Identification of Mycobacteria by High-Performance Liquid Chromatography

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Mycolic acids extracted from saponified mycobacterial cells were examined as *p*-bromophenacyl esters by high-performance liquid chromatography (HPLC). Standard HPLC patterns were developed for species of *Mycobacterium* by examination of strains from culture collections and other well-characterized isolates. Relative retention times of peaks and peak height comparisons were used to develop a differentiation scheme that was 98% accurate for the species examined. A rapid, cost-effective HPLC method which offers an alternative approach to the identification of mycobacteria is described.

Complex high-molecular-weight β-hydroxy fatty acids with a long alkyl chain at the α position are characteristic of mycolic acids of species of mycobacteria. Mycolic acids have been shown by various methods of fatty acid analysis to be species specific (2, 3, 7, 9, 10). By using two-dimensional thin-layer chromatography-mass spectrometry, 11 distinct structural groups of mycolic acids were identified among 50 recognized species of *Mycobacterium* (10). Gas chromatography-mass spectrometry chemical analysis of α-mycolic acid functional groups for 25 species of *Mycobacterium* demonstrated eight groups (7). Previously, we reported high-performance liquid chromatography (HPLC) patterns of the mycolic acids of seven species of mycobacteria that make up 90% of mycobacterial isolates in clinical laboratories (2, 3). We noted that mycolic acids emerge from the column either as a single, contiguous cluster of peaks or as multiple, discontinuous clusters of peaks (3). The purpose of this study was the examination of well-characterized isolates of slowly growing mycobacteria that produce single-clustered mycolic acid HPLC patterns and to demonstrate their species-specific nature.

MATERIALS AND METHODS

Mycobacteria. Standard HPLC patterns were determined by examination of culture collection isolates from the Trudeau Mycobacterial Culture Collection (TMC) or the American Type Culture Collection (ATCC) or from clinical isolates identified by the Mycobacteriology Identification Laboratory, Centers for Disease Control (CDC). Cultures examined in a training set were *Mycobacterium africanum* (TMC 5122 and 1 clinical isolate); *Mycobacterium asiaticum* (TMC 803, ATCC 25274, and 10 clinical isolates); *Mycobacterium bovis* (TMC 403 to TMC 405, TMC 407, TMC 409, TMC 410, TMC 412, TMC 602, TMC 605, TMC 606, and TMC 609); *Mycobacterium bovis* BCG, the bacillus of Calmette-Guérin (TMC 1002 [Birkhaug], TMC 1010 [Danish], TMC 1011 [Pasteur], TMC 1020 [Mexican], TMC 1021 [Australian], TMC 1024 [Glaxo], TMC 1025 [Prague], TMC 1030 [Connaught], TMC 1103 [Montreal], TMC 1108 [Pasteur], and 5 clinical isolates); *Mycobacterium gastri* (TMC

1456, ATCC 25157, ATCC 25159, ATCC 25162, and 12 clinical isolates); *Mycobacterium gordonae* (TMC 1319, TMC 1326, TMC 1327, ATCC 14490, and 10 clinical isolates); *Mycobacterium kansasii* (TMC 1203, TMC 1204, TMC 1214, TMC 1217, ATCC 12478, and 11 clinical isolates); *Mycobacterium marinum* grown at 33°C (obtained from N. Warren, Department of General Services, Richmond, Va.; identified as TI-796, TI-960, TM-909, and 9 clinical isolates); *Mycobacterium microti* (TMC 1601, TMC 1608, and TMC 1619); *Mycobacterium szulgai* (TMC 1328; cultures from E. Wolinsky, Cleveland Metropolitan General Hospital, Cleveland, Ohio identified as 954, 965, 995, and 10065; cultures from P. Jenkins, University Hospital of Wales, Cardiff, England, identified as 6555, 20886, and 25932; and 7 clinical isolates); and *Mycobacterium tuberculosis* (TMC 107, TMC 109 to TMC 112, TMC 116, TMC 120, TMC 124, TMC 125, TMC 302, TMC 305, TMC 307, TMC 309, TMC 314, TMC 320, TMC 321, TMC 323, TMC 326, TMC 331, ATCC 27244, and 10 clinical isolates). The HPLC patterns generated from the training set represented the standard patterns for the species.

Pure mycobacterial cultures in the training set were grown for 21 days at 35°C, except where indicated, on Lowenstein-Jensen slants in screw-cap tubes (20 by 150 mm). Conventional biochemical identification methods were used to confirm species (8).

Mycolic acid sample preparation. Conditions for the saponification of mycobacteria and derivatization of the mycolic acids to *p*-bromophenacyl esters have been described previously (3, 4). All TMC and ATCC cultures in the training set were subcultured and reprocessed to confirm reproducibility.

HPLC. An HPLC Beckman System Gold (Altex Division, Beckman Instruments, Inc., San Ramon, Calif.) was used with gradient elution chromatography stabilized with Beckman model 126 pumps. An IBM personal system II (model 50) computer with Beckman System Gold software controlled the instruments and processed the data. Samples were injected by using an Altex injector (model 210A), with a 20-μl sample loop. Mycolic acid samples were separated by using a Beckman C-18 ultrasphere-XL analytical cartridge column (4.6 mm by 7.5 cm) with a particle size of 3 μm. Detection of UV-absorbing esters was done with a

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Beckman model 166 detector set at 260 nm. The column was equilibrated with 98% methanol–2% methylene chloride. Solvent concentrations were programed to change after injection, over a duration of 1 min, to 80% methanol–20% methylene chloride. After this time, the solvent changed linearly to 35% methanol–65% methylene chloride over a 9-min interval with a flow rate of 2.5 ml/min. The mobile phase was then changed to the initial conditions of 98% methanol–2% methylene chloride over 0.5 min, and equilibration was continued for 1.5 min. The total run time was 12 min.

