NARASIN IN CATTLE AND CHICKEN TISSUES

Contents

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Narasin is extracted from samples with methanol in the case of lean, liver, or kidney and redistilled hexane in the case of skin or fat (skin not applicable to cattle). Tissue extracts are processed by carbon tetrachloride extraction in the case of lean, liver, or kidney and acetonitrile in the case of skin or fat. Subsequently all samples are purified by silica gel column chromatography and alumina column chromatography. Aliquots of the semipurified extracts in methanol are subjected to thin-layer chromatography on silica gel plates, and the antibiotic is detected on the TLC plates by bioautographic techniques using *Bacillus subtilis* as the assay organism. Narasin levels are estimated by comparing zones of inhibition from experimental samples to those from standard recoveries. Samples are authenticated by Polaroid photography.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

a. Tissue blender: Hamilton Beach Model 266, or equivalent, equipped with blender heads to fit a one-pint Ball Mason jar.

b. Centrifuge: International Centrifuge Model V, size 2, or equivalent.

c. Rotary vacuum evaporator: Rinco evaporator, or equivalent.

d. Thin-layer chromatography spreading board, applicator, and developing chambers: Brinkmann Instruments, Inc., Westbury, NY, or equivalents.

e. Photographic equipment: Polaroid Model MP3, or equivalent, and Type 55 Polaroid film.

f. Incubator maintained at 37° C.

g. Colorimeter: Bausch and Lomb Spectronic-20, or equivalent.

h. Water bath.

i. Grumbacher atomizer No. 836 sprayer, or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

a. Hexane, A.R., redistilled.

b. Methanol A.R.

c. Chloroform A.R.

d. Benzene A.R.

e. Carbon tetrachloride A.R.

f. Ethylene glycol monomethyl ether A.R.

g. Acetonitrile.

h. Chloroform A.R., containing 5% by volume of methanol A.R.

i. Chloroform A.R., containing 1% by volume of methanol A.R.

j. Silica gel 0.2 to 0.5 mm for column chromatography: Brinkmann Instruments, Inc.

k. Alumina, Alcoa F-20, for column chromatography.

l. Glass wool.

m. Sodium sulfate, anyhdrous, A.R.

n. Agar medium No. 23: Dissolve 0.69 g $K_2HPO_4 \cdot 3H_2O$, 0.45 g KH_2PO_4, 2.5 g yeast extract (Difco), 10 g glucose (cerelose), and 6.0 g agar-agar (Colab Ionagar No. 2S) in enough deionized water to give 1 L total volume. Autoclave the solution for 15-20 min at 121° C.

o. Thin-layer chromatography plates: Prepare a slurry of 32 g silica gel G in 74 mL of deionized water and apply to standard 20 × 20 cm glass plates at a thickness of 250 microns. Air-dry the plates for 10 to 15 min. Activate the plates in a drying oven by heating at 100° C for 1 hr. Following activation, allow the plates to cool slowly to room temperature.

p. Alternatively, commercial silica gel thin-layer chromatographic plates may be used, such as Quanta Q47G (Quantum Industries) or Analtech made with Woelm silica gel (Analtech, Inc.).

q. *Bacillus subtilis* stock suspension.

   i. Wash the growth of *Bacillus subtilis*, American Type Culture Collection No. 6633, from one penassay seed agar slant (culture medium No. 1, from Grove and Randall, Assay Methods of Antibiotics) with 3 to 5 mL sterile deionized water onto the surface of a Roux bottle containing 300 mL of penassay seed agar with 0.33% manganous sulfate added.

   ii. Incubate the culture for 1 week at 37° C.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

iii. Following incubation, wash the growth from the agar surface of the Roux bottle with approximately 50 mL of sterile deionized water.

iv. Transfer the wash to a sterile 250 mL centrifuge bottle and hold the organisms at 65° C in a water bath for 30 minutes.

v. Centrifuge the suspension and discard the supernatant liquid.

vi. Repeat the resuspension and discard the supernatant liquid.

vii. Repeat the resuspension and washing of the organisms three times.

viii. After the final wash, heat-shock the cells again by immersion in the 65° C water bath for 30 min and suspend them in 30 mL sterile deionized water.

