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

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Title: Screen for Nitroimidazoles by HPLC		
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Contents

A. INTRODUCTION

1. Theory

Dimetridazole and ipronidazole are anti-parasitic drugs that are rapidly converted to their hydroxy metabolites, hydroxydimetridazole (DMZOH) and hydroxyipronidazole (IPROH), in vivo. This procedure employs an extraction and cleanup that exploits their weakly basic character. A tissue sample is made basic and the metabolites are extracted into ethyl acetate. This extract is evaporated and the residue re-dissolved in 1N HCl. The HCl is partitioned with hexane/ethyl acetate to remove lipophilic interferences, then adjusted to pH 5. This solution is applied to a C-18 SPE column, and DMZOH and IPROH are eluted from the column with methanol. The eluate is evaporated and the residue redissolved in water and analyzed by HPLC using UV detection.

2. Applicability

This method detects DMZOH and IPROH in poultry and swine muscle at ≥1 ppb.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Balance - capable of weighing 50 g within 0.1 g, Cat. No. PB 1501S, Mettler.

b. Centrifuge tube - 250 mL polypropylene, Cat. No. 25350-250, Corning.

c. Centrifuge - Capable of holding 250 mL centrifuge tubes, model RC5C, Sorvall Instruments.

d. Round bottomed flask - 500 mL with 24/40 ground glass joint, Cat. No. 25285-500, Kimble.

e. Rotary evaporator - Labconco Inc.

f. Separatory funnel -125 mL, with stopper, Cat. No. 29049F-125, Kimble.

g. Scintillation vial - 20 mL, Cat. No. 74504, Kimble.

h. pH meter or pH indicator paper - (ColorpHast, Cat. No. 1.09590, EMD).

i. Pasteur pipet - Short, Cat. No. P5202-2, American Scientific Products.

j. C-18 SPE column - High capacity (1000 mg), Honeywell Burdick & Jackson.

k. Vacuum Manifold - Burdick & Jackson.

l. Glass culture tube - 13 x 100 mm, Cat. No. 73500, Kimble.

m. Graduated centrifuge tubes - 10 mL, Cat. No. 45164, Kimble.

n. N-Evap - Cat. No. 111, Organomation Inc.

o. Acrodisc LC-13 PVDF filter - 0.45 µm, Cat. No. 4450, Gelman.

p. Microfilterfuge filter - 0.2 µm nylon filter, Cat. # 7016-021, Rainin.

2. Instrumentation

a. Liquid Chromatograph consisting of an Agilent 1100 mobile phase pump, automated injector, column heater, and diode array detector.

b. Analytical Column - Agilent Hypersil ODS 5 µm 2.1 x 250 mm.

C. REAGENTS AND SOLUTIONS

1. Reagents

Note: Equivalent reagents may be substituted if necessary.

a. Ethyl acetate - Optima Grade, Burdick and Jackson.

b. Methanol - high purity, Burdick and Jackson.

c. Hexane - UV, high purity, Burdick and Jackson.

d. 18 megohm deionized (Milli-Q) water.

e. Chloroform - high purity, E & M Sciences.

f. Sodium chloride - Baker analyzed grade, J.T. Baker.

g. Potassium phosphate dibasic (K_2HPO_4) - Baker analyzed grade, J.T. Baker.

h. Ammonium phosphate monobasic ($NH_4H_2PO_4$) - Baker analyzed grade, J.T. Baker.

i. Concentrated hydrochloric acid - Baker analyzed grade, J. T. Baker.

j. Sodium hydroxide pellets - Baker analyzed grade, J. T. Baker.

k. Triethylamine - > 99% pure, Aldrich Chemical.

l. Acetonitrile - high purity, Burdick and Jackson.

2. Solutions

a. Hydrochloric Acid, 1N:

Dilute 83 mL of concentrated HCI to 1 L with Milli-Q water.

b. Sodium Hydroxide, 5N:

Dissolve 200 g of NaOH to 1 L in Milli-Q water.

c. Ammonium Phosphate Buffer, 0.05M:

Dissolve 5.75 g reagent grade $NH_4H_2PO_4$ in approx. 980 mL Milli-Q water.

Adjust pH to 7 with triethylamine and dilute to 1 L. Pass through a 0.4 µm filter before use.

d. HPLC mobile phase (12:88 acetonitrile:$NH_4H_2PO_4$ Buffer):

Combine 880 mL of 0.05M $NH_4H_2PO_4$ buffer with 120 mL of Acetonitrile and mix. Pass through a 0.4 µm filter before use. Note: This solution may also be prepared from component solutions using the HPLC gradient mixer.

D. STANDARDS

Note: Equivalent solutions may be prepared.

