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Title: Determination of Ivermectin, Doramectin, and Moxidectin by HPLC		
Revision: 04	Replaces: CLG-AVR.03	Effective: 03/28/2011

Contents

A. INTRODUCTION

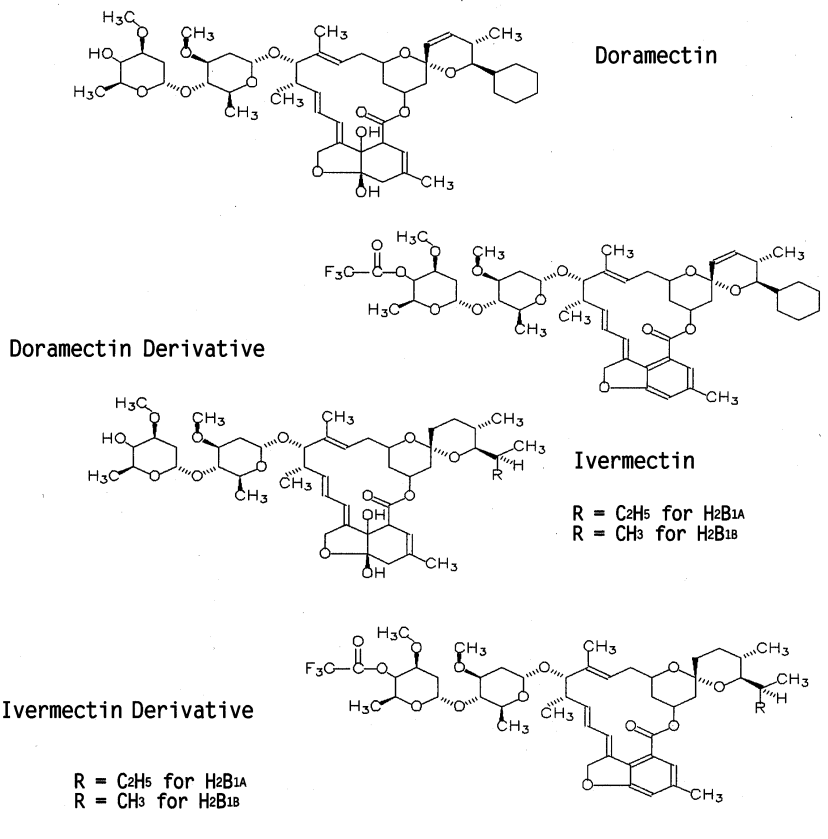
1. Theory

Moxidectin, ivermectin and doramectin are potent anthelmintics used in food animals to control parasitic infections. Moxidectin, ivermectin and doramectin are extracted from tissue with acetonitrile; extraneous substances are removed using alumina chromatographic cleanup. The analytes are determined by HPLC after formation of fluorescent derivatives with trifluoroacetic anhydride/1-methylimidazole.

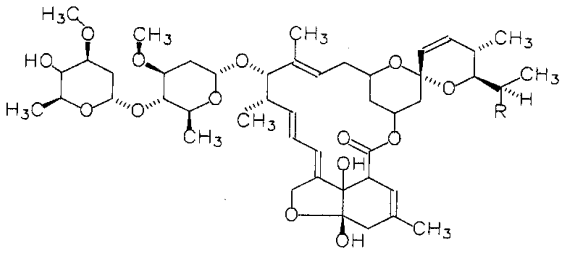
2. Applicability

The method is applicable to determination of Moxidectin, Ivermectin, and Doramectin in liver and muscle of bovine, ovine, porcine, caprine and equine species as well as their processed products at ≥ 7.5 ppb.

3. Structures

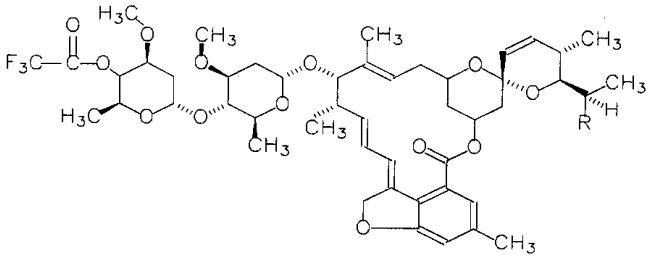


3. Structures (cont.)



Abamectin

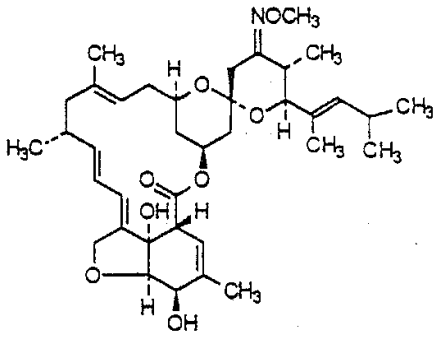
R = C_2H_5 for H_2B_{1A}
R = CH_3 for H_2B_{1B}



