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Title: Determination of Tilmicosin by HPLC/Ion Pairing		
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A. INTRODUCTION

1. Summary of Procedure

Tilmicosin extraction takes advantage of its basic character. In the initial liquid-liquid extraction, Tilmicosin remains in a weakly acidic aqueous solution while matrix impurities are removed in a non-polar organic phase. The final partition is performed after making the remaining aqueous phase basic. After partitioning with an organic solution, Tilmicosin is collected in the organic phase. The collected liquid is evaporated to dryness and reconstituted. Chromatographic analysis is performed by gradient elution HPLC using dibutylammonium phosphate as an ion-pair reagent with UV detection at 280 nm. Quantities are computed versus external standards.

2. Applicability

This method is suitable for the quantification of Tilmicosin in beef liver and kidney at ≥0.6 ppm and muscle tissue at levels ≥ 0.3 ppm.

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. Balance capable of weighing 10 grams within 0.1 gram, PB 300, Mettler.
b. 50 mL conical polypropylene centrifuge tube, 2098, Falcon.
c. Homogenizer, Polytron, PT10/35, Kinematica GmbH.
d. 50 mL Repipettor, P4970-50, Scientific Products.
e. Centrifuge capable of 2000 rpm, HN-SII, Damon/IEC.
f. Stirring rod, S8205-4, Scientific Products
g. 60 mL Dispenser, 3300–60E, Corning.
h. 250 mL separatory funnels, F7860-250, Kimble.
i. 20 mL Dispenser, 3300 – 20E, Corning.
j. 300 mL glass beakers, 14030, Kimax.
k. pH meter capable of measuring to 0.01 pH units, 611, Orion Research.
l. Combination pH electrode, 81-02, Orion Research.

m. N-Evap, 111, Organomation.

n. Vortex Mixer, 1290, Labline.

o. 5 mL pipets, 37000-5, Kimble.

p. 10 mL pipets, 37000-10, Kimble.

q. 20 mL pipets, 37000-20, Kimble.

r. Pasteur pipets, 72050, Kimble.

s. Vacuum aspirator with trap.

t. Platform mechanical shaker, Eberbach Corp.

u. 3 mL syringes, 309586, Becton-Dickinson.

v. 0.22 µm LC 13 PVDF acrodisc filter, 4450, Gelman.

w. 1 mL autosampler vials, 62111, Kimble.

x. 11 mm vial caps, 5061-3370, Hewlett Packard.

y. 11 mm cap crimper, 666011, Alltech.

z. 3 mL vials, 200644, Sun Brokers.

aa. Solvent filtration apparatus, 2001, Alltech.

bb. Membrane filters, 0.2 micron, 2034, Alltech.

cc. Stirrer, Corning, S8285A, Scientific Products.

dd. Stirring bar, S8309A, Scientific Products.

ee. Vacuum oven, Precision, N8045, Scientific Products.

ff. 1 mL syringes, 309602, Becton-Dickinson.

gg. Pipetter, capable of delivering 10 to 100 µL, P-1000, Ranin - Gilson Pipetman

2. Instrumentation

a. Liquid chromatograph consisting of a Hewlett Packard 1100 equipped with a quaternary pump and, a uv/vis detector.

b. Analytical column – 1) Adsorbosphere phenyl, 5 µm particle size, 4.6 x 250 mm (287542, Alltech) or 2) Waters Spherisorb Phenyl, 5 µm particle size, 4.6 x 250 mm (Z226106, Sigma-Aldrich)

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration dates of the compounds used.

1. Reagents

   a. Methanol, 9093-03, J.T. Baker.

   b. Sodium Chloride, 3624-01, J.T. Baker.

   c. Hexane, UV grade, 216-4, Burdick and Jackson.

   d. Sodium Carbonate, 3604-01, J.T. Baker.

   e. Chloroform, C606-4, Fisher.

   f. Concentrated phosphoric acid, 0262-03, J.T. Baker.

   g. Dibutylamine, D4, 495-2, Aldrich Chemical Co.

   h. Acetonitrile, UV grade, 015-4, Burdick and Jackson.

   i. Concentrated hydrochloric acid, 9535-03, J.T. Baker.

   j. Deionized water.

