JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Flunixin Residues in Milk after Intravenous Treatment of Dairy Cattle with ^{14}C-Flunixin

WILLIAM F. FEELY,*,† CHERYL CHESTER-YANSEN,† KENNETH THOMPSON,‡ JOHN W. CAMPBELL,§ PAMELA L. BONER,|| DAVID D. W. LIU,|| AND LOUIS S. CROUCH†

Schering-Plough Research Institute, Lafayette, New Jersey 07848, Schering-Plough Animal Health, Union, New Jersey 07083, Southwest Bio-Labs, Las Cruces, New Mexico 88005, and XenoBiotic Laboratories, Plainsboro, New Jersey 08534

Flunixin meglumine is used in veterinary medicine as an alternative to narcotic analgesics and as an antiinflammatory agent. Eight Holstein dairy cows were dosed intravenously once daily on three consecutive days with ^{14}C-flunixin meglumine at approximately 2.2 mg of flunixin free acid/kg of body weight. Milk was collected twice daily to determine the decline of the total radioactive residues (TRR) in milk and to identify or characterize residue components. TRR in milk declined rapidly and averaged 66, 20, and 14 ppb, respectively, for the first, second, and third milkings after administration of the last dose. Milk was extracted, and the extracts were examined for radioactive residues. Mean extractability of milk TRR was always greater than 80%. Flunixin and 5-hydroxyflunixin were identified by coelution with analytical standards using reverse phase HPLC. These two residues were the main radioactive residues found in milk and together accounted for 64, 37, and 44% of the extractable residues, for the first, second, and third milkings, respectively, after administration of the last dose. The presence of 5-OH flunixin in milk was confirmed by HPLC/MS/MS.

KEYWORDS: Flunixin meglumine; 5-hydroxyflunixin; flunixin; HPLC/MS/MS; total radioactive residues

INTRODUCTION

Flunixin (SCH 14714) *N*-methylglucamine (NMG, meglumine) (2-[[2-methyl-3-(trifluoromethyl)phenyl]amino]-3-pyridinecarboxylic acid as the NMG salt) is used in veterinary medicine as an alternative to narcotic analgesics and an antiinflammatory drug. Banamine and Finadyne are the injectable flunixin-NMG trade names for the formulation approved for use in cattle and horses in the U.S. and for cattle, swine, horses, and dairy cattle in the E.U., respectively.

Flunixin is presently being developed by Schering-Plough for use in dairy cattle in the U.S. The clinical dose is 2.2 mg of flunixin free acid/kg of body weight per day for up to 3 days. This report describes a total residue depletion and metabolism study in dairy cattle which is one of several studies required by regulatory agencies, such as the Food and Drug Administration, Center for Veterinary Medicine (FDA-CVM in the U.S.) or the Center for Veterinary Medicinal Products (CVMP in the E.U.), to register a veterinary pharmaceutical. One purpose of a total residue depletion study performed in food animals is to choose a marker residue. The marker residue may be the drug, a metabolite, or a derivative that is selected to allow the concentration of the total drug related residue in the edible tissue of animals treated with the drug to be monitored. After selection of the marker residue, a regulatory surveillance (determinative) method is developed and validated to allow quantitation of the concentration of marker residue in commodities from food animals. Samples from the total residue depletion study may be analyzed using the determinative method to obtain the ratio of the marker residue to the total radioactive residue or the ratio is obtained by HPLC radiometric methods. The ratio is used in further studies which are performed to ascertain the time between dosing and when the tissues would be safe for human consumption.

Flunixin and related residues have been investigated previously by others in urine, plasma, serum, and milk from treated animals. Flunixin was quantified in horse urine by gas chromatography (*1*). Flunixin residues and an hydroxylated metabolite were investigated in horse urine and plasma by gas chromatography/mass spectrometry (*2*). In other reports, HPLC with UV detection was used to quantify flunixin in horse urine (*3*) and in cow plasma (*4*). Thin-layer chromatography was used to detect flunixin in horse serum (*5*). Other experiments describe the identification of radioactive 2′-OH methylflunixin, 4′-OH flunixin, and 5-OH flunixin in cattle and in rats (*6*).

One study described a method for the determination of flunixin in cow milk and plasma by HPLC with UV detection

* To whom correspondence should be sent. Telephone: (973) 940-4111. Fax: (973) 940-4159. E-mail: william.feely@spcorp.com.
† Schering-Plough Research Institute.
‡ Schering-Plough Animal Health.
§ Southwest Bio-Labs.
|| XenoBiotic Laboratories.

