CLG-FLX2.00		Page 1 of 14
Title: Confirmation of Flunixin Residues in Bovine Liver by HPLC/ESI-MS/MS		
Revision: 00	Replaces: NA	Effective: 3/24/03

Contents

Approval Signatures on file

A. INTRODUCTION

1. Theory

   This method utilizes the extracts from the determinative method (CLG-FLX1). The extracts are evaporated, redissolved in 400 µL mobile phase, filtered and analyzed by High Performance Liquid Chromatography/Electrospray Ionization MS/MS (HPLC/ESI-MS/MS). Confirmation is based on comparison of MS/MS product ion rations 259/279 and 264/279 in the sample against those obtained for a standard.

2. Applicability

   This method is applicable to beef liver.

B. EQUIPMENT

Note: Equivalent equipment may be substituted for the following:

1. Apparatus
   a. Test tubes or centrifuge tubes - 15 mL.
   b. Vortex test tube mixer - Fisher Vortex, Genie 2.
   c. Filters - 0.2 µm nylon acrodisc.
   d. Syringe – 20 µL, Hamilton gastight.
   e. Disposable pipettes – 5 3/4 inches, borosilicate glass, Kimble #72050.
   f. Nylon Syringe Filter.
   g. Autosampler vials - 1.8 mL.
   h. Glass volumetric flask - Kimax, Class A 100 mL, VWR.
   i. Glass volumetric pipettes - Class A, 5 and 10 mL, VWR.
   j. N-Evap® - Organomation, Model No. 111.

2. Instrumentation
   a. Mass spectrometer - Thermoquest/FinniganTSQ-700.
   b. HPLC equipped with a binary pump and auto-injector – Hewlett Packard (HP)/Agilent Model 1100.
   c. Analytical column - Zorbax Rx-C8, 2.1 mm x 150 mm, 5 µm.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents/solutions may be substituted for the following:

1. Reagents

   a. Methanol - HPLC grade.

   b. Water - HPLC Grade.

   c. Formic acid – ACS reagent grade.

   d. Ammonium Acetate (NH_4OAc) - Greater than 98% purity.

2. Solutions

   a. 0.01 M ammonium acetate:

      Add 0.77 g ammonium acetate to a 1 liter volumetric flask and dilute to volume with HPLC grade water.

   b. 0.4% formic acid:

      Add 4 g of formic acid to a 1 liter flask and dilute to volume with HPLC water.

   c. LC Mobile Phase:

      Methanol: 0.4% formic acid: 0.01 M NH_4OAc (75:24:1). Mix well and before using.

D. STANDARDS

1. 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 the analytical standard. If purity is less than 100%, make corrections based on the actual purity provided.

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

      Weigh sufficient amount of flunixin-NMG analytical standard, equivalent to 50 mg of flunixin free acid, into a 100-mL class A volumetric flask. Dissolve the material, and dilute to volume with methanol. Stock solution A is stable for six months if stored at $< -10°C$.

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

      Pipet 10 mL of stock solution A using a class A pipette into a 100 mL class A volumetric flask and dilute to volume with methanol. Stock solution B is stable for six months if stored at $<$-10°C.

c. Working External Standard (0.125 µg/mL equivalent to 125 ppb):

Dilute 10 µL of Stock Solution B with 4.0 mL of HPLC mobile phase. Use this solution for the system suitability as well as the external standard for HPLC/MS confirmation. This solution is made up daily.

2. Stability and storage - see 1.a, 1.b, and 1.c. above.

E. SAMPLE PREPARATION

1. Using an N-Evap® set at 60 -70º C, evaporate 1.0 mL of each extract of sample, recovery and tissue blank in a 15 mL test tube from the determinative method, and reconstitute to 400 µL with HPLC mobile phase. Vortex and filter the solution through a 0.2 µm nylon syringe filter directly into a 1.8 mL glass autosampler vial.

2. Inject an appropriate aliquot into the HPLC-MS for analysis.

F. ANALYTICAL PROCEDURE

1. Instrument Parameters

Note: The instrument parameters listed here are examples of one set of suggested optimization parameters. Others may yield equivalent results. The analyst should optimize parameters for the instrument being used.

a. HPLC conditions:

Mobile Phase	See Section C.4.c.
Flow rate	0.30 mL/min
Injection volume	20 µL
Run Time	15 minutes

b. Mass Spectrometer Parameters:

EM Voltage	1200
Collision offset	-31.9
Multiplier Gain	7
Capillary voltage	20.9
Tube lens	77.7
Auxiliary flow	30

Sheath flow	75
Capillary temp	250
CID offset	-15
Spray voltage	4.80
Collision cell pressure	3.3 mT

c. Data Acquisition Selected Reaction monitoring mode—Precursor ion 297

Ion 259, scan width 0.6d, scan time 0.1sec.

