SOP No: CLG-NIMZ2.00		Page 1 of 12
Title: Confirmation of Nitroimidazoles by ESI – LC/MS/MS		
Revision .00	Replaces: NA	Effective: 02/07/2005

Contents

A. INTRODUCTION

1. Theory

This method utilizes ESI-LC/MS/MS operating in MRM mode to detect nitroimidazole metabolites in extracts from the FSIS screening method CLG-NIMZ1. Hydroxy-dimetridazole (DMZOH) and hydroxy-ipronidazole (IPROH) in the LC effluent are subjected to electrospray ionization to produce protonated molecular ions of m/z 158 and 186, respectively, which are isolated and subjected to secondary ionization and monitored for selected daughter ions. Confirmation of analyte identity in a test sample extract is based on comparison of its retention time and daughter ion relative abundances against those recorded for a reference standard.

See section K.1. for postulated daughter ion fragmentation pathways.

2. Scope

This method is applicable to swine and poultry muscle extracts from CLG-NIMZ1. It has been shown to reliably confirm DMZOH and IPROH in extracts containing ≥ 20 ng analyte.

B. EQUIPMENT

Note: An equivalent may be substituted for any equipment listed below.

1. Apparatus

a. Analytical column - Phenomenex RP-18, 150 x 4.6mm column, 3µ particle size

b. Guard column - Phenomenex SecurityGuard cartridge C18, 4mm x 3.0mm ID.

2. Instrumentation

a. HPLC - Waters Alliance 2695 Model HPLC.

b. MS/MS - Micromass QuattroMicro tandem mass spectrometer.

c. ESI Probe - Waters ESI Probe Assembly, Part Number M955015DC6.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents may be substituted if necessary.

1. Reagents

a. Water - 18 megaohm Millipore grade, filtered before use.

b. Acetonitrile - LC grade, Burdick & Jackson.

c. Formic acid - Fluka Chemika.

2. Solutions

   a. 0.1% Formic acid in water:

      Add 1 mL of formic acid to water. QS with water to 1 liter and filter through a 0.45 µm filter before use.

   b. 0.1% Formic acid in acetonitrile:

      Add 1 mL of formic acid to acetonitrile. Adjust volume to 1 L with acetonitrile.

D. STANDARDS

Refer to method NIMZ1, Section D for source, preparation instructions, and stability of standards.

Standards required specifically for confirmation include:

Fortification Solution A, 5 µg/mL DMZOH, IPROH (D.2.c).

Fortification Solution B, 10 µg/mL DMZOH, 20 µg/mL IPROH (D.2.d).

External Standard Solution, 0.05 µg/mL DMZOH, IPROH (D.2.e).

More concentrated standard solutions may be prepared if necessary to confirm analytes detected at much higher levels than the screening method's target levels.

E. SAMPLE PREPARATION

Refer to method CLG-NIMZ1, section E, for sample preparation instructions.

F. ANALYTICAL PROCEDURE

1. Prepare necessary extracts for analysis. Refer to method CLG-NIMZ1, section F, for sample extraction, and cleanup procedures necessary to produce extracts for confirmatory analysis.

   For confirmatory analysis, sample set must contain a concurrently run tissue blank and recovery in addition to sample(s).

2. Set up and tune LC/MS system.

   Note: The following instrument conditions reflect optimal conditions for the specific instruments used to develop this method. It may be necessary to modify these parameters to optimize performance of any given instrument.

   a. HPLC conditions

Flow rate	0.4 mL/min
Flow ramp	2.00
Column Temperature	25 °C
Mobile Phase	40/60 0.1% formic acid in water/ 0.1%

	formic acid in acetonitrile
Run Time	10 minutes
Injection Volume	50 µL

b. MS Tuning Parameters

ESI polarity	positive
Capillary Voltage	3 kV
Cone Voltage	18 V
Extractor Voltage	2.00 V
RF Lens	0.1
Source Temperature	140 °C
Desolvation Temperature	450 °C
Desolvation Gas Flow	650 L/hr
Cone Gas Flow	150 L/hr
MS1 Low Mass Resolution	15.0
MS1 High Mass Resolution	15.0
Ion Energy	10.5
Entrance Lens	-5
Collision Gas Flow	18
Exit Lens	3
MS^2 Low Mass Resolution	14.5
MS^2 High Mass Resolution	14.5
Ion Energy	20
Multiplier Voltage	650 V