Abamectin Derivative

R = C_2H_5 for H_2B_{1A}
R = CH_3 for H_2B_{1B}

3. Structures (cont.)



Moxidectin

B. EQUIPMENT

Note: Equivalent apparatus and/or instrumentation may be substituted

1. Apparatus
   a. N-EVAP - Model 112, Organomation Assoc. Inc.
   b. Centrifuge - Sorvall model T-6000B, DuPont Co.
   c. Mechanical shaker - Eberbach model 610 equipped with shaker box model 6040, Thomas Scientific.
   d. Vortex mixer - Fisher Scientific.
   e. Extraction columns - Fisher Scientific Prep Sep-R (empty), Cat. No. P449R, Fisher Scientific.
   f. 50 mL screw cap centrifuge tubes - Cat. No. 05-558-12B, Fisher Scientific.
   g. 50 mL polypropylene centrifuge tubes - Cat. No. 222-3937-G80, Evergreen Scientific International Inc.
   h. EDP Plus Micropipet - Rainin Instruments Inc.
   i. Eppendorf Pipettor - Cat. No. 4789, Brinkman Instrument Inc.
   j. Eppendorf Combitips - 5.0 mL and 12.5 mL, Brinkman Instrument Inc.

      SPE Cartridges *(*In-house prepared C18 cartridge*)* - Place a small silanized glass wool plug into the neck of a 5.75"- disposable transfer pipet. Add 0.1 ± 0.01 g C18 bulk packing material into the disposable pipet. Tap gently to settle.
   k. Glass test tubes - 16 x 100 mm, 20 x 150 mm, and 12 x 75 mm, Fisher Scientific.
2. Instrumentation
   a. Liquid Chromatography System
      i. Agilent 1100 Series Quaternary Pump, Agilent Technologies, Inc.
      ii. Agilent 1100 Series Autosampler, Agilent Technologies, Inc.
      iii. Agilent 1100 Series Vacuum Degasser, Agilent Technologies, Inc.
      iv. Agilent 1100 Series Fluorescence detector, Agilent Technologies, Inc.
      v. Agilent 1100 Series Column Compartment, Agilent Technologies, Inc.
      vi. Zorbax ODS 4.6 mm x 15 cm C18 analytical column, Agilent Technologies, Inc.
      vii. Brownlee Columns Spheri-5 RP-18, 30 mm x 4.6 mm guard column, 5 micron particle size, Perkin Elmer.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted if necessary.

1. Reagents

   a. Acetonitrile LC Grade

   b. Alumina - Neutral type WN-3, Activity grade 1, Sigma Chemical Co. Dry at 135 ± 5 °C for at least 24 hours prior to use.

      Prepare deactivated alumina for column chromatography. Alumina should be 12% deactivated For example: Add 24 g distilled/deionized water to 176 g alumina. Mix by shaking until there are no visible lumps. Store deactivated alumina at room temperature in a tightly closed container. Use within one week after opening.

      Prepare alumina columns by weighing 2.0 ± 0.2 grams of deactivated alumina into an empty Prep-Sep column.

   c. 1-Methylimidazole - redistilled (99+ %), Cat. No. 33,609-2, Aldrich Chemical Co.

   d. Trifluoroacetic anhydride (TFAA) - (99+ %), Cat. No. 10,623-2, Aldrich Chemical Co.

   e. Methanol - HPLC Grade.

   f. Water - HPLC grade.

2. Solutions

   a. 1-Methylimidazole - 1:1 v/v 1-methylimidazole/acetonitrile:

      Add 1 part acetonitrile to 1 part 1-methylimidazole.

      To forty samples, add 5 mL of acetonitrile to 5 ml of 1-methylimidazole and mix.

