United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health and Science

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Title: DETERMINATION OF FLUNIXIN IN CATTLE LIVER BY HPLC		
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A. INTRODUCTION

1. Theory

   Flunixin, an anti-inflammatory drug present in tissues as flunixin or its acid conjugates is converted to free flunixin by acid-catalyzed hydrolysis. The free flunixin is isolated by partitioning with organic solvent and the sequential use of silica gel, C_{18}, and ion exchange chromatography, and quantitated by reverse-phase high performance liquid chromatography (HPLC) with UV detection.

2. Applicability

   This method is used for determination of flunixin in bovine liver.

B. EQUIPMENT AND INSTRUMENTATION

Equivalent equipment may be substituted for those listed in this method

1. Balance, analytical: Sartorius A120S and BP210D, sensitive to 0.0001 g.

2. Balance, top loading: Shimadzu E83200D, sensitive to 0.01 g

3. Centrifuge: Centra-7R, International Equipment Company, capable of 2000 RPM and holding 50-mL glass centrifuge tube

4. Evaporator: nitrogen evaporator, N-Evap®, Organomation, Model No. 111

5. Glass or plastic beakers, which will fit into the solid phase extraction tank to collect waste eluant

6. Glass centrifuge tube: Pyrex® or Corex® round-bottom screw-cap, 29-mm, 50-mL centrifuge tube

7. Glass disposable Pasteur pipettes: 9 inches, VWR Cat. No. 14672-380

8. Glass volumetric flask: Kimax, class A, 10 mL, 25 mL, 50 mL, and 100 mL, 1000 mL VWR

9. Glass volumetric pipette: Kimax, class A, 1 mL, 2 mL, 4 mL, 5 mL, 10 mL and 25 mL, VWR

10. Glass graduated cylinders: 10 mL, 25 mL, 50 mL, 100 mL, 200 mL, 250 mL, 500 mL and a 1 L mixing cylinder with stopper VWR,

11. Heating Block: Pierce Reach-Therm™ III (Pierce Cat. No. 18935 H) with three aluminum

heating blocks holding at least twelve 29-mm. 50-mL centrifuge tubes. The heating block is custom drilled from a blank aluminum block (Pierce Cat. No. 18810 H)

12. pH meter. Corning 340 pH meter, capable of 0.01 pH unit sensitivity

13. Pipette: automatic pipettor capable of accurately delivering 200-µL and 400-µL volume

14. Scintillation vials: 20 mL

15. Solid phase extraction (SPE) vacuum manifold: Supelco Cat. No. 5-7030

16. Solid phase extraction (SPE) cartridges and accessories:
    a. Silica Cartridge: Waters Sep-Pak® Vac 6 cc, 1 g, Cat. No. WAT0036910.
    b. Silica Gel: J.T. Baker, Silica Get for Flash Chromatography (40 µm), Cat. No. 7024-01.
    c. C18 Cartridge: Waters Sep-Pak Vac 6 cc, 1 g, Cat. No. WAT036905.
    d. SCX Cartridge: Applied Separations, Benzensulfonic SCX, 500-mg/3 mL, Cat. No. 2323.
    e. Reservoir
       Cartridges: An empty 25-mL cartridge.
       Cartridge Adapters: Supelco Cat. No. 5-7020

17. Test tubes or centrifuge tubes 15 mL.

18. Vortexer: Vortexer-2, and multi-tube vortexer, VWR Cat. No. 58816-115.

19. Filter membrane. 0.2 micron HPLC filter membrane

20. 0.2 µm filter Acrodisc Gelman PVDF Membrane, Cat. No. PN 4450

21. Syringe filter barrel can be attached to filter acrodisc (21) Lab Depot Cat. No. BD309602 (1 mL).

22. HPLC System
    a. Pump: Agilent model 1050 series pump, capable of running isocratic solvent.
    b. Detector: Agilent model 1050 series UV detector, capable of operating at 330 nm.
    c. Autosampler: Agilent model 1050 series autosampler or equivalent, capable of injecting 75 µL.
    d. Analytical Column: Hypersil C-18, 5 µm, 4.6 mm x 250 mm (Bodman Cat. No. HC 15-255).

e. Guard Column: Brownlee column; RPI8, 7 µm, 3.2 x 15 mm (Bodman Cat No. 0711-0092).

f. Column Heater: Agilent model 1050 series column heater capable of operating at 40°C.

g. Data System: Agilent Chemstation

h. LC Vial. - 1.8 ml Vial, Wheaton, Cat. No. 224799-01SP.

i. Vial Cap. - 11 mm snap cap with PTFE/Silicone Liner, Wheaton, Cat. No. 242776.

j. Flat Bottom Limited Volume Inserts. - Agilent, Cat. No. 5181-3377.