1. Source:

Each new lot of standard should be prepared as stated below and compared to current standards before use.

a. Hydroxydimetridazole (DMZOH) can be obtained from Australian Government Analytical Laboratories, National Analytical Reference Laboratory, Pure Substance Reference Materials Group.

b. Hydroxyipronidazole (IPROH) is available from RIKILT-Institute of Food Safety, Dept. Analysis & Development, The Netherlands.

2. Preparation of Standards

a. DMZOH stock solution, 0.5 mg/mL:

Weigh 25 mg DMZOH (to nearest 0.1 mg) into a 50 mL volumetric flask and dilute to volume with ethyl acetate.

b. IPROH stock solution, 0.5 mg/mL:

Weigh 25 mg IPROH (to nearest 0.1 mg) into a 50 mL volumetric flask and dilute to volume with ethyl acetate.

c. Fortification Solution, 5 µg/mL DMZOH and IPROH:

Pipet 1.00 mL of DMZOH and IPROH stock solutions into a 100 mL volumetric flask, and dilute to volume with ethyl acetate.

d. External Standard Solution, 0.05 µg/mL DMZOH and IPROH:

Pipet 10 µL of Fortification Solution into a test tube. Evaporate under nitrogen to dryness. Redissolve in 1 mL water. Prepare fresh each day of use.

3. Storage Conditions: All standards should be kept tightly closed and refrigerated when not in use.

4. Stability: Standards 2.a - 2.c are stable for at least one year. Standard (2.d) must be

freshly prepared each day.

E. SAMPLE PREPARATION

Pass tissue through a meat grinder (5/32" plate) and mix, or blend in a food processor until homogeneous. Store frozen until the sample is ready for extraction.

F. ANALYTICAL PROCEDURE

1. Extraction

   a. Add 10 g of K_2HPO_4, 10 g NaCl, and 50 ± 1 g of thawed tissue to a 250 mL plastic centrifuge tube.

      Note: Prepare a negative and positive control at this time. Weigh two blank tissues (tissue shown to contain no analyte or interfering chromatographic peaks). Fortify one at 1 ppb level by adding 10 µL of Fortification Solution.

   b. Add 100 mL ethyl acetate and shake by hand or mechanical shaker for 1 minute.

   c. Centrifuge at 1500 rpm for 5 minutes. Decant supernatant into a 500 mL round bottom flask being careful to not decant the salts.

   d. Repeat steps b and c, combining the extracts.

   e. Evaporate the ethyl acetate to an oily residue using a rotary evaporator with a water bath temperature of 50-55 °C.

   f. Add 2 mL of 1 M HCl and 2.5 mL of ethyl acetate to each flask and swirl contents.

   g. Transfer extract to a 125 mL separatory funnel containing 15 mL of hexane.

   h. Repeat step f and add the second HCl/ethyl acetate rinse to the hexane.

   i. Gently swirl or rotate separatory funnel for 1 minute, to partition analytes across interface, taking care to avoid forming large emulsions. Allow layers to separate. After this period, attempt to disrupt any emulsions that remain by carefully swirling the separatory funnel.

   j. Collect bottom aqueous layer in a scintillation vial or small beaker. Leave behind any emulsion.

      Note: If a sizeable emulsion layer remains at this point, transfer to a 50 mL centrifuge tube and centrifuge at 2500 rpm for 10 minutes. Add the bottom aqueous layer from the centrifuge tube to the previously collected aqueous solution.

   k. Carefully adjust to pH 4.8 - 5.2 by the dropwise addition of 5N NaOH or 1N HCl.

      Caution! Increased volumes of extract applied to the SPE column may result in decreased DMZOH recoveries. Analyst should take care not to overshoot the

pH endpoint.

2. Solid Phase Extraction

   a. Attach a Baker high capacity C18 SPE column to vacuum chamber.

   b. Using a mild vacuum to assist drainage, pass the following through the column:

      i. 10 mL methanol
      ii. 10 mL chloroform
      iii. 10 mL methanol
      iv. 20 mL distilled water

      Do not let the column fully drain at any time during this step.

   c. When water meniscus is within 1/8 inch of C18 bed add sample extract and allow to flow through column by gravity or gentle vacuum until fully drained. Apply vacuum for several seconds to remove residual water from the column.

   d. Elute analytes from column with 3 mL of methanol using mild vacuum. Collect eluate in a 13 X 100 mm glass culture tube using an N-Evap maintained at 50 ± 5 °C, and evaporate samples to dryness using a gentle stream of N_2.

   e. Add 1.0 mL water and mix thoroughly.

   f. Filter solution through 0.22 µm syringe filter into a glass HPLC vial for analysis.

3. HPLC Analysis

   a. Recommended Instrumental Settings and Conditions.

      Note: Actual settings may be adjusted, if necessary to optimize performance.

