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Title: Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS		
Revision: 04	Replaces: CLG-FLX4.03	Effective: 03/15/21

Contents

A. INTRODUCTION

1. Background / Summary of Procedure

Flunixin, an anti-inflammatory drug present in tissues as flunixin or its acid conjugates, is converted to free flunixin by acid-catalyzed hydrolysis. After neutralizing the acidic mixture, the free flunixin is extracted with ethyl acetate and the extract is purified by passing through a SCX cation exchange cartridge. Flunixin is eluted from the cartridge with ammonium methanol, evaporated to dryness, reconstituted in 50% methanol / water, and quantitated by High Performance Liquid Chromatography/ Electrospray Ionization MS/MS (HPLC/ESI-MS/MS). An external standard curve based on the precursor ion (m/z 297) fragmenting to the product ion m/z 279 is used for quantitation.

2. Applicability

This method is suitable for the determination and confirmation of flunixin in bovine liver and muscle at levels ≥ 62.5 ppb and at levels ≥ 12.5 ppb and in porcine liver and muscle at ≥ 15.0 ppb and at ≥ 12.5 ppb, respectively.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Blender - Model No. 51BL31, Waring Inc.

b. Balance, analytical - Sensitive to 0.1 mg, Model No. A120S, Sartorius.

c. Balance, top loading - Sensitive to 0.01 g, Model No. E83200D, Shimadzu.

d. Centrifuge - Capable of 700 x g and holding 50 mL glass centrifuge tubes, Model No. Centra-7R, International Equipment Company.

e. Evaporator - Nitrogen evaporator, N-Evap®, Model No. 111, Organomation.

f. Glass centrifuge tube - Pyrex® round-bottom screw-cap, 29 mm, 50 mL centrifuge tube, Cat. No. 21023-401, VWR.

g. Polypropylene centrifuge tubes - 50 mL conical, with closures, Cat. No. 352070, Blue Max.

h. Glass disposable Pasteur pipettes - 9-inch, Cat. No. 14672-380, VWR.

i. Glass volumetric flask - Class A, 10, 50, 100, and 1000 mL, Kimax.

j. Glass graduated cylinder - 500 mL.

k. Heating Block - Pierce Reach-Therm™ III (Cat. No. 18935 H, Pierce) 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 (Cat. No. 18810 H, Pierce).

l. pH meter - Capable of 0.01 pH unit sensitivity, Model No. 340 pH meter, Corning.

m. Pipettes - Automatic pipettors capable of accurately delivering 300 µL to 5 mL volume.

n. Repipetters - Bottle top dispensers of appropriate volume.

o. Spatula - stainless steel or plastic.

p. Test tube racks - for 15 and 50 mL tubes.

q. Solid Phase Extraction Vacuum Manifold - Cat. No. 57030-U, Supelco.

r. Trap for SPE Vacuum Manifold - Side arm flask with tubing, Cat. No. 57120-U, Supelco.

s. SCX Cartridges - Benzenesulfonic acid cation exchange (SCX), 500-mg/3 mL, Cat. No. 2323, Applied Separations, Allentown, PA.

t. Reservoirs - Empty polypropylene SPE tubes (no frits) 60 mL, Cat. No. 57022, Supelco.

u. Cartridge Adapters - For 3 mL tubes, Cat. No. 57020-U, Supelco.

v. Disposable glass culture tubes - 15 mL, 16 x 100 mm, Cat. No. 73500 16100, Kimble.

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

x. Mobile Phase Filtration Apparatus - Cat. No. 58062-U, Supelco.

y. Membrane Filter Disks - 0.45 µm pore size, 47 mm, nylon, HPLC mobile phase membrane filter, Cat. No. HNWP 047 00, Fisher.

z. Whatman Mini-Uniprep syringeless filters - Standard, PTFE, 0.45 µm, Cat. No. 09-923-28, Fisher.