C. REAGENTS AND SOLUTIONS

Equivalent reagents and solutions may be substituted for those listed in this method. Deionized, distilled water, or water of equal purity should be used.

1. Reagents

Commercially Available Solvents and Reagents

a. Ethyl acetate (EtOAc). HPLC grade.

b. Hexane, HPLC grade.

c. Methanol (MeOH), HPLC grade.

d. Ammonium Hydroxide, (NH_4OH) 30%, J.T. Baker Cat. No. 9721-33.

e. Hydrochloric acid (HCl), concentrated, J.T. Baker Cat. No. 9535-05.

f. Sodium Chloride Crystals (NaCl), J.T. Baker Cat. No. 3624-01.

g. Sodium Hydroxide Pellets (NaOH), J.T. Baker Cat, No. 3728-05.

h. Sodium Phosphate, Dibasic (Na_2HPO_4), Reagent Grade.

i. PIC® A reagent (tetrabutylammonium phosphate), Waters Part No. WAT085101. For stability, follow manufacturer's information.

j. Liquid nitrogen.

2. Solutions

a. 6 N Hydrochloric Acid (HCl):

Mix equal volumes of concentrated hydrochloric acid and water. This solution is good for one year at room temperature.

b. 20% (w/w Sodium Hydroxide NaOH):

Dissolve 200 g of sodium hydroxide into 800 mL of high purity water and mix well. This solution is good for one year at room temperature

c. 0.01 M Sodium Phosphate Dibasic (Na_2HPO_4):

Dissolve 1.42 g of sodium phosphate dibasic into a 1-L volumetric flask of high purity water and mix well.

d. Ethyl Acetate/Hexane (30/70, v/v)

Mix 300 mL of ethyl acetate with 700 mL of hexane. This solution is stable for one yearat room temperature

e. Methanol/Water (5/95, v/v)

Mix 50 mL of methanol with 950 mL of water. This solution is stable for six months at room temperature.

f. Methanol/Water (40/60, v/v)

Mix 400 mL of methanol with 600 mL of water. This solution is stable for six months at room temperature.

g. Ammonium Hydroxide/Methanol (10/90 v/v)

Mix 20 mL of ammonium hydroxide with 180 mL of methanol. This solution should be made fresh with each use.

h. HPLC Mobile Phase, 0.005 M Tetrabutyl Ammonium Phosphate / Methanol (46.5 /53.3 v/v):

Measure 465 mL of water in a 500 mL graduated cylinder. Remove approximately 50 mL of water from the graduated cylinder and save. Add the Pic A solution from the bottle. Rinse the Pic A bottle with the 50 mL water taken out from the graduated cylinder. Measure 535 mL of methanol in the 1000 mL mixing cylinder. Add Pic A solution to the methanol.

Mix well and degas the solvent by vacuum or helium purge if desired. The solution may also be filtered through an aqueous 0.2 micron HPLC filter membrane prior to degassing. This solution is good for one month at room temperature

D. STANDARDS

1. Source

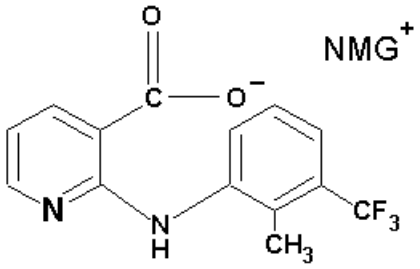
a. Flunixin Analytical Standard: Schering-Plough, P.O. Box 3182, Union, NJ 07083-1982. Cat. No. SCH 14714 and U.S. Pharmacopeia 12601 Twinbrook Parkway, Rockville MD 20852.Cat. No. 27460-7.