c. MRM Functions

DMZOH	Dwell (secs)
158.1 > 139.6	0.30
158.1 > 111.8	0.30
158.1 > 93.5	0.30

IPROH	Dwell (secs)
186.00 > 168.10	0.30
186.00 > 127.70	0.30
186.00 > 121.60	0.30

d. Tune MS analyzer using Fortification Solution A directly injected into ESI source.

3. Inject confirmation series in the following sequence:

   a. External standard

   b. Recovery

   c. Blank

   d. Samples

If the blank shows evidence of carryover contamination from the recovery, reinject until it is eliminated. If more than one sample is to be confirmed in this case, follow sample with blank solvent or tissue injections to verify absence of carryover before injecting the next sample.

4. Chromatograms

   See Section K.2

G. CONFIRMATION

Note: If possible, use the external standard as the reference for confirmation. If matrix interferences affect ion abundance ratios (ratios of sample and positive control match, but neither matches external standard), use of that control as the reference is acceptable.

1. Analyze MS data for DMZOH and IPROH in each injection:
   a. Generate ion chromatograms for all daughter ions.
   b. Determine average retention times and peak areas or peak heights for those ions.
   c. Calculate ion abundance ratios relative to the most abundant daughter ion in the reference.

2. Confirmation of an analyte's presence in a test sample requires:
   a. The retention time of the peak of interest is within 2% of that observed for the reference.
   b. At least two ion abundance ratios calculated for the test sample agree with those of the reference within ± 20% (relative).
   c. The signal to noise ratio of all ion chromatogram peaks used to calculate abundance ratios in the reference and the sample is at least 3:1.
   d. The blank control shows no traces of the analyte.

3. Absence of analyte in the test sample at levels ≥ the positive control may be assumed if:
   a. The test sample fails to meet requirements specified in G.2.a-c.
   b. The positive control can be confirmed.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, gloves, and lab coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Acetonitrile	Highly Flammable. Can form toxic cyanide vapors upon decomposition. Irritating to skin, eyes and mucus membranes.	Use under fume hood. Keep tightly closed and away from flame or heat. Avoid breathing vapor.
Formic Acid	Corrosive to skin	Avoid contact with skin.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic solvents	See above	Collect waste in tightly sealed container, segregating chlorinated from non-chlorinated solvents. Store in a cool, well ventilated, storage area for disposal in accordance with local, state and Federal regulations.
Formic Acid	See above	

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analytes	*Analytical Range*[1]	*False Positive Rate*	*False Negative Rate*
DMZOH	≥ 20 ng	0%	0%
IPROH	≥ 20 ng	0%	0%

[1] Amount of analyte present in final 1 mL extract from screening method.

2. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

      i. Phase I: Standards - Analyze at least one external standard on each of two different days.

      ii. Phase II: Analyst's self-fortified samples - Analyze duplicate recoveries (blank muscle tissues fortified at the screening method's minimum

proficiency level (MPL)) for each species/compound pair of interest, on at least two different days. Sets must include required method controls in addition to samples. Use a different tissue to prepare recoveries on each day of analysis.

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

iii. Phase III: Check samples for analyst accreditation.

(a) 6 unknowns, using swine or poultry muscle, or both. At least one, but no more than two of the unknowns must be negative, and the remainder fortified at or near the screening method's MPL for the tissue used.

(b) Report analytical findings to Quality Assurance Manager (QAM).

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

b. Acceptability criteria.

Refer to section I.1 above.

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One sample weekly per analyst as sample analyzed.

ii. Records are to be maintained for review.

b. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

4. Sample Acceptability and Stability

Refer to method CLG-NIMZ1, section I.5

5. Sample Set

a. Each sample set must include:

i. a tissue blank

ii. a recovery containing both analytes at the screening method's MPL or other level consistent with the expected analyte concentration in the sample.

iii. Sample(s) to be confirmed.