2. Instrumentation LC/ESI-MS/MS System

a. Pumps - Two Varian Prostar model 210 pumps, Master and Slave.

b. Autosampler with column heating compartment - Varian Prostar model 410 autosampler.

c. Source - Varian Electrospray ionization.

d. Detector - Model 1200L quadrupole MS/MS, Varian.

e. Analytical Column - Eclipse XDB-C18 2.1 x 150 mm, 5 µm, Cat. No. 993700-902, Agilent, or Acquity UPLC BEH C18 2.1 x 50 mm, 1.7 µm, Cat No. 186002350, Waters

f. Guard Column - Brownlee cartridge column, RPI8, 7 µm, 3.2 x 15 mm, Cat. No. 0711-0092 and NewGuard holder (complete), Cat. No. 0715-0001, Chrom TechItem 1 – name, model, Cat. No. (if available) & Company

C. REAGENTS AND SOLUTIONS

Note: Equivalent standards / solutions may be substituted.

1. Reagents

a. Ethyl acetate (EtOAc) - HPLC grade, Cat. No. 230-4, Burdick & Jackson.

b. Methanol (MeOH) - HPLC grade, Cat. No. 100-4, Burdick & Jackson.

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

d. Hydrochloric acid (HCl) - Concentrated, Cat. No. 9535-05, J.T. Baker.

e. Phosphoric acid (H_3PO_4) - 85% in water, Cat. No. 438081-500ML, Sigma.

f. Formic acid (CH_2O_2) - 98 - 100% purity, Cat. No. 27001-500ML-R, Sigma.

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

h. Water – Millipore water (deionized distilled).

2. Solutions

a. 6N Hydrochloric Acid (HCl):

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

b. 20% Sodium Hydroxide (NaOH):

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

c. Methanol / Water (50/50, v/v):

Mix 500 mL of methanol with 500 mL of water. This solution is stable for six months at room temperature.

d. 0.1% Phosphoric Acid / Methanol:

Pipet 1 mL of phosphoric acid into a 1 L volumetric flask. Fill to volume with methanol. This solution is stable for one year at room temperature.

e. Ammonium Hydroxide / Methanol (10/90 v/v):

Mix 20 mL of 28-30% ammonium hydroxide solution with 180 mL of methanol. This solution is stable for 6 months at room temperature.

f. 1% Formic Acid / Methanol:

Pipet 1 mL of formic acid into a 100 mL volumetric flask. Fill to volume with methanol. This solution is stable for 6 months at room temperature.

g. HPLC Mobile Phase:

i. Aqueous Mobile Phase (0.4% formic acid in water):

Into a 1 L volumetric flask, fill halfway with Millipore water. Pipet 4 mL of formic acid. Fill to volume with Millipore water. Mix and filter through a 0.45 µm nylon filter. This solution is stable for 1 month at room temperature.

ii. Organic Mobile Phase (0.18% formic acid in 4:5 acetonitrile:methanol):

Into a 1 L bottle add 400 mL acetonitrile and 500 mL methanol. Pipet 1.6 mL of formic acid. Mix and filter through a 0.45 µm nylon filter. This solution is stable for 1 month at room temperature.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. In-house prepared standards shall be assigned an expiration date that is no later than the stability stated in the method.

Note: The analyst may prepare different standard volumes and/or concentrations to cover the range of interest.

1. Standard Information

Common Name:	Flunixin-N-methyl glucamine salt (NMG), or flunixin meglumine
Chemical Name:	2-[{2-Methyl-3-(trifluoromethyl)-phenyl} -amino]-3-pyridinecarboxylic acid (as NMG salt), $C_{21}H_{28}F_3N_3O_7$.
Molecular Weight:	491 (Free Acid MW is 296).
Source:	Cat. No. SCH 14714, Schering-Plough, Union, NJ and Cat. No. 27460-7, U.S. Pharmacopoeia, Rockville, MD.and Cat No. 42461-84-7, Sigma Aldrich, Saint Louis,MO.
Storage:	Desiccated at room temperature.

