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# Determination and Confirmation of 5-Hydroxyflunixin in Raw Bovine Milk Using Liquid Chromatography Tandem Mass Spectrometry

PAMELA L. BONER,*,† DAVE D. W. LIU,† WILLIAM F. FEELY,‡ MATTHEW J. WISOCKY,† AND JINN WU†

XenoBiotic Laboratories, Inc., 107 Morgan Lane, Plainsboro, New Jersey 08536, and Schering-Plough Research Institute, Lafayette, New Jersey 07848

A method was developed and validated to determine 5-hydroxyflunixin in raw bovine milk using liquid chromatography tandem mass spectrometry (LC/MS/MS). The mean recovery and percentage coefficient of variation (%CV) of 35 determinations for 5-hydroxyflunixin was 101% (5% CV). The theoretical limit of detection was 0.2 ppb with a validated lower limit of quantitation of 1 ppb and an upper limit of 150 ppb. Accuracy, precision, linearity, specificity, ruggedness, and storage stability were demonstrated. A LC/MS/MS confirmatory method using the extraction steps of the determinative method was developed and validated for 5-hydroxyflunixin in milk from cattle. Briefly, the determinative and confirmatory methods were based on an initial solvent (acetone/ethyl acetate) precipitation/extraction of acidified whole milk. The solvent precipitation/extraction effectively removed incurred ($^{14}$C) residues from milk samples. The organic extract was then purified by solid phase extraction (SPE) using a strong cation exchange cartridge (sulfonic acid). The final SPE-purified sample was analyzed using LC/MS/MS. The methods are rapid, sensitive, and selective and provide for the determination and confirmation of 5-hydroxyflunixin at the 1 and 2 ppb levels, respectively.

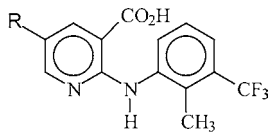
**KEYWORDS: Flunixin meglumine; 5-hydroxyflunixin; LC/MS/MS; determinative method; confirmatory method**

## INTRODUCTION

Flunixin (SCH 14714; **Figure 1**) is a nonsteroidal antiinflammatory agent approved for use worldwide in cattle. Flunixin (2-[[2-methyl-3-(trifluoromethyl)phenyl]amino]-3-pyridinecarboxylic acid) is administered intravenously (IV) as the meglumine salt (trade names Banamine and Finadyne). Flunixin is presently being developed by Schering-Plough for use in dairy cattle in the U.S. (*1*). Because flunixin is being developed for use in dairy cattle, methods for regulatory surveillance of flunixin residues in bovine milk were needed.

A metabolism study, conducted in cattle treated by IV administration of a nominal dose of 2.2 mg/kg [$^{14}$C]flunixin for 3 consecutive days, indicated that 5-hydroxyflunixin was the major residue in bovine milk (*1*). Consequently, 5-hydroxyflunixin (**Figure 1**) has been established as the marker residue to be used for monitoring of flunixin residues in bovine milk (*1*).

Previously, a method for determination of flunixin at 1 ppb in bovine tissues by high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS) was developed and validated at XenoBiotic Labs (P. Boner, manuscript in preparation). A method for determination of parent flunixin in milk by LC and confirmation by gas chromatography (GC)/MS has been reported (*2*). However, no method for the determination of the marker residue, 5-hydroxyflunixin, was available at the 1 ppb level. The potential worldwide use of flunixin in dairy cattle necessitated the development of methods to determine and confirm the presence of flunixin in milk by monitoring its marker residue at the 1 and 2 ppb levels, respectively. The determinative method was developed to allow regulatory organizations to ensure that flunixin residues remain below established tolerances. The confirmatory method was developed to rule out false positive responses in the determinative assay.




flunixin; R=H

5-hydroxyflunixin; R=OH

**Figure 1.** Structures of flunixin and 5-hydroxyflunixin (free acids).

* To whom correspondence should be addressed. Tel: (609)799-2295. Fax: (609)799-7497. E-mail: pboner@xbl.com.
† XenoBiotic Laboratories, Inc.
‡ Schering-Plough Research Institute.

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## MATERIALS AND METHODS

**Chemicals, Materials, and Solvents.** Reference standard 5-hydroxyflunixin was obtained from Schering-Plough Research Institute (SPRI) (Union, NJ). Complete characterization and identification (including chemical purity) of the analytical standard was provided by SPRI. Flunixin NMG, cephalonium, eprinomectin, and tilmicosin were also provided by SPRI. Bacitracin zinc, chlortetracycline hydrochloride, fenbendazole, lasalocid sodium, levamisole hydrochloride, penicillin G sodium, monensin sodium, oxytetracycline dihydrate, sulfamethazine sodium, and tylosin tartrate were purchased from Sigma-Aldrich (St. Louis, MO). Flavomycin (bambermycins) was obtained from Magellan Laboratories (Morrisville, NC). Laidlomycin propionate potassium salt was obtained from Syntex (Springfield, MO). Ceftiofur was from Pharmacia Upjohn (Kalamazoo, MI). All solvents used were of HPLC or HRGC grade. Only reagents of recognized analytical grade were used. Water was purified on site using a Barnstead NANOPure II Water Purification System (resistivity $\geq$ 16.7 M$\Omega$ cm) or obtained from EM Science (Gibbstown, NJ). Acetonitrile, acetone, ethyl acetate, and methanol were obtained from EM Science. Acetic acid, ammonium hydroxide, hydrochloric acid, and phosphoric acid were obtained from J. T. Baker (Phillipsburg, NJ). Benzenesulfonic acid cation exchange (SCX) cartridges (1 g packing) were purchased from Varian (Mega Bond Elut) (Harbor City, CA). Alternate cation exchange solid phase extraction (SPE) cartridges were Spe-ed benzenesulfonic SCX (Applied Separations, Allentown, PA) and Baker bond aromatic sulfonic acid (J. T. Baker).

