GENTAMICIN IN PORCINE TISSUE BY HPLC

Contents

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This is a sensitive, specific, reproducible HPLC method for the qualitative determination of gentamicin in porcine tissue. Concentrations as low as 0.4 ppm can be determined without interference from other chemical substances. Tissues are extracted with 1N sulfuric acid and cleaned up using column chromatography and liquid-liquid solvent extraction, followed by Sep-Pak® chromatography. The identification of gentamicin is obtained by post column, isocratic HPLC using a retention time standard (netilmicin).

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B. EQUIPMENT

1. Apparatus

a. Blender, commercial dual-speed model: Waring, or equivalent.

b. Centrifuge: Sorvall RC-5B, fitted with model SS-34 rotor; DuPont Instruments, or equivalent.

c. Centrifuge: Damon UV-IEC; IEC Division, International Equipment Company, or equivalent.

d. Centrifuge tubes: 10 mL conical, graduated; Pyrex® , or equivalent.

e. Centrifuge tubes: 50 mL conical, graduated; Pyrex® , or equivalent.

f. Centrifuge tubes: 50 mL plastic; Sorvall by DuPont, or equivalent.

g. Fluorometer: Kratos model FS970, or equivalent, equipped with:

   i. 5 μL flow cell.

   ii. Overload reset—FSA986.

   iii. Computer interface—FSA987.

h. Glass chromatographic column (id = 10.5 mm, length = 20 cm), fitted with Teflon stopcock; SGA Scientific, Inc., or equivalent.

i. Glass wool: Pyrex wool filtering fiber; Corning Glass Works, or equivalent.

j. Balance, Analytical: Mettler model H-51; Fisher Scientific Co., or equivalent.

k. Balance, Top-loading: Mettler model P1200N; Fisher Scientific Co., or equivalent.

l. pH meter: Equipped with Sensorex SG900C combination electrode; Corning Scientific Instruments, or equivalent.

m. Pipetman: Adjustable digital microliter pipets (P20, P200, P1000); Gilson, or equivalent.

n. Plastipak syringes: 20 cc; Becton, Dickinson and Co., or equivalent.

o. PRP-1-HPLC analytical column (id = 4.1 mm, length = 150 mm, particle size = 10 μm); Hamilton Co., or equivalent.

p. Polytron: Willems, Brinkmann Instruments, or equivalent, equipped with:

   i. Homogenizer—model PT 10-35 basic assembly complete.

   ii. Generator—model PT 20-ST (sawtooth).

q. Reaction coil: Stainless steel tubing (1.6 mm O.D. × 0.23 mm I.D. × 1.5 m long); Waters Associates, or equivalent.

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B. EQUIPMENT (Continued)

r. Solvent clarification kit: Waters Associates, or equivalent, consisting of:

i. Glass filter apparatus.

ii. Filters for aqueous solvents: 0.45 μm (catalog #85117).

s. Stirrer, magnetic: Versamix; Fisher Scientific Co., or equivalent.

t. Tee, low dead volume mixing: #1-1HBZ; Parker Co., or equivalent;

u. Thermomix: Model 1419, heating and circulating unit with 5 L water bath; B. Braun or equivalent.

v. Vortex: Model K-55-G; Fisher Scientific Co., or equivalent.

2. Instrumentation

High-pressure liquid chromatograph system: HPLC, Waters Associates. Comprised of the following components coupled via the Interlink® System:

a. System controller: Model 720.

b. Data module: Model 730.

c. Solvent delivery system: Model 6000A.

d. Intelligent sample processor (WISP): Model 710B.