2. Preparation of Standard Solution(s)

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

Weigh 8.3 mg flunixin-NMG analytical standard equivalent to 5.0 mg of flunixin free acid into a 10 mL volumetric flask, dissolve the material, and dilute to the mark with methanol. This stock standard is stable for 6 months at less than -10 ºC.

b. Intermediate Stock Solution (5 µg/mL as free acid):

Pipet 500 µL of the stock solution into a 50 mL volumetric flask and dilute to the mark with methanol. This stock standard is stable for 6 months if stored at less than -10 ºC.

c. Fortification Solution (250 ng/mL as free acid):

Pipet 5 mL of the intermediate stock solution (5 µg/mL) into a 100 mL volumetric flask. Dilute to the mark with 50% methanol / water. Fortification solution stored in the freezer at less than -10 ºC is stable for six months.

d. LC/MS Matrix-Matched Standard Curve Solutions for liver:

Weigh 2 g of blank liver and carry through hydrolysis and extraction steps F.2.a - h. Clean up four 10 mL aliquots of the resulting extract using steps F.2.j -k. Add Fortification standard solution (D.2.c) to tubes and reconstitute with 50:50 methanol / water, as shown in the following Tables:

Bovine Liver

LC/MS STD Calibration Level	Vol. Fortification Solution, µL	Vol. 50:50 MeOH/water (µL)	Flunixin free acid (ng/mL)	ppb equivalent
0	0	2000	0	0
1	125	1875	15.6	62.5
2	250	1750	31.3	125
3	500	1500	62.5	250

Porcine Liver

LC/MS STD Calibration Level	Vol. Fortification Solution, µL	Vol. 50:50 MeOH/water (µL)	Flunixin free acid (ng/mL)	ppb equivalent
0	0	2000	0	0
1	30	1970	3.75	15.0
2	60	1940	7.50	30.0
3	120	1880	15.0	60.0

Vortex and filter according to steps F.2.m. – n. below.

e. LC/MS Standard Curve Solutions for muscle:

Precondition glass culture tubes before making standard curve. See section F.2.i for precondition step for glass culture tubes.

Dilute Fortification standard solution (D.2.c) in 4 tubes to 2 mL with 50:50 methanol/water, as shown in the following Table:

LC/MS STD Calibration Level	Vol. Fortification Solution, µL	Vol. 50:50 MeOH/water (µL)	Flunixin free acid (ng/mL)	ppb equivalent
0	0	2000	0	0
1	25	1975	3.1	12.5
2	50	1950	6.3	25
3	100	1900	12.5	50

E. SAMPLE RECEIPT AND PREPARATION

Cut tissue into chunks and thoroughly homogenize using a Waring blender. Store tissue at < -10 °C when not processing.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

   a. Weigh 2.00 ± 0.10 g of samples and blank tissue homogenate (liver or muscle) into a 50 mL round-bottom screw top centrifuge tube. Record the weights to three significant figures.

      i. For a 125 ppb bovine liver recovery, fortify with 1000 µL of fortification solution (D.2.c.). Label the tube and cap to prevent possible cross contamination.

      ii. For a 30 ppb porcine liver recovery, fortify with 240 µL of fortification solution (D.2.c.). Label the tube and cap to prevent possible cross contamination.

      iii. For a 25 ppb bovine or porcine muscle recovery, fortify with 200 µL of fortification solution (D.2.c). Label the tube and cap to prevent possible cross contamination.

2. Extraction Procedure

   a. In a well-ventilated hood, add 8 mL of 6N HCI to each tube including samples and controls. Using a spatula, scrape the tissue down into the liquid.

   b. 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 - 120 °C within 15 min. After at least 2 hours of heating at 95 - 120 °C, allow cooling to ambient temperature. Tubes can be immersed into a cold water bath to accelerate the cooling process.