2. Solutions

   Use deionized water unless otherwise noted.

   a. 1N hydrochloric acid:

      Add approximately 83 mL concentrated hydrochloric acid and dilute to 1 L with water.

   b. Sodium chloride solution, 10%:

      Add 100 g of sodium chloride to a graduated cylinder and dilute to 1 L with water.

   c. Chloroform:hexane solution, 2:1:

      Add 1 L of chloroform to 500 mL of hexane.

   d. Sodium carbonate solution, 0.5 M:

      Add 53 g of sodium carbonate to a graduated cylinder and dilute to 1 L with water.

   e. Dibutylammonium phosphate (DBAP) buffer solution, 1 M:

      Add 168 mL dibutylamine to 700 mL of water. Slowly add approximately 120 mL concentrated phosphoric acid to adjust the pH of this solution to 2.5-2.6. Dilute solution to 1 L with water. Filter under vacuum.

f. DBAP buffer solution, 0.05 M:

Add 25 mL of the 1M DBAP buffer to a 500 mL graduated cylinder. Dilute to 500 mL with water.

g. Mobile Phase A:

In a graduated cylinder, add 500 mL acetonitrile and dilute to 950 mL with water. Adjust the pH to 2.5 with phosphoric acid. Dilute to 1 L with water. Pass solution through a 0.2 µm filter as needed.

h. Mobile Phase B:

Adjust pH of 1 L of water to 2.5 with phosphoric acid. Pass solution through a 0.2 µm filter as needed.

i. Mobile Phase C:

Dilute 80 mL 1M DBAP solution to 1 L with water. Pass solution through 0.2 µm filter as needed.

j. Mobile Phase Premix:

Combine 550 mL of Mobile Phase A, 300 mL of Mobile Phase B, and 150 mL of Mobile Phase C.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependent on the expiration date of the components used. In-house prepared standards shall be assigned an expiration date that is no later than the expiration date of the earliest expiring component or no later than the stability stated in the method, whichever ends soonest.

1. Standard Information

a. Tilmicosin primary standard is available from Sigma-Aldrich, Product #33864

2. Preparation of Standard Solution(s)

a. Stock standard (120 µg/mL):

Accurately weigh 12.0 mg of Tilmicosin, compensating for purity, and transfer to a 100 mL volumetric flask. Dissolve and dilute to volume with methanol. Tilmicosin stock standard solutions are stable for three months when stored at 2-8 ºC in a light protected environment. Store in an amber glass container.

b. Working standards (24 µg/mL):

Dilute 20 mL of stock standard to 100 mL with methanol. Tilmicosin working standard solutions are stable for 1 month when stored at 2-8 C in a light protected environment. Store in an amber glass container.

3. Preparation of External Calibration Curve

a. HPLC external standards at 12.0, 6.0, 3.0, and 1.5 µg/mL are prepared for each set as follows and are stable for 2 weeks when stored at 2-8 °C :

i. 12.0 µg/mL:

Pipette 1.0 mL of the 24 µg/mL tilmicosin working standard solution and 1.0 mL of 0.05 M DBAP solution into a 50 mL Falcon tube. Vortex.

ii. 6.0 µg/mL:

Pipette 1.0 mL of the 12 µg/mL HPLC standard curve solution and 1.0 mL of the 1:1 MeOH: 0.05 M DBAP solution into a 50 mL Falcon tube. Vortex.

iii. 3.0 µg/mL :

Pipette 1.0 mL of the 6.0 µg/mL HPLC standard curve solution and 1.0 mL of the 1:1 MeOH: 0.05 M DBAP solution into a 50 mL Falcon tube. Vortex.

iv. 1.5 µg/mL:

Pipette 1.0 mL of the 3.0 µg/mL HPLC standard curve solution and 1.0 mL of the 1:1 MeOH: 0.05 M DBAP solution into a 50 mL Falcon tube. Vortex.

E. SAMPLE PREPARATION

Sample tissue should be cool and soft before processing. Cut tissue pieces from various locations, avoiding fat. Place liver tissue in a blender and muscle tissue in a Robot Coupe or grinder. Process tissue just long enough to produce a homogenous blend without warming the tissue. Place samples in a freezer after preparation.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

a. Accurately weigh 10 ± 0.1 g of sample into a 50 mL polypropylene centrifuge tube for a tissue blank, a recovery, and a check sample if needed. Fortify the recovery as follows:

i. 0.5 mL of the 24 µg/mL solution for a 1.2 ppm (1.2 µg tilmicosin / g tissue)

ii. 0.25 mL of the 24 µg/mL solution for a 0.6 ppm (0.6 µg tilmicosin / g tissue)

2. Extraction Procedure

a. Thaw samples for 15 to 20 minutes. Accurately weigh 10 ± 0.1 g of sample into a 50 mL polypropylene centrifuge tube.

b. Add 30 mL of methanol and shake for 5 minutes on a mechanical shaker.

c. Centrifuge samples for 10 minutes at 2000 rpm.