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C. REAGENTS AND SOLUTIONS

Reagent and Solution List

All reagents/chemicals are of analytical grade.

a. Acetic acid.

b. Ammonium hydroxide.

c. Chloroform, distilled in glass: Burdick & Jackson, or equivalent.

d. IRC-50 (Amberlite®) cation exchange resin, chloride form, 20-50 mesh: Mallinckrodt, or equivalent.

e. 1-Pentane sulfonic acid, sodium salt: Eastman Kodak Co., or equivalent.

f. Methyl alcohol, distilled in glass: Burdick & Jackson, or equivalent.

g. 2-Mercaptoethanol, 98%: Aldrich Chemical Co., or equivalent.

h. Milli-Q water from water purification system: Millipore Corporation.

i. o-Phthalaldehyde (OPT), 98%: Alfa Products, or equivalent.

j. Potassium tetraborate powder ($K_2B_4O_7 4H_2O$).

k. Sep-Pak® (C_{18}): Waters Associates.

l. Sodium sulfate, anhydrous.

m. Sodium hydroxide pellets.

n. Sulfuric acid.

o. Tetrabutylammonium hydroxide: 40 wt. % aqueous solution; Aldrich Chemical Co., or equivalent.

p. HPLC mobile phase (prepare daily).

   i. Combine 300 mL methanol with 700 mL water.

   ii. Add 6.5 mL of tetrabutylammonium hydroxide solution.

   iii. Stir with a stir bar on a magnetic stir plate for 3 min.

   iv. Assemble solvent clarification kit as shown in the manufacturer's instructions, using an aqueous filter (0.45 μm) between the funnel and filter support screen.

   v. Immediately prior to use, filter the solution through the solvent clarification apparatus, under vacuum, while stirring the filtrate with a stir bar on the magnetic stirrer. Mobile phase is ready for use.

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C. REAGENTS AND SOLUTIONS

q. o-Phthalaldehyde reagent (OPT) (prepare daily).

i. Weigh 60 mg of o-phthalaldehyde (OPT).

ii. Add 1 mL methanol.

iii. Mix solution with vortex mixer until OPT is dissolved.

iv. Add 0.2 mL 2-mercaptoethanol to the OPT solution and mix on a vortex mixer.

v. Add the resultant solution to 100 mL of 0.4M aqueous potassium tetraborate solution.

vi. Mix with a stir bar in the magnetic stirrer for 2 min.

vii. Filter the resultant solution through the solvent clarification assembly with a 0.45 μm filter under vacuum while stirring with a stir bar on the magnetic stirrer.

viii. Protect this reagent solution from light by wrapping the container with tin foil and cover the OPT reagent with a blanket layer of nitrogen. Reagent is now ready for use.

r. Ion-exchange resin (Amberlite® —IRC-50—ammonia phase).

i. Add 75 g of IRC-50 (Amberlite®) resin to 300 mL of 2N ammonium hydroxide in a 500 mL Erlenmeyer flask.

ii. Mix by swirling stoppered flask for 1 min. Let stand overnight at room temperature before use. This resin slurry may be stored up to 2 weeks in a stoppered flask at room temperature.

s. Sep-Pak® elution solvent.

i. Add 2 mL methanol to 98 mL water.

ii. Weigh 7.1 g of anhydrous sodium sulfate.

iii. Weigh 0.95 g of 1-pentane sulfonic acid sodium salt.

iv. Measure 1 mL acetic acid, using a 1 mL volumetric pipette.

v. Combine reagents i through iv and stir to dissolve the solids, using a magnetic stirrer.

vi. Assemble solvent clarification kit, using an aqueous filter (0.45 μm).

vii. Filter solution through solvent clarification assembly while stirring with the magnetic stirrer under vacuum. Reagent is ready for use (use within 6 weeks).

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D. STANDARDS

1. Source

a. Gentamicin C components (C_1, C_{1a}, C_2): Compound Distribution, Corporate Research Div., Schering Corporation.

b. Gentocin® : Gentamicin sulfate veterinary solution (50 mg gentamicin base/mL); Schering Corporation.

c. Netilmicin sulfate (Sch 20569): Schering Corporation.