STOPPING POINT – Samples may be stored at 2 - 8 ºC up to 3 days.

c. Adjust the pH of the HCI hydrolysate to pH 9.5 (9.30 - 9.70) by slowly adding approximately 6 mL of 20% NaOH and gently mixing. Check the pH of the solution with a pH meter and add additional 20% NaOH or 6N HCI if necessary. Rinse the pH meter probe with methanol and then with water between samples.

d. Add 10 mL of ethyl acetate to each tube, cap and vortex at high speed for 1 min. (Alternatively, shake the test tube rack along the long axis of the tubes for 5 minutes).

e. Centrifuge the tubes for 5 min at approximately 900 g.

f. Using a long-stem Pasteur pipette, transfer the upper ethyl acetate extract to another properly labeled, clean 50 mL polypropylene centrifuge tube. Care should be taken not to transfer any of the lower dark layers.

g. Repeat the 10 mL ethyl acetate solvent partition three more times and combine the ethyl acetate fractions (step g.) and adjust the volume to 40 mL with ethyl acetate using the graduations on the tube. Discard aqueous layer.

h. Cap the tube and mix thoroughly.

STOPPING POINT: Extracts are stable up to three days at 2 - 8 ºC.

i. Precondition 15 mL disposable glass culture tubes by vortexing the tubes containing 2 mL of a 1% formic acid in methanol for about 30 sec, pouring out the residual solution, and allowing the tubes to dry.

j. SCX Cartridge Cleanup

  i. Attach 60 mL reservoirs to SCX column cartridges on a vacuum manifold.

  ii. Condition the cartridges as follows:

    (a) Add 6 mL water and allow to drain to near the bed surface. Do not allow column to run dry.

    (b) Add 6 mL 0.1% phosphoric acid in methanol and allow to drain until 1 mL remains above the bed.

  iii. Transfer 10 mL of ethyl acetate extract from step (F.2.i) into the 60 mL reservoir and mix with an equal volume of 0.1% phosphoric acid in methanol and let the mixture flow through the SCX cartridge. Do not allow the cartridge to run dry.

  iv. Rinse the cartridge with 5 mL of ethyl acetate followed by 5 mL of methanol. Discard the eluate.

  v. Elute the residue with 12 mL of methanolic ammonium hydroxide solution (C.2.e) into a preconditioned 15 mL disposable glass culture tube (j) at about 1 drop per second. Ensure complete elution by observing only air passing through the column.

k. Evaporate the NH_4OH / MeOH eluate to dryness under a stream of nitrogen or air using an N-Evap® in a < 70 °C water bath.

l. Reconstitute the residue in 2 mL of 50% methanol / water to yield the final extract.

m. Vortex for approximately 10 seconds.

n. Filter a portion of the extract to an autosampler vial for LC/MS/MS analysis using 0.45 µm PTFE filters.

Note: Samples are stable up to five months if stored in the refrigerator (2-8 ºC).

3. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

a. HPLC Mobile Phase Gradient Table:

Run time (min)	Flow rate (mL/min)	(A) Aqueous (%)	(B) Organic (%)	Switching valve
0:00	0.40	55	45	Waste
9:00	0.40	55	45	MS
13:00	0.40	55	45	Waste
13:30	0.40	5	95	
13:50	0.55	5	95	
17:50	0.55	5	95	
18:00	0.55	55	45	
23:00	0.55	55	45	
23:10	0.40	55	45	

b. UPLC Mobile Phase Gradient Table

Run time (min)	Flow rate (mL/min)	(A) Aqueous (%)	(B) Organic (%)	Switching valve
0:00	0.65	55	45	Waste
0.42	0.65	65	35	MS
5:53	0,65	65	35	Waste
5:64	0.65	5	95	
6:36	0.55	5	95	
6:42	0.55	55	45	
8:00	0.55	55	45	

c. Mass Spectrometric Parameters:

Nebulizing gas pressure	50 psi
Needle voltage	5000 V
Shield voltage	600 V
Housing temperature	50 °C
Drying gas temperature	400 °C
Drying gas pressure	30 psi
Capillary voltage	44 V
Manifold temperature	42 °C
CID gas pressure	3 mTorr
Detector voltage	1300 V
Injection volume	20 µL
Column temperature	40 °C
Ion mode	Positive
Precursor ion m/z	297
Product ions m/z	279, 264, 259

Quantitate using the signal area from the precursor ion fragmenting to the most abundant product ion (m/z 279) using multiple reaction monitoring.