6. Sensitivity

Minimum proficiency level (MPL): 20 ng analyte in 1 mL sample extract.

J. WORKSHEET

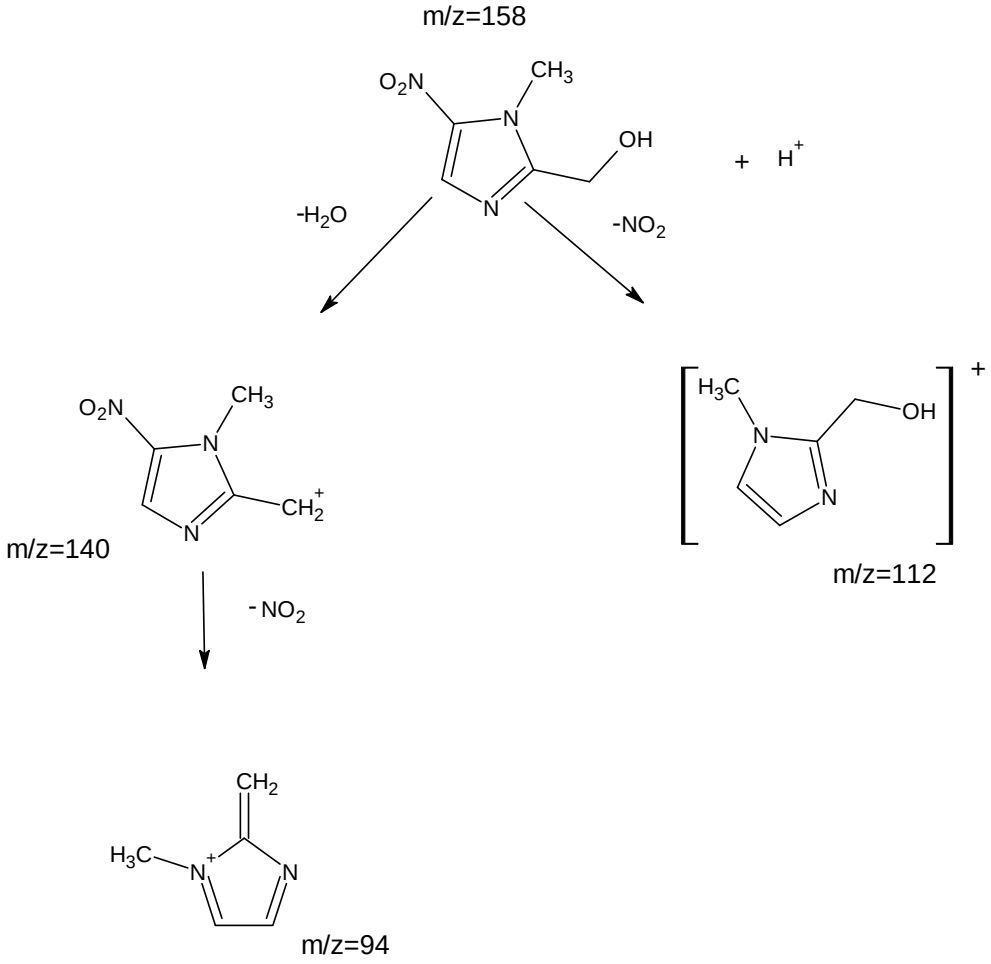
NITROIMIDAZOLE CONFIRMATION

Analyst				**Method Title**							
Analyst Code											
Date Began											
Date Ended											
Compound											
Standard ILN											
		Ion Abundances			**Ion Ratios**						
Sample	Ret Time										
Standard											
Recovery											
		Ion Abundances			**Ion Ratios**			**Ret Time Correlation**		**Ratio**	
Sample	Ret Time							Std Ret Time	Rec Ret Time	**Match**	
Range for Standard								Range for Recovery			
Ion	-20%	20%						Ion	-20%	20%	

