milk concentration for each group is presented in **Table 4**. The milk concentrations for 5-hydroxyflunixin for all cows for time points between 72 and 156 h either were below the limit of detection or were not detected. These values are not presented in **Tables 1**−**4**. The milk concentrations for 5-hydroxyflunixin for each group of cows (based on milk production) are discussed below.

Residue Depletion for Group I Cows (Average Milk Production >14.4 kg per Milking). The concentrations for 5-hydroxyflunixin measured in the milk from the first milking (12 h) after the last administration of drug from group I cows (**Table 1**) ranged from 1.56 to 30.4 ppb. By 36 h (third milking) after the last administration of Banamine injectable solution, the 5-hydroxyflunixin milk concentrations had depleted to levels <2 ppb for seven of the eight cows. It took 48 h postdose (fourth milking) for the residues of 5-hydroxyflunixin to deplete to values <2 ppb for one cow.

Residue Depletion for Group II Cows (8.7 kg per Milking < Average Milk Production <14 kg per Milking). The concentrations for 5-hydroxyflunixin measured in milk collected 12 h (first milking) after the last administration of drug (**Table 2**) ranged from 8.08 to 40.6 ppb. For all cows, the milk concentrations for 5-hydroxyflunixin depleted to values <2 ppb in 36 h (third milking) after the last administration of Banamine injectable solution.

Residue Depletion for Group III Cows (Average Milk Production <8.7 kg per Milking). For group III cows (**Table 3**), the concentrations for 5-hydroxyflunixin measured in milk collected 12 h (first milking) after the last administration of drug ranged from 8.62 to 40.2 ppb. The milk concentrations for 5-hydroxyflunixin depleted to values <2 ppb in 36 h (third milking) postdose for all cows.

Overall Residue Depletion. The concentrations for 5-hydroxyflunixin measured in milk collected 12 h (first milking) after the last administration of drug ranged from 1.56 to 40.6 ppb

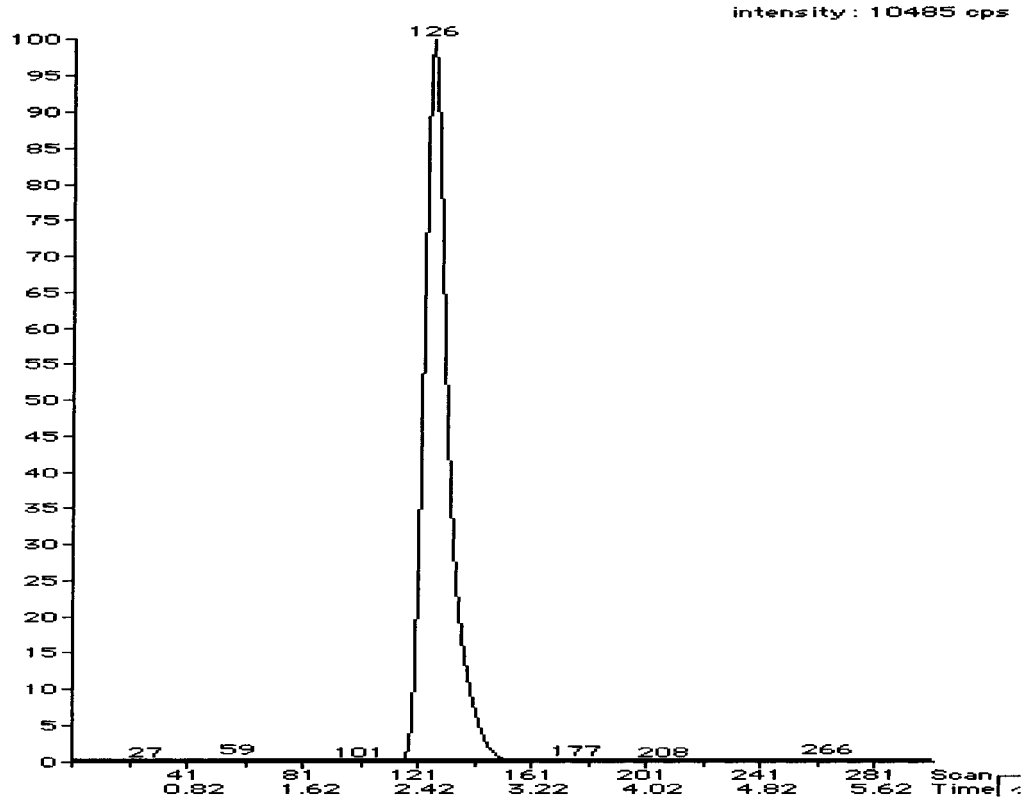

Figure 2. Standard equivalent to 50 ppb of 5-hydroxyflunixin in milk.