d. Decant supernatant into a 300 mL beaker.

e. Rinse the centrifuge tube with another 30 mL of methanol. Completely resuspend the sample with a stirring rod or spatula. Shake for 5 minutes. Centrifuge again for 10 minutes at 2000 rpm. Combine the resulting supernatant with that in the beaker.

f. Add 60 mL of the 10% solution of sodium chloride to the extract in the beaker.

g. Check pH of the extract solution. If necessary, adjust pH dropwise with 1 N HCl to below 6.0. Pour solution into a 250 mL separatory funnel.

h. Add 50 mL of hexane to the aqueous extract. Shake for 15 seconds, venting regularly. Allow phases to separate completely. Aspirate and discard the hexane layer.

i. Repeat partition with another 25 mL of hexane. Shake for 15 seconds, venting regularly. Allow phases to separate completely. Transfer the aqueous layer to a 300 mL beaker.

j. Adjust the pH of the aqueous extract to 9.0 ± 0.4 pH units with 0.5 M sodium carbonate solution. Addition of 0.9 mL of this solution will adjust this pH to around 8.6. If pH exceeds 9.4, adjust downward with 1 N HCl. Pour solution into a separatory funnel.

k. Add 20 mL of 2:1 chloroform:hexane to the aqueous extract. Shake for 15 seconds, venting regularly. Allow phases to separate completely. Collect the chloroform: hexane layer in a 50 mL polypropylene tube.

l. Add another 20 mL of 2:1 chloroform:hexane to the aqueous extract. Shake for 15 seconds, venting regularly. Allow phases to separate completely. Combine the chloroform:hexane layer with that in the polypropylene tube.

m. Using an N-Evap under nitrogen, concentrate the collected organic extract to dryness. Maximum water bath temperature is 40 ºC.

n. Dissolve the dry residue in 1 mL of methanol. Vortex tube for 15 seconds. Add 1 mL of 0.05 M dibutyl ammonium phosphate to the polypropylene tube. Vortex tube for 30 seconds.

o. Centrifuge extracts for 10 minutes at 2000 rpm.

p. Pass the samples through a 0.22 µm acrodisc filter. Extracts must be clear at this step. Refilter samples that appear cloudy after filtration. Samples stored at this stage are stable for 14 days if kept in a refrigerator and protected from light.

3. Instrumental Settings

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

a. Instrumental Settings – System may be adjusted to insure optimum response.

Column – Adsorbosphere Phenyl, 5 µm particle size (4.6 x 250 mm)

Pre-column – Adsorbosphere Phenyl, 5 µm particle size

Column Temperature - 47 ºC

Flow rate – 1.5 mL/min.

Detector – 280 nm

b. HPLC gradient elution parameters

Time (min.)	**% Mobile Phase**		
	A	**Premix**	
0	100	0	
3	100	0	
4	0	100	
15	0	100	
16	100	0	
21	100	0	

c. HPLC analysis

Inject a consistent volume (approximately 10 µL). If necessary, dilute the extracts so that the amount of analyte falls within the linear range of the external standard curve.

4. Injection sequence (if applicable)/ Example Sample Set,

a. External Standards

b. Recovery

c. Check Sample, if necessary

d. Blank

e. Samples and/or Dilutions

f. External Standard or Recovery

G. CALCULATIONS / IDENTIFICATION

1. Quantitative

a. Using linear regression, construct a standard curve by plotting Concentration in µg/mL (x), versus LC peak amount (y) for all external standards. Do not force the curve through the origin.

Acceptable correlation coefficient for standard curve: ≥0.9950.

b. Compute ppm Tilmicosin in each sample using the formula:

PPM Tilmicosin = $(y-b)\ (V_{SE})\ (DF) / (m)\ (W_S)$ Where

y = the observed amount for the injected sample.

m, b = the slope and intercept of the standard curve calculated in step a.

V_{SE} = Final volume of sample extract, in mL.

W_S = Weight of sample matrix in grams.

DF = Any dilution factor (Volume of diluted aliquot / Volume of aliquot) that might be applied to the sample extract.

H. SAFETY INFORMATION AND PRECAUTIONS

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

2. Hazards

Caution: Pregnant women should not carry out this analysis.