10.1021/jf0258609 CCC: $22.00
Published on Web 11/06/2002

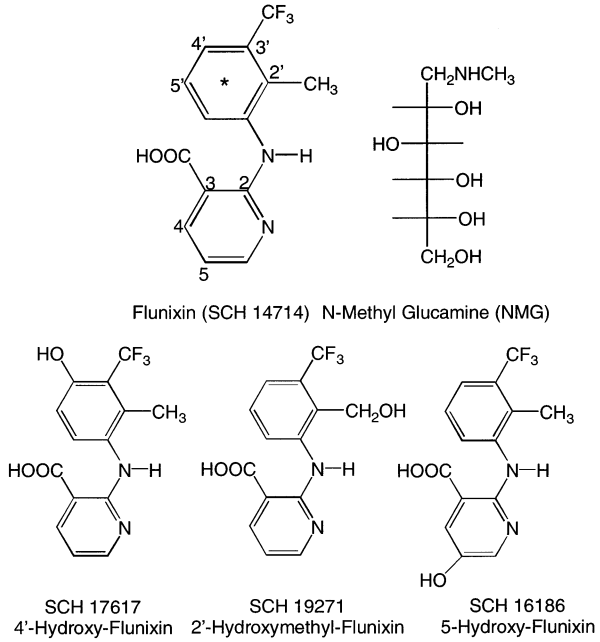

Figure 1. Structures of ^{14}C-flunixin-NMG, 5-OH flunixin, 4′-OH flunixin, and 2′-(hydroxymethyl)flunixin.

(*7*). In another study (*8*), one lactating cow was given flunixin intravenously at a dose of 2.2 mg/kg. Flunixin was quantitated, using HPLC with UV detection, in milk at a concentration level of 1.88 ppb at 16 h postdose. However, higher levels were obtained after additional treatment with β-glucuronidase with residues ranging from 7.34 ppb (16 h postdose) to 1.34 ppb (24 h postdose). Indeed, a C-1 β-glucuronide flunixin metabolite was found by HPLC/MS/MS in the urine of greyhound dogs (*9*).

A preliminary study indicated that 5-OH flunixin was present in milk from cattle treated with ^{14}C-flunixin-NMG (Casciano and Charles, personal communication). However, to our knowledge, no mention has been made of 5-OH flunixin in cow milk in the literature. The present study is the first to describe the detection of 5-OH flunixin in cow milk and to characterize flunixin residue components in milk.

The objectives of the study were (1) to determine the decline of the total radioactive residues (TRR) of flunixin in milk following ^{14}C-flunixin-NMG treatment of lactating dairy cows. (2) to determine the identity of or characterize the metabolites in milk, to ensure that there were no milk-specific residues of flunixin that have not been tested for toxicity, and (3) to select the marker residue and determine the ratio of marker to total residue by analyzing radioactive milk samples by the determinative method.

MATERIALS AND METHODS

Test Articles. Placebo Banamine formulation was received from Schering-Plough (Kenilworth, NJ). ^{14}C-Flunixin-NMG (200 mCi, >99.4% purity by HPLC and >98.5% purity by TLC) was supplied by Amersham Life Science Inc. (Arlington Heights, IL) and was used less than 2 months after analysis at Amersham. Nonradiolabeled flunixin-NMG (>99.6% purity) was received from Schering-Plough (Rathdrum, Ireland). The structure and radiolabel position for flunixin are shown in **Figure 1**.

Analytical Standards. In addition to the flunixin-NMG mentioned above, the following analytical standards (**Figure 1**) were obtained from Schering-Plough (Kenilworth, NJ) for use in the chromatographic characterization of flunixin and related compounds in milk: 5-hydroxyflunixin (5-OH flunixin, >98% purity); 4′-hydroxyflunixin (4′-OH flunixin, >93% purity); 2′-(hydroxymethyl)flunixin (2′-OH methylflunixin, >99% purity).

^{14}C-Flunixin Dose Solution Formulation. The dosing solution was prepared to be similar to the commercial formulation (Banamine), which contains 50 mg of flunixin free acid/mL or 83 mg of flunixin-NMG/mL of formulation. Placebo Banamine (no active ingredient), ^{14}C-flunixin-NMG, and nonradiolabeled flunixin-NMG were mixed and sterile filtered at the Schering-Plough Research Institute. Formulated material was sterile filtered in a negative pressure, microbiological hood through a 0.22 μm nylon filter attached to a Waters HPLC solvent filtering apparatus. All glassware was washed with methanol and allowed to air-dry overnight in the hood. Formulated material was filtered directly into a 1 L sterile amber bottle. Formulated material was stored at 2−8 °C and was sterile filtered again just prior to dosing.

The concentration, homogeneity, specific activity, purity, and stability of ^{14}C-flunixin-NMG in filter sterilized dosing solutions was determined by liquid scintillation counting (LSC) and high-performance liquid chromatography (HPLC).

The final concentration of flunixin as free acid in the final dose solution was 48.88 mg/mL (97.76% of target concentration). Specific activity was 8492 dpm/μg as free acid. HPLC analyses before and after dosing indicated that the radiopurity of flunixin in the dosing solution was >99% in all cases and thus remained stable during the in-life phase of the study. For LSC, multiple aliquots were taken, at different times, from the top, middle, and bottom of the 1 L bottle. The LSC analyses indicated that the dosing solution was homogeneous.

In-Life Study Design. The in-life phase of the study was conducted at Southwest Bio-labs (SBL, Las Cruces, NM). A group of 12 lactating Holstein cows, 2−10 years of age, was obtained from Gonzalez Dairy (Mesquite, NM) and transported to SBL. Cows were acclimated to their surroundings for about 22 days before the initiation of dosing. All animals received periodic physical examinations to assess their clinical status and acceptability for the study. Cows were initially housed in outdoor pens with overhead roofing. They were then placed in individual metabolism cages the day before dosing began. During acclimation and during the study all animals were allowed ad libitum access to water and to a mixture of feed concentrate and alfalfa hay.