      Note: *During hot, humid months, storing the 1-Methylimidazole in a dessicacator inside a refrigerator may extend its operating shelf life.*

   b. TFAA - 1:1 v/v trifluoroacetic anhydride/acetonitrile:

      Add 1 part of acetonitrile to 1 part trifluoroacetic anhydride.

      For forty samples, add 5 mL of acetonitrile and 5 mL of trifluoroacetic anhydride and mix.

D. STANDARDS

1. Source

   Note: Alternative sources are permissible if similar quality standards are available.

   a. Ivermectin standard, Cat. No. L-640,471-076P004, Merck Manufacturing

Division, West Point, PA.

b. Abamectin standard, Cat. No. L-676,863-038A003, Merck Manufacturing Division, West Point, PA.

c. Doramectin standard, Cat. No. UK-67,994, Pfizer, Groton, CT.

d. Moxidectin standard, Cat. No. 301423, Fort Dodge Animal Health.

2. Preparation

a. Stock solution:

Follow manufacturer's instructions accompanying their standards to obtain a stock solution of approximately 125 ± 1 µg/mL in acetonitrile.

b. Moxidectin, ivermectin and doramectin ~~F~~fortification solution (0.50 µg/mL):

Add 1 mL each of moxidectin, ivermectin and doramectin stock solutions to a 250 mL volumetric flask and bring to volume with acetonitrile and mix.

c. Abamectin Internal standard (IS) fortification solution (0.50 µg/mL):

Add 1mL of the abamectin stock solution to a 250 mL volumetric flask and bring to volume with acetonitrile and mix.

3. Storage and Stability (if not included with preparation)

a. Store stock solution in freezer < -10 ºC.

b. Fortification solutions may be stored at room temperature.

c. Stability:
Fortification solution: 90 days.
Stock solution: 1 year.

E. SAMPLE PREPARATION

Process samples of liver and muscle until homogeneous. All samples are stored refrigerated or frozen until analyzed.

Note: Remove excess fat from tissue before homogenization.

F. ANALYTICAL PROCEDURE

1. Sample extraction

a. Weigh 2.5 ± 0.2 g ground tissue into a 50 mL polypropylene centrifuge tube.

b. Add 8 mL acetonitrile.

c. Fortification:

i. Each sample, external standard curve, matrix blank, recovery sample and

any internal check sample should be fortified with 150 µL of abamectin (IS) solution (30 ppb).

ii. Each recovery sample should also be fortified with 75 µL of the moxidectin, ivermectin and doramectin fortification solution (15ppb).

iii. Each internal check sample should also be fortified with 0 µL or 37.5 -300 µL of the moxidectin, ivermectin and doramectin fortification solution.

iv. The external standard curve solutions should be fortified at 0.0 ppb, 7.5 ppb, 15 ppb, 30 ppb and 60 ppb with the moxidectin, doramectin and ivermectin fortification solution. Evaporate the external standard curve solutions under a gentle stream of nitrogen on the N-Evap, reconstitute in 2.5 mL of acetonitrile and proceed to step (m).

d. Vortex for about 30 secs and centrifuge for 3 min at 1500 RPM.

e. Pour acetonitrile eluent through a deactivated alumina column and collect eluate in a glass tube.

f. Repeat extraction with additional 8 mL acetonitrile, centrifuge and decant through alumina column combining eluates.

g. Transfer the glass tubes to an N-Evap and evaporate acetonitrile under a gentle stream of dry nitrogen or dry air while maintaining the water bath at 65 ± 5 °C.

h. Reconstitute the dried sample using 0.5 mL acetonitrile. Vortex to mix.

i. For muscle tissue - Add 2 mL acetonitrile and proceed to step (m).

j. For liver tissue –Pre-wet the SPE column or cartridge with 1.0 mL acetonitrile. Discard and wash.

k. Load the 0.5 mL sample from step (h) onto the wet SPE cartridge. **(The columns must not dry out at any time or the Avermectin recoveries will be low).** Collect the eluate in a test tube.

l. Add 2 mL of acetonitrile to the sample tube and mix. Add to the SPE column. Collect the eluate in the same container as the initial 0.5 mL eluate.

m. Add 200± 10 µL 1-methylimidazole/acetonitrile reagent to the eluate and vortex at least 10 sec.

n. Add 200 ± 10 µL TFAA/acetonitrile reagent and vortex at least 10 sec.

o. Allow the sample to derivatize in the dark for a minimum of 15 minutes before HPLC analysis.