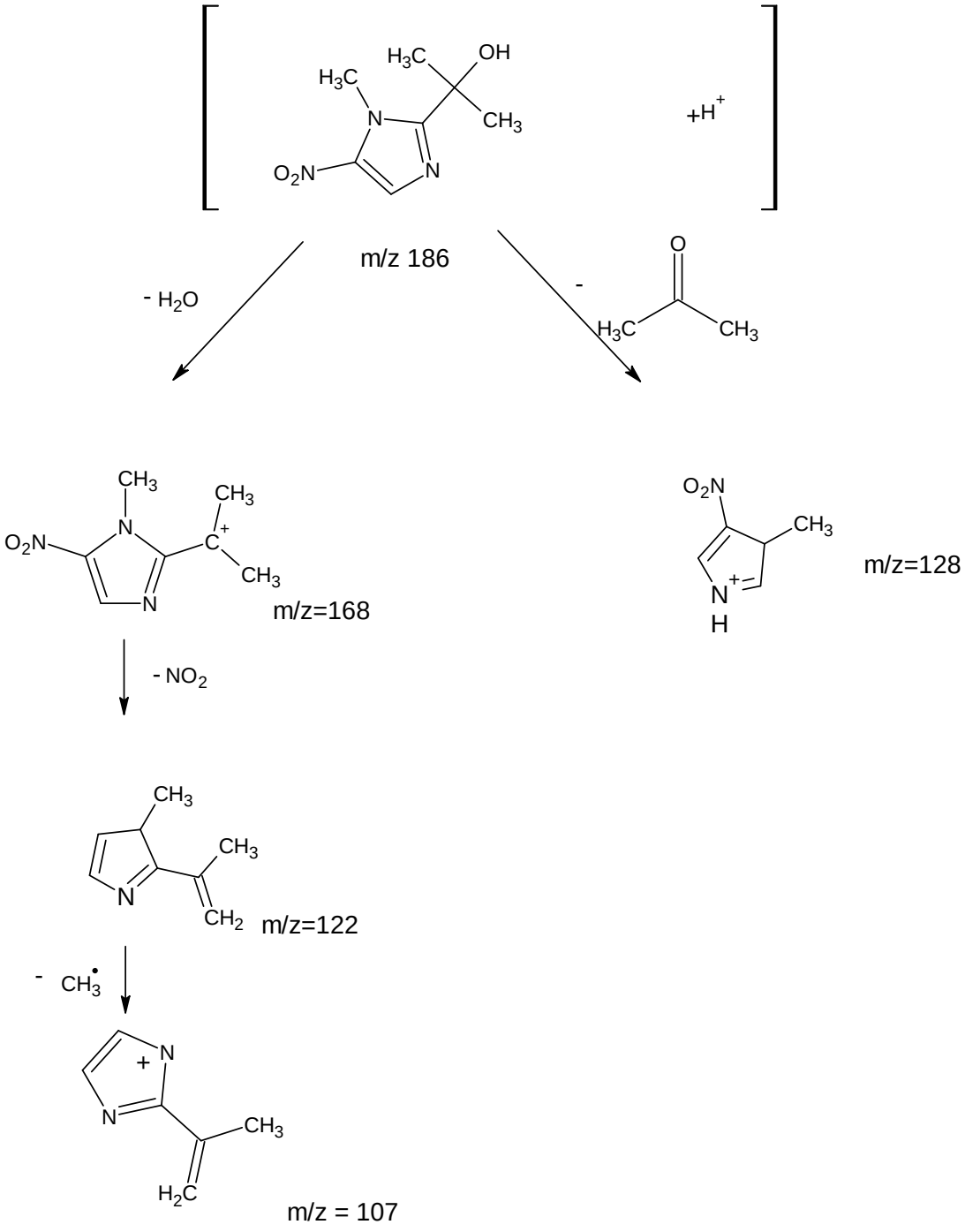
K. Appendix

1. Postulated Daughter Ion Fragmentation Pathways for hydroxy-dimetridazole and hydroxy-ipronidazole

Hydroxy-dimetridazole and fragments

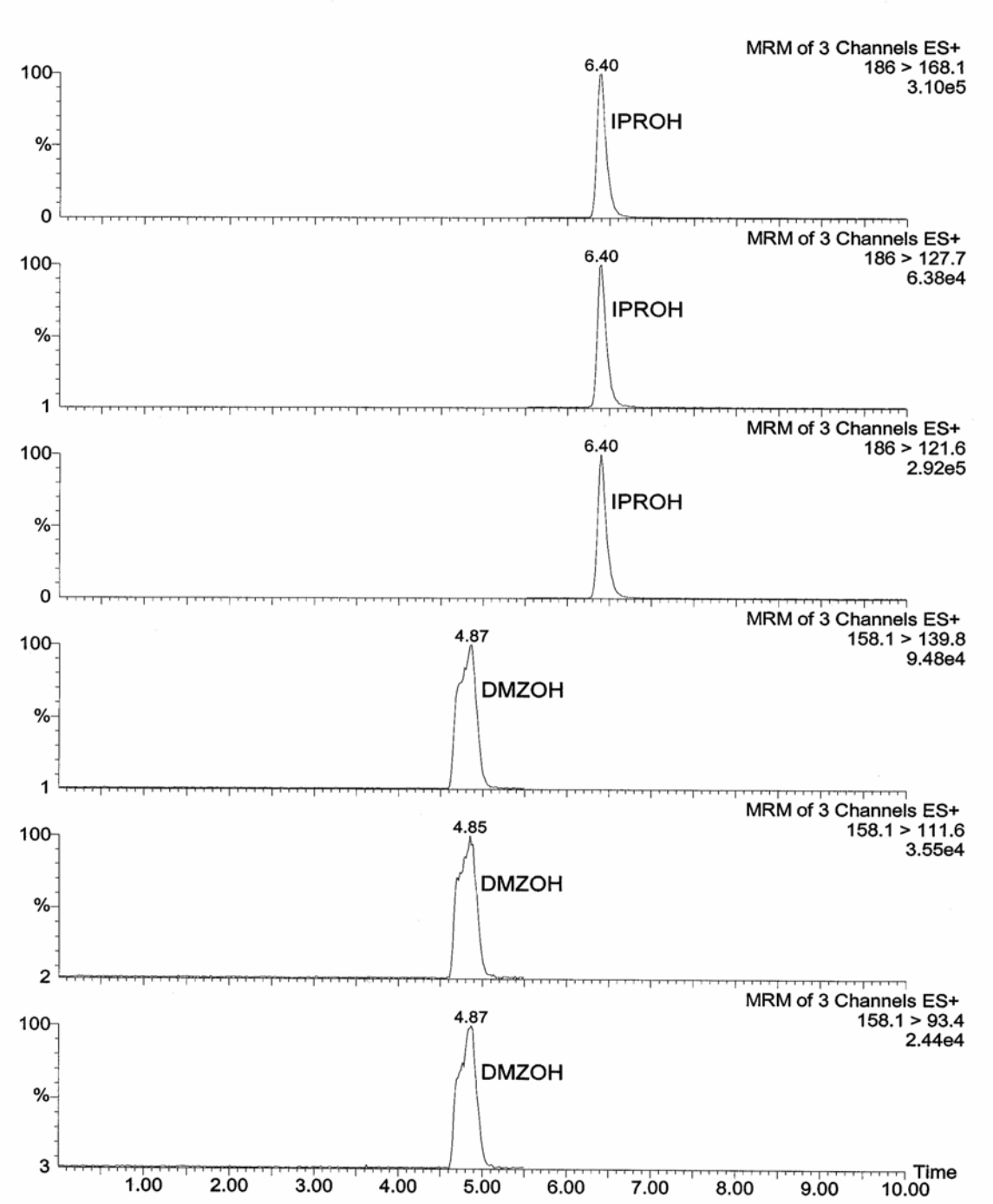


Hydroxy-ipronidazole and its fragments



2. Daughter Ion Chromatograms

2 ng DMZOH, IPROH injected (40 ng in 1 mL extract)



Approvals

Approved by	*Date Approved*
Eric Flynn	January 28, 2005
Gina McLeroy	January 31, 2005
Bill Koscinski	January 28, 2005
Jess Rajan	January 28, 2005
Charles Pixley	February 1, 2005
Phyllis Sparling	January 31, 2005

Approval records on file.