b. Common Name: Flunixin-N-methyl glucamine salt (NMG)

c. Chemical Name: 2[[2-Methyl-3-(trifluoromethyl)phenyl]amino]--3-pyridinecarboxylic acid (as NMG salt)

d. Storage: Ambient temperature

e. Chemical Structure:



NMG: N-Methylglucamine

2. **Preparation of Standard Solutions**

Flunixin N-methyl glucamine salt (NMG salt, $C_{21}H_{28}F_3N_3O_7$, MW 491 and free acid, $C_{14}H_{11}F_3N_2O_{2,\ MW}$ 296), is used as an analytical standard. If the purity is less than 100%, make corrections based on the actual purity provided.

a. Stock Solution A (500 µg/mL free acid):

Weigh 83.0 mg flunixin-NMG analytical standard equivalent to 50 mg of flunixin free acid into a 100-mL volumetric flask, dissolve the material, and dilute to the mark with methanol. Stock standard A is stable for 6 months at less than -10ºC.

b. Stock Solution B (50 µg/mL free acid):

Pipet 10 mL of stock solution A into a 100-mL volumetric flask and dilute to the mark with methanol). The stock standard B is stable for 6 months at less than -10ºC.

c. Fortification Solution

i. Dilute 5 mL of stock solution B with methanol to 100 mL using a Class A volumetric pipette and 100-mL flasks. Conc: 2.50 µg/mL.

Fortification solution stored in the freezer at less than –10°C is stable for six months.

d. HPLC Standard Curve Solutions

Prepare a serial dilution of stock solution B (50 µg/mL) with mobile phase using a volumetric pipette and flask as shown in the following Table:

HPLC STD	Vol. (mL)	Dilute to Vol. (mL	Flunixin acid µg/mL	ppb equivalent
Stock C1	10: Stock B	50	10.000	2000 ppb
Stock C2	5: Stock B	50	5.000	1000 ppb
A	25: Stock C2	50	2.500	500 ppb
B	25: HPLC STD A	50	1.250	250 ppb
C	25: HPLC STD B	50	0.625	125 ppb
D	25: HPLC STD C	50	0.313	63 ppb
E	0: HPLC STD 0	50	0.000	0 ppb

Stored in the refrigerator (approximately 2-8ºC) these solutions are stable for one month.

E. SAMPLE PREPARATION

To ensure the integrity of the sample and representative sampling, the following sample preparation procedures (E.1 and E.2) are recommended:

1. Sampling (Preparation of Laboratory Sample)

   Cut entire liver into chunks and homogenize one half for the ELISA screen. Store the other half frozen (lower than -10ºC) until further processing for the HPLC analysis.

2. Preparation of Liver Tissue

   Freeze the collected liver sample in liquid nitrogen and finely grind with liquid nitrogen in a Hobart ® grinder, or appropriate grinder. Sublimate samples in a freezer overnight. Keep several sub-samples frozen and place them in a separate package until further processing. Sub-samples should be homogeneous before sampling. One packet is removed from the freezer for analysis, used and discarded. If a second analysis is needed, a fresh packet is used that has not been thawed.

   SAFETY NOTE: Liquid nitrogen will cause burns on contact with skin.

F. ANALYTICAL PROCEDURE

1. Sample Extraction and Cleanup Procedures

   a. Each sample should be homogeneous. .

   b. Weigh 2.00 ± 0.20 g of tissue homogenate into a 50-mL Pyrex® or Corex® round-bottom screw-cap centrifuge tube. Record the weights to three significant figures.

   c. *Fortification of recovery should be performed during this step*. For a 125 ppb (tolerance level) recovery, fortify 2-g of control liver with 100 µL of fortification solution (D.2.c.i). Label the tube and cap to prevent possible cross contamination.

      SAFETY – Cracked or damaged tubes should be discarded.

   d. In a well-ventilated hood, add 8 mL of 6 N HCl to each tube. Using a spatula, scrape the tissue, if any, off the tube side wall down to the bottom of the tube. Cap the tube. SAFETY – 6N HCl is a corrosive liquid. Use only in a fume hood.

   e. Place the sample tubes in a heating block preheated to 110°-120°C. The temperature of the heating block will gradually drop to 95°C when the tubes are in the heating block. The temperature will rise to 110°-115°C within 15 min. After 2 hours of heating at 95°-115°C, remove the tubes and allow cooling to ambient temperature. Tubes can be immersed into a cold water bath to accelerate the cooling process.