Mobile phase	See Section C.2.d
Flow rate	0.24 mL/minute
Column Temperature:	40 °C
Detector wavelength	312 nm
Sample Bandwidth	20 nm
Reference wavelength	440 nm
Reference Bandwidth	40 nm
Injection Volume	100 µL
Run time	20 minutes (or as needed to clear late eluting interferences).

b. Minimum Required Sensitivity:

The instrument must demonstrate a signal to noise ratio of >20 for both analytes when 100 µL of combined external standard solution is injected.

c. Injection sequence

i. 0.05 µg/mL external standard.

ii. Negative control (tissue blank).

iii. Positive control(s) run with sample set.

iv. Sample extracts.

v. At end of run, re-inject standard and/or fortified control to verify consistency of instrument performance.

Note: Additional standards and controls may be added to the sequence if conditions warrant.

d. Chromatograms. See Section K.1

G. CALCULATIONS

1. Construct appropriate baselines and measure retention times and peak heights for all chromatographic peaks of appropriate shape (peakwidth, skew) eluting in the vicinity of the DMZOH and IPROH peaks seen in the external standard.

2. A sample is screen positive for an analyte only if the following conditions are met:

a. The retention time of the sample peak matches that of the target compound in the nearest standard or fortified control within ± 3%.

b. The peak height of the analyte detected is at least 40% of that measured for the external standard.

3. A sample that fails to meet screen positive criteria may be reported as screen negative if

a. No interfering peaks large enough to obscure presence of an analyte peak are present.

b. The positive control meets screen positive criteria.

H. HAZARD ANALYSIS

1. Required Protective Equipment — Safety glasses, plastic gloves, and laboratory coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Ethyl Acetate, Hexane, Methanol, Chloroform, Acetonitrile	Highly flammable. Explosive hazard, vapors, mixed with air, will explode if ignited. Irritating to skin and mucus membranes. Inhalation of high concentration will cause narcosis, unconsciousness.	Keep tightly closed and away from fire. Avoid breathing vapor. Use under fume hood.
Hydrochloric Acid	Corrosive to skin.	Avoid contact with skin.
Sodium Hydroxide	Irritating to mucus membranes. Corrosive.	Avoid breathing vapors.
Rotary Evaporation	May result in implosion.	Check glassware for flaws. Always leave some air bleeding into the round-bottomed flask.
Solid Phase extraction	May result in implosion.	Do not exceed a vacuum of 10 inches Hg.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic Solvents	See Above.	Collect waste in a 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 Standard

Analyte	Species	Detection level	False negative rate
IPR-OH	Poultry, Swine	≥ 1 ppb	No false negatives in controls fortified at minimum detection level
DMZ-OH	Poultry, Swine	≥ 1 ppb	

2. Critical Control Points and Specifications

Record		*Acceptable Control*
a.	Losses of analyte during evaporation	Maintain a water bath temperature of 50-55 °C for the rotary evaporator.
b.	Back extraction equilibration time	Allow the two liquid phases to equilibrate at least 20 minutes.
c.	Neutralization pH must be 5.0 ± 0.2	Adjust the pH to 5.0 ± 0.2 using a calibrated pH meter or pH indicator paper.

3. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

      i. Phase I: Prepare and analyze external standard curves at the following concentrations:

         (a) 0.00 µg/mL (0 ppb equivalent)

         (b) 0.025 µg/mL (0.5 ppb equivalent)

         (c) 0.05 µg/mL (1 ppb equivalent)

         on each of two different days.

      ii. Phase II: Analyst's self-fortified samples - For poultry and swine muscle, analyze the following sample set on at least two different days, using a different blank muscle tissue each day of analysis:

         Sample set will consist of one blank tissue and 3 recoveries prepared by adding 10 µL of Fortification Solution to the same blank tissue.

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

      iii. Check samples for analyst accreditation.

(a) 6 samples blind to the analyst, fortified at the minimum proficiency level for the tissue(s) used. Tissues from either species, or both, may be used for this phase. At least one, but no more than two of the unknowns must be negative.

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

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

b. Acceptability criteria.

Refer to section I.1 above.

4. Controls and Intralaboratory Check Samples

a. Daily Controls

i. Frequency: Minimum one positive and one negative control, run with each sample set.

ii. Records are to be maintained for all findings

b. Check Samples

i. Frequency: Minimum one blind recovery (level unknown to analyst) per analyst per week when samples are being analyzed.

ii. Records are to be maintained for all findings.

c. Acceptability Criteria

Refer to Section I.1. If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Muscle

b. Sample size on receipt: ≥450 g

c. Condition on receipt: Frozen

d. Sample storage: Store samples frozen at all times.