**Raw Whole Milk.** Bovine whole raw milk samples from [$^{14}$C]-flunixin-treated cattle were obtained from a previously conducted carbon-14 depletion study (*1*). In addition, control whole raw milk samples were obtained from six different dairy cattle (Southwest Bio-Labs; Las Cruces, NM).

**Preparation of Standards, Fortification Solutions, and Calibration Curve.** The 5-hydroxyflunixin standard solutions were prepared in methanol at approximately 500 $\mu$g/mL (corrected for purity) and stored at or below $-10$ °C. Intermediate stock solutions of 50 $\mu$g/mL 5-hydroxyflunixin were prepared by a 10-fold dilution of the stock solution in methanol.

Fortification solutions of 5-hydroxyflunixin were prepared by serial dilution of the intermediate stock solution with 20% MeOH in $H_2O$ covering a 0.02$-$3 $\mu$g/mL range (equivalent to 1$-$150 ppb 5-hydroxyflunixin in milk).

A seven point calibration curve was prepared from 0.25 to 250 ng/mL of 5-hydroxyflunixin (equivalent to 0.5$-$500 ppb 5-hydroxyflunixin in milk) for the determinative assay. For the confirmatory assay, two absolute standards of 8 and 40 ng/mL (equivalent to 2 and 10 ppb 5-hydroxyflunixin in milk, respectively) were prepared. The calibration curve standard solutions were prepared by volumetrically diluting the intermediate stock solution with a solution of 50% MeOH in $H_2O$.

**Preparation of Untreated Control Samples.** For each analysis, untreated control milk served as matrix blanks and was processed and analyzed as described for incurred and fortified samples.

**Preparation of Fortified Samples.** Fortified milk was prepared by adding working standards to control milk. Control milk was fortified with 5-hydroxyflunixin at concentrations of 1, 10, 37.5, 50, 75, and 150 ppb for the determinative method. Control milk was fortified at 2 ppb 5-hydroxyflunixin for the confirmatory method.

**Sample Preparation Procedure (Determinative and Confirmatory).** A 2 $\pm$ 0.1 g of milk sample was mixed with 1.5 mL of 0.1 N HCl in a 15 mL disposable polypropylene centrifuge tube and vortexed for 3 min. For the fortified samples, 100 $\mu$L of an appropriate working standard solution was added to each tube prior to addition of HCl. To each tube was then added 6 mL of acetone:ethyl acetate (1:1). Tubes were capped and vortexed for 20 s at high speed and then centrifuged for 3 min at approximately 2000*g*. Supernatants were then transferred to clean, graduated, 50 mL polypropylene centrifuge tubes. The solvent partition was repeated three more times, and extracts were combined into the polypropylene centrifuge tubes. Volumes were adjusted to 30 mL using the graduations on the tube with acetone:ethyl acetate (1:1), and the extracts were capped and vortexed. Subsamples (7.5 mL) of the extracts were transferred into 15 mL glass centrifuge tubes and concentrated on a turbo-evaporator at 50 °C to approximately 1 mL. Ten milliliters of 0.1% $H_3PO_4$, in methanol, was added to each tube, and the tubes were capped and vortexed. Clean 25 mL reservoirs were fitted to preconditioned SCX column cartridges. Each SCX column was conditioned with 2 column volumes of water followed by 2 column volumes of 0.1% phosphoric acid in methanol, and approximately 4 mL of the 0.1% phosphoric acid in methanol was allowed to remain above each column bed. Sample extracts were delivered directly to the top of the appropriate column bed, and solvent was allowed to drain to waste by gravity or with a low vacuum ($\leq$5 in Hg). At the end of the sample loading, a high vacuum ($\geq$15 in Hg) was applied for approximately 5 s to draw off the remaining solvent. 5-Hydroxyflunixin residues were eluted with 5 mL of $NH_4OH$:MeOH (10:90, v:v) into 15 mL disposable glass centrifuge tubes. $NH_4OH$:MeOH eluates were evaporated to dryness at 50 °C under a gentle stream of nitrogen. After approximately 20 min, the sides of each tube were washed with methanol and then were dried completely. One milliliter of 50% methanol:water was added to each tube, and tubes were capped and vortexed for $\sim$30 s. Dissolved samples were transferred to 1.5 mL