SAFETY – Take precautions with the handling of closed systems under high temperatures or pressures with corrosive liquids and gases. Recommend safety shields or hoods.

STOPPING POINT – Samples may be stored in refrigerator (2-8ºC) up to 3 days

f. Adjust the pH of the HCl hydrolysate to pH 9.50-9.70 by slowly adding approximately 8 mL of 20% (w/w) NaOH and gently vortexing. Check the pH of the solution with a pH meter and add additional 20% (w/w) NaOH or 6 N HCl if necessary. Rinse the pH meter probe with water between samples.

SAFETY – Conduct this procedure under a fume hood. Care must be used to avoid spattering of samples.

g. Add approximately 2 g of sodium chloride to the test tube. A pre-calibrated measuring scoop or cup can be used

h. Add 10 mL of ethyl acetate to each tube, cap and vortex at high speed for 1 min. Centrifuge the tube for 5 min at approximately 2000 rpm (approximately 1400 rcf). Using a long-stem Pasteur pipette, pipet the upper ethyl acetate extract to another properly labeled, clean 50-mL glass centrifuge tube. Care should be taken not to transfer any of the lower dark layer. Retain the pipette for later use in the following three cleanup steps

i. Repeat the 10-mL ethyl acetate solvent partition three more times and combine the ethyl acetate fractions.

j. Evaporate the combined ethyl acetate extracts to dryness under a stream of nitrogen using N-Evap® in a 60-70°C water bath. Be sure that all samples are completely dry before reconstitution (next step). A slightly oily residue may still be present.

STOPPING POINT – Cap or Parafilm® seal the tubes. Samples are stable up to three days at refrigerated temperature (2-8ºC).

k. Add 10 mL of hexane to each tube, cap and vortex to mix. Some insoluble residues will be evident.

STOPPING POINT –Samples may be stored overnight in refrigerator (2-8°C).

l. Silica Cartridge cleanup

i. Mount a 1-g silica cartridge on the SPE manifold. Add approximately 100 mg of 40-µm silica gel on top of the column. The loose silica on top of the

column is used to prevent clogging during sample loading. Condition the column with two reservoir volumes (≈ 5 mL) of hexane gravimetrically or under low vacuum. Do not allow the second hexane solvent to drain below the column bed (≈ 1-2 mL above the column bed). Attach a 25-mL reservoir cartridge on top of the column.

ii. Pipet the hexane fraction into the reservoir. Open the valve and collect the eluant into a waste beaker. Apply low vacuum as needed to increase flow. Do not let the hexane drain below the column bed. If the column becomes clogged (no flow), close off the valve and insert the Pasteur pipette through the reservoir to the top of the silica column bed. Gently disperse the loose silica to break up the insoluble residues of the hexane extract. Open the valve and apply low vacuum as needed to maintain a flow rate of 0.4 mL-0.8 mL/min. This is to prevent air blockage between the reservoir and the column.

iii. Wash the centrifuge tube with 5 mL of 30% ethyl acetate/hexane (v/v), vortex, and pipette the solvent into the reservoir with the valve closed. Wash the centrifuge tube with 10 mL of 30% ethyl acetate/hexane (v/v) and add the wash solution to the column reservoir. Use Pasteur pipette to start the flow as described in step b. Open the valve and apply low vacuum (0.4-0.8 mL/min) to collect the eluant into a waste beaker. Close the valve when all the wash solvent has drained just above the column bed. Pipet 5 mL of ethyl acetate into the reservoir. Use the Pasteur pipette to start the flow again. Open the valve to apply low vacuum as needed to maintain a flow rate of 0.4-0.8 mL/min, and collect the eluant into a waste beaker. Once all the solvent elutes are off the column, apply high vacuum and air dry the silica column for at least 15 min.

iv. Close the valve. Wash the centrifuge tube with 8 mL of 0.01 M sodium phosphate buffer and pour the buffer into the reservoir. Start the flow with the Pasteur pipette. Open the valve and make sure to collect the eluant into a 20mL scintillation vial using moderate vacuum as needed to maintain a flow rate of 1-2 mL/min. Add another 10 mL of 0.01 M sodium phosphate into the reservoir and collect the eluant into the same vial.