A total of 8 dairy cows, weighing between 490 and 592 kg, were randomly selected from the original 12 animals for the study. Animals were machine-milked twice daily. Control milk was obtained from each animal at the morning milking before administration of the first dose. Animals were dosed intravenously in the jugular vein once per day, just after the morning milking, for three consecutive days with ^{14}C-flunixin-NMG at a target rate of 2.2 mg of flunixin free acid/kg of body weight. The actual dose administered was 2.0−2.1 mg/kg. Milk was collected from all cows for nine consecutive days, evenings, and mornings, after administration of the first dose. The collected whole milk was weighed and subsampled for total radioactive residue analysis. Other subsamples were stored frozen (ca. ≤ 17 °C) for additional analyses.

All cows lost weight slightly during the study. Weight loss is normally expected for animals held in metabolism cages due to the decrease in feed consumption. All cows maintained a steady production of milk during the study (data not shown).

Liquid Scintillation Counting. Milk samples required no further processing prior to analysis for total radioactivity. Three aliquots (10 mL) of each milk sample were weighed and directly combined with 10 mL of Insta-Gel Plus scintillation cocktail (Packard Instrument Co.) and counted on a Packard model 2550 TR/AB (LSC). The minimum detectable concentration for LSC analysis was 0.1 ppb.

Packard 2500TR or 2700TR (LSC) were also used for determination of total radioactivity in milk as well as in milk extracts and HPLC fractions. Background CPM was automatically subtracted before DPM calculation by the counter software, and background vials were counted for 10 min. Quench correction was performed by the external standard method. For milk and extracts, background was subtracted by counting a vial containing a volume of solvent or buffer similar to the volume of milk or extract being counted. The cocktail used was Packard Instagel-XF (10 mL). For HPLC fractions, the background sample was the vial containing the initial 1 min fraction collected. For HPLC, the cocktail used was Packard Ultima Gold (6 mL).

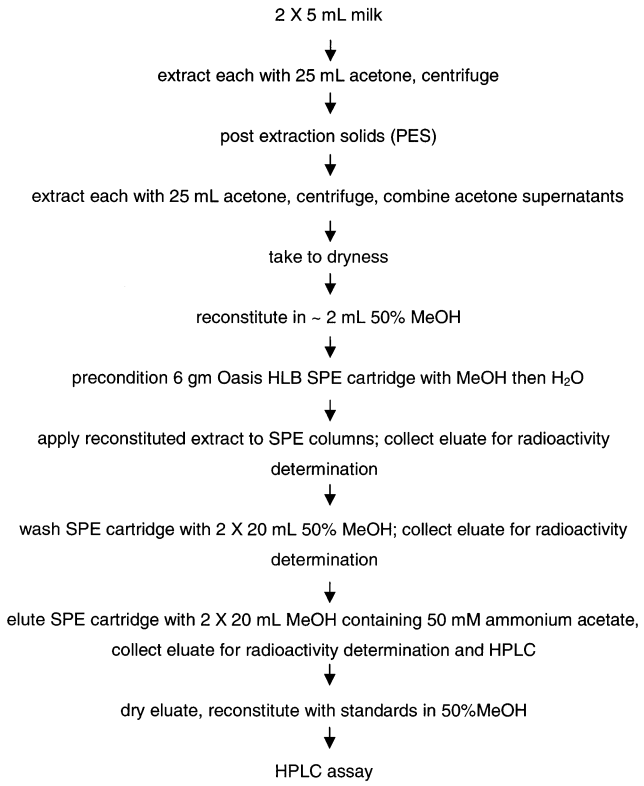

Figure 2. Milk extraction scheme.

Solvent Extraction of Milk and Purification of Extractable Residues by Solid-Phase Extraction. Milk samples from the first three milkings after the last dose were chosen for extraction. An outline of the solvent extraction and solid-phase extraction (SPE) procedure is shown in **Figure 2**.

Milk samples (5 mL aliquots) were assayed in duplicate by adding each sample to 10 mL of Instagel-XF contained in separate 20 mL glass scintillation vials for determination of total radioactivity content. For extraction, milk samples (5 mL aliquots) were transferred in duplicate into individual 50 mL plastic centrifuge tubes (10 mL total for each sample). The milk samples contained in the plastic centrifuge tubes were mixed and extracted with 25 mL of acetone and centrifuged for 5 min. The acetone supernatants were transferred and combined into a single 500 mL round-bottom flask. The acetone extraction was repeated on the postextraction solids (PES) a second time. All acetone extracts were combined. The masses of the PES were inconsequential and were therefore not analyzed.

The acetone extracts were taken to dryness on a rotary evaporator; sometimes CH_3CN was used to facilitate drying. The acetone extracts were dissolved in a minimal volume of 50/50 MeOH/H_2O (~2 mL).