NOTE: This stock suspension is stored at 4°-5° C for use.

r. *Bacillus subtilis* inoculum: Prepare the inoculum fresh daily by diluting the stock suspension with sterile deionized water to obtain a 20% light transmittance at 530 nm using a Spectronic-20 colorimeter.

s. A 2 mg/mL solution of INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride).

t. Sodium bicarbonate A.R.

u. Deacidified methanol A.R.: Add 1.0 g sodium bicarbonate A.R. to 100 mL methanol A.R. and mix. Filter off the excess sodium bicarbonate through Whatman No. 2 filter paper.

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards

Standard solutions.

a. Weigh a quantity of narasin standard to contain 10 mg of narasin activity.

b. Transfer the standard quantitatively to a 100 mL volumetric flask and dissolve it in deacidified methanol. Dilute to the mark with deacidified methanol and mix thoroughly. This standard contains 100 μg/mL of narasin activity.

c. Pipette 5 mL of 100 μg/mL standard into a 50 mL volumetric flask with a volumetric pipette. Dilute to the mark with deacidified methanol and mix thoroughly. This standard contains 10 μg/mL of narasin activity.

d. Pipette 5 mL of 10 μg/mL standard into a 50 mL volumetric flask with a volumetric pipette. Dilute to the mark with deacidified methanol and mix thoroughly. This standard contains 1.0 μg/mL of narasin activity.

2. Storage Conditions

Stock solutions should be stored tightly stoppered at 4° C.

3. Shelf Life Stability

Standard soluitions 2.b.-2.d are stable at least 5 days when stored under refrigeration as described in section 3.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

1. Lean, Liver, and Kidney Tissue Extraction and Purification

a. Weigh 50 g ground or blended tissue sample into a one-pint extraction jar. Prepare negative control samples and standard recoveries for assay with each set of samples.

   i. Weigh appropriate control tissue into one-pint blender jars.

   ii. Add standard narasin (1 μg/mL in methanol) by pipette to give the desired concentration in the recovery samples.

   NOTE: One negative control (no narasin added), one recovery sample containing 5 ng/g wet tissue (5 ppb), one recovery sample containing 10 ng/g (10 ppb), and one recovery sample containing 20 ng/g (20 ppb) are processed with each set of the samples.

   Control and recovery samples are extracted and chromatographed exactly as described for the corresponding tissue.

b. Add 2 mL methanol per gram of tissue.

c. Blend sample until uniform (approximately 30 sec).

d. Transfer sample to 250 mL centrifuge bottle and centrifuge for 10 min at 2500 rpm.

e. Transfer the supernate (measure via a graduated cylinder) to a 250 mL separatory funnel.

f. Extract the supernate with three 25 mL potions of carbon tetrachloride. Combine the carbon tetrachloride fractions in a 125 mL evaporating flask and evaporate to dryness by rotary vacuum evaporation, using a water bath at 50°-60° C.

g. Prepare silica gel column for chromatography. Prepare a separate column for each sample.

   i. Place approximately 10 mL of chloroform into a 14 X 240 mm glass chromatographic column. Insert a glass wool pledget and tamp with a glass stirring rod to eliminate air bubbles.

   ii. Add 10 mL (measured with a 10 mL graduated cylinder) of 0.2 to 0.5 mm silica gel to the column and follow with 5 mL chloroform.

   iii. Stir the silica gel with a glass rod to eliminate air bubbles; then let the column stand until the silica gel settles.

   iv. Add approximately 5 mL (measured with a 5 mL graduated cylinder) of anhydrous sodium sulfate to the column, layering it carefully to avoid disturbance of the silica gel surface.

   v. Drain the chloroform to the top of the sodium sulfate.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE

h. Dissolve the sample from step f in 20 mL of chloroform and charge the silica gel column with solution at a flow rate of 3-4 mL per min. Rinse the evaporating flask with additional 20 mL and 10 mL aliquots of chloroform and transfer the rinses to the column. Discard the chloroform effluent.