STOPPING POINT – Combined sodium phosphate solutions may be stored overnight in refrigerator (2-8°C).

m. C18 Cartridge Cleanup

i. Mount a 1-g (6 cc) C_{18} column on the SPE manifold. Condition the column with one column volume (≈ 5 mL) of methanol followed. by two additional column volumes (total of ≈ 10 mL) of water. Leave approximately 1-2 mL of water above the column bed. Attach the 25-mL cartridge reservoir to the top of the column.

ii. With the valve closed, pour the aqueous sodium phosphate eluant from the scintillation vial into the C18 cartridge reservoir. Open the valve, apply low vacuum (0.4-0.8 mL/min), and drain the eluant into a waste beaker. Wash the vial with 10 mL of water and pour into the column reservoir. Drain the eluant into the waste beaker. Pipet 5 mL of 5% MeOH/H_2O (v/v) into the reservoir and wash the column. Drain the eluant into a waste beaker. Start the flow as needed using the Pasteur pipette.

iii. With the valve again closed, add 16 mL of 40% MeOH/H_2O (v/v) into the reservoir. Start the flow with a Pasteur pipette. Open the valve and collect the eluant into a 20-mL scintillation vial using low vacuum as needed to maintain a flow rate of 0.4-0.8 mL/min. Ensure complete elution by observing only air passing through the column.

STOPPING POINT – Samples may be stored overnight in refrigerator (2-8°C).

n. SCX Cartridge Cleanup

i. Mount a 500-mg SCX column on the SPE manifold. Condition the column with one column volume (≈3 mL) of methanol and two column volumes (total of ≈6 mL) of water). Leave approximately 1 mL water above the column bed. Attach the 25 mL cartridge reservoir on top of the column.

ii. Pour the 40% MeOH/H_2O (v/v) eluant from the scintillation vial into the reservoir. Start eluant flow. Open the valve and apply low vacuum as needed to maintain a flow rate of 0.4-0.8 mL/min, and drain the eluant into a waste beaker. Wash the vial with 10 mL of methanol and load onto the column. Drain the eluant into a waste beaker.

iii. Put a 15-mL test tube or centrifuge tube on a rack to collect eluant. Add 12 mL of 10% NH_4OH/MeOH (v/v) into the reservoir. Start the flow with a Pasteur pipette. Collect the eluant into the test tube with moderate vacuum (≈1-2 mL/min). Ensure complete elution by observing only air passing through the column.

SAFETY – Conduct this procedure in a fume hood.

STOPPING POINT – Samples may be stored overnight in refrigerator (2-8°C).

iv. Concentrate the NH_4OH/MeOH eluant to near dryness under a stream of nitrogen using N-Evap® in a 60-70°C water bath. Wash the side wall of the test tube with approximately 0.5-1 ml of methanol and evaporate to dryness. Accurately add 5.0 mL methanol to each tube using a 5.0 mL Class A

pipette. Vortex to mix contents of each tube. Using a 4.0 mL Class A pipette, accurately transfer 4.0 mL of solution from each tube to a similarly numbered tube identified for HPLC analysis. The remaining solution will be used for LC/MS/MS confirmation. Contents of tubes designated for the HPLC determination and the LC/MS/MS confirmation are evaporated to dryness. The LC/MS/MS confirmation tubes, originally containing 1mL of methanol, are capped after evaporation. Store in the freezer at less than -10ºC The HPLC determination tubes, originally containing 4 mL of methanol, are reconstituted in 320 µL of HPLC mobile phase. Vortex to mix contents. Filter extract through a 0.2 micron filter Acrodisc attached to a Syringe filter barrel into an HPLC autosampler vial glass insert. The sample is ready for HPLC analysis.

STOPPING POINT – Samples are stable up to five months if stored if stored in refrigerator (2-8°C).

2. Instrumental Settings: These settings represent one approach to the detection of Flunixin in liver extracts. Other optimizations may yield equivalent results.

