Agricultural Analytical Chemistry
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DETERMINATION OF APRAMYCIN IN SWINE TISSUE

AM-AA-CA-R037-AB-755
(Supersedes Procedure 5801622)

I. PRINCIPLE

Samples are treated with potassium hydroxide and heated. After cooling, the samples are blended with trichloroacetic acid, the precipitate is removed by centrifugation, and the supernate is passed over an ion exchange column. Subsequently, the column is washed and the apramycin is eluted with ammonium hydroxide. The samples are taken to dryness and then reconstituted in a small amount of water. An aliquot is spotted on a silica gel plate, developed and bioautgraphed using Bacillus subtilis as the test organism.

II. REAGENTS

A. Potassium hydroxide A. R., 0.1 N, 0.05 N

B. Trichloroacetic acid A. R., 25°/o (w/v)

C. Sodium hydroxide A. R., 50°/o (w/v)

D. Ammonium hydroxide A. R., 1.0 N

E. Methanol A. R.

F. Chloroform A. R.

G. Amerlite CG 50 Li+ cycle - Slurry new CG-50 resin (Amberlite CG-50 AR, 100-200 Mesh)) in 1.0 N H_2SO_4 for a period of three hours. Wash the resin with distilled water until the pH of the wash water is above 5.0. Add LiOH slowly, and with stirring, until the pH of the slurry remains between 7.0 and 8.0. Allow the slurry to stand overnight. Wash the resin with distilled water at least ten times. Adjust the pH of the resin-water slurry to 7.0 with 1.0 N H_3PO_4. Store the slurry in tightly sealed glass jars until ready for use.

H. Thin-layer Chromatographic Plates - Woelm Silica Gel G 250 micron precoated TLC plates from Analtech, Inc., or equivalent.

I. Glass Wool

J. Agar Medium No. 23 N/1 - Dissolve 0.69 g K_2HPO_4; 0.45 g KH_2PO_4; 2.5 g yeast extract (Difco); 10.0 g glucose (cerelose); and 15.0 g Noble Agar (Difco), in deionized water to give one liter total volume. Autoclave the solution for 15-20 minutes at 121°C. Adjust the pH to 6.0 just before use.

K. Agar Medium No. 23 N/2 - Dissolve 0.69 g K_2HPO_4; 0.45 g KH_2PO_4; 2.5 g yeast extract (Difco); 10.0 g glucose (cerelose); and 6.0 g Noble Agar (Difco) in deionized water to give one liter total volume. Autoclave the solution for 15-20 minutes at 121°C. Adjust the pH to 6.0 just before use.

L. Bacillus subtilis stock suspension - Wash the growth of Bacillus subitilis, 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 of sterile deionzied water onto the surface of a Roux bottle containing 300 ml of penassay seed agar with 0.03 percent manganous sulfate added. Incubate the culture for one week at 37°C. Following incubation, wash the growth from the agar surface of the Roux bottle with approximately 50 ml of sterile deionized water. Transfer the wash to a sterile 250 ml centrifuge bottle and hold the organisms at 65°C in a water bath for 30 minutes. Centrifuge the suspension and discard the supernatant liquid. Repeat the resuspension and washing of the organisms three times. After the final wash, heat shock the cells again by immersion in the 65°C water bath for 30 minutes and suspend them in 30 ml of sterile deionized water. This stock suspension is stored at 4° to 5°C for use.

M. Bacillus subtilis inoculum - Prepare the inoculum by diluting the stock suspension with sterile deionized water to obtain a 20 percent light transmittance at 530 nm using a Spectronic-20 colorimeter. This suspension may be used for one week when stored under refrigeration.

N. A 2 mg/ml solution of INT (2(p-Iodophenyl)-3-p-nitrophenyl-5-phenyl Tetrazolium Chloride). Dissolve the INT in 10% of final volume of methanol and make to volume with deionized water.

O. Standard Solutions

1. Weigh a quantity of standard apramycin base to contain 100 mg of apramycin activity.

2. Quantitatively transfer the weight standard to a 100-ml volumetric flask and dissolve in deionized water. Dilute to the mark with water and mix thoroughly. This standard solution contains 1,000 mcg/ml of apramycin activity and is stable at least 30 days when kept under refrigeration.

3. Pipette 1 ml of 1,000 mcg/ml standard into a 100-ml volumetric flask with a volumetric pipette. Add a few

drops of concentrated ammonium hydroxide and dilute to the mark with water and mix thoroughly. This standard contains 10 mcg/ml of apramycin activity.