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Tilmicosin	Allergen. Eye irritant. May cause increased heart rate.	Wear protective gloves, lab coat, & eye protection.
Methanol Acetonitrile Hexane	Highly flammable. Explosive hazard. Vapors mixed with air will explode if ignited. Irritating to skin and mucous membranes. Inhalation of high concentrations will cause narcosis and unconsciousness.	Keep tightly closed and away from fire. Use under a fume hood. Avoid breathing vapors.
Chloroform	Exposure to this solvent affects the nervous system, heart, liver, kidney, and is an embryonic toxin. Possibly a carcinogen.	Wear protective gloves, lab coat, and eye protection.
Sodium Carbonate	Eye and mucous membrane irritant.	Wear protective gloves and eye protection.
Dibutylamine	Toxic, mutagenic, corrosive and combustible. Destructive to upper respiratory tract, eyes, and skin.	Wear protective gloves, lab coat, and eye protection. Always handle under a hood. **Do not breathe vapor*. Pregnant women should not work with this chemical.***
Liquid-liquid partition	Possible exposure to solvents.	Wear protective gloves, lab coat, and eye protection. Vent separatory funnels frequently while under agitation.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Compound	*Acceptable Recovery (%)*	*Repeatability (% CV)*	Minimum Level of Applicability (ppm)
Tilmicosin (in kidney)	70-110	≤15	0.6
Tilmicosin (in liver and muscle)	60-100	≤15	0.6 (liver) 0.3 (muscle)

Acceptable correlation coefficient for standard curve: $\geq$ 0.9950.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Protect standard solutions from light	Protect from light.

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week per analyst when samples analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Condition upon Receipt

   Cool or frozen

J. APPENDIX

1. References (if available)

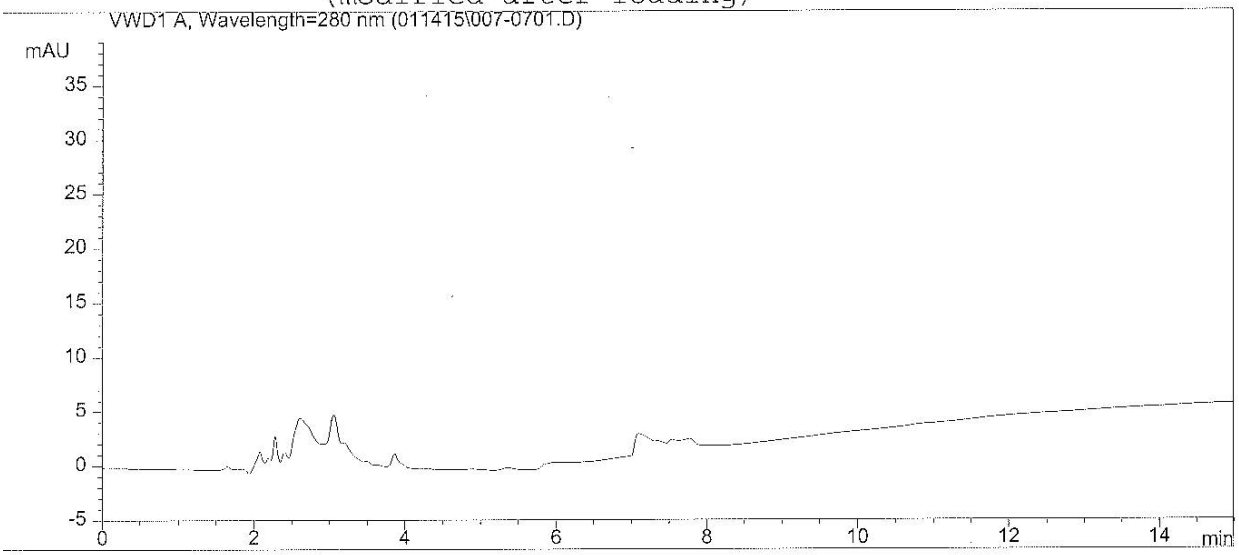
   Eli Lilly and Company, AM-AA-CR-R175-AA-791 Revision: 0