For each reconstituted extract sample, a 50 mL plastic centrifuge tube was placed in a vacuum manifold to receive the SPE eluates. For each extract, a 6 g Waters (Milford, MA) Oasis (hydrophilic−lipophilic balance, HLB) SPE cartridge was preconditioned with ~20 mL of methanol (MeOH) followed by ~20 mL of H_2O; vacuum was applied, and the eluates were discarded. Each residue sample dissolved in 50/50 MeOH/H_2O was then applied to the preconditioned HLB cartridge. The evaporation flasks were then washed with about 20 mL of 50/50 MeOH/H_2O, and the washes were applied to the cartridges. Vacuum was applied, the resulting cartridge application eluate was collected, and the volume was measured. Duplicate 5 mL aliquots of each application eluate were taken, dissolved in Instagel-XF in 20 mL glass scintillation vials, and counted for radioactivity content.

For each sample, the 50 mL plastic centrifuge tube was replaced and the HLB cartridge was washed with an additional 2 × 20 mL of 50/50 MeOH/H_2O; vacuum was applied, and the resulting 50/50 MeOH/H_2O eluate was collected.

The volume of the 50/50 MeOH/H_2O eluate was measured; 2 × 5 mL aliquots were taken, dissolved in Instagel-XF in 20 mL glass scintillation vials, and counted for radioactivity content.

The 50 mL plastic centrifuge tube was again replaced, and the HLB cartridge was eluted with 2 × 20 mL of 50 mM ammonium acetate in MeOH; vacuum was applied, and the resulting eluate was collected. The volume of the eluate was measured; two separate 5 mL aliquots were taken, dissolved in Instagel-XF in 20 mL glass scintillation vials, and counted for radioactivity content.

HPLC Radiometric Analysis of Milk Residues. The methanolic 50 mM ammonium acetate eluate from the extraction procedure was transferred to a 250 mL round-bottom flask. The tube was rinsed with MeOH, the rinsate was transferred to the flask, and the combined eluate and MeOH rinsate were dried on a rotary evaporator. Methanol was added to the flask, and the residue was transferred to a 15 mL plastic centrifuge tube followed by multiple MeOH washes of the flask to the tube. The solution was taken to dryness by evaporation under nitrogen in a Zymark (Hopkinton, MA) TurboVap LV evaporator. The dried residue samples were removed from the turboevaporator and fortified with flunixin-NMG and 5-OH flunixin standards dissolved in a minimal amount of MeOH. These standards were injected separately to establish their retention times. The fortified samples were then dissolved in 400 μL of 50/50 MEOH/H_2O; 100 μL was taken for determination of radioactivity content by LSC, and 100−175 μL was analyzed by HPLC. Eluate samples were collected in 1 min fractions for 35 min; each fraction was dissolved in liquid scintillation cocktail and counted by LSC.

HPLC Analysis. For analysis of the methanolic 50 mM ammonium acetate eluate, HPLC was performed at 45 °C with a Perkin-Elmer (Norwalk, CT) 2000 pump and a MAC-MOD (Chadds Ford, PA) Zorbax C8 5 μm analytical column (250 × 4.6 mm). A Waters Resolve C8 guard pak was placed in-line directly before the analytical column. The column was eluted with 45/55 methanol (MeOH)/H_2O (both MeOH and H_2O contained 10 mM ammonium acetate) at a flow rate of 1 mL/min. An elution program with the following parameters was used: 0−15 min, 45/55 MeOH/H_2O; 15−20 min, increase to 100% MeOH; 20−25 min, hold at 100% MeOH; 25−30 min, decrease to 45/55 MeOH/H_2O; 30−35 min, hold at 45/55 MeOH/H_2O. Under these conditions, 5-OH flunixin eluted at 10−12 min and flunixin eluted at 16−18 min.

The analytical column was connected to a PE 235C UV diode array detector, attached to a PE Turbochrom version 6.1 data acquisition system. The UV detector was set at 285 nm. For milk sample analyses, radioactivity was collected by using an ISCO (Lincoln, NE) Foxy 200 fraction collector. The 1 min fractions were collected into 7 mL glass scintillation vials. The SPE application eluates and the 50/50 MeOH/H_2O eluates were not analyzed by HPLC.

HPLC−MS/MS Analysis. A regulatory surveillance determinative method, using an external standard calibration curve, for the quantitation of 5-OH flunixin in milk was developed and is the subject of a separate report (P. Boner, manuscript in preparation). Briefly, 2 g samples of milk were acidified with 0.1 N HCl followed by precipitation/extraction of whole milk using a mixture of acetone/ethyl acetate. The acetone/ethyl acetate extract was concentrated followed by addition of 0.1% H_3PO_4 in methanol. Cleanup of the acidified extract was accomplished using a Varian (Harbor City, CA) strong cation-exchange (SCX) SPE column. The eluate from the SCX column was concentrated to dryness, followed by addition of 50/50 MeOH/H_2O and centrifugation. Quantitation of the 5-OH flunixin residues was accomplished by HPLC-MS/MS of the 50% methanol solution.