Column:	Hypersil 120 C18, 5 µm particle size (4.6 mm i.d. x 250 mm)
Guard Column:	Brownlee RP C18 guard column (3.2 mm id x 15 mm, 7 µm particle)
Column Temperature:	40 °C for both analytical and guard columns
Mobile Phase:	See section C.2.h.
Flow rate:	1.0 mL/min
Injection Volume:	75 µL
UV Wavelength Monitored:	330 nm
Run Time:	35-40 min
Approximate Retention Time Range of Flunixin:	20-30 min

Retention time may deviate up to 30 seconds from the most recent injection of a sample containing flunixin.

3. **HPLC Analysis**

a. Equilibrate HPLC system with mobile phase at 1.0 mL/min for at least 30 min before any injections.

b. Inject standard curve series. Perform routine system suitability. (See F.6 of Analytical Procedure.)

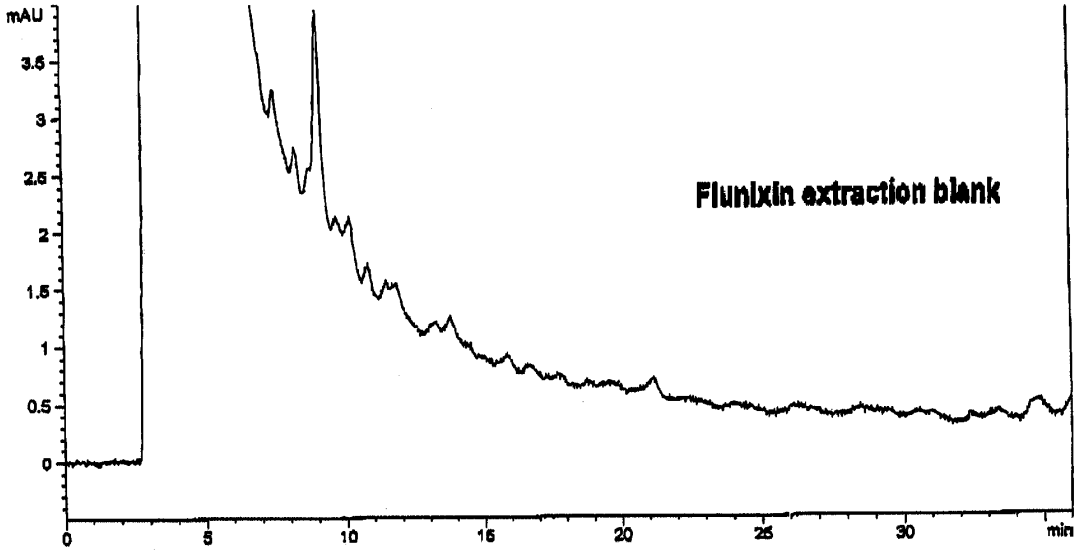
c. Inject a maximum of 12 sample extracts including a control blank and a fortified control between standards and a check sample.

d. Inject at least one standard at 125 ppb at the end of the run to assure the flunixin retention time and peak shape are still acceptable.

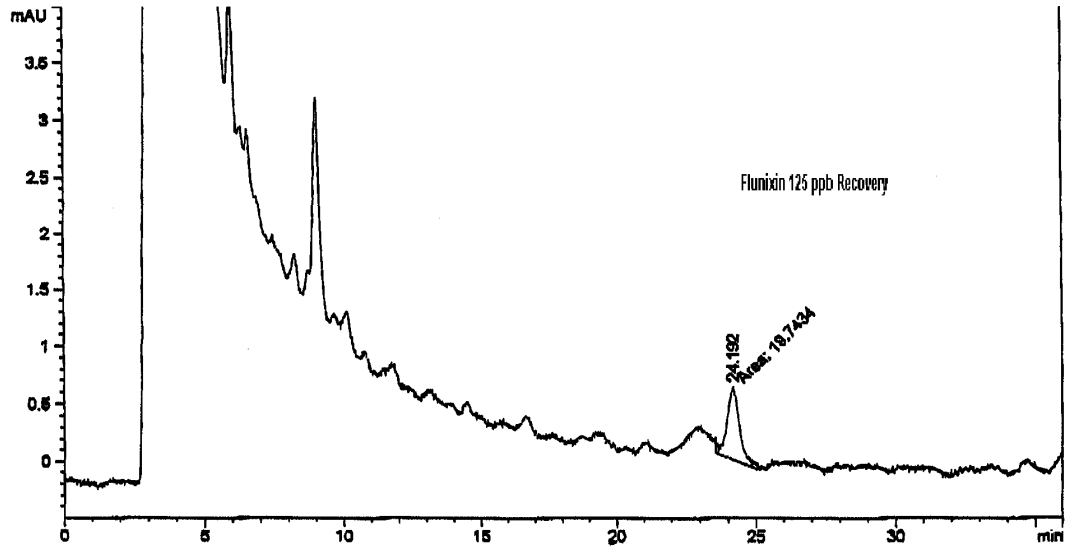
e. Dilute and re-inject sample extracts whose response exceeds the range covered by the linearity curve.

