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Title: Determination Of Tilmicosin By HPLC/Ion Pairing		
Revision .02	Replaces: .01	Effective: 8/25/05

Contents

A. INTRODUCTION

1. Theory

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 in the HPLC mobile phase. 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.

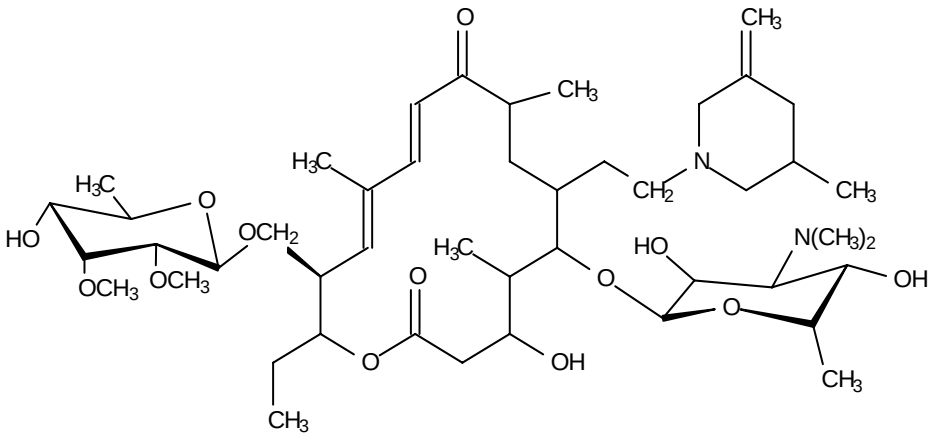
Reference: Eli Lilly and Company, AM-AA-CR-R175-AA-791 Revision: 0

Caution: Pregnant women should not carry out this analysis.

2. Applicability

This method may be used for quantitative determination of Tilmicosin in beef liver, kidney, and muscle tissue.

3. Structure



Tilmicosin

B. EQUIPMENT

1. Apparatus

   Equivalent apparatus may be substituted.

   a. Balance capable of weighing 10 grams within 0.1 gram (Mettler – Model Number PB 300)

   b. 50 mL conical polypropylene centrifuge tube (Falcon – Catalog Number 2098)

   c. Homogenizer, Polytron (Kinematica GmbH – Model Number PT10/35)

   d. 50 mL Repipettor (Scientific Products – Catalog Number P4970-50)

   e. Centrifuge capable of 2000 rpm (Damon/IEC – Model Number HN-SII)

   f. Stirring rod (Scientific Products – Catalog Number S8205-4)

   g. 60 mL Dispenser (Corning – Catalog Number 3300–60E)

   h. 250 mL separatory funnels (Kimble – Catalog Number F7860-250)

   i. 20 mL Dispenser (Corning – Catalog Number 3300 – 20E)

   j. 300 mL glass beakers (Kimax – Catalog Number 14030)

   k. pH meter capable of measuring to 0.01 pH units (Orion Research – Model Number 611)

   l. Combination pH electrode (Orion Research – Model Number 81-02)

   m. N-Evap (Organomation Model 111)

   n. Vortex Mixer (Labline Model 1290)

   o. 5 mL pipets (Kimble – Catalog Number 37000-5)

   p. 10 mL pipets (Kimble – Catalog Number 37000-10)

   q. 20 mL pipets (Kimble – Catalog Number 37000-20)

   r. Pasteur pipets (Kimble – Catalog Number 72050)

   s. Vacuum aspirator with trap

   t. Platform mechanical shaker (Eberbach Corp.)

   u. 3 mL syringes (Becton-Dickinson – Catalog Number 309586)

   v. 0.22 µm LC 13 PVDF acrodisc filter (Gelman – Product Number 4450)

   w. 1 mL autosampler vials (Kimble – Catalog Number 62111)

   x. 11 mm vial caps (Hewlett Packard – Part Number 5061-3370)

   y. 11 mm cap crimper (Alltech – Catalog Number 666011)

z. 3 mL vials (Sun Brokers – Catalog Number 200644)

aa. Solvent filtration apparatus (Alltech – Catalog Number 2001)

bb. Membrane filters, 0.2 micron (Alltech – Catalog Number 2034)

cc. Stirrer, Corning (Scientific Products – Catalog Number S8285A)

dd. Stirring bar (Scientific Products – Catalog Number S8309A)

ee. Vacuum oven, Precision (Scientific Products – Catalog Number N8045)

ff. 1 mL syringes (Becton-Dickinson – Catalog Number 309602)

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

2. Instrumentation

An equivalent instrument may be substituted.

a. Liquid chromatograph consisting of a Hewlett Packard 1050 equipped with a quaternary pump, a uv/vis detector and a Hewlett Packard model 3396 integrator. At least a tertiary pump is required to perform the gradient described in F.3. Other suitable chromatographic conditions are described in the reference document.