Chromatography was performed at 40 °C with a Waters Alliance 2690 separations module connected to a Zorbax Eclipse XDB-C18 5 μm analytical column (150 × 2.1 mm). A Brownlee (Santa Clara, CA) RP18 guard column was placed in-line directly before the analytical column. The column was eluted with 60% of 0.4% acetic acid in H_2O−35% of 0.2% acetic acid in CH_3CN−5% MeOH at a flow rate of 0.3 mL/min. A Perkin-Elmer Sciex triple quadrupole, model API 365, mass spectrometer, using the positive turbo-ion spray interface, was used for mass analysis. The limit of quantitation (LOQ) for the method was 1 ppb. The ionic transition of the ion at *m*/*z* 313 (parent ion $[M + H]^+$) to the ion at *m*/*z* 295 ($[M - H_2O + H]^+$) was monitored.

RESULTS

Total Radioactive Residue Levels in Milk. The TRR (expressed as ng of ^{14}C-flunixin free acid equiv/g or ppb) was

Table 1. Total ^{14}C-Flunixin Residues in Milk Concentration in ppb for Milkings Conducted after Administration of the First Dose

	dose 1:[a] day 1		dose 2: day 2		dose 3: day 3		day 4		day 5		day 6		day 7		day 8		day 9	
animal no.	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
2896	94	41	111	31	63	13	7	4	3	2	3	2	2	2	2	5	3	2
2899	57	19	75	20	54	20	12	5	4	3	3	3	2	2	2	2	2	2
2900	29	8	41	8	53	9	4	2	1	1	1	1	1	2	1	1	1	1
2901	41	12	47	9	56	10	5	2	2	2	2	2	1	1	1	1	1	1
2902	32	7	49	10	64	10	5	2	2	1	2	2	1	1	1	1	1	1
2903	120	33	97	45	142	87	67	32	17	9	7	5	5	5	4	4	3	3
2905	45	17	66	9	71	9	5	3	2	2	2	2	2	1	1	1	1	1
2906	25	5	34	6	26	5	3	1	1	1	1	1	1	1	2	1	1	1
mean	55	18	65	17	66	20	14	6	4	3	3	2	2	2	2	2	2	2

[a] Each flunixin dose was administered before the collection of the second milking of the day.

Table 2. Summary Data: Extraction and SPE of Radioactive Residues in Milk

	1st milking after last dose mean % TRR 8/8 animals	2nd milking after last dose mean % TRR 8/8 animals	3rd milking after last dose mean % TRR 3/8 animals
application eluate	1.57	4.72	3.58
50% MeOH/H_2O eluate	11.96	20.98	18.23
50 mM ammonium acetate in MeOH eluate	75.49	73.90	58.77
tot. % recovery	89.02	99.60	80.58

determined for the twice daily milk samples for all animals over 9 days. **Table 1** shows the TRR data for all animals after administration of each dose. The mean TRR for the first milking after the second dose were slightly higher than after the first dose. However, no further increase was seen for the third dose. For the first three milkings after administration of the last dose (day 3 and after), individual TRR levels ranged from 3 to 142 ppb (**Table 1**). Except for animal no. 2903, TRR levels were less than 71 ppb for all animals at the first milking after administration of the last dose. By the fourth milking after administration of the last dose (day 4, second) total residue levels in all animals, except for animal no. 2903 (32 ppb), were less than or equal to 5 ppb. Therefore, TRR levels declined rapidly in milk.

Analysis of Milk Extracts. Milk samples, following the third dose, which contained sufficient radioactivity for analysis were chosen for radioprofiling by HPLC. These samples were from the first three milkings after the last dose on day 3, first and second milkings (8 samples each), and 3 samples from day 4, first milking (**Table 1**).

Table 2 summarizes mean recovery values from SPE for extracted milk samples. Three fractions were obtained, the application eluate, the 50/50 MeOH/H_2O eluate, and the methanolic 50 mM ammonium acetate eluate. SPE columns were washed with 50/50 MeOH/H_2O to remove interfering oily material. Because of the amount of oily material present and the low levels of radioactivity present, the 50/50 MeOH/H_2O washes were not examined by HPLC.

The mean total recovery for all milk samples at the first, second, and third milkings after the last dose was respectively, 89, 100, and 81%. The majority of radioactivity was eluted with 50 mM ammonium acetate in MeOH. Average percentages of milk radioactivity recovered in this fraction for the first, second, and third milkings were respectively 75, 74, and 59%.

Figures 3−**5** display HPLC UV chromatograms (top panels) and profiles of eluted radioactivity (bottom panels) for extracted milk samples from animal no. 2903 for the first, second, and third milking after the last dose. These results demonstrated that the two major peaks of radioactivity coeluted with the 5-OH flunixin and flunixin standards which had been added to the extracts. For all other samples analyzed by HPLC, similar results were observed (data not shown).