4. Pipette 5 ml of the 10 mcg/ml standard solution into 40 ml volumetric flask and make to volume with deionized water. This standard contains 1.25 mcg/ml of apramycin activity.

III. EQUIPMENT AND SUPPLIES

A. Meat Grinder - Hamilton Beach, Model 223 or commercial type grinder

B. Tissue Blender - Waring-type blender such as Hamilton Beach, Model 266 equipped with blender heads to fit a one-half pint Mason jar or Tissuemizer , homogenizer, Tekmar Co., or equivalent.

C. Centrifuge - International centrifuge Model V, size 2, with either a No. 239 or No. 267 horizontal head (equipped for 250 ml glass bottles) or equivalent.

D. Sample Heating Equipment, steam chest at atmospheric pressure, autoclave, or boiling water bath

E. Hot plate, Corning PC 100, or steam table

F. Chromatography columns - 10 mm i.d. x 250 mm (with 100-250 ml reservoir, suggested) and Teflon stopcocks

G. Hot water bath

H. Incubator maintained at 30°C

I. Colorimeter -Bausch and Lomb Spectronic-20 or equivalent

J. Thin-layer chromatography developing chambers

K. Photographic equipment - Polaroid Model MP3 or equivalent and Type 55 P/N Polroid film

L. Schleicher and Schuell No. 588 filter paper, or equivalent

M. Chromatography spray flask

N. Artist's atomizer - type sprayer (such as Grumbacher atomizer No. 836)

O. Vortex Mixer - such as Vortex-Genie, Scientific Industries, Inc.

IV. PROCEDURE

A. Preparation of Standard Recoveries

Prepare negative control samples and standard recoveries for assay with each set of experimental samples.

1. Weigh 25 grams of the appropriate control tissue into blender jars.

2. Add the standard solution of apramycin (1.25 mcg/ml in water) by pipette to give the desired concentration in the recovery samples. One negative control (no apramycin added), and three recovery samples containing 0.05, 0.1 and 0.2 mcg apramycin per gram of tissue (1, 2, and 4 ml of the 1.25 mcg/ml standard) respectively are to be processed with each set of experimental samples. The standard solution may be stirred into the tissue with a stainless steel spatula and rinsed with the added extraction solution.

3. Control and recovery samples are extracted as described for the experimental samples.

B. Sample extraction and cleanup

1. Weigh a representative, previously ground tissue sample (25 g) into a suitable glass container compatible with the type of blender to be used.

   NOTE: Although Pyrex containers are preferred, one-half pint Mason jars may be used when only Waring type blenders are available.

2. For lean, kidney, fat and skin tissues, add 4 ml of 0.1 N KOH per gram of tissue (100 ml). For liver tissue samples, add 4 ml of 0.05 N KOH per gram.

3. Blend until uniform and rinse blender head into the sample container with deionzied water.

4. Cover each sample container with commercial grade aluminum foil.

5. Heat the samples to greater than 85°C for at least 20 minutes in a steam chest, autoclave, or boiling water bath.

   NOTE: 30-40 minutes in an Arnold steamer or 10 minutes in an autoclave set at 121°C with slow exhaust have been found to be satisfactory.

   CAUTION: If Mason jars are being used, do not place directly on solid metal surface to prevent breakage. Use a woven wire shelf or cake cooling rack.

6. Punch a small hole in the aluminum foil and cool the samples to near room temperature in a refrigerator (30-40 minutes) or by partial immersion of the sample containers in warm then cold running tap water.

7. Add 1 ml of 25% trichloroacetic acid per gram of tissue (25 ml).

8. Blend until slurry is uniform.

9. Transfer the slurry to a 250 ml centrifuge bottle.

10. Centrifuge 15 minutes at 1700-1800 RPM.

11. Filter supernate through Schleicher and Schuell No. 588 filter paper into a 250 or 300 ml Erlenmyer flask.

    NOTE: If the sample will not filter through the filter paper--this is most likely to occur when the sample contains a large percentage of skin--then use four thicknesses of cheese cloth instead of filter paper.

12. Adjust the filtrate pH to 7.0±0.2 using 50 percent NaOH and 10 N HCl.

    NOTE: Be careful not to let pH go above 8.0.

13. Prepare a CG-50 (Li^+) column for each test and recovery sample to be processed.

    a. Place approximately 10 ml of deionized water into a 10 x 250 mm glass reservoir column. Insert a glass wool pledget and tamp it with a glass rod to eliminate air bubbles.

    b. Add CG-50 (Li^+) resin slurry to the column to provide a column bed approximately three cm in depth.

    c. Add 50 ml deionized water to the column and drain it to the top of the resin. Discard all effluent.