6. Sample Set
   a. Negative control - Tissue blank
   b. Positive control - Recovery containing analytes at Minimum Proficiency Level for species used
   c. Samples

7. Method Sensitivity
   a. Minimum proficiency level (MPL):

      Poultry and swine: 1 ppb DMZOH, IPROH.

J. WORKSHEET

See next page for example worksheet.

NITROIMIDAZOLE ANALYSIS

Analyst: _____ HPLC Inst. ID: _____

Date Started: _____ External Std ID: _____

Date Completed: _____ Fortification Std ID: _____

No.	Sample ID	Sample Wt. (g)	Peak Height		Results	Comments
			DMZOH	IPROH		
1	External Std	NA				
2	Blank					
3	Recovery					
4						
5						
6						
7						
8						
9						
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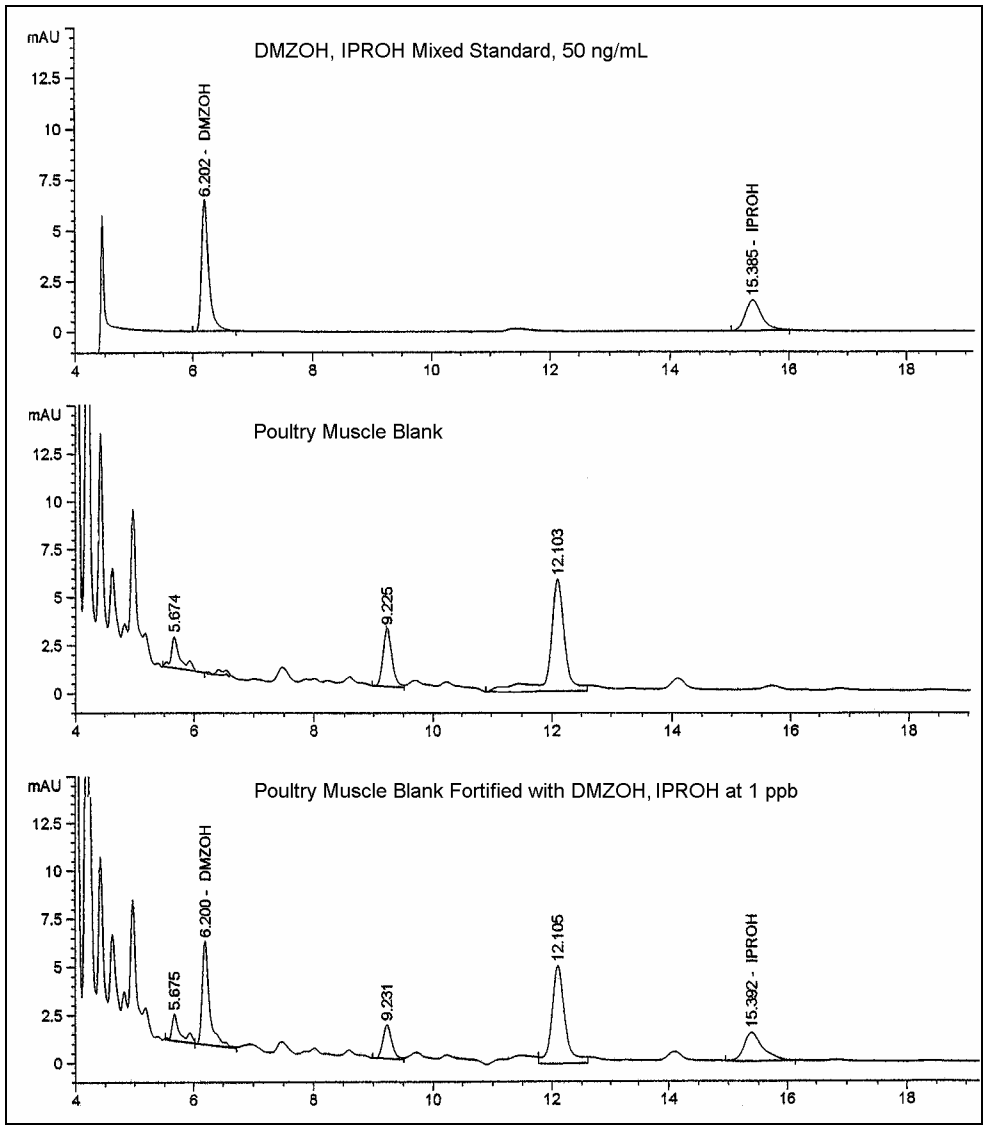
K. Appendix

1. Chromatograms



2. References

Determination of Nitroimidazole Metabolites in Swine and Turkey Muscle by Liquid Chromatography, Mallinson, E.T., Henry, A.C., & Rowe, L., Journal of AOAC International, Vol. 75, No. 5 (1992), pp790-96.

Approvals

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