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RAPIDVAP

BENCHNOTES

Standardized Method for HPLC Identification of Mycobacteria

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

Determination

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.

Suggested approach for visual identification:

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 the reference set having similar visual appearance and verify RRT values to determine species.

Apparatus

1. Certified biological safety cabinet
2. Certified chemical fume hood
3. HPLC equipped with a C18 end-capped column with integral guard column, packed with 3 μ l silica; and a column oven
4. Autoclave
5. Labconco RapidVap® Vacuum Evaporation System with lid heater (model 79000-01 series)
6. Labconco 13mm Block (model 74858-00)
7. Vortex type mixer
8. Adjustable micropipette
9. Safety pipetting device

Supplies

1. Pasteur pipettes, 5 3/4" disposable glass
2. Borosilicate glass culture tubes (13x100mm, new, defect-free, unwashed) with new, Teflon-lined screw caps
3. Polyester fiber-tipped applicators
4. Inoculation loops (ca. 10 μ l)
5. Dark amber glass reagent dispensing bottles; or 1.0ml and 5.0ml glass pipettes
6. Volumetric flasks, 50ml, 100ml, and 250ml
7. Dark amber glass bottles with caps, 1L, 50ml, and 100ml
8. 200 μ l tips for adjustable micropipette

9. Filtered dry air or nitrogen
10. pH paper (range pH 0 to pH 13) or litmus paper
11. 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. Derivatization reagent
p-bromophenylacetyl bromide (0.1mmol/ml) and dicyclohexyl-18-crown-6-ether (0.005 mmol/ml) in acetonitrile (not ACS-reagent grade)
7. Chloroform
UV cutoff 245nm, stabilized and packaged under nitrogen in a dark amber bottle
8. Dichloromethane (methylene chloride)
UV cutoff 233nm, stabilized and packaged under nitrogen in a dark amber bottle
9. Methanol
UV cutoff 210nm

Reagents for saponification, extraction, and derivatization of mycolic acids

1. Saponification reagent:

Potassium hydroxide	200g
Reagent-grade water	400ml
Methanol	400ml

 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.
2. Acidification reagent::

Reagent-grade water	400ml
Conc. Hydrochloric acid	400ml

 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.
3. Potassium bicarbonate reagent:

Potassium bicarbonate (KHCO ₃)	4g
Reagent-grade water	98ml
Methanol	98ml

 Add the KHCO₃ to the 98ml 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 re-solubilize before use.
4. Derivatization reagent
Prepare and store as described in the manufacturer's instructions.
5. Clarification reagent
Mix 100ml-acidification reagent with 100ml methanol. Store at ambient temperature in a convenient container.

Sample Diluent with Internal Standards.

1. As a suggested starting point, add 4mg of LMW-ISTD and 2mg of HMW-ISTD to 50ml dichloromethane in a 50ml volumetric flask (8 and 4µl/100µl, 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 100mm tube with 2.0ml saponification reagent. Cap tightly and mix vigorously using vortex mixer for 20 seconds.

B) Saponification and extraction procedure

1. Autoclave tubes for a minimum of 1 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 2.0ml of Chloroform.
3. Add 1.5ml of acidification reagent. Cap tightly and vigorously mix tubes with vortex mixer for 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.

Parameters:

Temp: 80-90°C

Vortex Motion: 25-50%

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

Time: 15-20 min

C) Derivatization to *p*-bromophenacyl esters

1. Add 0.1ml of Potassium bicarbonate reagent to the dry sample.
2. Use the RapidVap System to dry the Potassium bicarbonate from the sample.
Parameters:
Temp: 80-90°C
Vortex Motion: 25-50%
Vacuum: greater than 20" Hg but less than 29" Hg
Time: 15-20 min
3. Cool the sample to ambient temperature or below and add 1.0ml of Chloroform, followed by 50µl 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 1.0ml, 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 1ml 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) 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.*

Additional information:

<http://www.usershome.com/hplcusersgroup/>

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