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

c. Precolumn – Adsorbosphere phenyl, 5 µm particle size (Alltech, #96045)

d. Precolumn cartridge holder (Alltech, #80101)

C. REAGENTS AND SOLUTIONS

1. Reagents

An equivalent may be substituted for any reagent listed below.

a. Methanol (J.T. Baker, #9093-03)

b. Sodium Chloride (J.T. Baker, #3624-01)

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

d. Sodium Carbonate (J.T. Baker, #3604-01)

e. Chloroform (Fisher, #C606-4)

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

g. Dibutylamine (Aldrich Chemical Co., #D4,495-2)

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

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

j. Distilled deionized water.

2. Solutions

Use distilled deionized water unless otherwise noted. Larger volumes may be prepared in the same proportions.

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 prior to use.

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 prior to use.

i. Mobile Phase C:

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

D. STANDARDS

1. Source

   a. Tilmicosin primary standard is available from Elanco Animal Health, a Division of Eli Lilly and Company, Greenfield, IN 46140-0708.

   b. The Tilmicosin primary standard should be dried according to the following procedure immediately prior to use: Weigh approximately 40 mg of the standard into an actinic glass bottle; remove the lid and cover the mouth of the bottle with a piece of filter paper, secured with a rubber band; and place the bottle in a vacuum oven for three hours at 60 ºC and a vacuum of 26 inches of mercury. After at least three hours, remove the primary standard from the oven and let cool to room temperature in a desiccator.

2. Preparation

   a. Stock standard (120 µg/mL):

      Accurately weigh 12.0 mg of dried Tilmicosin, compensating for purity, and transfer to a 100 mL volumetric flask. Dissolve and dilute to volume with methanol.

   b. Working standards (24 µg/mL):

      Dilute 20 mL of stock standard to 100 mL with methanol.

   c. HPLC external standards at 12.0, 6.0, 3.0, and 1.5 µg/mL are prepared for each set as follows:

      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.

3. Storage and Stability:

   Fresh Tilmicosin spiking solutions should be prepared monthly. Store these solutions in a light protected environment at 2-8 ºC. Wrap in aluminum foil to reduce light induced degradation or store in colored (amber) glass container.

   Tilmicosin stock standard solutions are stable for three months when stored at 2-8 ºC in a light protected environment.

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. Extraction

   a. Thaw samples for 15 to 20 minutes. Accurately weigh 10 ± 0.1 g of sample into a 50 mL polypropylene centrifuge tube. Include a tissue blank and a recovery with each sample set. Fortify the recovery as follows:

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

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

      Quantitate using an external standard curve as described in D.2.c.

   b. If the sample(s) was previously homogenized add 30 mL of methanol and shake for 5 minutes on a mechanical shaker.

      If the sample(s) was not previously homogenized add 30 mL of methanol to the sample(s) and homogenize for 15 seconds. Rinse homogenizer between samples. Shake sample(s) for 5 minutes on a mechanical shaker.

   c. Centrifuge samples for 10 minutes at 2000 rpm.

   d. Decant supernatant into a 300 mL beaker. From this point, perform all steps under conditions protecting samples from bright light, especially sunlight.

   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 a 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.

2. 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

3. HPLC gradient elution parameters

   Mobile Phase A – 50% acetonitrile in water, pH = 2.5

   Mobile Phase B – water, pH = 2.5

   Mobile Phase C – 0.08 M dibutyl ammonium phosphate

Time (min.)	% Mobile Phase		
	A	B	C
0	100	0	0
3	100	0	0
4	55	30	15
15	55	30	15
16	100	0	0
21	100	0	0

4. HPLC analysis

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

5. Chromatogram – See **K. Appendix**

G. CALCULATIONS

1. Quantitative

   a. Using linear regression, construct a standard curve by plotting Concentration in µg/mL (x), versus LC peak height (y) for all 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 peak height 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, plastic gloves, and laboratory coat.

2. Hazards

Reagents	*Hazard*	*Recommended Safety Procedures*
Tilmicosin	Allergen. Eye irritant. May cause increased heart rate.	Wear plastic 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 plastic gloves, lab coat, and eye protection.
Sodium Carbonate	Eye and mucous membrane irritant.	Wear plastic gloves and eye protection.
Dibutylamine	Toxic, mutagenic, corrosive and combustible. Destructive to upper respiratory tract, eyes, and skin.	Wear plastic 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 plastic gloves, lab coat, and eye protection. Vent separatory funnels frequently while under agitation.

