LASALOCID IN BOVINE TISSUE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Ten-gram aliquots of ground liver are extracted with 40 mL acetonitrile. A 23.5 mL portion of the extract is washed with hexane and the acetonitrile layer is evaporated to dryness under nitrogen at 55°-65° C. 1 mL of water saturated with the HPLC mobile phase and 2 mL of the HPLC mobile phase are added to the residue. After vortexing and centrifugation, a portion of the organic (top) layer is removed, and an aliquot is analyzed by HPLC on two 25 cm Partisil PXS 10/25 (Whatman) columns. The effluent is monitored by fluroescence with excitation set at 310 nm and emission at 440 nm.

The peak height is determined and the quantity of lasalocid is calculated from an external standard curve run on the same day. The fortification is done on acetonitrile enzyme deactivated liver sample because of lasalocid's instability on spiking into liver. Lasalocid is stable in livers from dosed animals.

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B. EQUIPMENT

1. Apparatus

a. 80 mL glass centrifuge tubes.

b. Polytron: Brinkman Industries, equipped with small blending heat or equivalent.

c. 50 mL polypropylene tubes: Corning.

d. Centrifuge: Damon/IEC Model HN-s equipped with six-place fixed rotor or equivalent.

e. N-Evap, Model 111: Organomation Assoc., Inc., or equivalent.

f. Maxi Mix: Themolyne, Sybron Corp.

g. Pre-injection column: Whatman Partisil PXS 10/25 (10 micron microparticulate silica, 25 cm, 4.6 mm id).

h. Analytical column: Two Whatman Partisil PXS 10/25 in series.

i. 100 μL syringe.

j. Sep-Pak silica cartridge: Waters Associates #51900, or equivalent.

k. Usual glassware.

2. Instrumentation

HPLC Chromatograph: Waters Associates, Model 244, or equivalent, with Schoeffel FS970 LC fluorometer, detector, Wisp 710B sample processor, and a strip chart recorder (Perkin Elmer Model 056 1001).

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C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hexane—distilled in glass (Burdick and Jackson).

b. Methanol—distilled in glass (Burdick and Jackson).

c. Tetrahydrofuran—distilled in glass (Burdick and Jackson).

d. Acetonitrile—distilled in glass (Burdick and Jackson).

e. Ammonium hydroxide—ACS reagent grade.

f. Chloroform—distilled in glass (Burdick and Jackson), or equivalent.

g. HPLC mobile phase.

   i. Prepare solvent mixture A, adding the components in order of decreasing volume percents:

      (a) Hexane: 810 mL.

      (b) Tetrahydrofuran: 150 mL.

      (c) Methanol: 30 mL.

      (d) Ammonium hydroxide: 10 mL.

   ii. Mix thoroughly in separatory funnel and let stand until upper phase is clear (about 1 hr). Empty lower phase and discard. Reserve upper phase. Process three volumes (three separatory funnels) simultaneously.

   The mobile phase is stable for a minimum of one week.

h. Preparation of water saturated with HPLC mobile phase.

   i. Mix 50 mL water (deionized, distilled) with 100 mL HPLC mobile phase in a 250 mL separatory funnel. Shake vigorously for 30-40 sec.

   ii. Let sit until lower layer (water) is clear. Remove lower layer immediately prior to use.

   Prepare fresh each day.

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D. STANDARDS

1. Source

Standard lasalocid (mw 612.80), Animal Health Research, Hoffman LaRoche.

2. Preparation of Standards

Preparation of lasalocid standard solutions for standard curve and sample fortification.

a. 140 μg/mL lasalocid: Weigh exactly 72.66 mg lasalocid sodium salt standard into a 500 mL volumetric flask. Add THF to dissolve lasalocid and dilute to volume with THF.

b. 70 μg/mL lasalocid: Dilute 50 mL of standard 2.a to 100 mL with tetrahydrofuran.

c. 35 μg/mL lasalocid: Dilute 25 mL of standard 2.a to 100 mL with tetrahydrofuran.

For standard curve, dilute 100 μL of each standard to 4 mL with mobile phase representing 1.4, 0.7, and 0.35 ppm respectively.

3. Storage Conditions

Store stock solutions in refrigerator at 4° C in stoppered volumetric flasks sealed with parafilm.

4. Shelf Life Stability

Solutions may be kept for five months. Each week, decant a fresh supply of each solution into screw-capped culture tubes, seal with parafilm, and store at room temperature for use in sample fortification and generation of daily standard curves.