2. Preparation of Standards

a. Gentocin® standard solution: A 100 μg/mL base equivalent standard is prepared by adding 20 μL Gentocin solution (batch #OKMF115 P64883) to a 10 mL volumetric flaks and adding water to volume.

b. Gentamicin C component standard solutions.

i. C_{1a} sulfate (Sch 13707, batch 7017-119-I). A 100 μg/mL base equivalent standard is prepared in water.

ii. C_2 sulfate (Sch 13705, batch #7017-122-I). A 100 μg/mL base equivalent standard is prepared in water.

iii. C_1 sulfate (Sch 13706, batch #7017-92-II). A 100 μg/mL base equivalent standard is prepared in water.

c. Netilmicin standard and working standard solution.

i. A 1 mg/mL base equivalent standard solution is prepared by adding 17.15 mg of netilmicin sulfate (batch #NIM-O-N-40, potency 583 μg/mg base equivalents) to a 10 mL volumetric flask and adding water to volume.

ii. 1 mL of a 1 mg/mL base equivalent solution is placed in a 10 mL volumetric flask and brought to volume with water to give a 100 μg/mL base equivalent working standard.

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E. EXTRACTION PROCEDURE

1. Sample Extraction

a. Immediately prior to use, thaw tissue homogenate and weigh 10g into a plastic 50 mL Sorvall centrifuge tube.

b. Run a fortified and blank tissue with each set of samples. For a fortified tissue, add 40 μL of the Gentocin standard solution (100 μg/mL) to the thawed homogenate, using a Pipetman (P-200) syringe.

c. Add 12 mL 1N H_2SO_4 to the thawed homogenate.

d. Homogenize tissue mixture for 30 sec using the Polytron at low-speed setting.

e. Centrifuge the resulting homogenate for 10 min at 20,000 rpm in a Sorvall RC-5B centrifuge.

f. Decant upper aqueous solution (supernatant) into a glass beaker.

g. Add 10 mL water to the tissue pellet remaining in the tube.

h. Homogenize for 30 sec at low speed, using the Polytron.

i. Centrifuge for 10 min at 20,000 rpm in a Sorvall RC-5B centrifuge.

j. Decant the supernatant and combine with supernatant from step f.

k. Using the pH meter, adjust combined supernatant to pH 4.0 with 1N and/or 10N NaOH solution.

l. Centrifuge the resultant solution at 20,000 rpm for 10 min.

m. Decant the supernatant fluid.

n. The extract sample is now ready for the IRC-50 ammonia phase column.

2. Preparation of IRC-50 (Ammonia Phase) Column

a. Place a glass wool plug into the bottom of a 20 cm glass column.

b. Fill the column to give 10 mL bed volume with IRC-50-ammonia phase resin (refer to section C, Reagent and Solution List, item r).

c. Wash with 60 mL of water at 4 mL/min through column and discard the aqueous elute. Do not allow the column to run dry.

d. Place glass wool plug on top of column head.

e. Column is now ready for use.

3. Column Chromatography of Tissue Extracts

a. Place the tissue extract (1.n) onto the top of the IRC-50 (ammonia phase) column (2.e).

b. Adjust the flow rate of the column eluate to 4 mL/min. Allow column to run until about 1 mm of liquid remains on top of upper glass wool plug on the column.

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E. EXTRACTION PROCEDURE (Continued)

c. Discard aqueous eluate.

d. Eluate column with 5 mL of water.

e. Discard eluate.

f. Eluate the column with 2 mL of 0.5N NH_4OH.

g. Discard eluate.

h. Elute column with 10 mL of 1.0N NH_4OH and collect the resultant eluate into a clean 50 mL glass conical graduated centrifuge tube.

i. The eluate is ready for the liquid-liquid solvent extraction purification step.