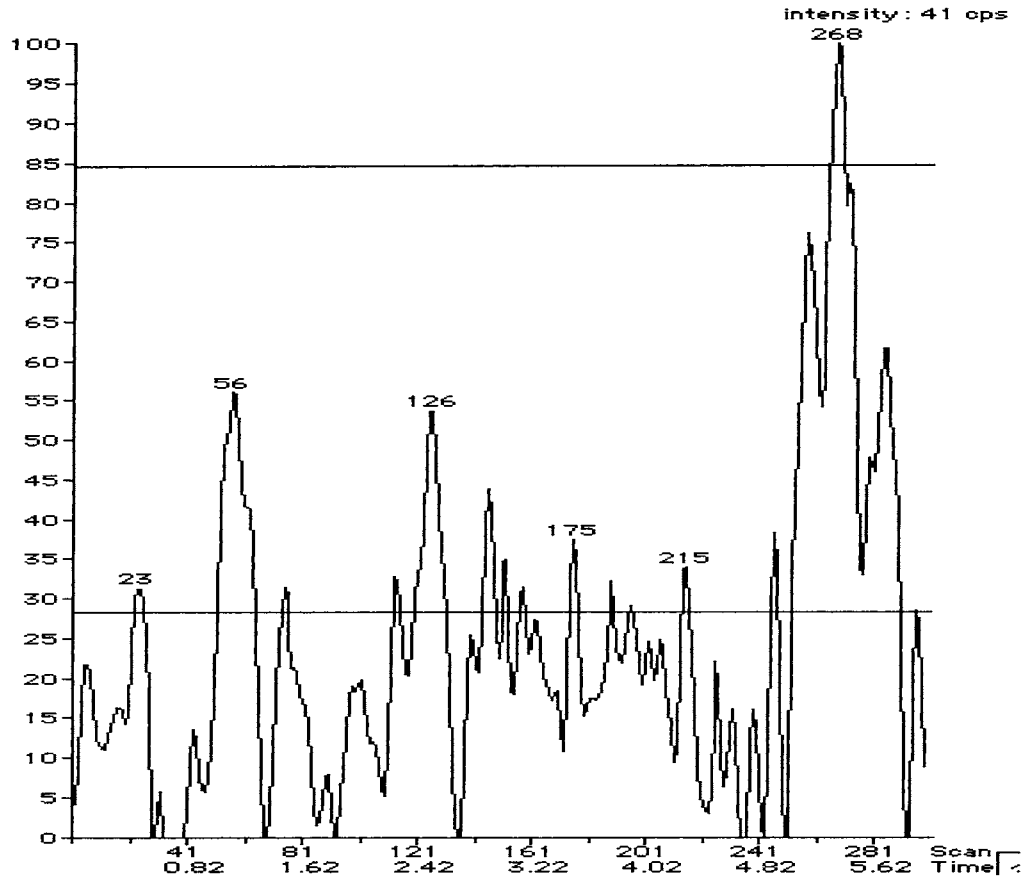

Figure 3. Control milk.