i. Develop the silica gel column with 200 mL of chloroform at a flow rate of approximately 4-6 mL/min. Discard the chloroform effluent.

j. Place an evaporating flask into position to receive the column effluent.

k. Elute the narasin from the column with 50 mL of chloroform:methanol (95:5) at a flow rate of 3-4 mL/min.

l. Evaporate the eluate to dryness by rotary vacuum evaporation using a water bath at 50°-60° C.

m. Prepare an alumina column for chromatography.

Prepare a separate column for each sample.

i. Place approximately 10 mL of chloroform into a 14 × 250 mm glass chromatographic column. Insert a glass wool pledget and tamp with a glass stirring rod to eliminate air bubbles.

ii. Add 5 mL of Alcoa F-20 chromatographic alumina (measured with a 5 mL graduated cylinder) and follow with 5 mL of chloroform. Be sure alumina has been washed from the sides of the column. Let the column stand until the alumina settles.

iii. Add approximately 2 mL of anhydrous sodium sulfate (measured with a 5 mL graduated cylinder) to the column, layering it carefully to avoid disturbance of the alumina surface.

iv. Drain the chloroform to the top of the sodium sulfate.

n. Place an evaporating flask into position to receive the column effluent.

o. Dissolve the sample from step l in 20 mL of chloroform:methanol (99:1) and charge the alumina column with this solution at a flow rate of 3-4 mL/min. Rinse the evaporating flask with additional 20 mL and 10 mL aliquots of chloroform:methanol (99:1) and transfer the rinses to the column. Collect the chloroform:methanol effluent.

p. Evaporate the eluate to dryness and transfer the sample to a 15 mL screw-cap sample bottle in 5 mL of redistilled hexane. Rinse the flask with an additional 5 mL of hexane and transfer the rinse to the sample bottle.

q. Evaporate the hexane under a mild stream of compressed air.

r. Dissolve the sample in methanol, using 0.02 mL methanol per 10 mL of supernate extracted.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

2. Skin Tissue Extraction and Purification

a. Weigh a ground or blended 50 g tissue sample into a one-pint extraction jar.

b. Add 4 mL of redistilled hexane per gram of tissue and place on the steam bath until the tissue is dissolved or is dissolved as much as possible. Stir occasionally to facilitate dissolution. About 15 to 20 min will usually suffice.

c. Centrifuge the sample 10-15 min at 2500 rpm.

d. Transfer the supernate (measure via a graduated cylinder) to a 500 mL separatory funnel.

e. Extract the supernate with five 1 mL/g tissue portions of acetonitrile. Combine the acetonitrile fractions in a 500 mL evaporating flask and evaporate to dryness by rotary vacuum evaporator, using a water bath at 50°-60° C.

f. Continue as described in steps 1.g-1.q above.

g. Dissolve the sample in methanol, using 0.01 mL methanol per 10 mL of supernate.

3. Fat Tissue Extraction and Purification

a. Perform steps described in 2.a-2.c above.

b. Transfer the supernate to a 500 mL separatory funnel.

c. Perform steps described in steps 2.e and 1.g-1.q above.

d. Dissolve the sample in 0.004 mL methanol/g tissue.

4. Thin-Layer Chromatography

Chromatograph the extracts from 1, 2, and 3 above on thin-layer plates as follows:

a. Apply 20 μL of methanol extract of negative control sample, standard recovery samples, and experimental samples to silica gel thin-layer plate. A forced-air hair dryer may be used to hasten evaporation of solvent during sample application.

NOTE: Up to 9 samples (1 negative control, 3 recoveries, and 5 samples) may be assayed on one thin-layer plate.

b. Add negative control and recovery samples to each thin-layer plate in order to ensure adequate standardization of the procedure.

c. Label the TLC plate and draw a line in the silica gel with a pencil on a scribe 16 cm from the bottom of the plate to mark the solvent front. Center the applied samples 1.5 cm from the bottom of the plate.

d. Line a TLC developing chamber with a sheet of filter paper or white blotter paper to improve saturation of the atmosphere in the chamber.

e. Prepare the developing solvent by mixing 80 parts carbon tetrachloride with 10 parts benzene and 6 parts ethylene glycol monomethyl ether.