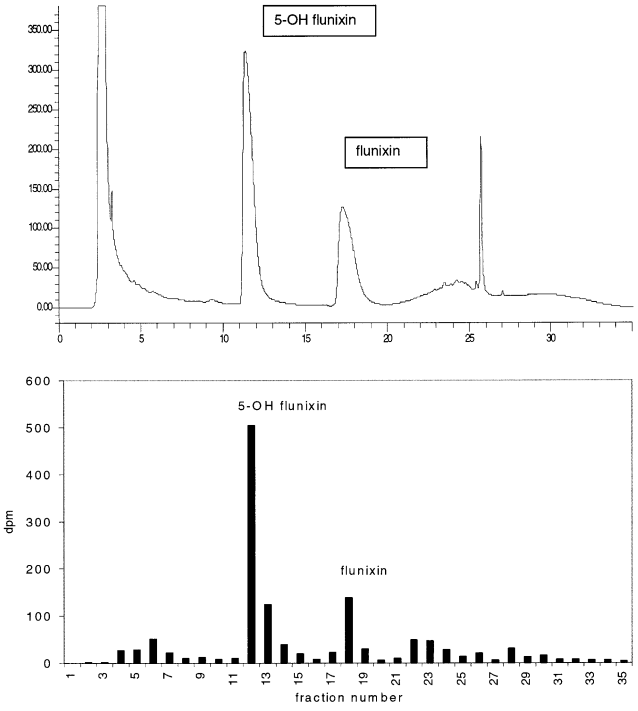

Figure 3. HPLC UV chromatogram (top) and histogram of eluted radioactivity (bottom) of milk sample no. 2903, first milking after administration of the last dose (day 3, 1st milking).

Table 3 summarizes the results for the 19 milk samples which were assayed by HPLC. Residue levels for 5-OH ^{14}C-flunixin ranged from 77 ppb to less than 0.5 ppb for the milk samples analyzed. Residue levels for ^{14}C-flunixin ranged from 27 ppb to less than 0.5 ppb. The concentrations in **Table 3** were calculated by multiplying the total radioactive residue levels (**Table 1**) by the percent of column radioactivity that eluted with either the 5-OH flunixin or flunixin peak during HPLC analyses. These calculations assume that the residues in the 50/50 MeOH/H_2O SPE eluates are similar to those in the methanolic 50 mM ammonium acetate in MeOH SPE eluates.

For the first milking after administration of the last dose, the major residue in milk was 5-OH ^{14}C-flunixin for all 8 animals

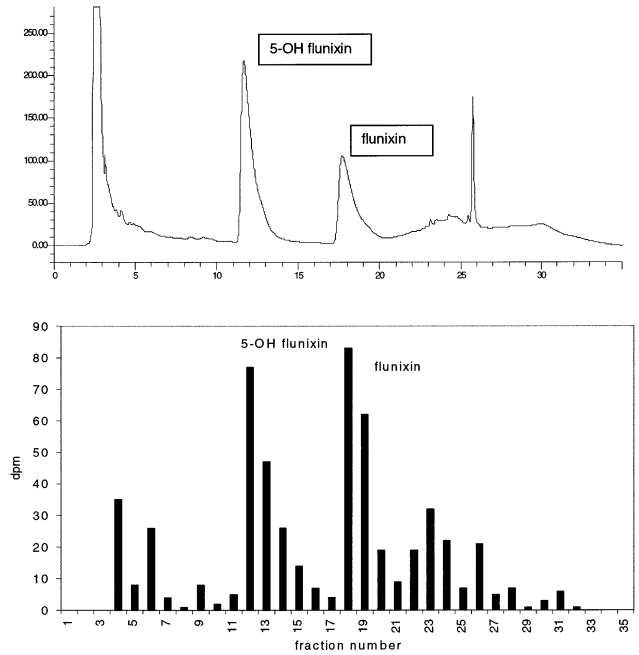

Figure 4. HPLC UV chromatogram (top) and histogram of eluted radioactivity (bottom) of milk sample no. 2903, second milking after administration of the last dose (day 3, 2nd milking).

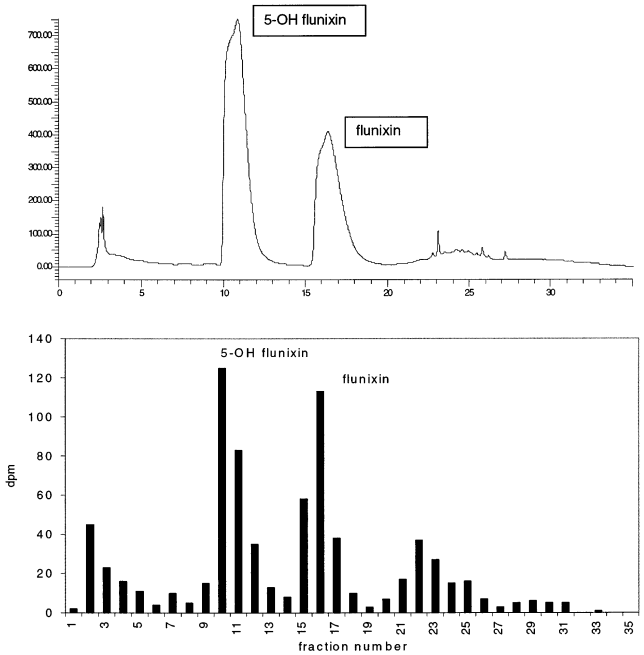

Figure 5. HPLC UV chromatogram (top) and histogram of eluted radioactivity (bottom) of milk sample no. 2903, third milking after administration of the last dose (day 4, 1st milking).

with a mean concentration of 32 ppb. The mean concentration of ^{14}C-flunixin was 12 ppb. In 9 out of 11 samples for the second and third milkings after administration of the last dose, the amount of 5-OH ^{14}C-flunixin was greater than or equal to the amount of flunixin present. For the second milking, the average concentration of 5-OH ^{14}C-flunixin and ^{14}C-flunixin was 5 ppb for both compounds. The average concentrations of 5-OH ^{14}C-flunixin and ^{14}C-flunixin for the three animals analyzed from the third milking were 9 and 7 ppb, respectively.