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E. EXTRACTION PROCEDURE

1. Decontamination of Apparatus and Glassware

a. Meat grinder: All parts of the grinder that come in contact with the tissue sample should be washed with hot soapy water, rinsed, air-dried, rinsed with ehtyl acetate, and air-dried. The hot soapy water wash should be repeated between sets of samples expected to contain different levels of lasalocid.

b. Polytron: After homogenizing a sample, wipe the remaining liver off the shaft, using a clean paper towel, and rinse the shaft successively in water 2 × 50 mL (in a 250 mL centrifuge bottle), 50 mL ethyl acetate, and 50 mL acetonitrile, with polytron set at medium speed for 10-15 sec for each rinse.

2. Preparation of Samples

a. Weigh 10.0 $\pm$ 0.05 g of partially defrosted liver sample into a clean 80 mL glass centrifuge tube. Select a blank liver sample for a second control and three levels of fortification.

b. Add 40 mL of acetonitrile to each weighed sample and homogenize for 15-30 sec.

c. Fortify the homogenized blank samples according to the following table:

Fortification Level (ppm)	*Standard Lasalocid*	*µL of Fortifying Solution/10 g sample*
1.4	D.2.a	100
0.7	D.2.b	100
0.35	D.2.c	100

d. Homogenize each sample again for 15-30 sec.

e. Centrifuge for 10 min at 2000-2500 rpm.

f. Using a 25 mL graduated cylinder, transfer a 23.5 mL aliquot into a 50 mL screwcapped polypropylene tube. (This represents ½ of the sample extract, assuming 10 g liver to contain approximately 14 mL water.)

g. Add 20 mL hexane.

h. Shake vigorously for 15-20 sec, with venting.

i. Centrifuge at 1500-2000 rpm for 10 min at room temperature.

j. Aspirate hexane layer and discard.

k. To remove interfering fat, repeat steps g-j.

l. Evaporate acetonitrile layer to less than 0.5 mL under nitrogen using N-Evap at 45°-60° C. *Do not overdry.*

m. Add 5 mL chloroform and filter through Sep-Pak silica cartridge and evaporate to dryness under nitrogen using N-Evap at 45°-60° C. Do not overdry.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

n. Add 2.0 mL HPLC mobile phase and vortex for 15-20 sec with the screw cap on.

o. Transfer the mixture into 15 mL stoppered glass centrifuge tube with *tapered bottom.*

p. Centrifuge at 1500-2000 rpm for 10 min.

q. Transfer about 200 μL *clear* upper layer into a capped plastic vial for HPLC analysis.

r. With each set of samples, process a control liver, reagent blank, and a 0.7 ppm fortified liver to determine recovery.

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F. ANALYTICAL QUANTITATION

1. HPLC Analysis

a. Follow SOP to HPLC with the following exceptions:

   i. Do not filter or de-gas mobile phase.

   ii. Do not filter samples.

   iii. Each day, transfer only enough mobile phase mixture for a daily run from the amber gallon bottle into the HPLC solvent bottle. Cover solvent bottle with aluminum foil.

b. Set the fluorescence spectrophotometer as follows:

   Excitation: 310 nm Slit: 8 nm
   Emission: 440 nm Slit: 8 nm
   Sensitivity: 10, 3, or 1 as needed to keep lasalocid peak on scale

   PM gain: Norm
   Response: Norm
   Mode: Norm
   Zero Suppression: Off

c. Equilibrate the entire system with mobile phase until a stable baseline is obtained (about 30-45 min). If the column is new, equilibrate with mobile phase until a constant retention time and fluorescent response for lasalocid are obtained (about 24 hr).

d. Measure the flow rate at the beginning of each day's samples and recheck periodically throughout the day.

e. Using Wisp 710B sample processor, inject (in duplicate) 20 μL portions each of sample extracts and mixed standards.

f. Retention time for lasalocid is about 6.5 min at a flow of 2.0 mL/min. Chart speed should be about 0.5 cm/min. Allow 10 min between injections.

g. With each set of samples, inject three different lasalocid standards before the set, and the same three standards after the set. The concentration of lasalocid injected should be above, below, and approximately the same concentration as expected to be present in the final dilution of the samples to be assayed.

ppm Lasalocid in Liver	*Expected Concentration Lasalocid per Final Dilution of Liver Extract*
0.35	35 μg/mL
0.7	70 μg/mL
1.4	140 μg/mL

h. If the HPLC system is not to be used for 8 hr or more, flush the entire system with hexane.