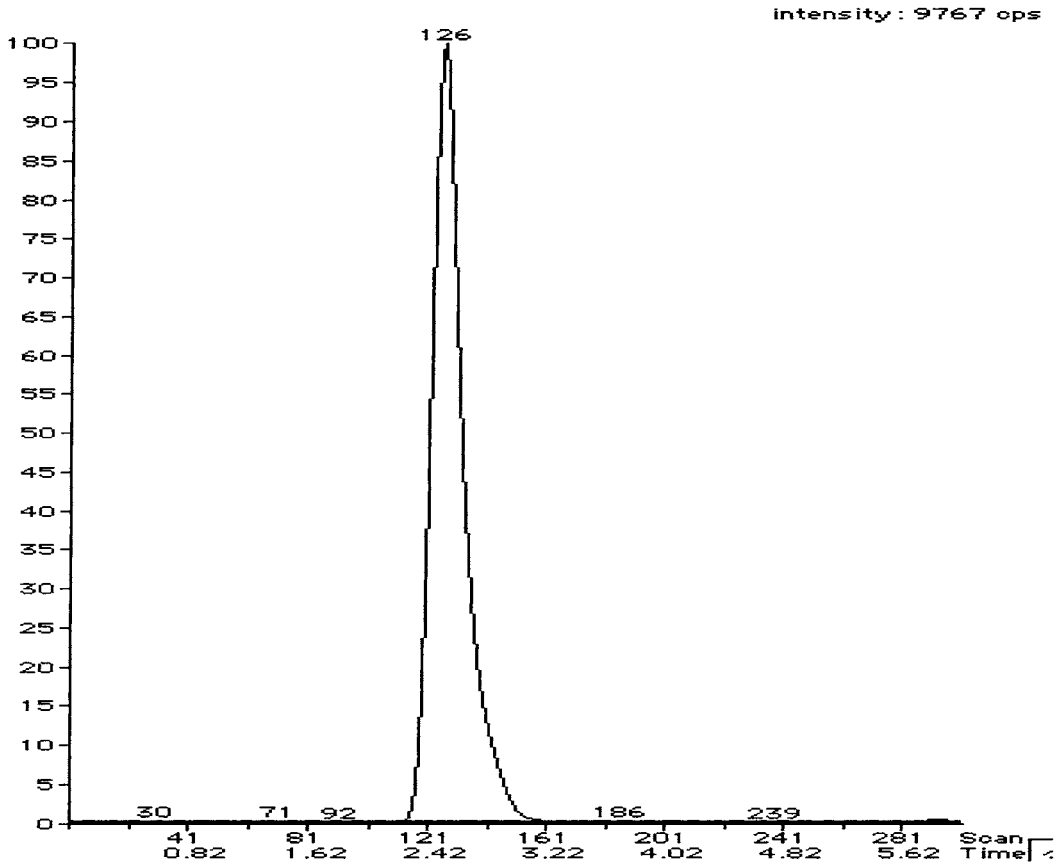

Figure 4. Control milk fortified at 50 ppb of 5-hydroxyflunixin.