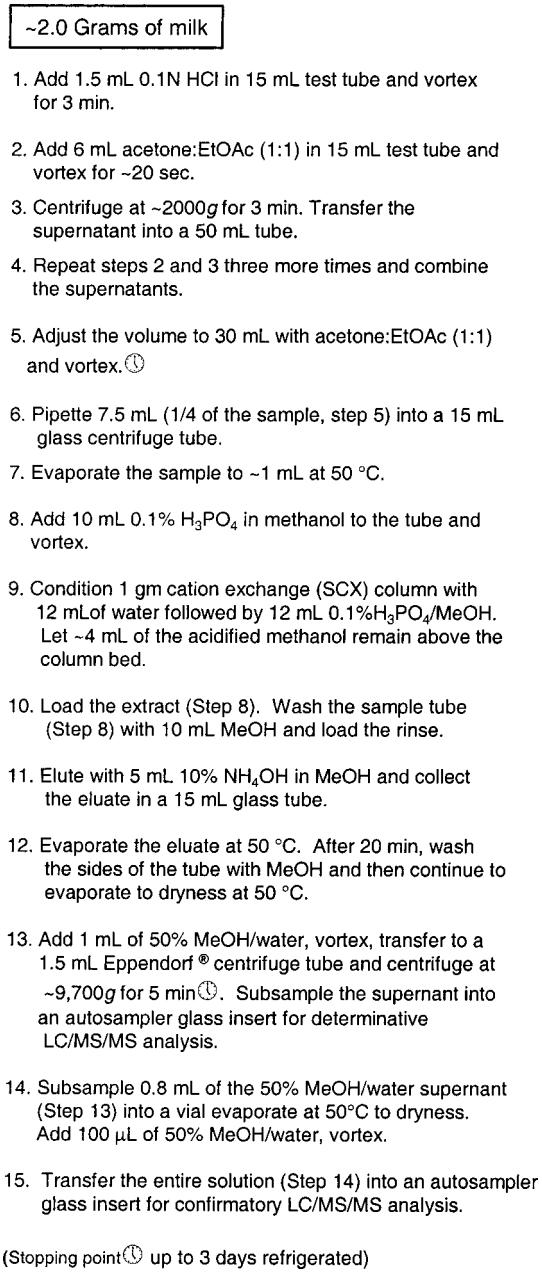


**Figure 2.** Flow diagram of the procedure to determine and confirm 5-hydroxyflunixin in bovine milk.

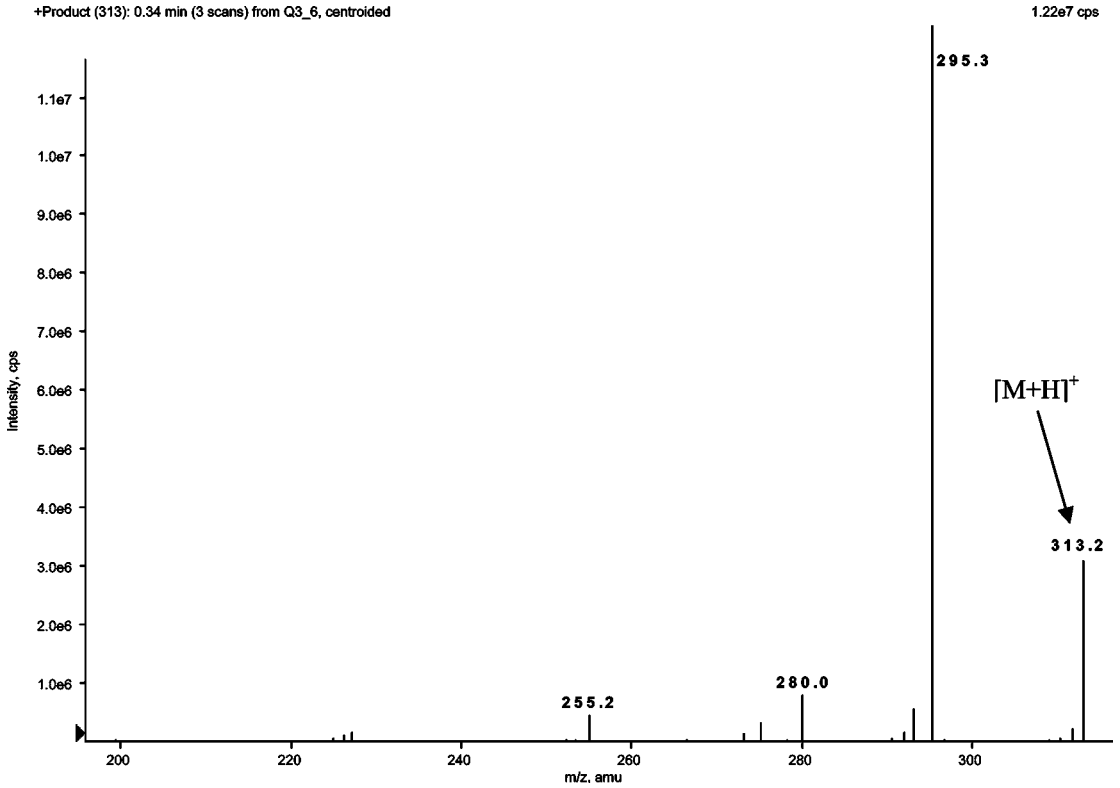


**Figure 3.** Product ion scan of a 5-hydroxyflunixin standard.

**Table 1.** HPLC Solvent Composition and Flow Rate

| run time (min) | flow (mL/min) | 0.4% acetic acid in $H_2O$ (%) | 0.2% acetic acid in $CH_3CN$ (%) | methanol (%) | switching valve |
|---|---|---|---|---|---|
| 0 | 0.3 | 60 | 35 | 5 | waste |
| 5.0 | 0.3 | 60 | 35 | 5 | MS |
| 7.8 | 0.3 | 60 | 35 | 5 | waste |
| 8.0 | 0.55 | 0 | 0 | 100 | |
| 11.0 | 0.55 | 0 | 0 | 100 | |
| 11.2 | 0.55 | 60 | 35 | 5 | |
| 15.2 | 0.55 | 60 | 35 | 5 | |
| 15.5 | 0.3 | 60 | 35 | 5 | |

Eppendorf tubes and centrifuged at approximately 9500*g* for 5 min. A subsample of each supernatant was transferred to glass autosampler inserts for the determinative LC/MS/MS analyses. For confirmatory analyses, 0.8 mL subsamples of each final supernatant was concentrated under $N_2$ to dryness and reconstituted with 100 $\mu$L of 50% MeOH in $H_2O$ and transferred to a glass autosampler insert. The determinative and confirmatory analyses were completed via reversed-phase HPLC with MS/MS detection. A flow diagram of the sample extraction procedure with appropriate stopping points is in **Figure 2**.