4. Purification of Tissue Extract by Liquid-Liquid Solvent Extraction

a. Add 10 mL chloroform to the column eluate (3.h).

b. Vortex mix for 15 sec.

c. Centrifuge the resultant mixture for 5 min at 1500 rpm with Damon IEC-UV centrifuge.

d. Pipette bottom chloroform layer and discard the chloroform.

e. Add 10 mL chloroform to remaining aqueous solution.

f. Vortex mix for 15 sec.

g. Centrifuge the resultant mixture for 5 min at 1500 rpm with Damon IEC-UV centrifuge.

h. Pipette bottom (chloroform) layer and discard the chloroform.

i. Dilute remaining aqueous solution to 45 mL with water.

j. Sample is now ready for Sep-Pak chromatography.

5. Sep-Pak® Chromatography

a. Pass 10 mL of methanol through the Sep-Pak C_{18} cartridge using a 20 mL syringe.

b. Discard eluate.

c. Pass 10 mL of water through in the same manner.

d. Discard eluate.

e. Fill the 20 mL syringe with the tissue extract (4.i).

f. Pass the tissue extract through the cartridge slowly (approximately 20 mL/min).

g. Discard the eluate.

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E. EXTRACTION PROCEDURE (Continued)

h. Repeat steps e-g until all the extract (45 mL) has been processed.

i. Pass 20 mL of water through the cartridge and discard eluate.

j. Pass 20 mL of methanol through the cartridge and discard eluate.

k. Pass 20 mL of water through the cartridge and discard eluate.

l. Pass 20 mL of air through the cartridge to remove any remaining liquid.

m. Using the elution solvent (refer to section C, Reagent and Solution List, item s) in the syringe, collect the initial 2 mL eluate from the cartridge in a 10 mL conical glass graduated tube.

n. Add 30 μL of a 100 μg/mL base equivalent aqueous solution of netilmicin.

Sample is now ready for HPLC analysis, and may be stored up to 48 hrs at ambient temperature.

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F. ANALYTICAL QUANTITATION

1. HPLC System

a. The individual components of the system are assembled using 1.6 mm od × 0.23 mm id stainless steel tubing. All systems are under the control of the Model 720 system controller except for the detector and the post column derivatization system.

b. The HPLC system operation programming uses the appropriate standardized procedure as detailed in the Waters Associates, Inc., Data Module (No. IM82908, November 1979, Rev.8), System Controller (No. 82477, March 1980) and WISP (No. IM83697, April 1980) instruction manuals.

c. Operation of the fluorometric detector and accessories is in accordance with Kratos Schoeffel Instrument, FS970 L.C. Fluorometer instruction/ maintenance manual (11.22.78). The output from the detector is fed via Kratos computer interface FSA987 to the Waters Data Module.

d. The post column derivatization system is comprised of a Waters Model 6000A pump, low dead volume mixing tee, and solvent reservoir containing o-phthalaldehyde reagent (refer to section C, Reagent and Solution List, item q). The reagent solution enters the mixing tee at 180 degrees to the column effluent and the mixed liquids exit the T, to the reaction coil, via the stem exit of the tee.

e. The reaction coil is connected directly to the fluorometer detector. The effluent from the detector is collected in a waste container (2 L Erlenmeyer flask).

2. HPLC System Operating Conditions

a. Mobile phase (refer to section C, Reagent and Solution List, item p) flow rate: 0.6 mL/min.

b. Sample volume injected: 50 μL.

c. Column (PRP-1 column) operated at 24° C by immersion in water bath (refer to section B.1, item u).

d. o-Phthalaldehyde reagent solution (refer to section C, Reagent and Solution List, item q) flow rate: 0.4 mL/min.

e. Reaction coil at ambient temperature.

f. Detector conditions: Excitation wavelength 335 nm, emission wavelength 418 nm, emission filter KV418, absorbance range 0.2 μA, suppression Hi, time constant 7 sec automatic overload reset (FSA986) 0.5 min.

g. Data module chart speed: 0.5 cm/min.

h. Sample run time: 20 min.

i. Void volume of system: 1.6 mL.

3. Reference

NADA 103-037 and 91-191, Schering.