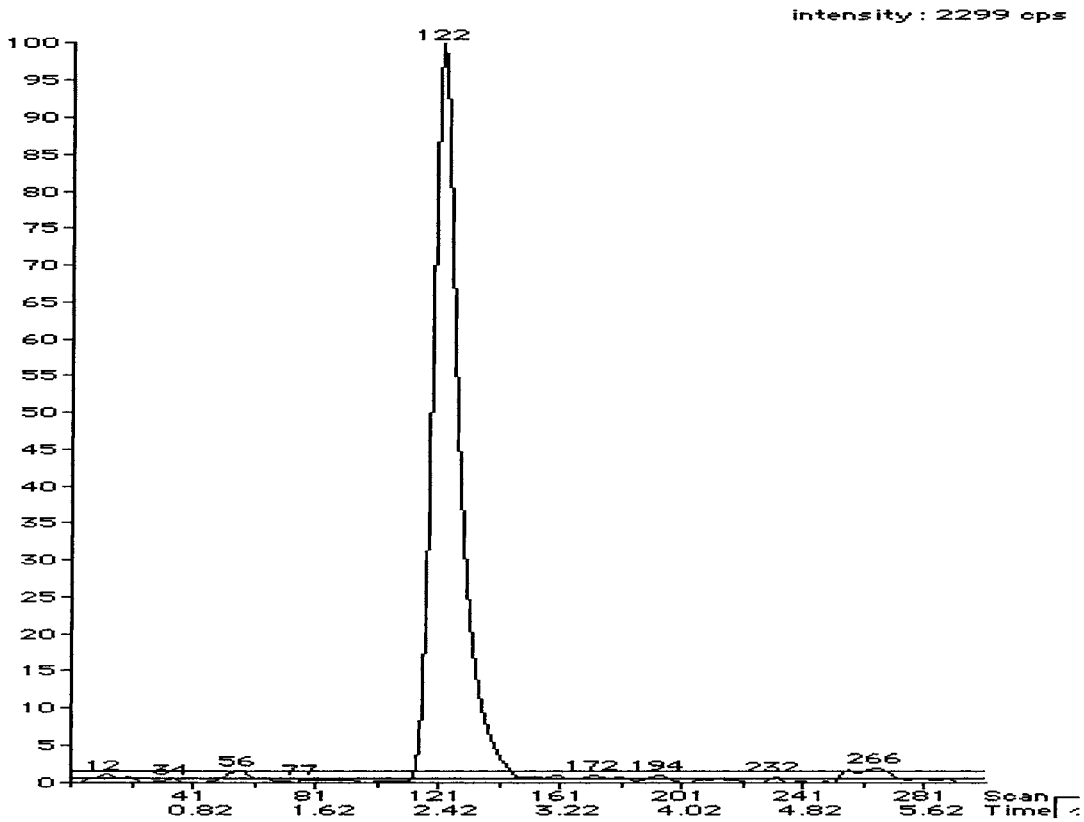

Figure 5. Milk containing incurred residues of 5-hydroxyflunixin, 10.1 ppb.

for all cows. By 36 h (third milking) after the last administration of the drug, the milk concentrations for 5-hydroxyflunixin rapidly depleted to levels <2 ppb for 24 of the 25 cows. By 48 h (fourth milking), the milk concentrations for 5-hydroxyflunixin had depleted <2 ppb for all cows. There were no significant differences in the average milk concentrations for 5-hydroxyflunixin for cows in the three groups even though the average milk productions were slightly different for each group of cows (see **Table 4**).

Calculation of Withdrawal Period Using the FDA/CVM Guidelines. The milk concentrations for 5-hydroxyflunixin (**Tables 1**−**4**) were used to calculate the withdrawal period by applying the statistical tolerance limit procedure (*8*). The withdrawal period provides an interval within which the concentrations of 5-hydroxyflunixin are at the maximum permitted concentration (2 ppb) or below for 99% of treated cows and the confidence level is 95%. In performing the statistical analyses, all of the concentrations for 5-hydroxyflunixin for all cows that were at the LOQ or above were used. The triplicate concentrations for 5-hydroxyflunixin from each time point were treated as independent measurements for each cow, and each cow was treated independently. The log concentrations for 5-hydroxyflunixin for each cow were fitted using a linear least-squares fit (*9*). The withdrawal period was established from the regression lines from each cow. The FDA/CVM withdrawal period was established as 36 h (or three milkings at 12 h intervals).

Calculation of Withdrawal Period Using the EMEA/CVMP Guidance Document. Milk concentrations for 5-hydroxyflunixin reported in **Tables 1**−**4** were corrected for recovery [corrected concentration (ng/g) = measured concentration (ng/g)/average percent recovery (90.4%, overall average during method validation)]. Milk concentrations for 5-hydroxyflunixin were subjected to statistical analysis using the time-to-safe-concentration (TTSC) approach listed in the guidance document for the determination of withdrawal periods for milk (*10*). With this approach, the triplicate concentrations for 5-hydroxyflunixin from each time point for each cow were averaged, and each cow was treated independently. The time-to-safe-concentration is identified as the first time the concentration for 5-hydroxyflunixin is at the MRL (40 μg/kg) or below and stays below the MRL at later times. The resulting TTSC points for all of the cows were used to calculate the average, standard deviation, and tolerance limit. Using the EMEA/CVMP guidelines, the withdrawal period was calculated to be 24 h.

ACKNOWLEDGMENT

We thank the following contributors: Dr. Louis Crouch of Schering-Plough Research Institute for helpful discussions and review of the reports; William Feely of Schering-Plough Research Institute, Dr. Pamela L. Boner, Dr. David D. W. Liu, and Matthew Wisocky of XenoBiotic Laboratories Inc. for developing the determinative assay.

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Received for review December 13, 2002. Revised manuscript received April 4, 2003. Accepted April 16, 2003.

JF026219+