**LC/MS/MS Analysis Conditions (Determinative Assay).** HPLC was performed with a Waters Alliance 2690 Separations system, equipped with a 2.1 mm × 150 mm Zorbax Eclipse XDB-C18 column (5 $\mu$m; MAC-MOD Analytical, Chads Ford, PA) and protected by a 3.2 mm × 15 mm Brownlee RP-18 Newguard guard column. The column temperature was 40 °C, the autosampler was 5 °C, and the injection volume was 20 $\mu$L. The mobile phase consisted of 0.4% acetic acid in Omnisolv water (A), 0.2% acetic acid in acetonitrile (B), and HRGC grade methanol (C). The solvents were mixed in proportions shown in **Table 1**. A six port injection valve (Valco Instruments) and actuator control module (Valco Instruments) controlled the flow of solvent and sample to the MS. Column effluent at the peak region (~6.0−7.8 min) was delivered to the MS source. Prior to and after peak elution (before 6.0 min and after 7.8 min, respectively), column effluent was diverted to waste and 0.2% acetic acid in acetronitrile was delivered to the MS source via a second isocratic pump at 0.3 mL/min. A Perkin-Elmer Sciex Triple Quadrapole LC/MS/MS Spectrometer, model API 365 equipped with the Positive Turbo-Ion Spray interface, was used to collect quantitative and confirmatory data. The instrument was operated in the positive-ion mode and optimized to monitor the transition from the precursor ion (*m*/*z* 313.1) to the product ion (*m*/*z* 295.1) in the multiple reaction monitoring (MRM) mode. A typical product ion scan from a 5-hydroxyflunixin standard is shown in **Figure 3**. Instrument variables were set as follows: ion spray voltage, 5500 V; ion source temperature, 400 °C; nebulizer and curtain gas flows, 14 and 10 units, respectively; collision energy, 29 eV; orifice plate, 35 V; and quad 2 rod offset, −36 V. Mac-Quan, version 1.5, was used for quantitative analysis.

**LC/MS/MS Analysis Conditions (Confirmatory Assay).** The column and HPLC conditions as described for the determinative assay, but with an injection volume of 90 $\mu$L, were used. The LC/MS/MS, described above, was operated in the positive-ion mode with the orifice and ring energy optimized to maximize the formation of *m*/*z* 295.1 ($M + H - H_2O$) from parent *m*/*z* 313.1 in Q1. The instrument was further optimized to monitor the ionic transition from the precursor ion (*m*/*z* 295.1) to product ions at *m*/*z* 280 $(M + H - H_2O - CH_3)^+$, 275 $(M + H - H_2O - HF)^+$, 252 $(M + H - H_2O - CH_3 - CO)^+$, and 226 $(M + H - H_2O - 2HF - CO - H)^+$ in the MRM mode. A product ion scan of *m*/*z* 295 is shown in **Figure 4**. Ion spray voltage, source temperature, nebulizer, and curtain gas values were the same as those for the determinative assay. The collision energy was 44 eV with the orifice plate set at 55 V and quad 2 rod offset at −51 V. Mac-Quan, version 1.5, was used to measure peak area response.

**System Suitability (Determinative and Confirmatory Assay).** System suitability assessment was performed prior to injection of standard curve series. Three (or greater) replicates of 0.5 (determinative) or 8 ng/mL (confirmatory) 5-hydroxyflunixin were injected to ensure that all area responses were greater than 2000 counts for accurate quantitation. Three (or greater) replicates of a middle range standard were also injected for the determinative assay to ensure reproducibility of area responses of ≤5% RSD. The retention time for 5-hydroxyflunixin was approximately 6.7 min, and within a day, the retention time variation did not differ more than ±10 s from the mean for either determinative or confirmatory assays.