Ion 264, scan width 0.6d, scan time 0.1 sec.

Ion 279, scan width 0.6d, scan time 0.1 sec.

2. MS Optimization

a. Perform tune and calibration of instrument after each source cleaning.

b. Perform full MS scan via flow injection analysis of 1.25 ng of flunixin in mobile phase and obtain the centroid of the precursor ion (297). An MS/MS scan of 1.25 ng flunixin flow injected using the previously measured precursor ion is performed. Use the centroids of the product ions (259, 264, 279) obtained for subsequent analyses. The 125 ppb external standard may be used for this optimization.

c. Inject 125 ppb external standard (0.125 μg/mL) into the HPLC. Sufficient sensitivity should be present (>20x background signal) for the m/z = 279 ion. If necessary, dilute sample extract to match the concentration of external standard.

d. Compute ion ratios.

3. Confirmation Analysis

a. For sample analysis use the following sequence:

i. External standard

ii. Fortified blank

iii. Solvent blank

iv. Control tissue

v. Samples

Note: Normally no carry-over is observed in the solvent blank. If a

flunixin response is noted, re-inject another solvent blank. If no flunixin is observed follow each sample with a solvent blank. If a flunixin response is observed in the second solvent blank, troubleshoot the HPLC, retaining the flunixin samples (stored at 2 - 8º C) until the system is restored. Use a needle wash routinely to eliminate possible carry over.

4. Confirmation Criteria

   a. Tissue blank has no confirmable target compound.

   b. The retention time of the unknown must be ± 3% of the external standard.

   c. All three ion products (259, 264, 279) must be present.

   d. The ion ratios 259/279 and 264/279 must match those of the external standard within a relative difference of ± 10%.

   e. Operational criteria for sample repeat injection

      i. For unknown samples that will not confirm and system suitability has not been compromised, simply repeat the injection along with a pure standard.

      ii. If upon re-injection the sample still fails to confirm, repeat the extraction.

      iii. If upon re-extraction the sample fails to confirm, the sample should be reported as non-detected for flunixin.

G. CALCULATIONS (not applicable)

H. HAZARD ANALYSIS

1. Method Title -- Confirmation of Flunixin Residues in Bovine Liver by HPLC/ESI-MS/MS.

2. Required Protective Equipment - Safety glasses, disposable gloves, lab coats.

3. Hazards

Reagents / Solutions	Hazard	Recommended Safe Procedure
Methanol	Flammable & poisonous	Wear gloves, work in fume hood.
Formic acid	Dangerously caustic to skin, causes albuminuria and hematuria	Wear gloves, work in hood. Use protective eyewear.

4. Disposal Procedures

Reagents / solutions	Hazard	Recommended Safe Procedure
HPLC Mobile phase containing formic acid, ammonium acetate and methanol	Formic acid caustic to skin. causes albuminuria and hematuria	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, State, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standards
   a. No false positives from blank tissues.
   b. No false negatives at 125 ppb (as calculated using determinative method).

2. Critical Control Points and Specification

Record	Acceptable Control
Reconstituted extract	Filter prior to the injection into HPLC-MS for analysis.

3. Readiness To Perform (FSIS Training Plan)
   a. Familiarization
      i. Phase 1: Standards. Prepare a standard solution containing flunixin at concentration equivalent to 125 ppb in sample extracts. Analyze by HPLC/ ESI-MS/MS, and obtain three product ions:

259, 264 and 279. Repeat analysis on three different days to verify parameters.

ii. Phase II: Analyst fortified samples. Conduct analyses of one control tissue and one control tissue fortified with 125 ppb on three separate days. Confirmation is achieved by comparing the ion ratios 259/279 and 264/279 of the fortified samples to that of the standard. Phases I and II can be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) A minimum of 6 check samples blind to the analyst given by supervisor/Quality Assurance Manager (QAM) quality manager. At least one check sample should be blank. Recoveries should be spiked at 125 ppb level.