Inspection of radioprofiles indicated there were at least 8 other residue fractions present in the analyzed milk samples, four eluting before 5-OH flunixin and four eluting after flunixin

Table 3. 5-OH ^{14}C-Flunixin and ^{14}C-Flunixin Residue Levels for the First Three Milkings after the Last Dose

	animal no.	TRR (ppb)	HPLC % 5-OH flunixin	5-OH flunixin (ppb)[a]	HPLC % flunixin	flunixin (ppb)[a]
first milking	2896	63	31	20	25	16
	2899	54	34	18	32	17
	2900	53	41	22	14	7
	2901	56	48	27	15	8
	2902	64	61	39	15	10
	2903	142	54	77	15	21
	2905	71	54	38	12	9
	2906	26	46	12	14	4
mean		66	46	32	18	12
second milking	2896	13	12	2	18	2
	2899	20	23	5	23	5
	2900	9	12	1	10	1
	2901	10	18	2	14	1
	2902	10	11	1	41	4
	2903	87	32	28	31	27
	2905	9	25	2	14	1
	2906	5	2	0[b]	9	0
mean		20	17	5	20	5
third milking	2896	7	5	0	18	1
	2899	12	23	3	18	2
	2903	67	37	25	29	19
mean		29	22	9	22	7

[a] ppb were calculated by multiplying the TRR values by the percentage of radioactivity in the corresponding HPLC analysis identified as either 5-OH flunixin or flunixin. The mean percent recovery from HPLC for all samples was 107%. [b] Zero is reported if rounded values are less than 0.5 ppb.

(**Figures 3−5**). For all 3 milkings, the mean concentration of each of these residue fractions was always less than the mean concentrations of 5-OH ^{14}C-flunixin and ^{14}C-flunixin (data not shown). Further analyses of the radioprofiles of milk extracts, after addition of 4′-OH flunixin and 2′-OH methylflunixin, were performed. From these HPLC analyses it appeared that 4′-OH flunixin coeluted with radioactivity in fraction nos. 6 and 7 and 2′-OH methylflunixin coeluted with radioactivity collected in fraction no. 9 (Figures 3−5). Residue levels for these two compounds were always less than 8 ppb in all milk samples analyzed (data not shown).

HPLC−MS/MS Analyses. **Table 4** summarizes the concentration values of 5-OH flunixin in milk obtained using the determinative HPLC/MS/MS method versus the HPLC radiometric method. Comparison of the values shows that the two methods for quantitation of 5-OH flunixin were comparable. **Table 4** also contains the calculated ratios of 5-OH flunixin/TRR derived using the values obtained with the determinative method and with the radiometric method. The average ratio of 5-OH/total radioactive residue, obtained using values from the determinative method, for the first, second, and third milkings after the last dose were 0.48, 0.20, and 0.22 (**Table 4**). The average ratio of 5-OH/TRR, obtained using values from the radiometric method, for the first, second, and third milkings after the last dose were 0.46, 0.19, and 0.22 (**Table 4**).

DISCUSSION

The residues present in milk after completion of the maximum proposed clinical dosing regimen are of the greatest concern for human food safety. This study was performed to investigate the metabolism of flunixin in lactating dairy cows treated with ^{14}C-flunixin NMG. Residue levels in milk declined rapidly. A major residue, 5-OH flunixin, was positively identified by LC/MS/MS. Another major residue, flunixin, was characterized by

Table 4. Calculation of Ratio of 5-OH Flunixin/Total Radioactive Residue for the First Three Milkings after the Last Dose

	animal no.	ppb 5-OH flunixin[a]	ppb 5-OH ^{14}C-flunixin[b]	ppb TRR[c]	ratio 5-OH/ TRR[d]	ratio 5-OH ^{14}C/TRR[e]
first milking	2896	22	20	63	0.35	0.32
	2899	18	18	54	0.33	0.33
	2900	26	22	53	0.49	0.42
	2901	28	27	56	0.50	0.48
	2902	40	39	64	0.63	0.61
	2903	66	77	142	0.46	0.54
	2905	47	38	71	0.66	0.54
	2906	11	12	26	0.42	0.46
mean		32	32	66	0.48	0.46
second milking	2896	2	2	13	0.12	0.12
	2899	4	5	20	0.18	0.23
	2900	1	1	9	0.11	0.12
	2901	2	2	10	0.17	0.18
	2902	2	1	10	0.18	0.11
	2903	22	28	87	0.26	0.32
	2905	2	2	9	0.22	0.25
	2906	12	0[f]	5	0.32	NA
mean		5	5	20	0.20	0.19
third milking	2896	BLQ[g]	0	7	NA[h]	0.05
	2899	2	3	12	0.19	0.23
	2903	16	25	67	0.24	0.37
mean		6	9	14	0.22	0.22