3. Disposal Procedures

Reagents	*Hazard*	*Recommended Safety Procedures*
Organic solvents	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.
Dibutylamine	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

Compound	*Acceptable Recovery (%)*	*Repeatability (% CV)*	*Analytical Range (ppm)*	Minimum Proficiency Level (ppm)
Tilmicosin (in liver and kidney)	70 - 110	≤15	≥0.6	0.6
Tilmicosin (in muscle)	70 - 110	≤15	≥0.3	0.3

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

The Measurement Uncertainty and Method Detection Limit should be recalculated yearly or whenever a major change in the method occurs. For example: Change in personnel analyzing samples or new equipment received - detector, spectrophotometer, column.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample weight, 10 $\pm$ 0.1 g	Use a calibrated balance.
b.	Protect standard solutions from light	Protect from light.
c.	Prior to hexane extraction, pH should be < 6	Use a calibrated pH electrode.
d.	Prior to 2:1 chloroform:hexane extraction, pH should be 9.0 ± 0.4	Use a calibrated pH electrode.
e	N-Evap water bath temperature not to exceed 40 ºC	Use a calibrated thermometer.

3. Readiness To Perform (FSIS Training Plan)

a. Familiarization

i. Phase I: Standards

On 3 separate days, run a set of standards at the following concentrations: 0, 1.5, 3.0, 6.0 and 12.0 µg/mL.

ii. Phase II: Fortified samples

On 3 separate days, run fortified matrix standards in the following matrices and at the specified fortification levels:

(a) beef muscle at 0, 0.3, 0.6 and 1.2 ppm;

(b) beef liver and kidney at 0, 0.6, 1.2 and 2.4 ppm.

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

iii. Phase III: Check samples for analyst accreditation.

(a) For each tissue type, analyze one blank, 0.6 ppm recovery for muscle (1.2 ppm for liver and kidney), and 8 unknown samples fortified between 0 and 1.2 ppm for muscle and 0 and 2.4 ppm for liver and kidney.

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

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

4. Intralaboratory Check Samples

   a. System, minimum contents.

      i. Frequency: 1 check sample per week as samples analyzed.

      ii. Records are to be maintained.

   b. Acceptability criteria.

      Refer to 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. Matrices: Beef muscle, kidney, and liver.

   b. Sample size upon receipt: ~ 40 g.

   c. Condition upon receipt: Frozen.

   d. Sample storage: Frozen at ≤ -10 ºC.

6. Sample Set

   a. For quantitative analysis, the batch size is 1-20 samples and must include a blank beef tissue extract, a 0.6 ppm fortified tissue recovery for muscle (1.2 ppm for liver and kidney).

7. Sensitivity

   a. Minimum proficiency level (MPL): 0.3 ppm beef muscle

      0.6 ppm beef liver and kidney

J. WORKSHEET

The following Tilmicosin Worksheet can be reproduced and/or photocopied.

WORKSHEET: TILMICOSIN

Analyst: _____ Date Started: _____ Date Completed: _____

INSTRUMENT CONDITIONS

Column: _____Column Temp: _____°C
Mobile Phase Gradient as Described in Section F.3? Yes/No
Wavelength:_____nm Attenuation:_____Chart Speed:_____Flow Rate:_____mL/min._____

Ext. Standard Conc. μg/mL	Peak Height	Regression Analysis
		r =
		m =
		b =
		y =

BE SURE THAT EXTRACTS ARE CLEAR PRIOR TO INJECTION ON THE HPLC

Internal Lab Number	Form Number	Sample Size (g)	Injection Volume (μL)	Peak Height (mm)	Dilution Factor	TIL (ppm)
Recovery						
Blank						