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F. ANALYTICAL QUANTITATION (Continued)

2. Automatic Integration Devices

Laboratory instrumentation that automatically provides peak area or peak heights can be used in the method. The technique used should be common for all the samples and the calculation should reflect techniques used in height or area.

3. Retention Time or Volume

a. The retention volume or time will shift based on the history of the column. The standards are run at the start and end of a series and are used to determine responses as well as retention time.

b. Variation in retention time is also influenced by the time-marking process, either manual or automatic. This also is resolved by inspection of multiple standards.

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G. CALCULATIONS

1. Procedure

Calculation of lasalocid in sample: Using area data or peak height from 1.40, 0.70, and 0.35 ppm standard lasalocid injections, construct a linear standard curve based on the formula $y = mx + b$, where x is the peak area or height and y is ppm. Calculate regression coefficient and standard error of estimate for these data. Calculate recovery from a spiked sample included in every set. Correct sample values for recovery.

2. Reference

NADA 96-298V, Hoffman LaRoche.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Analysis of Lasalocid in Bovine Liver		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		*Hazards*	*Recommended Safe Procedures*
	C. Reagents		
	Hexane Methanol Tetrahydrofuran Acetonitrile Ammonium hydroxide	These solvents are typically very flammable or corrosive. The vapors are extremely irritating to the skin, eyes, and respiratory system.	These solvents should only be handled in an efficient fume hood. Excepting acetonitrile, the other solvents are used to prepare the HPLC mobile phase. This may be done in a fume hood away from any heat-producing device.
4. Disposal Procedures	Organic solvent mixtures	See above	The large volume may be reduced by partial evaporation in a fume hood and the remaining solvent can be stored in waste cans until disposed of by the contractor or in-house specialist.

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J. QUALITY ASSURANCE PLAN

1. Performance Standards

Compound	*Analytical Range (ppm)*	*Acceptable Recovery (%)*	*Repeatability % CV*
Lasalocid	0.025-1.4	80-110	≤ 10

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. Sample weight	10.0 g ± 0.05 g
b. Acetonitrile volume	40.0 mL
c. Blend time	20 sec ± 5 sec
d. Volume transferred	23.5 mL
e. Evaporation	No overdrying
f. Vortex time	20 sec ± 5 sec
g. Upper layer	Clear

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—0.35, 0.70, 1.40 ppm. Standard curves prepared on three separate days.

ii. Phase II: Duplicate recoveries (self-fortified samples) on three separate days on blanks and at three different levels (each day) within the analytical range, 0.35-1.40 ppm.

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

iii. Phase III: Check samples for analyst accreditation.

(a) 14 samples submitted by FSIS Science & Technology Western Laboratory. (Samples submitted by supervisor if only one laboratory is performing this analysis.)

(b) Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Refer to section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents:

  i. Initially, at least one check sample biweekly per analyst.

  ii. Random replicates or blind check samples chosen by the supervisor and/or the Laboratory QA Officer.

  iii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QA Officer for:

    (a) All replicate findings.

    (b) CUSUM control chart on differences between replicates.

    (c) All percent recoveries.

    (d) For all recoveries—the running average, standard deviation, and coefficient of variation on last acceptable 10.

b. Acceptability criteria.

If unacceptable values are obtained, then:

  i. Stop all official analyses for that analyst.

  ii. Investigate and identify probable cause.

  iii. Take corrective action.

  iv. Repeat Phase III of section J.3 above if cause was analyst-related.

5. Sample Acceptability and Stability

a. Matrix: Liver.

NOTE: Method has been extended to poultry fat and skin.

b. Sample receipt size: Varied; enough to obtain matrix required for all quantitative and confirmation tests and reserve sample.

c. Condition upon receipt: Frozen.

d. Sample storage:

  i. Time: ≤ 1 yr.

  ii. Condition: Frozen.

6. Sample Set

a. Blank tissue (control blank).

b. Blank tissue fortified at 0.7 ppm.

c. Samples processed the same day.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

7. Sensitivity

a. Lowest detectable level (LDL): 0.025 ppm.

b. Lowest reliable quantitation (LRQ): 0.35 ppm.

c. Minimum proficiency level (MPL): 0.35 ppm.