14. Filter the pH 7.0 extract from step 12 through S and S No. 588 filter paper into the prepared column.

15. Adjust flow rate to approximately 5 ml/minute. Drain to top of resin.

16. Wash the flask filter and column with 100 ml boiling deionized water and drain to the top of the resin. Discard all column effluents up to this point.

17. Place a 50-ml beaker into position to receive the column eluate.

18. Carefully elute the column with 20 ml of 1 N NH_4OH at the rate of approximately 3 ml per minute. Rinse the column with an additional 5 ml of NH_4OH and retain the combined eluates.

19. Evaporate the combined column eluates to 0.5-1 ml by adding 2 or 3 small carborundum boiling chips and gentle boiling on a hot plate or heating on a steam bath with a gentle stream of clean, dry air or nitrogen.

NOTE: Foaming may occur with some samples. Avoid splattering.

20. Quantitatively transfer the residue to a small sample vial or 15 ml conical test tube with the aid of 3 x 1 ml rinses with 1 N NH_4OH.

21. Evaporate the sample to dryness by heating on a steam bath or warm water bath and directing a continuous stream of clean, dry air or nitrogen over the sample surface.

22. Reconstitute the sample in 0.10 ml deionized water.

NOTE: Rinsing of the sides of the container can be facilitated by the use of a vortex mixer.

C. Thin-layer Chromatography

1. Spot 20 microliters of the sample on a silica gel thin-layer chromatographic plate. A stream of warmed air may be used to facilitate spotting. Keep spot size <1.0 cm in diameter.

2. Develop the thin-layer chromatographic plate in a saturated tank using the developer methanol:chloroform:concentrated ammonium hydroxide (55:15:50). Development time, approximately 2 hours.

3. Subsequent to development, allow the thin-layer plate to air dry thoroughly.

D. Preparation of Bioautographs

NOTE: Prepare the bioautographs essentially according to the method of Kline and Golab, J. Chromatog. 18, 409 (1965).

1. Melt Media Nos. 1 and 2 (items J and K under REAGENTS) in a steam bath or autoclave.

2. Fix the thin-layer plate from step 3, section B, above, by spraying the surface with Medium No. 1 (item J) using an artist's atomizer-type sprayer attached to the laboratory compressed air supply. Spray until the entire plate is well coated (approximately 20 ml).

3. Place the TLC plate in a plexi-glass holder (or other suitable container that will prevent contamination of the plate and drying out of the agar during incubation).

4. Cool 50 ml of melted Medium No. 2 (item K) to 52°C in a 125-ml Erlenmeyer flask and inoculate the agar with 0.1 percent (1 drop) of Bacillus subtilis inoculum suspension from item M under REAGENTS. Mix quickly by swirling.

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

NOTE: Pouring of the seed agar must be done rapidly and carefully to insure an even agar overlay before the agar solidifies.

6. Allow the plate to cool until the agar sets. Leave the cover of the plate holder off to permit moisture to escape for a period of approximately 1 hour.

7. Place the cover on the plate holder and incubate at 30°C overnight.

8. Subsequent to incubation, spray the plate with a solution of INT (item N under REAGENTS). Allow the color to develop for a period of approximately 1-2 hours. Additional sprayings (1 or 2) applied subsequent to the initial spraying being absorbed into the agar are helpful in increasing the rate and degree of color contrast.

E. Estimation of Antibiotic Concentration

1. After incubation, locate the apramycin zones on the bioautograph at the R_f of approximately 0.55.

2. Estimate apramycin concentration by visually comparing zone sizes from experimental samples to zone sizes from standard recovery samples.

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

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

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

3. If permanent records of bioautographs are desired, label the TLC plates for identification and photograph by 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.

V. DISCUSSION

A. Sensitivity

1. Test sensitivity when standard apramycin is applied to the TLC plates in water solution is approximately 0.1 mcg.

2. The lower limit of test sensitivity for standard recoverie processed by the extraction procedures described is approximately 50 ng of apramycin per gram of fresh tissue (50 parts per billion).

3. The assay is to be regarded as semiquantitative and is mor applicable to verifying absence of apramycin at 0.1 ppm in fresh tissue than to exact measurement of apramycin levels

B. Specificity

Swine kidney tissue was fortified with 0.1 ppm apramycin and 0.5 ppm of either lincomycin, virgimamycin, furazolidone, nitrofurazone, tylosin, oxytetracycline, chlorotetracycline, streptomycin, or neomycin. No interference with the detection o apramycin was observed from any of the compounds examined.

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References: Method 5801622 and Notebook 34W