DETERMINATIVE METHOD

E. EXTRACTION PROCEDURE (Continued)

f. Add the solvents to the developing chamber prior to placing the filter (or blotter) paper in the tank. Prepare the tank 1 to 2 hr prior to introduction of the thin-layer plates in order to ensure saturation of the paper and atmosphere.

g. Develop the thin-layer plate in the chamber, allowing the solvent front to move up to the line previously scored on the plate.

h. Remove the plate from the chamber and allow the plate to air-dry on the laboratory bench.

5. Preparation of Bioautographs

Bioautographs are prepared according to the method of Kline and Golab, *J. Chromatog.*, 18, 409 (1965).

a. Melt No. 23 agar in a steam bath.

b. Pour approximately 50 mL of melted agar into a 250 mL Erlenmeyer flask and fix the TLC plate by spraying the surface with the agar, using an artist's atomizer-type sprayer (Grumbacher atomizer No. 836) attached to the laboratory compressed air supply.

c. Place the TLC plate in a plexiglass holder (or other suitable container that will prevent contamination of the plate) and allow the plate to come to room temperature.

NOTE: This step is necessary to prevent too rapid solidification of the seeded agar overlay.

d. Cool 60 mL of melted No. 23 agar to 52° C in a 125 mL Erlenmeyer flask.

NOTE: Cool slowly to prevent solidification of the agar before it can be inoculated and poured.

e. Inoculate the agar with 0.2% of *B. subtilis* inoculum (refer to section C, Reagent and Solution List, item r). Mix quickly by swirling.

f. Pour this seeded agar over the surface of the TLC plate.

NOTE: Pouring of the seed agar must be done rapidly and carefully to ensure an even agar overlay before the agar solidifies. Also, the closer to using only 40 mL of the agar to pour the bioautograph, the greater the sensitivity.

g. Allow the plate to cool until the agar sets.

h. Cover the TLC plate holder to prevent contamination and to prevent evaporation of moisture from the plate.

i. Incubate the bioautograph overnight (16-18 hr) at 37° C.

j. Subsequent to incubation, spray the plate with a solution of INT (refer to section C, Reagent and Solution List, item s). Allow the color to develop for a period of 1-2 hr. An additional spraying applied after the first spraying has been absorbed into the agar is helpful in increasing the rate and degree of color contrast.

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION

1. Estimation of Concentration

a. After incubation, locate the narasin zones on the bioautograph at the R_f of 0.3 to 0.4.

b. Estimate narasin concentration by visually comparing zone sizes from experimental samples to zone sizes from standard recovery samples.

   i. Negative samples are reported as no activity at a sensitivity of the lowest positive standard recovery.

   ii. Positive samples are reported as the concentration of the corresponding standard recovery level or as a range between two standard recoveries.

   NOTE: Narasin levels obtained in this manner are regarded as approximate rather than absolute values.

c. If permanent records of bioautographs are desired, label the plates for identification and photograph by either transmitted or reflected light.

   NOTE: Polaroid photography of bioautographs has the advantage of producing a print immediately so that a satisfactory print may be obtained before destruction of the bioautograph.

d. Test sensitivity when standard narasin is applied to the TLC plates in methanol solution is 30 to 50 ng.

e. Test sensitivity of standard recoveries processed by the extraction procedures described is approximately 5 ng narasin per gram of fresh tissue (5 ppb).

f. The assay must be regarded as semiquantitative and is more applicable to verifying absence of narasin than to exact measurement of narasin levels.

2. References

Ely LIlly and Co. Procedure 5801652 (1975).

Elanco, NADA 118-980.

Kline and Golab, *J. Chromatog.*, 409 (1965).