4. Sample Chromatograms

Representative HPLC chromatograms are shown below



(a) Chromatograms of blank beef liver tissue



(b) Chromatogram of 125 ppb (1x) flunixin recovery

5. System Suitability

Perform the following system suitability at initial HPLC setup:

As a check of the HPLC system, system suitability may be evaluated before or during sample analysis as follows:

a. Inject at least three of a middle range standard. The percent relative standard deviation of the area peak response should be ≤ 5%.

b. Peak asymmetry of a middle range standard should be ≤ 2. The degree of tailing or asymmetry is measured by the peak asymmetry factor A_S. Symmetrical peak has an A_S of 1.0.

A_S = CB/AC

where:

AC = peak width from start of peak to center at 10% peak height.

CB = peak width from center to end of peak at 10% peak height

c. For one day's analysis the retention time for flunixin should not vary more than ±30 seconds from the mean or most recent injection of Flunixin.

d. The UV detector should be capable of providing approximately 10 to 1 signal-to-noise ratio when the lowest level of flunixin standard (0.313 µg/mL) is injected on column.

G. CALCULATIONS

1. Standard Curve

Generate a linear standard curve using at least 4 concentrations of standard curve solutions (0, 62.5, 125, and 250 ppb standards (see D.2.4)). Construct a linear regression standard curve using flunixin peak area response as the "y" coordinate and flunixin-free acid standard concentration (µg/mL) as the "x" coordinate on the basis of following formula

$$Y = mx + b$$

Where: y = peak area; x = concentration (ppb); m = slope, and b = y intercept. Calculate sample results (µg/mL) using the linear regression standard curve.

Convert the resulting sample extract concentration to ppb by multiplying the calculated extract concentration by the final mL volume of 0.4 mL) and dividing by the sample weight in grams as indicated below. The result is then expressed to at least two decimal places. This is expressed in the equation below:

(C x V) / W = ppm concentration of Flunixin in tissue

Where: C = Sample concentration in extract (µg/mL)
V = Final volume of extract in mL.
W = Sample weight in grams

H. HAZARD ANALYSIS

1. Required Protective Equipment — Lab coat, gloves, safety glasses, Plexiglas shield and fume hood.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
E.2	Liquid nitrogen causes burns on contact with skin.	Use gloves, face shield, and lab coat
F.1.d and F.1.f.	Corrosive liquids [6N HCl and 20% (w/w) NaOH].	Use safety glasses and gloves. Use a fume hood.
F.1.e.	Possibly explosive (Heating samples in sealed tubes to 110-120ºC).	Use Plexiglas shield.
Primary standard (D.1).	Skin irritation and possible pulmonary edema after prolonged inhalation of this compound.	Use safety glasses and gloves when handling the primary standard. Avoid breathing crystals.
F.1.i, F.1.n.iii-iv	EtOAc , and NH_4OH/MeOH	Harmful vapor, use fume hood.

3. Disposal Procedures

Solvent waste should be disposed in the appropriate solvent container. Perform evaporation steps in a well-ventilated hood.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range*	*Acceptable Recovery*	*Acceptable Repeatability (CV)*
Flunixin	62.5 –250 ppb		
	< 100 ppb	60-110%	≤ 20%
	≥ 100 ppb	80-110%	≤ 20%

2. Standard curve should give a correlation coefficient (r^2) $\geq$ 0.990

3. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample Weight (F.1.b)	2.00 ±0.20 g
b. pH of Hydrolysate (F.1.f)	9.50-9.70
c. Phase Transfer (F.1.h)	Care should be taken to avoid transferring lower dark layer.
d. Column Elution (F.1.l-n)	0.4-0.8 mL/min.
e. Column Bed (F.1.l.i.,m.i.,n.i.)	Keep wet between changes of elution solvent except silica gel cartridge drying step (F.1.l.iii.).
f. Silica Solid Phase Extraction Sample Loading (F.1.j and F.1.k)	(a) Remove all ethyl acetate from the sample tube prior to reconstitution with hexane (b) Do not use vacuum during sample loading.
g. Silica cartridge clean up (F.1.l.iii)	Ensure complete drying of SPE column
h. Glassware	50-mL hydrolysis tube and cap are washed sequentially with alconox and distilled water and air-dried. All other glassware is disposable.