Note: Derivatized samples decompose on exposure to strong light.

p. Inject on HPLC.

2. Instrumental Settings

Note: System may be adjusted to insure optimum response.

a. Mobile phase: 3:97 v/v water/methanol.

b. Flow rate: 1.8 mL /min.

c. Column temp.: 30 °C.

d. Injection vol.: 50 µL - As determined by detector/integrator conditions.

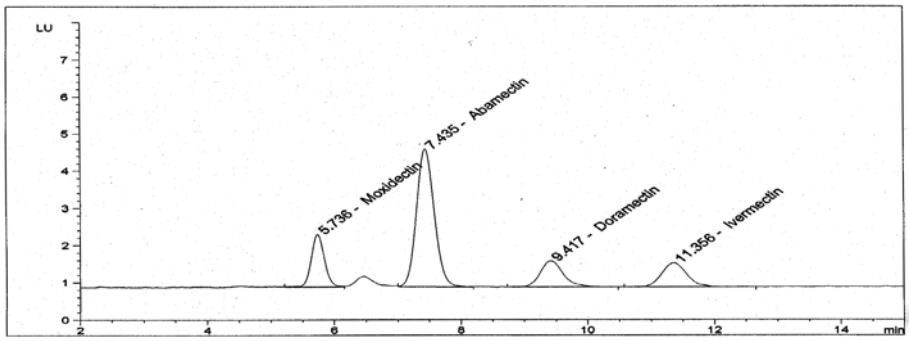
e. Run time: 15 min.

f. Detector settings:

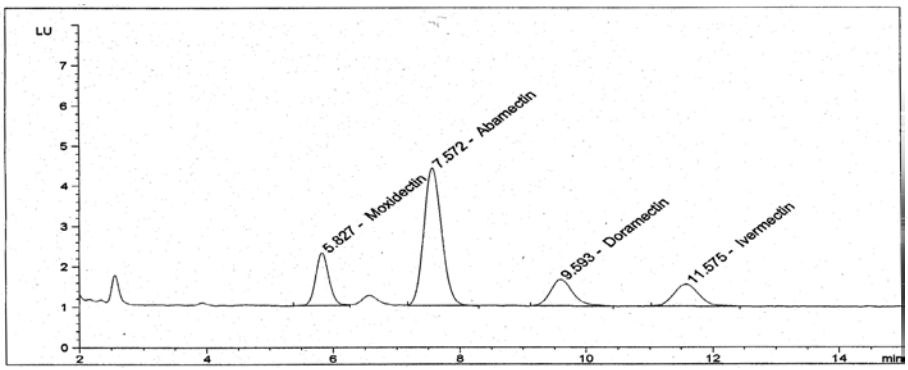
i. Excitation wavelength 365 ± 20 nm.

ii. Emission wavelength 465 ± 20 nm.

3. Sample Chromatograms



a. External Standard of Moxidectin (7.5 ppb), Abamectin (15 ppb), Doramectin (7.5 ppb), and Ivermectin (7.5 ppb).



b. Beef Liver fortified with Moxidectin (7.5 ppb), Abamectin (15 ppb), Doramectin (7.5 ppb), and Ivermectin (7.5 ppb).

G. CALCULATIONS

Quantitation is performed by measuring peak area. Each set is accompanied by an external standard curve at 0, 7.5,15, 30, and 60 ppb.

Measure peak area of abamectin, ivermectin, moxidectin, and doramectin peaks in the standards and calculate the peak area ratios.

$$\text{Peak Area Ratio} = \frac{\text{Moxidectin, Ivermectin or Doramectin Peak Area}}{\text{Abamectin Peak Area}}$$

Construct a linear regression line using the ratios and standard concentrations. The correlation coefficient should be >0.995.

The equation is $y = mx + b$, where

- $x =$ Ivermectin, Moxidectin, or Doramectin concentration (ppb).
- $y =$ Ivermectin, Moxidectin, or Doramectin/Abamectin peak area ratio.
- $m =$ slope
- $b =$ y-intercept

Incurred tissue ivermectin, moxidectin, or doramectin concentrations should be calculated using this regression line.