[a] Values obtained by the determinative HPLC/MS/MS method. [b] Values obtained by HPLC radiometric method (**Table 3**). [c] Values obtained from LSC analyses (**Table 1**). [d] ppb 5-OH flunixin from determinative method/ppb total residue. [e] ppp 5-OH flunixin from radiometric HPLC analyses/ppb total residue. [f] Zero is reported if rounded values are less than 0.5 ppb. [g] BLQ = below the limit of quantitation (for calculation of mean, zero was used). [h] NA = not applicable.

co-chromatography with standard. Two other minor residues were also characterized using standards, 4′-OH flunixin and 2′-OH methylflunixin. Comparison of the concentration values of 5-OH flunixin in milk, obtained using the determinative HPLC/MS/MS method versus the HPLC radiometric method, showed that the two methods were comparable. Therefore, this is consistent with the assumption that the residues in the 50/50 MeOH/H_2O and methanolic 50 mM ammonium acetate SPE eluates have the same approximate residue composition, at least in regard to 5-OH ^{14}C-flunixin.

In the study by Rupp et al. (*8*), using only one cow, up to three-fourths of the total flunixin was present as a β-glucuronide. This was determined by treatment with β-glucuronidase. In the present study, using eight cows, it does not appear that flunixin could be conjugated to this extent. However, treatment with β-glucuronidase was not performed. It is possible that some glucuronide was present in some of the unidentified peaks eluting near the void of the HPLC column. Other residue components present in milk were always present in concentrations less than that of either 5-OH flunixin or flunixin.

HPLC profiles of radioactive flunixin residues found in milk were compared with residues found in the previous rat metabolism study and were found to be similar (*6*). Therefore, metabolite residues were considered to be adequately tested toxicologically.

In summary, the presence of 5-OH flunixin in milk was confirmed by HPLC/MS/MS. The concentration of 5-OH flunixin in milk and the ratio of 5-OH flunixin to total radioactive residue were comparable using either radiometric detection or detection by HPLC/MS/MS. In 15 of 19 milk samples examined, the most abundant identified residue was 5-OH flunixin. Therefore, 5-OH flunixin was chosen as the marker residue in milk.

LITERATURE CITED

(1) Johansson, M.; Anler, E. L. Gas Chromatographic Analysis of Flunixin in Equine Urine After Extractive Methylation. *J. Chromatogr*. **1988**, *427* (1), 55.

(2) Jaussaud, P.; Courtot, D.; Guyot J. L.; Paris, J. Identification of a Flunixin Metabolite in the Horse by Gas Chromatography−Mass Spectrometry. *J. Chromatogr*. **1987**, *423*, 123.

(3) Higgins, A. J.; Lees, P.; Sharma, S. C.; Taylor, J. B. Measurement of Flunixin in Equine Inflammatory Exudate and Plasma by High Performance Liquid Chromatography. *Equine Vet. J.* **1987**, *19* (4), 303.

(4) Odensvik, K.; Johansson, I. M. High Performance Liquid Chromatography Method for Determination of Flunixin in Bovine Plasma and Pharmacokinetics After Single and Repeated Doses of the Drug. *Am. J. Vet. Res.* **1995**, *56* (4), 489.

(5) Pemberton, A. D.; Slater, J. S.; Milne, E..M. An Improved TLC Method for the Detection of Flunixin in Equine Serum. *Res. Vet. Sci.* **1989**, *47* (3), 406.

(6) Clement, R. P.; Simmons, R. D.; Christopher, R. J.; Charles, S. F.; Casciano, C. N.; McCullough, C. B.; Lamendola, J. F.; Cayen, M. N. Design and Conduct of Studies to Meet Residue Chemistry Requirements: Residue Depletion and Metabolism of Flunixin in Cattle. In *Xenobiotics and Food-Producing Animals*; Hutson, D. H., Hawkins, D. R., Paulson, G. D., Struble, C. B.; Eds.; American Chemical Society: Washington, DC, 1992; Chapter 4, pp 37−48.

(7) Neff-Davis, C. A.; Bosch, K. An HPLC Method for the Determination of Flunixin in Bovine Plasma and Milk. *J. Vet. Pharmacol. Ther*. **1985**, *8* (3), 331.

(8) Rupp, H. S.; Holland, D. C.; Munns, R. K.; Turnipseed, S. B.; Long, A. R. Determination of Flunixin in Milk by Liquid Chromatography with Confirmation by Gas Chromatography/Mass Spectrometry and Selected Ion Monitoring. *J. AOAC Int.* **1995**, *78* (4), 959.

(9) Brady, T. C.; Kind, A. J..; Hyde, W. H..; Favro, M.; Hill, D. W.. Isolation, Purification, and Structural Characterization of Flunixin Glucuronide in the Urine of Greyhound Dogs. *Drug Metab. Disp*. **1998**, *26* (4), 294.

Received for review July 30, 2002. Revised manuscript received September 27, 2002. Accepted October 1, 2002.

JF0258609