Standardized Method for HPLC Identification of Mycobacteria

Principle: Identify Mycobacteria by analysis of mycolic acids, using high performance liquid chromatography.

Equipment

- Labconco RapidVap Vacuum System with lid heater (7900002)
- Labconco 13mm block (7458500)
- Diaphragm vacuum pump (7393000)
- Certified Biological safety cabinet
- Certified chemical fume hood
- Autoclave
- HPLC equipped with a C18 end-capped column, packed with 3 ul silica and a column oven
- Vortex type mixer
- Adjustable micropipette
- Safety pipetting device

Supplies

- Pasteur pipettes, 5 3/4" disposable glass
- Borosilicate glass culture tubes (13 x 100mm, new, defect-free, unwashed) with new, Teflon-lined screw caps
- Polyester fiber-tipped applicators
- Inoculation loops (ca. 10µl)
- Dark amber glass reagent dispensing bottles; or 1 ml and 5 ml glass pipettes
- Volumetric flasks, 50 ml, 100 ml, and 250 ml
- Dark amber glass bottles with caps, 1 L, 50 ml, and 100 ml
- 200µl tips for adjustable micropipette
- Filtered dry air or nitrogen
- pH paper (range pH 0 to pH 13) or litmus paper
- Autosampler vials and caps

Reagents

Except where noted, chemicals and solvents must be American Chemical Society (ACS) reagent grade[20, 23]. Additionally, solvents must be HPLC grade (recommend filtered). Use ACS or United States EPA-Type II or equivalent reagent-grade water.

1. Hydrochloric acid
2. Potassium hydroxide
3. Potassium bicarbonate
4. US EPA-registered, tuberculocidal disinfectant (a phenolic compound is recommended)
5. Synthetic compounds High- and low-molecular weight internal standards (HMW-ISTD and LMW-ISTD)
6. Derivation reagent p-bromophenylacyl bromide (0.1 mmol/ml) and dicyclohexyl-18-crown-6-ether (0.005 mmol/ml) inacetonitrile (not ACS-reagent grade)
7. Chloroform UV cutoff 245 nm, stabilized and packaged under nitrogen in a dark amber bottle
8. Dichloromethane (methylene chloride) UV cutoff 233 nm, stabilized and packaged under nitrogen in a dark amber bottle
9. Methanol UV cutoff 210 nm

Reagents for saponification, extraction, and derivatization of mycolic acids

Saponification reagent

Potassium hydroxide: 200 g

Reagent-grade water: 400 ml

Methanol: 400 ml

While stirring, slowly add the KOH to the water in a glass beaker, using an ice bath to cool the mixture. Continue stirring until the KOH has dissolved. When cooled, add the methanol. Store in a convenient container at ambient temperature until used.

Acidification reagent

Reagent-grade water: 400 ml

Concentrated Hydrochloric acid: 400 ml

While stirring, slowly add 400ml of concentrated HCL to the water in a 1L beaker. Store in a convenient container at ambient temperature until used

Potassium bicarbonate reagent

Potassium bicarbonate (KHCO3): 4 g

Reagent-grade water: 98 ml

Methanol: 98 ml

Add the KHCO3 to the 98 ml of water in a suitable container. Stir until dissolved, then add the methanol and stir. Store at ambient temperature in a convenient container. If precipitation occurs, heat to 35° C 1° C to resolubilize before use

Derivatization reagent

Prepare and store as described in the manufacturer's instructions.

Clarification reagent

Mix 100 ml of acidification reagent with 100 ml methanol. Store at ambient temperature in a convenient container.

Sample Diluent with Internal Standards

As a suggested starting point, add 4 mg of LMW-ISTD and 2 mg of HMW-ISTD to 50 ml dichloromethane in a 50 ml volumetric flask (8 and 4 µl/100 µl, respectively). Store at 4 C in a tightly capped dark amber bottle.

Sample Preparation Procedure

A) Cell Harvesting

1. Remove a sample of bacteria from the medium using sterile polyester swab or transfer loop
2. Add bacteria to a 13 x 100 mm tube with 2 ml of saponification reagent. Cap tightly and mix vigorously using a vortex mixer for 20 seconds

B) Saponification and extraction procedure

1. Autoclave tubes for a minimum of one hour at 121° C, 15 psi. The tops of the tubes should be covered with aluminum foil to ensure adequate heat transfer for decontamination. Cool to ambient temperature or below
2. Add 2 ml with the following parameters of Chloroform.
3. Add 1.5 ml of acidification reagent. Cap tightly and vigorously mix tubes with a vortex mixer for a minimum of 20 seconds. Allow layers to separate for 20-30 seconds. If the bottom layer remains turbid, mix again for 30-60 seconds. If still turbid, proceed
4. Using a glass Pasteur pipette, remove the bottom Chloroform layer containing mycolic acids and transfer to a new tube. Be careful not to transfer any of the upper aqueous layers. Samples may be capped and stored at 4-6° C overnight, if necessary
5. Use the RapidVap Vacuum Evaporation System to dry the sample with the following parameters

   Temperature: 80-90° C

   Vortex Motion: 25-50%

   Vacuum: greater than 20" Hg but less than 29" Hg

   Time: 15-20 minutes

C) Derivatization to *p*-bromonphenacyl esters

1. Add 0.1 ml of Potassium bicarbonate reagent to the dry sample.
2. Use the RapidVap System to dry the Potassium bicarbonate from the sample with the following parameters

   Temperature: 80-90° C

   Vortex Motion: 25-50%

Vacuum: greater than 20" Hg but less than 29" Hg

Time: 15-20 minutes

3. Cool the sample to ambient temperature or below and add 1.0 ml of Chloroform, followed by 50 µl of Derivatization reagent. Cap the tube and mix vigorously using the vortex mixer for 30 seconds
4. Using a heat block, heat the sample at 85-105° C for a minimum of 20 minutes. After the first 30-60 seconds of heating, check the volumes. If the volume in any tube appears to be less than 1ml, cool the tube to ambient temperature or below. Adjust the Chloroform to 1 ml, recap with new cap and reheat the sample

D) Clarification by liquid-liquid extraction

1. Cool the samples to ambient temperature or below and add 1 ml of clarification reagent. Recap
2. Mix each tube vigorously for a minimum of 20 seconds using the vortex mixer and allow the layers to separate (5-10 seconds, minimum)
3. Remove the bottom Chloroform layer with a glass Pasteur pipette and transfer to a new tube

E) Visual Identification

After final evaporation, run samples on a HPLC and visually interpret the chromatographic patterns. The results are interpreted visually by manually comparing the sample chromatogram with the laboratory reference pattern to determine a match.

1. Determine relative retention times for all peaks in the chromatograms.
2. Separate chromatograms into groups of single, double, distinct triple cluster and multi-peak cluster patterns
3. Match chromatograms with those in reference set having similar visual appearance and verify RRT values to determine species

E) Completion and storage

1. Evaporate to dryness in the RapidVap System and cap tightly
2. Store the sample at 4-6° C in the dark until ready for HPLC analysis

Reference

U.S. Public Health Service Centers for Disease Control and Prevention. Department of Clinical Microbiology, Arizona State Laboratory. Microbial Diseases Laboratory, California Department of Health Services Tuberculosis Reference Laboratory, Veterans Affairs Medical Center Microbiological Services Division, Texas Department of Health.

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