4. Sample Set

   a. Matrix-matched external standards for calibration curve (blank plus 3 levels: 62.5, 125 and 250 ppb) for bovine liver, (blank plus 3 levels: 15.0, 30.0, and 60.0 ppb) for porcine liver, and external standards (without extracted matrix) (blank plus 3 levels: 12.5, 25 and 50 ppb) for bovine or porcine muscle.

   b. One tissue blank.

   c. One recovery at 125 ppb for bovine liver, 30 ppb for porcine, or 25 ppb for bovine or porcine muscle.

   d. One intra-laboratory check sample(if needed).

   e. Up to 22 samples for muscle and up to 21 samples for liver.

G. DECISION CRITERIA / CALCULATIONS

1. Determinative Criteria

   a. Create a calibration curve using external standards (matrix-matched standards for liver; neat standards for muscle). The correlation coefficient must be ≥ 0.995.

2. Confirmation Criteria

   a. The tissue blank has no confirmable flunixin.

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

   c. All three ion products (m/z = 279, 264, 259) must be present.

   d. The ion ratios 259/279 and 264/279 must match those of the external standard within a relative difference of ± 20%.

   e. Analyte must have a signal to noise of at least 3:1 by visual inspection.

3. Calculations

   a. Using linear regression analysis, calculate the slope, intercept, and correlation coefficient of a standard curve constructed by plotting peak areas versus concentration (ng/mL) for all the injected standards. The correlation coefficient must be ≥ 0.995.

   b. The concentration of flunixin can be calculated using the following equation:

   ppb flunixin = $\frac{(A-B)\times D}{C\times E}$ where

   A = flunixin peak area.

B = intercept from the calibration curve.

C = slope of the calibration curve.

D = volume (mL) = (ethyl acetate extract volume / aliquot volume) x final volume =(40 mL / 10 mL) x 2 mL = 8 mL.

E = weight of the tissue sample.

c. Calculate results when the following conditions are met:

i. The correlation coefficient for the standard curve is ≥ 0.995.

ii. The recovery of the positive control falls within the limits specified in section I.1.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Personal Protective Equipment — Safety glasses, disposable gloves, lab coat.

2. Hazards

Consult all Safety Data Sheets (SDS) associated with the method.

3. Disposal Procedures

Follow federal, state and local regulations

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	Tissue	Analytical Range	Recovery
Flunixin	Bovine Liver Porcine Liver	62.5 – 250 ppb 15 – 60 ppb	60 – 110%
Flunixin	Bovine or Porcine Muscle	12.5 – 50 ppb	60 – 110%