(b) Report analytical findings to Supervisor/QAM.

(c) Notification from QAM is required to commence official sample analysis.

4. Intralaboratory check samples

a. System, minimum contents.

i. Frequency: One sample per week as samples analyzed.

ii. Records are maintained.

b. Acceptability criteria. Refer to section I.1 above.

If unacceptable results are obtained, then:

i. Stop sample analysis.

ii. Take corrective action.

5. Sample set must include:

a. External standard

b. Recovery containing analyte to be confirmed at 125 ppb level.

c. Tissue blank.

d. Sample(s).

6. Sensitivity

a. Minimum Proficiency Level (MPL): 125 ppb.

J. WORKSHEET

An example of a worksheet, on the following page, can be copied for use.

FLUNIXIN LC CONFIRMATION WORKSHEET

Analyst: _____
Date Started: _____
Date Completed: _____
Set Number: _____
Reviewed by: _____
(Initials & date): _____

SPECIFICATIONS:
Instrument Used (LC-MS): _____
Mobile Phase: _____
Injection Volume: _____
Ca. ng Std/Rec. Injected: _____
Run Time: _____

Sample Number	Form Number	Lab Number	Tissue Code	Dilution Factor	FLX RT (Min) m/z 279	Sample RT VS Rec/Std (±3%)	Ion Ratio m/z (259/279)	Ion Ratio % (vs std) (± 10%)	Ion Ratio m/z (264/279)	Ion Ratio % (vs std) (± 10%)	Result (±)