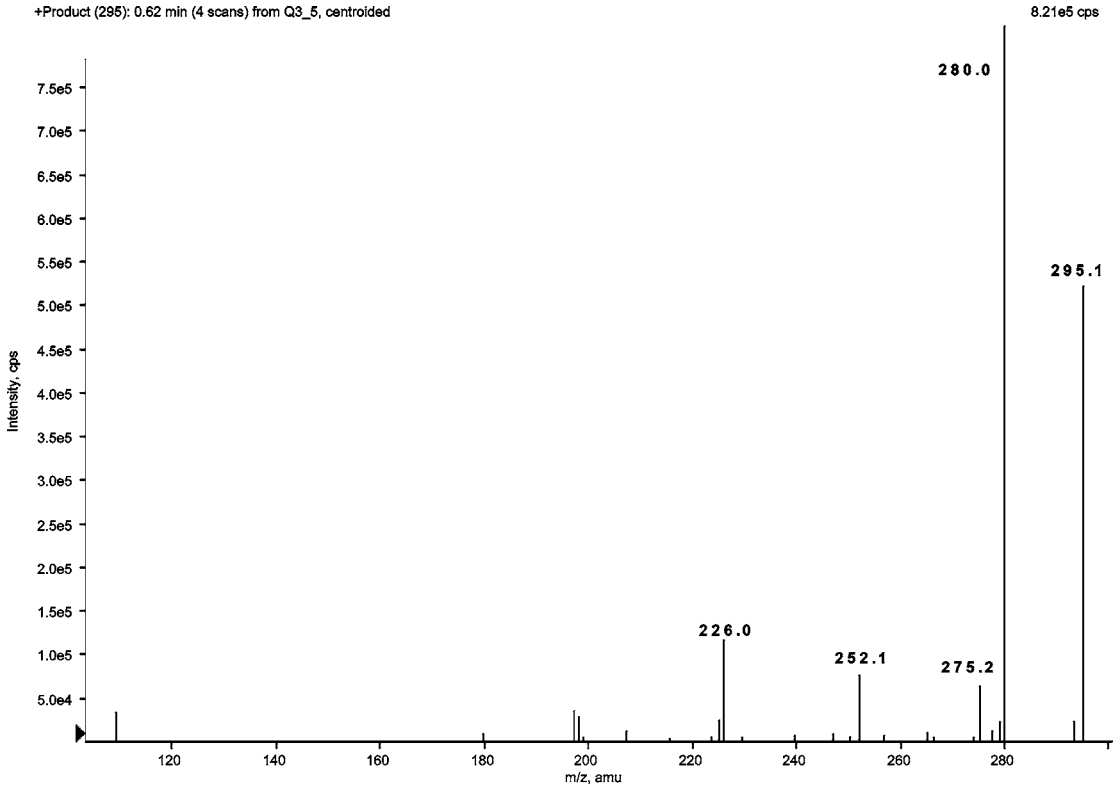


**Figure 4.** Product ion scan of *m/z* 295 $(M + H - H_2O)^+$ of a 5-hydroxyflunixin standard.

**Analysis Sequence.** System suitability was routinely performed before the injection of standard curve series. An absolute standard curve series was injected, followed by processed samples, control, fortified samples, followed by an additional absolute standard curve series.

**Quantitation (Determinative).** Sample concentrations were determined using a regression of standard peak area on standard concentration. The resulting sample extract concentration was converted to ppb (ng flunixin/g milk) by multiplying by the final (mL) take-up volume by four to take into account that only one-fourth of the ethyl acetate/acetone extract was used for the SPE workup and dividing by the sample weight in grams. The result was expressed to the whole number. The formula was expressed as the following equation:

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

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 one-fourth of the methanolic eluate, and $W$ = total milk weight (g).

## RESULTS AND DISCUSSION

**Determinative Assay.** *Accuracy (Recovery).* Percentage recoveries generated during the validation of the method as a measure of accuracy are presented in **Table 2**. Average recoveries between 97.6 and 107% (**Table 2**) were obtained for the various fortification levels in milk (**Table 2**). Individual recoveries across all fortification levels were between 93.7 and 111% (data not shown). The average recovery for milk over all fortification levels was 101%, covering a range of 1−150 ppb ($n$ = 35). Typical chromatograms resulting from determinative assay of fortified and incurred samples are shown in **Figure 5**.

*Precision.* The between day (intermediate) precision over all fortification levels was 5.4% (CV) (**Table 2**). The within day precision (repeatability) was 3.5% (CV) for the 1 ppb fortification level and 1.0−3.9% (CV) for higher fortification levels.

**Table 2.** 5-Hydroxyflunixin Milk Method Determinative Method Validation Results

| between day | | | | within day | | | |
|---|---|---|---|---|---|---|---|
| fortification level (ppb) | avg % recovery | %CV | N | fortification level (ppb) | avg % recovery | %CV | N |
| 1 | 99.3 | 5.7 | 7 | 1 | 102 | 5.7 | 3 |
| 10 | 97.6 | 6.4 | 7 | 10 | 99.5 | 6.4 | 3 |
| 37.5 | 99.1 | 5.8 | 7 | 37.5 | 98.4 | 5.8 | 3 |
| 75 | 107 | 5.2 | 7 | 75 | 108 | 5.2 | 3 |
| 150 | 102 | 4.0 | 7 | 150 | 104 | 4.0 | 3 |
| overall | 101 | 5.4 | 35 | overall | 102 | 2.9 | 15 |

Between day assay precision ranged from 4 to 6.4% for all fortification levels.

*Linearity and Range.* The relationship between detector response (peak area) and concentration of 5-hydroxyflunixin was linear and reproducible over the measured concentration range (0.25−250 ng 5-hydroxyflunixin/mL). Least-squares linear regression consistently yielded coefficients of determination ($r^2$) > 0.99. The effective linear concentration range of the method is from 1 to 150 ng/g.

*Ruggedness.* The ruggedness of the determinative method was evaluated by samples fortified at two concentrations utilizing cation exchange cartridges from two different manufacturers and replicate analytical HPLC columns within the manufacturer. Results demonstrated that alternate cation exchange cartridges or analytical columns could be used to prepare and analyze samples (data not shown).