K. APPENDIX

1. Chromatograms

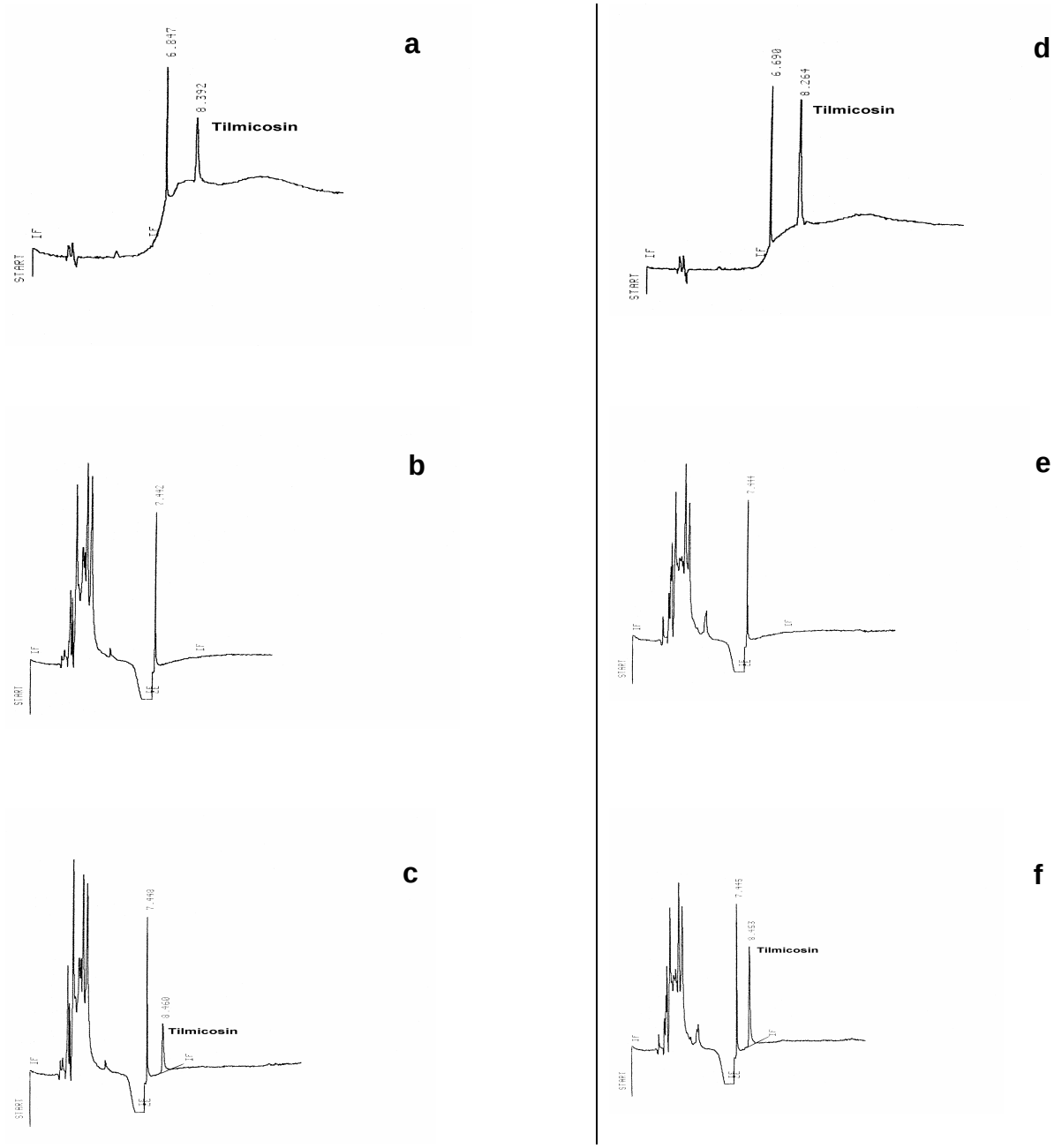
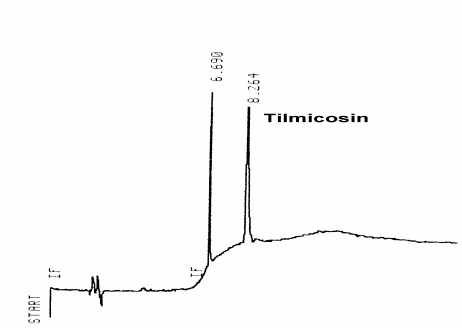


Figure 1. Chromatograms for 0.6 ppm external standard (a), beef muscle blank (b), 0.6 ppm fortified beef muscle (c), 1.2 ppm external standard (d), beef liver blank (e), and 1.2 ppm fortified beef liver (f).

a



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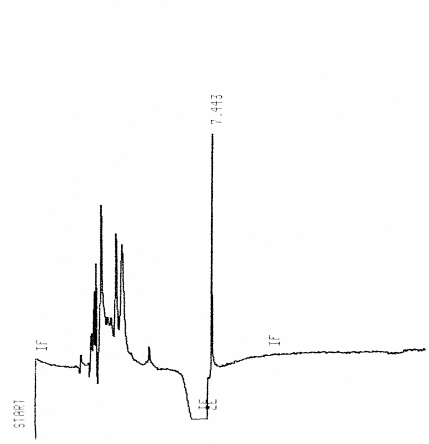

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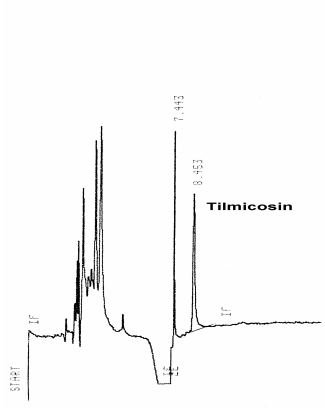

Figure 2. Chromatograms for 1.2 ppm external standard (a), beef kidney blank (b), and 1.2 ppm fortified beef kidney (c)

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Approved by:	Date approved:
Tom Mallinson	8/1/05
Terry Dutko	8/2/05
Jess Rajan	8/1/05
Charles Pixley	8/1/05
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Approval records on file.