4. Readiness To Perform

a. Analyst Training

i. Phase I: Standards-Duplicate standard curve on each of 3 consecutive days, which will include the following:

(a) 0 ppb

(b) 62.5 ppb

(c) 125 ppb

(d) 250 ppb

ii. Phase II: Fortified samples. Over a period of 3 different days using bovine liver at 4 different levels of 0, 62.5, 125 and 250 ppb, respectively.

NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III. Check samples for analyst accreditation.

(a) The analyst is to receive six unknown bovine liver samples incurred or fortified between 0 to 250 ppb levels.) At least one sample must be a blank These samples will be prepared and provided by Supervisor or Quality Assurance Manager (QAM).

(b) Report analytical findings to Supervisor. Supervisor will forward results to the laboratory QAM.

(c) A letter from QAM is required to commence official analysis.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

i. Take corrective action

5. Intralaboratory Check Samples

a. Frequency: One check sample weekly per analyst when samples are analyzed.

b. Records are to be maintained for:

i. All % recoveries.

ii. Running average, standard deviation, and CV for recoveries.

6. Sample Acceptability and Stability

a. Sample receipt size: approximately 1 kg.

b. Sample storage: In freezer at ≤ -10ºC until analyzed.

7. Standard and Extract Stability

a. Sample extracts: stable for up to five months if stored at 2-8°.

8. Sample Set

a. Each set should include

(i) One tissue blank,
(ii) One fortified tissue recovery at 125 ppp (tolerance) level.
(iii) Samples

9. Sensitivity

a. Lowest detectable level (LDL): 31.3 ppb.

b. Lowest reliable quantitation (LRQ): 62.5 ppb.

c. Minimum proficiency level (MPL): 62.5 ppb.

J, WORKSHEET

Example of a worksheet, on the following page, may be copied for use.

FLUNIXIN DETERMINATIVE FORM

Analyst: _____
Date Started: _____
Date Completed: _____
Set Number: _____
Reviewed By Initials/Date: _____

External Standard Data

HPLC Std	Standard Number	Conc., µg/mL	Ret. Time	Peak Area
A		2.500		
B		1.250		
C		0.626		
D		0.313		

m = _____
b = _____
r^2 = _____

Sample FRZ-_____ to Holding FRZ-_____
Holding FRZ-_____ to FRZ-_____

	Day One	Day Two
Heating Block Temp. (110-120 C)		
N-Evap Temperature (60-70 C)		

HPLC Conditions	Run time :	
	Injection vol. :	
	Pressure :	
	Flow rate :	
	Column Temp:	
	Mobile Phase :	
	Column Type:	
Detector Conditions	UV Signal (l):	
	Sensitivity (AUFS):	
	Attenuation:	

Sample Analysis Data

Sample No.	Lab. No.	Form No.	Sample Wt. (2.00 ± 0.20 g)	Sample pH (9.50-9.70)	Flunixin (Peak Area)	Flunixin (Ret. Time)	Dilution Factor	Flunixin µg/mL	Flunixin ppm	Recovery %
1	0.10 ppm Recovery									
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										

K. REFERENCE

Alice M. Bova, Schering-Plough Research Institute, Lafayette, NJ; and David Lui and Matt Wisocky, XenoBiotic Laboratories, Inc., Plainsboro, NJ.
High performance Liquid Chromatography method for the Determination of the Marker Residue (Flunixin) in the Target Tissue (Liver) of Cattle Treated with Flunixin-NMG (March 19, 1998).

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Title: DETERMINATION OF FLUNIXIN IN CATTLE LIVER BY HPLC		
Revision: 00	Replaces: NA	Effective:11/4/02

Approved by:	Date
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