2. Intralaboratory Check Samples

a. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

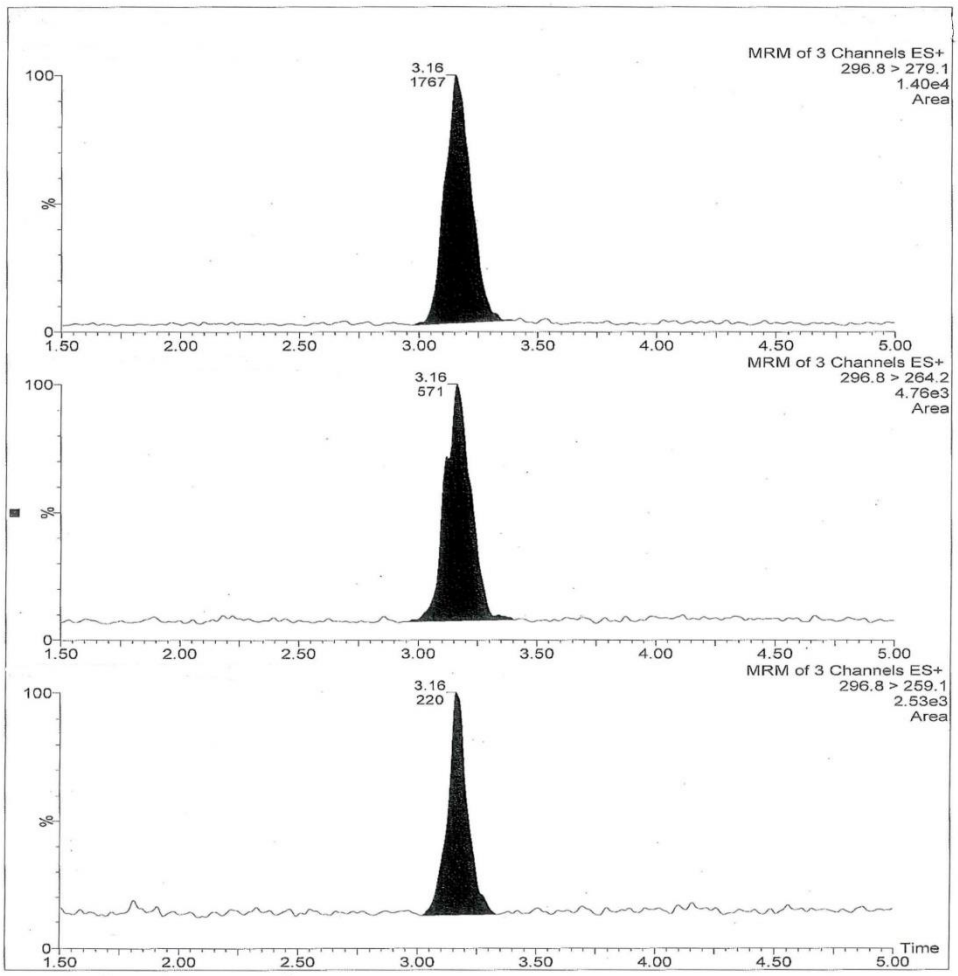
J. APPENDIX

1. References

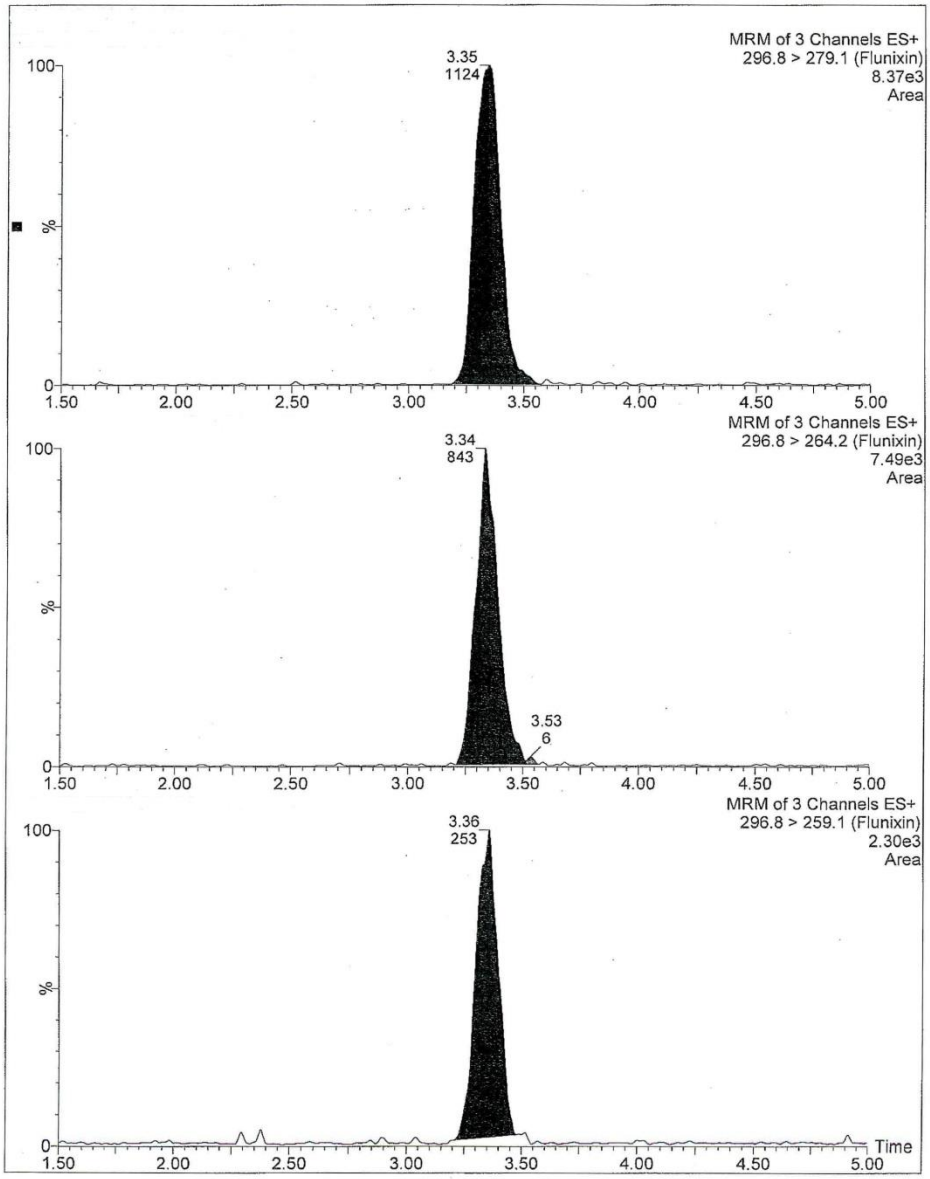
Boner, Pamela L. et al, J. Agric. Food Chem. 2003, 51, 7555-7559.