K. CHROMATOGRAMS AND STRUCTURAL INFORMATION

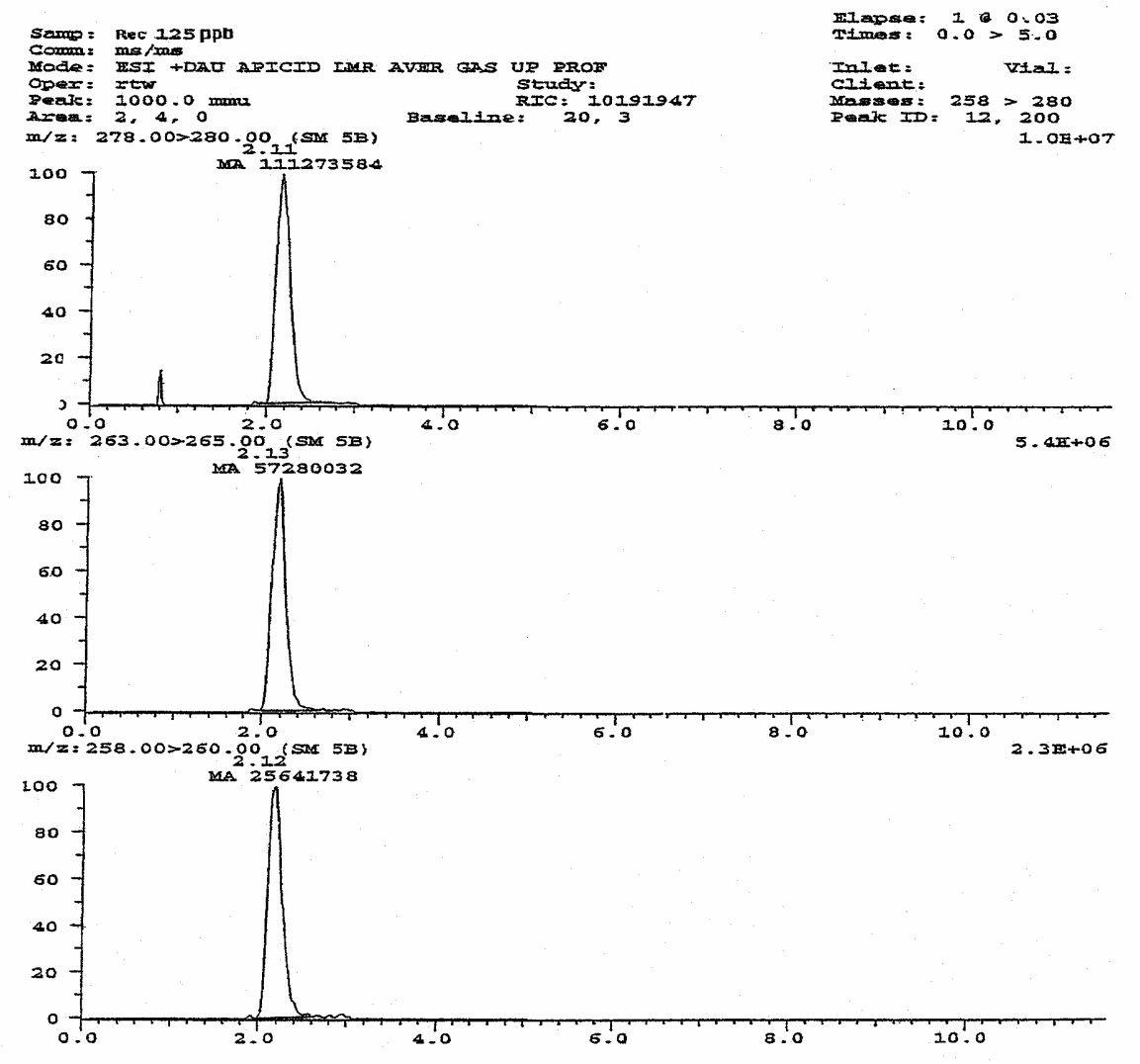

Figure 1. HPLC/ESI-MS/MS of 125 ppb flunixin recovery (0.125 μg/mL) of beef liver.

