HPLC SEPARATION GUIDE

Identification of Mycobacterium . Intracellulare by HPLC and Computer Driven Pattern Recognition System

The cell envelopes of all Mycobacteria contain high molecular weight -branched, B-hydroxy fatty acids components called mycolic acids. All known mycolic acids have the basic structure:

$$R_2CH(OH)CHR_1COOH$$

where R_1 is a C_{20} to C_{24} linear alkane and R_2 is a more complex structure of 30 to 60 carbon atoms that may contain various numbers of carbon-carbon double bonds and/or cyclopropane rings, methyl branches or oxygen functions such as C=O, CH_3, OCH=COOH. The actual structure of mycolic acids varies by families and species.

Proper identification of microbacterial species is very crucial for clinical application. Traditional methods of identification, which are generally slow, have been used for several years. New methods of identification such as DNA probes have greatly increased the speed of identification; however, the methods are applicable to only a few species of Mycobacteria. Gas chromatographic (GC) technique is another method that has been very effective for the identification of only the short-chain fatty acids. Fragmentation residues of the larger mycolic acids during GC analysis can interfere with pattern recognition and, as such, can preclude accurate quantitation.

A new approach using the technique of high performance liquid chromatography (HPLC) ha sproven most effective for the analysis of both the short and the long-chain fatty acids[1], but has never been automated to provide species identifications with high confidence levels until recently[2].

We report here a reversd-phase HPLC method that results in fast and reproducible identification of Microbacterial species. Separation time of 10 minutes or less is achievable using the newly developed Sherlock HPLC software* (MIDI, Newark, DE) and a specially designed SMT-FatSep analytical column. The Sherlock HPLC software is fully integrated, using an external calibration standard, preforming automated peak naming (by equivalent chain length, ECL, values: Table 1), comparing composition of unknowns to a stored databse by pattern recognition, and reporting the identification along with a similarity index.

The mycolic acids are derivatized for either Ultraviolet (UV) or Fluorescence detection, using p-Bromophenacyl Bromide or 4-Bromomethyl-6, 7-Dimethoxycoumarin, and then extracted into chloroform. UV detection works quite well with large sample concentration, such as 50 mg/mL. Fluorescence detector is ideal for increased sensitivity (up to 1,000 times better) and can be applied to direct identification from raw sample[3]. MIDI offers a method and library for each of the detectors. Figure 1 and Table 1 show typical HPLC chromatogram and Sherlock sample report, respectively, of Mycobacterium intercellular.

Figure 1 and Table 1: on page 2

Column Specifications:

Particle:	Spherical silica, 3 μm
Pore Size:	120Å
Surface Area:	170 m^2/g
% Carbon:	12%
pH range:	1-12

Sherlock HPLC software was developed by Microbial ID, Inc. (MIDI) Newark, DE
*SMT wishes to thank Mr. Michael Waddington of MIDI for providing the Sherlock HPLC software used for the method development.

1. Minnikin et. al. Arch. Michrobiol
2. Waddington, M., MIDI Application Notes, May, 1996.
3. Jost et. al. J. Clin. Microbiol, 1995, 33, 1270

SEPARATION METHODS TECHNOLOGIES
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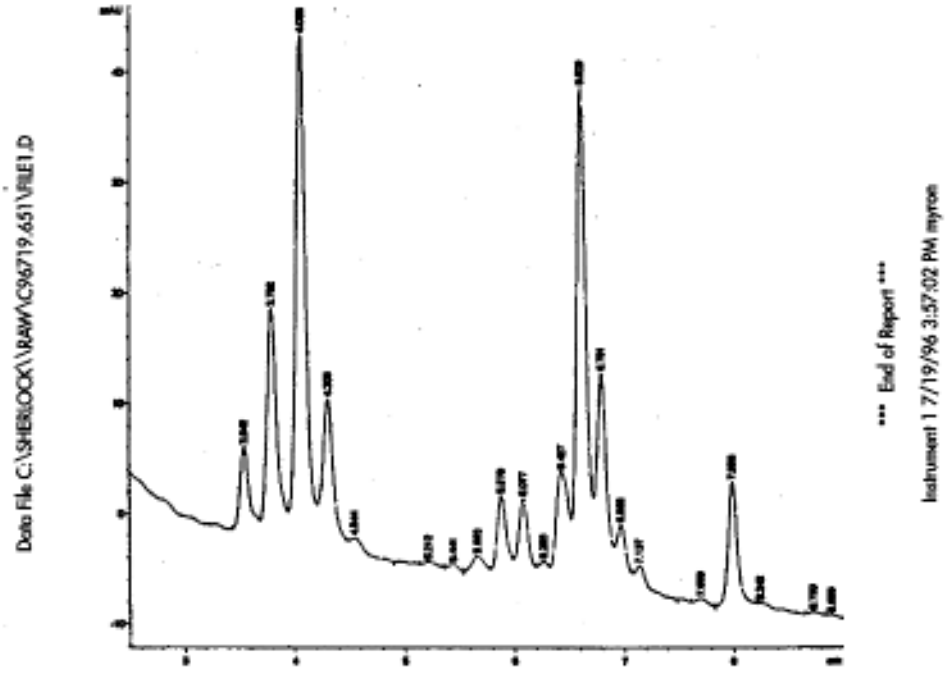
Figure 1: HPLC Chromatogram of Mycobacterium Intracellulare