*Specificity.* Controls were free from interference. **Figure 5** shows typical chromatograms that resulted from the determinative method. Sixteen animal health compounds, in addition to the parent drug, were evaluated for potential interference in the LC/MS/MS assay. These included bacitracin zinc, chlortetracycline hydrochloride, fenbendazole, lasalocid sodium, levamisole hydrochloride, monensin sodium, oxytetracycline dihydrate,

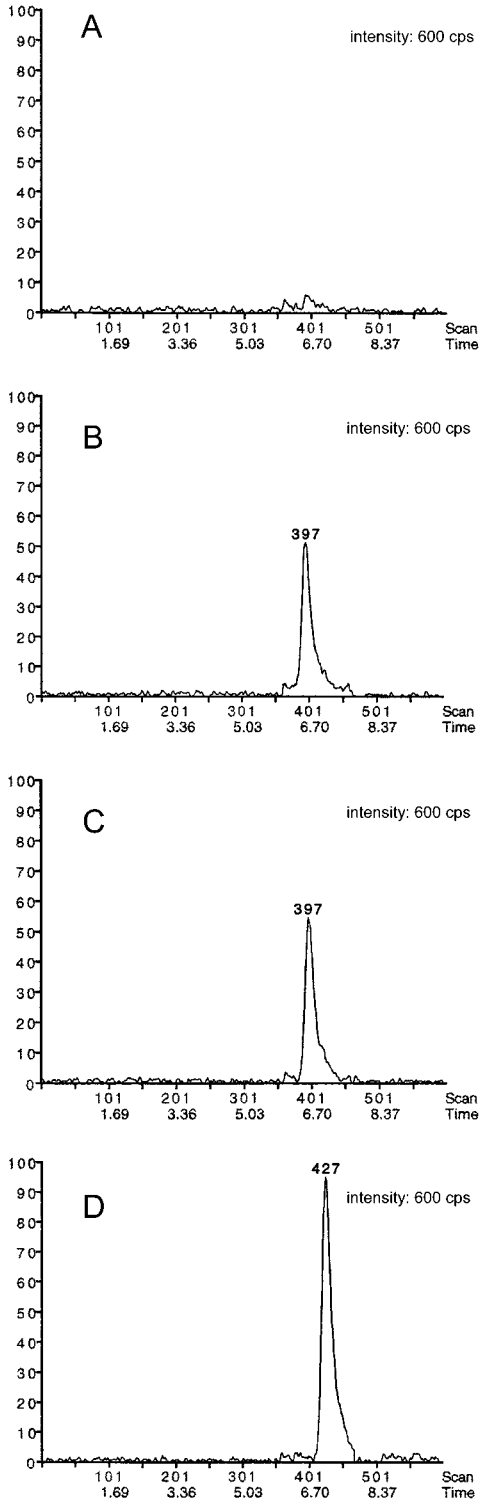


**Figure 5.** Representative determinative LC/MS/MS chromatograms of (**A**) control milk; (**B**) calibration standard at 0.5 ng/mL (equivalent to 1 ppb); (**C**) fortified milk at 1 ppb; and (**D**) incurred milk at 1.3 ppb.

penicillin G sodium, sulfamethazine sodium, tylosin tartrate, ceftiofur sodium, flavomycin (bambermycins), laidlomycin propionate potassium salt, tilmicosin, eprinomectin, and cephalonium. None of the animal health compounds, or flunixin, showed a LC/MS/MS response above the limit of detection (LOD) under the conditions used—thus confirming the specificity of the method.

*Extraction Efficiency.* Concentrations of [$^{14}$C]-5-hydroxyflunixin present in incurred bovine milk were analyzed by the determinative method. Analysis of samples from two intervals in replicates of five produced numbers with an intraday precision of 2.2% (CV, data not shown). Radioactivity in the acetone/ethyl acetate and methanol/water extract was analyzed by liquid scintillation counting as part of the radiovalidation. Approximately 96% of the ($^{14}$C) residues in the incurred samples were extracted into the acetone/ethyl acetate extract (**Table 3**). Approximately 77% of the ($^{14}$C) residues remained in the methanol/water extract. Analysis of the methanol/water extracts by the LC/MS/MS determinative method showed that approximately 48−50% of the ($^{14}$C) residues in the two incurred samples was 5-hydroxyflunixin. Approximately 50% of the initial TRR was comprised of ($^{14}$C) residues other than 5-hydroxyflunixin. These data agree with those obtained in the metabolism study (*1*).

**Table 3.** 5-Hydroxyflunixin Milk Method Determinative Method Radiovalidation Results

| | incurred milk sample—interval | | | |
|---|---|---|---|---|
| | milk | | | |
| | day 2 PM[a] no. 2901 | | day 2 PM[a] no. 2903 | |
| fraction | %TRR[b] | ppb[b] | %TRR[b] | ppb[b] |
| initial milk sample | NA | 56 | NA | 142 |
| acetone/EtOAc extractable | 96 | 54 | 97 | 137 |
| MeOH/$H_2O$ extract | 77 | 43 | 78 | 111 |
| ppb of 5-hydroxyflunixin determined by LC/MS/MS[c] | NA[e] | 28 | NA[e] | 67.7 |
| % 5-hydroxyfllunixin in TRR (flunixin equivalent)[d] | 50 | NA[f] | 48 | NA[f] |

[a] First milking following last dose. [b] Values were obtained by LSC counting, based on initial TRR and ppm values from SPRI study; see ref *1*; $n = 5$ for acetone/EtOAc; $n = 3$ for 50% MeOH/$H_2O$. [c] Values were obtained by the determinative method (LC/MS/MS). [d] % 5-Hydroxyflunixin in TRR (flunixin equivalent) = ppb of 5-hydroxyflunixin/TRR × 100. [e] Not applicable % 5-hydroxyflunixin in TRR shown below. [f] Not applicable ppb 5-hydroxyflunixin shown above.