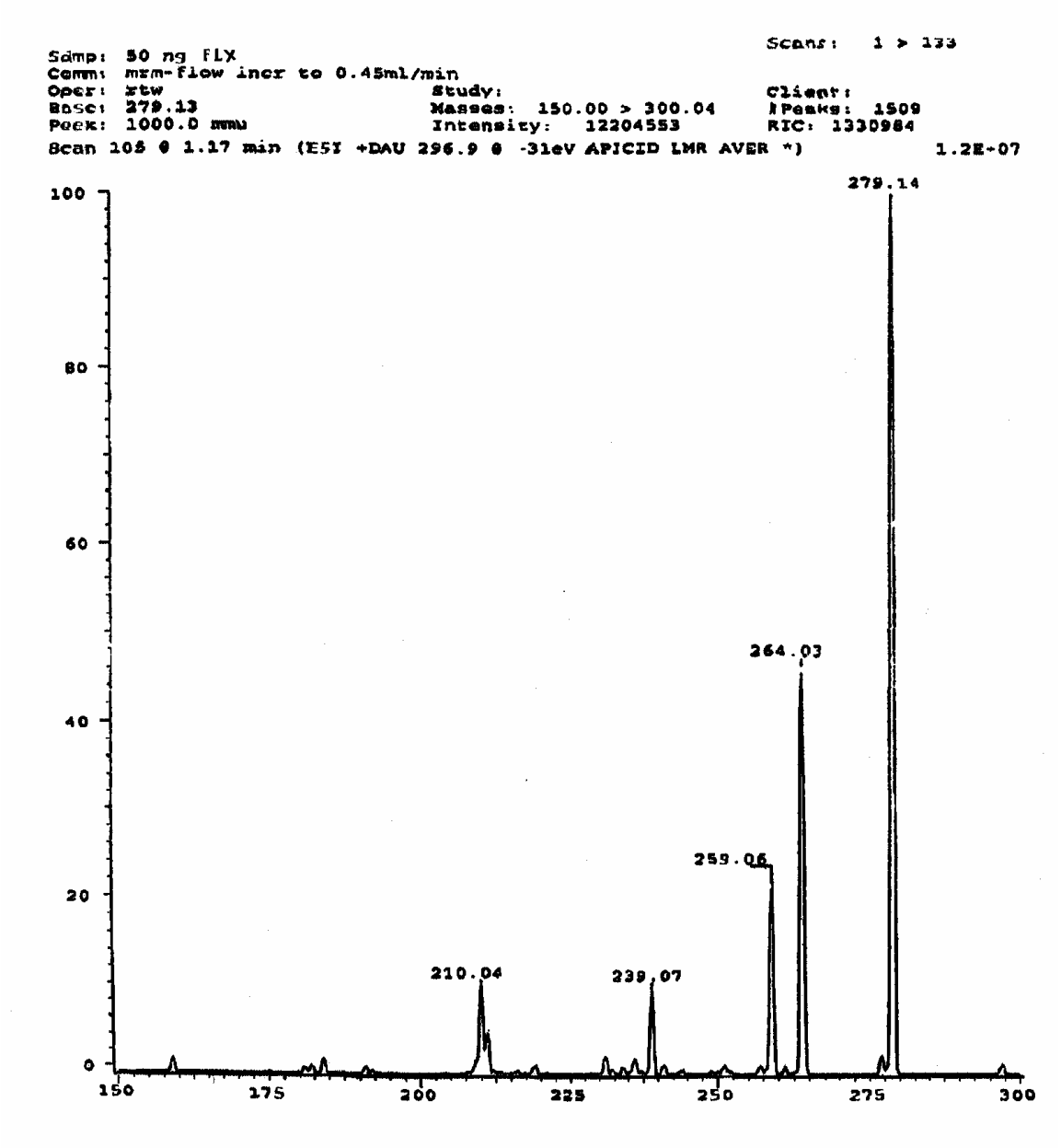

Figure 2. HPLC/ESI mass spectrum of flunixin

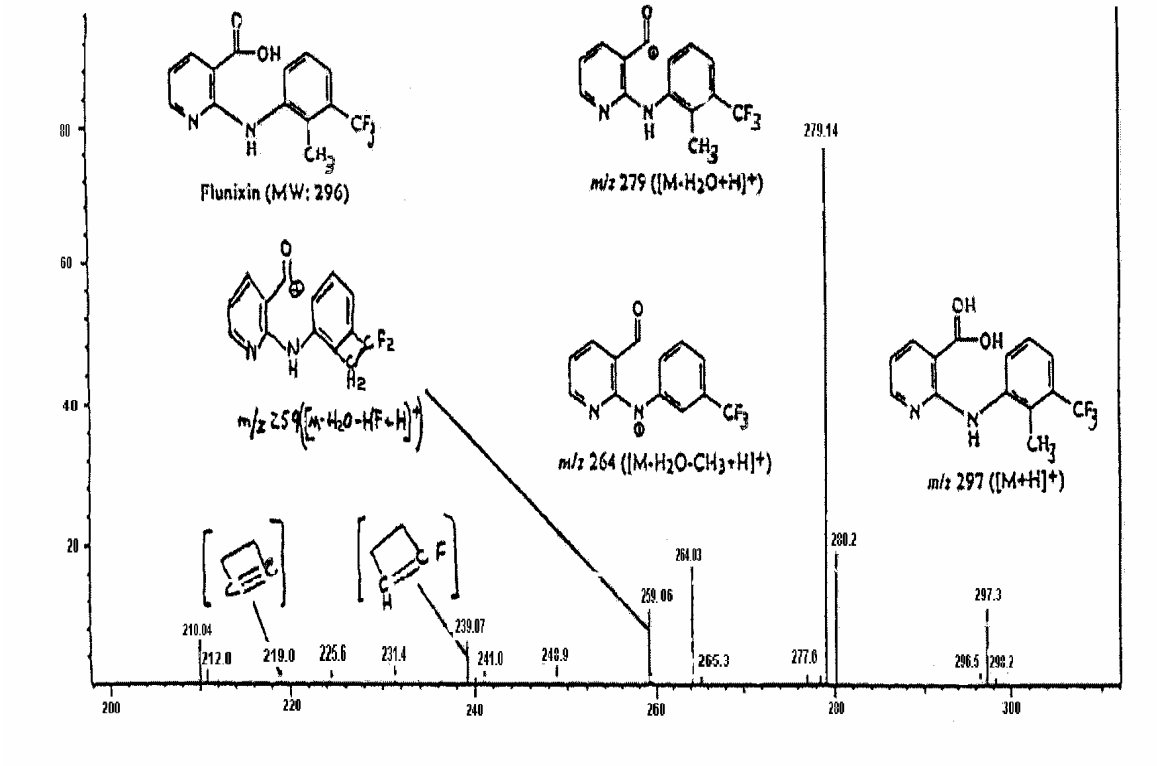

Figure 3. Tentative fragmentation pattern of flunixin

CLG-FLX2.00		Page 14 of 14
Title: Confirmation of Flunixin Residues in Bovine Liver by HPLC/ESI-MS/MS		
Revision: 00	Replaces: NA	Effective: 3/24/03

Approved by:	Date
Milo Stubban	03/11/03
Thomas Mallinson	03/06/03
Terry Dutko	03/10/03
Jess Rajan	03/13/03
Charles Pixley	03/10/03
Phyllis Sparling	03/13/03