Note: Peak heights may be substituted for peak areas if chromatographic peaks display sufficient symmetry.

Note: *Should a sample prove to have a concentration that is greater than the highest standard used in the calibration curve, the sample should be re-analyzed. To keep the sample concentration bracketed by the calibration curve, the sample weight may be reduced to as little as 1.0 g or the calibration curve may be extended such that the highest calibration standard is greater than the calculated concentration of the sample.*

H. SAFETY INFORMATION AND PRECAUTIONS

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

2. Hazards

Reagent	*Hazard*	*Recommended Safe Procedures*
Acetonitrile, Trifluoroacetic Anhydride, 1-methylimidazole	Flammable and corrosive, may cause skin or respiratory irritation.	Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.
Ivermectin Abamectin	Weak teratogen and possible mutagen	Handle with extreme caution.
Doramectin	Severe explosion hazard if in powdered form.	Handle with extreme caution.
Moxidectin	May cause skin or respiratory irritation. The toxic effects of this material have not been fully evaluated.	Work in a well ventilated area. Store material in a secure, dry, cool well ventilated room.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic solvents and Avermectin solutions	See above	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 Standard

Analyte	*Analytical Range*	*Acceptable Recovery (%)*	*Acceptable Repeatability (CV (%))*
Ivermectin	≥ 7.5 ppb	60 -120	< 20
Doramectin	≥ 7.5 ppb	60 -120	< 20
Moxidectin	≥ 7.5 ppb	60 -120	< 20

Acceptability criteria:

a. Correlation coefficient ≥ 0.995.

b. Mean recovery for each species in range of 60 -120%.

c. Repeatability for each species < 20%.

d. No false positive or false negative results.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample weight	2.5 g ± 0.2 g.
b.	Alumina deactivation level	12%
c.	1-methylimidazole/acetonitrile volume	200 µL ± 10 µL
d.	TFAA/acetonitrile volume	200 µL ± 10 µL
e.	Condition of SPE column after sample loading (F.1.k.)	Do not allow SPE column to run dry
f.	Sample derivatization (F.1.o.)	In dark for a minimum of 15 minutes

3. Readiness To Perform

a. Familiarization

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

(a) 0 ppb

(b) 7.5 ppb

(c) 15 ppb

(d) 30 ppb

(e) 60 ppb

ii. Phase II: Fortified samples - 3 replicates at 0, 7.5, 15, 30 and 60 ppb of liver, muscle, and/or processed products of any applicable species (bovine, ovine, porcine, caprine and equine) over a period of 3 different days.

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

iii. Phase III: Check samples for analyst accreditation.

(a) 8 samples fortified between 0 and 60 ppb. Samples should include liver, muscle, and processed products. At least one but no more than two of the 8 should be blank.

(b) Samples submitted by the Quality Assurance Manager (QAM) or supervisor.

(c) Authorization from the QAM and Supervisor is required to commence official sample analysis.

b. Acceptability criteria.

Refer to I.1.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One check sample per week per analyst when samples are analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I.1.

If unacceptable values are obtained, then:

i. Stop all official sample analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrices: Liver, muscle, and processed product.

b. Species: Bovine, ovine, porcine, caprine, and equine.

c. Sample size: 16 oz. minimum

d. Condition on receipt: Frozen/semi-frozen/shelf stable

e. Sample storage:

i. Time: 6 months

ii. Condition: Frozen

6. Sample Set

a. External standard curve at 0, 7.5, 15, 30, and 60 ppb

b. Blank

c. Recovery

d. Samples

7. Sensitivity

Minimum Level of Applicability: 7.5 ppb.

J. WORKSHEET

[Reserved]

K. APPENDIX

1. References

a. De Montigny, Pierre, Jung-sook, K. Shim and Pivnichny, J. V., J. Pharm. and Biomedical Anal., Vol. 8 No. 6, pp. 507-511, (1990).

b. Doherty, Steven J., Fox, Allen and Fink, David W., J.A.O.A.C. Vol. 73, No. 6, pp. 931-934, (1990).

c. Prabhu, Sunil V., Wherner, Teresa A., Egan, Richard S. and Tway, Patricia C., J. Agric. Food Chem., Vol. 39, pp. 2226-2230, (1991).

L. APPROVALS AND AUTHORITIES

1. Approvals on file.

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