Chromatographic Conditions

Column: SMT-**FatSep (70x4.6mm)**
Mobile Phase: A=MeOH B=CH2Cl2(20-65%B, 1-10min: 2%B 10.5min: 4.5min Hold)
Flow: 2.5mL/min
Detector: UV, 260nm

Table 1: Sherlock Sample Report for Above Sample

ID: 1 M-CALIB (Ken's Cal Mix, 7/10/96, MW Date of run: 19-JUL-96 15:38:50
Bottle: 1 CALIBRATION [MYCOUV]

RT	Area	Ar/Ht	Respon	RCL	Name	%	Comment 1	Comment 2
2.200	20000000	0.020	. . .	20.770	SOLVENT PEAK	. . .	> Max area	
3.542	77274	0.093	1.000	34.000	RCL 34.000	. . .		
3.782	207406	0.100	. . .	36.462	. . .	. . .		
4.063	460417	0.099	. . .	39.137	. . .	. . .		
4.305	135739	0.109	. . .	41.515	. . .	. . .		
4.566	14471	0.119	. . .	43.876	. . .	. . .		
5.212	4455	0.147	. . .	50.459	. . .	. . .		
5.461	5237	0.098	. . .	52.717	. . .	. . .		
5.663	16126	0.122	1.000	54.900	RCL 54.900	2.42	Peak match 0.0224	
5.879	73349	0.095	. . .	56.569	. . .	. . .		
6.077	73336	0.099	1.000	58.092	RCL 58.092	11.01	Peak match 0.0225	
6.263	19399	0.088	. . .	59.471	. . .	. . .		
6.427	102693	0.125	. . .	60.693	. . .	. . .		
6.609	452181	0.094	1.000	62.069	RCL 62.069	67.88	Peak match -0.0306	
6.791	197135	0.099	. . .	63.366	. . .	. . .		
6.968	62035	0.101	1.000	64.650	Sum In Feature 1	9.31	Peak match -0.0205	RCL 64.650
7.137	27365	0.148	. . .	66.057	. . .	. . .		
7.699	5492	0.112	. . .	70.732	. . .	. . .		
7.986	114146	0.096	. . .	73.119	. . .	. . .		
8.242	8866	0.270	. . .	75.247	. . .	. . .		
8.730	6908	0.132	. . .	79.308	. . .	. . .		
8.899	6489	0.132	1.000	80.715	RCL 80.715	0.97	Peak match -0.0213	
9.080	8400	0.247	1.000	83.755	Sum In Feature 6	1.26	Peak match -0.0008	RCL 83.755
9.505	10122	0.100	1.000	88.200	RCL 88.200	1.52	Peak match -0.0505	
9.627	12658	0.121	1.000	90.250	Sum In Feature 8	1.90	Peak match 0.0316	RCL 90.250
9.771	11078	0.091	1.000	92.882	RCL 92.882	1.66	Peak match 0.0164	
9.916	13763	0.139	1.000	95.400	RCL 95.400	2.07	Peak match -0.1532	
10.102	13536	0.160	. . .	103.665	. . .	. . .		
10.254	14978	0.273	1.000	110.000	RCL 110.000	. . .		
*******	62035	. . .	. . .	. . .	SUMMED FEATURE 1	9.31	RCL 64.650	RCL 65.437
*******	8400	. . .	. . .	. . .	SUMMED FEATURE 6	1.26	RCL 83.755	RCL 84.641
*******	12658	. . .	. . .	. . .	SUMMED FEATURE 8	1.90	RCL 89.417	RCL 90.250

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Xhr Ref	RCL Deviation	Ref RCL Shift
20000000	2155133	666189	30.91	666189	0	. . . . .	. . . . .

GOOD PEAK MATCHING: PEAK POSITION MATCHING ERROR (RMS) IS 0.0549.