*LOD and Limits of Quantitation (LOQ).* Duplicate extractions from six control milk samples and duplicate injections per sample extract (a total of 24 analyses) were made. Baselines at the peak region of 5-hydroxyflunixin (ca. 6.7 min) were integrated and quantified against the standard curve. The average concentration measured for control milk samples was 0.12 ppb with a standard deviation (SD) of 0.02 ppb. The theoretical LOD, defined as LOD = $\bar{X}$ + (3 × SD), was 0.18 ppb for milk. The theoretical LOQ, defined as LOQ = $\bar{X}$ + (10 × SD), was 0.32 ppb for milk. The validated LOQ was 1 ppb.

*Assay Timing and Repeats.* Approximately 8 h was required to prepare 24 samples for LC/MS/MS analysis. A typical sample set consisted of 21 unknowns, a control, a fortified control at low and high levels, and a set of seven standards injected before and after the unknowns. If repeat analyses were necessary, another subsample of the ethyl acetate extract was subjected to the remaining method steps.

**Stability.** *In-Process Extract Stability.* Fortified milk sample extracts (50% methanol/water) were analyzed on the day of extraction, ∼24 h after they were stored at room temperature and approximately 3 days after they were stored at ∼5 °C (data not shown). Samples prepared for LC/MS/MS analyses were not stable for 24 h when stored at room temperature. However, refrigerated samples were stable after ∼3 days of storage. Thus, the methanol/water LC/MS/MS extracts should be refrigerated at all times until analysis. Only one-quarter of the acetone/ethyl acetate extract was carried through the analysis, allowing for reanalysis of the sample if desired. Following 3 days of storage

at ~5 °C, the acetone/ethyl acetate extract was carried through the method and analyzed (data not shown). Results demonstrated that the acetone/ethyl acetate extracts were stable over the 3 day interval when stored at ~5 °C.

*Freeze/Thaw Stability.* Freeze/thaw stability was evaluated after three freeze/thaw cycles. Each cycle consisted of thawing the fortified and incurred milk samples at room temperature and returning to the freezer (~ −20 °C) for approximately 24 h. Recoveries of fortified samples were poor (<54%) after three freeze/thaw cycles; however, analysis of incurred samples after three freeze/thaw cycles resulted in accurate and reproducible data. Organic solvent in the fortification solution, although only ~100 μL, could have resulted in irreversible entrapping of the 5-hydroxyflunixin within the aggregated milk protein molecules.

*Stock Solution Stability.* Calibration standard solutions, prepared during method development, were analyzed at the initiation of the validation study. Area responses obtained from the analysis of these solutions were compared to those of the freshly prepared calibration standard solutions. Area responses from the stored and fresh solutions differed by less than 10% and indicated that the stock solutions were stable for at least 6 weeks at refrigerator temperatures (data not shown).

**Confirmatory Assay.** 5-Hydroxyflunixin in fortified and incurred samples was confirmed by LC/MS/MS in the ESI (+) mode. Confirmation of the presence of 5-hydroxyflunixin was carried out by optimizing the formation of *m*/*z* 295 from *m*/*z* 313, followed by MRM of *m*/*z* 295 to *m*/*z* 275 $(M + H - H_2O - HF)^+$, 252 $(M + H - H_2O - CH_3)^+$, 226 $(M + H - H_2O - 2HF - CO - H)^+$, and the base peak *m*/*z* 280 $(M + H - H_2O - CH_3)^+$ (**Figure 4**). Confirmatory ions were summarized in the form of the ratio of each daughter ion to the *m*/*z* 280 base peak. Two sets of standards, incurred, and fortified (QC) samples were analyzed to determine the precision of the procedure.

Results of the confirmatory assay analyses of the 5-hydroxyflunixin absolute standards are listed in **Table 4**. Fragment ion ratios, expressed as a percentage of the *m*/*z* 280 base peak, ranged from 6.3 to 13% for the monitored ions. The overall mean and SD of standards were 12 ± 0.64, 7.0 ± 0.37, and 8.9 ± 0.34% at *m*/*z* 226, 252, and 275, respectively, with an interassay precision (CV) for all ions of ≤5.3%.

Confirmatory assay ion ratios of control milk fortified with 2 ppb of 5-hydroxyflunixin are listed in **Table 4**. Ion ratios, expressed as a percentage of the *m*/*z* 280 base peak, ranged from 7.1 to 13% for the monitored ions. The overall mean and SDs were 12 ± 1, 7.9 ± 0.98, and 8.7 ± 0.53% at *m*/*z* 226, 252, and 275, respectively, with an interassay precision (CV) for all ions of ≤12.4%.

Confirmatory assay analyses of 5-hydroxyflunixin in incurred milk obtained from cattle following intravenous administration of flunixin are listed in **Table 4** (*1*). Milk from two animals that contained 3.2 and 6.8 ppb of 5-hydroxyflunixin was confirmed. Fragment ion ratios, expressed as a percentage of the *m*/*z* 280 base peak, ranged from 6.8 to 13% for the monitored ions. The overall mean and SDs ($n = 10$) were 12 ± 0.67, 7.6 ± 0.56, and 8.8 ± 0.42% at *m*/*z* 226, 252, and 275, respectively, with an interassay precision (CV) for all ions of ≤7.4%.