2. Chromatograms/spectra

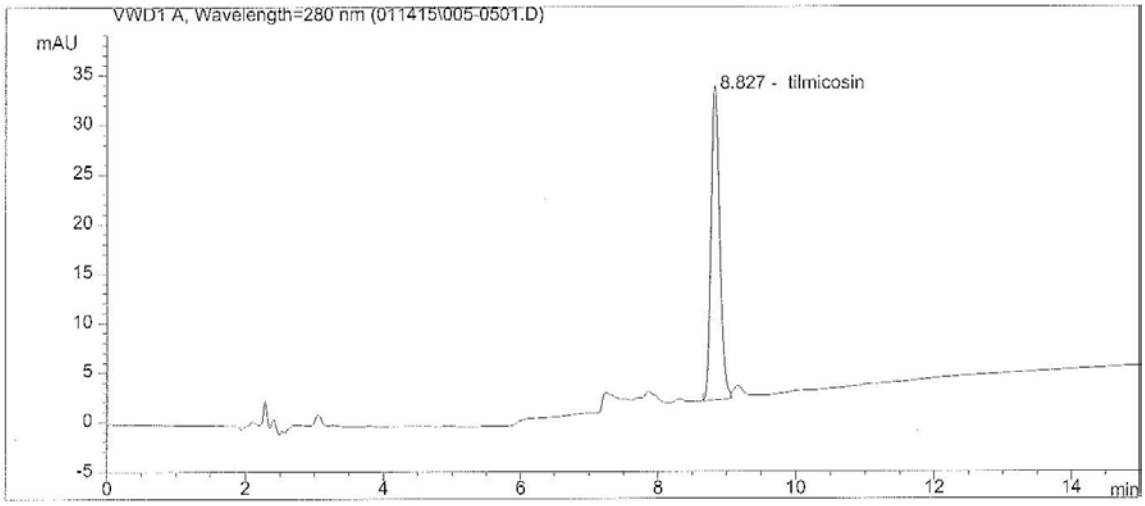
   a. Chromatograms

      i. Blank chromatogram

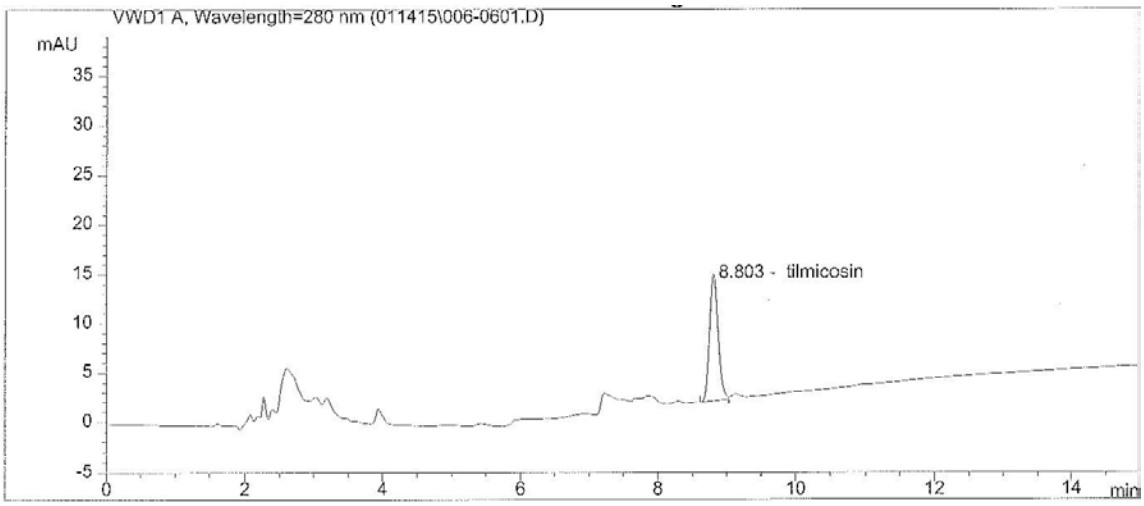
(modified after loading)



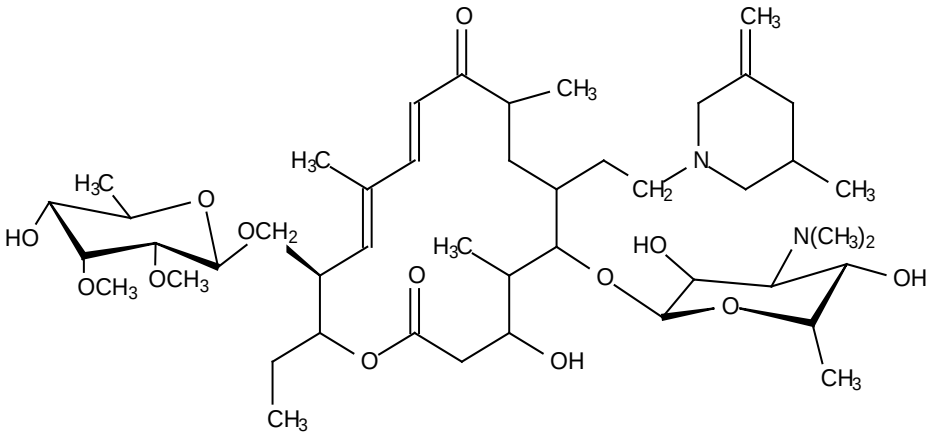
ii. External standard



iii. Recovery sample



b. Structure



K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Staff