2. Chromatograms/spectra

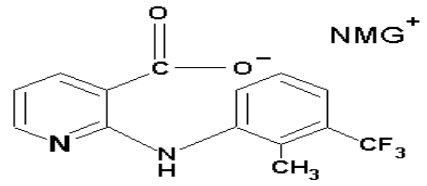
a. porcine liver at 15.0 ppb



b. Porcine muscle at 12.5 ppb

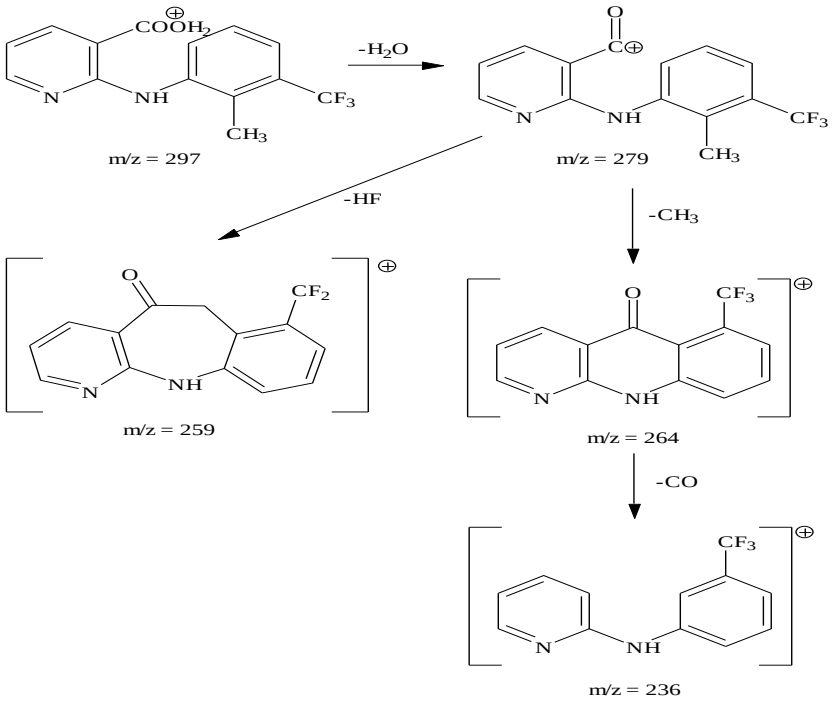


3. Structure



NMG: N-Methylglucamine

4. Fragmentation patterns



K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Staff.