Control milk samples were also analyzed by the confirmatory assay for background interference (**Table 5**). Endogenous (background) interference at *m*/*z* 280 from the control milk samples was 18% of the response for the 2 ppb-fortified sample and ranged from 0.70 to 3.3% for the other ions.

**Table 4.** Overall Summary of 5-Hydroxyflunixin Milk Method Confirmatory Method Validation Results of Standards, Fortified Controls, and Incurred Samples

| | | | % area ratio at *m*/*z* (relative to *m*/*z* 280) | | |
|---|---|---|---|---|---|
| analysis set | sample ID | average RIC $R_t$ (min)[a] | 226 | 252 | 275 |
| | | standards | | | |
| set 1 | 1 | | 11 | 7.4 | 8.8 |
| | 2 | | 12 | 7.1 | 8.8 |
| | 3 | | 12 | 7.4 | 9.2 |
| | 4 | | 12 | 7.0 | 8.6 |
| set 2 | 1 | | 12 | 7.0 | 9.2 |
| | 2 | | 13 | 6.7 | 8.5 |
| | 3 | | 13 | 6.3 | 9.4 |
| | 4 | | 12 | 6.8 | 8.6 |
| | avevage | 7.00 | 12 | 7.0 | 8.9 |
| | SD | 0.06 | 0.64 | 0.37 | 0.34 |
| | CV % | 0.9 | 5.3 | 5.3 | 3.8 |
| | | fortifications | | | |
| set 1 | QC-1 | | 11 | 7.1 | 9 |
| | QC-2 | | 11 | 8.5 | 8.8 |
| | QC-3 | | 12 | 6.5 | 9.1 |
| | QC-4 | | 10 | 9.6 | 8.9 |
| | QC-5 | | 11 | 8 | 8.9 |
| set 2 | QC-1 | | 13 | 7.4 | 8.7 |
| | QC-2 | | 12 | 7.1 | 7.7 |
| | QC-3 | | 12 | 8.7 | 9.2 |
| | QC-4 | | 13 | 8.4 | 7.9 |
| | average | 6.98 | 12 | 7.9 | 8.7 |
| | SD | 0.05 | 1.0 | 0.98 | 0.53 |
| | CV % | 0.72 | 8.3 | 12.4 | 6.1 |
| | | incurred | | | |
| set 1 | 1[b] | | 12 | 7.8 | 8.7 |
| | 1 | | 11 | 7.8 | 9.4 |
| set 2 | 1 | | 13 | 8.1 | 7.8 |
| | 1 | | 12 | 6.8 | 8.9 |
| | 1 | | 12 | 7.5 | 8.8 |
| | 2 | | 12 | 7.1 | 9.2 |
| | 2 | | 13 | 7.6 | 8.7 |
| | 2 | | 13 | 7.3 | 8.9 |
| | 2 | | 12 | 7.4 | 8.8 |
| | 2 | | 13 | 8.8 | 8.7 |
| | average | 7.00 | 12 | 7.6 | 8.8 |
| | SD | 0.04 | 0.67 | 0.56 | 0.42 |
| | CV % | 0.57 | 5.6 | 7.4 | 4.8 |

[a] Retention time of *m*/*z* 280. [b] Incured samples 1 and 2 were determined to contain 3.2 and 6.8 ppb of 5-hydroxyflunixin, respectively.

**Table 5.** Signal-to-Noise Threshold Analysis of Control Samples by LC/MS/MS Confirmatory

| | | peak area at *m*/*z* | | | |
|---|---|---|---|---|---|
| analysis set | sample ID | 226 | 252 | 275 | 280 |
| set 1 | control 1 | 497 | 446 | 314 | 5095 |
| | control 2 | 403 | 437 | 31 | 2220 |
| | control 3 | 303 | 387 | 48 | 943 |
| set 2 | control 4 | 175 | 193 | 0 | 698 |
| | control 5 | 340 | 363 | 0 | 941 |
| average | ($n = 5$) | 344 | 365 | 79 | 1979 |
| LOD (abundance) (average × 3) | | 1032 | 1095 | 237 | 5937 |
| LOD (%) relative to *m*/*z* 280 peak at 2 ppb[a] | | 3.1 | 3.3 | 0.70 | 18 |

[a] The average area of ions at *m*/*z* 280 of nine measurements of the 5-hydroxyflunixin-fortified control milk at 2 ppb = 32 982.

**Signal-to-Noise Threshold.** Calculations of the signal-to-noise ratios are summarized in **Table 5**. Signal-to-noise values for control samples, expressed as three times the average background response, were 1032, 1095, 237, and 5937 at *m*/*z* 226, 252, 275, and 280, respectively. The S/N results, expressed

as a percentage of the average base peak response at 2 ppb, were 3.1, 3.3, 0.7, and 18.0% at *m*/*z* 226, 252, 275, and 280, respectively, based on an average ion area of 32 982 (*m*/*z* 280) for the 2 ppb-fortified milk samples.

The method reported herein is a rapid and sensitive procedure for the determination and confirmation of low concentrations of 5-hydroxyflunixin marker residue in bovine milk. The determinative method has a validated LOQ of 1 ng/g and an LOD of 0.2 ng/g in bovine milk. The procedure was also shown to possess a sufficient level of ruggedness, such that cation exchange SPE supports from different manufacturers did not result in significant differences in determined values. Potential interference from several other animal health compounds and flunixin was investigated and showed no interference. The overall determinative method recovery of at all fortification levels was 101 ± 5%.

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