HPLC pattern interpretation. A specially prepared high-molecular-weight internal standard compound (Ribi Immunochem Research, Inc., Hamilton, Mont.) that was used to standardize the real retention times of peaks to the relative retention times (RRTs) of the peaks has been described previously (3). The locations of identical or unique peaks in the same species and in different species were determined by comparison of RRTs. Numbers were arbitrarily assigned to peaks on the basis of their progressive order of emergence from the column. Peaks with the same RRT were assigned the same number in the different species.

HPLC identification scheme. HPLC patterns from the training set were grouped by species, and peaks were assigned numbers as noted above. Obvious species differences in RRTs of peaks, total number of peaks, and peaks with the same RRT but with quantitative differences in peak size were noted. The use of RRTs alone to evaluate peak presence was not sufficient to differentiate all species. Therefore, comparable peaks were used to calculate peak height ratios. For each chromatogram, the height of a single peak was sequentially compared with all other peak heights by dividing the height of the peak that emerged first by the height of the peak that emerged next in the pattern. This process was repeated for all peaks in sequence until all ratios were determined. All peak height ratios were compared to determine which were the most definitive for a specific species. A simple flow chart decision scheme was designed by using characteristic peak height ratios as an identification method. Six peak height ratios were used to design the flow chart: peak 1 divided by peak 4, peak 2 divided by peak 3, peak 3 divided by peak 4, peak 3 divided by peak 5, peak 4 divided by peak 6, and peak 4 divided by peak 7. Calculated peak height ratio values were compared with the standard peak height ratio values in the flow chart sequentially, progressing from the top to the bottom of the chart. Subsequent peak height ratios were calculated only if the prior ratio failed to define the species.

Evaluation sets of mycobacteria. The flow chart was developed for the 11 species of mycobacteria represented in the training set. The flow chart was tested with two evaluation sets of mycobacterial strains, designated CDC and Texas. Strains in the evaluation sets were different from each other and from those in the training set. The CDC evaluation set consisted of clinical isolates that were previously identified by the CDC laboratory. These cultures were recovered from storage at −80°C, inoculated into 7H9 broths, and grown at 35°C. After 7 days, subcultures were prepared on Lowenstein-Jensen medium and incubated for an additional 21 days at 35°C before use. The following species (number of cultures) were included: *M. tuberculosis* (29 strains), *M. gordonae* (33 strains), *M. marinum* (9 strains), *M. szulgai* (22 strains), *M. bovis* (4 strains), *M. bovis* BCG (5 strains), *M. gastri* (6 strains), *M. kansasii* (13 strains), and *M. asiaticum* (8 strains).

The Texas evaluation set consisted of clinical isolates that were referred to the Texas Department of Health (TDH) for identification. Whole cells for HPLC analysis were removed directly from the original culture submitted to the TDH without additional incubation or subculture. Listed by species (number of cultures), they were as follows: *M. tuberculosis* (290 strains), *M. kansasii* (193 strains), *M. gordonae* (161 strains), *M. szulgai* (19 strains), and *M. marinum* (7 strains). The analysis conditions for HPLC, sample preparation, and instrumentation used by the TDH laboratory were exactly the same as those described for the CDC laboratory, with the exception that a Beckman autosampler (model 507) was used for sample injection by the TDH laboratory.

RESULTS

Advances in commercially available instrumentation and column technology allowed standardization of the HPLC analysis of mycolic acids to a run time of 12 min for each sample. In practical application, a single standardized culture specimen grown at 35°C on Lowenstein-Jensen medium was examined in less than 2 h. Standardization by subtraction of the absolute retention times of the peaks from the absolute retention times of the high-molecular-weight standards produced RRTs that were reliable for selecting comparable peaks (Fig. 1). In fact, RRTs compensated for all ambient conditions that affected HPLC analysis. Examination of a common peak, labeled 7, in 103 different chromatograms demonstrated a mean ± standard deviation RRT reproducibility of 1.22 ± 0.01 min. The real-time measurements for this peak recorded over a 6-month period revealed a low value of 1.16 min and a high value of 1.25 min, an overall difference of 0.09 min.

A unique HPLC pattern definitive of *M. tuberculosis* was not found among species of the *M. tuberculosis* complex. Species of *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti* produced similar HPLC patterns. Comparison of *M. bovis* BCG with members of the *M. tuberculosis* complex showed that only *M. bovis* BCG isolates produced a specific HPLC pattern with additional peaks that emerged early (Fig. 1). RRTs of the peaks for most of the species included in this study were the same except for those of species in the *M. tuberculosis* complex, which had peaks 3 to 9, and *M. marinum*, which had two additional peaks before the peak labeled 1. Usually, *M. marinum* was easily recognized by the presence of additional peaks, but because isolates showed peaks 1 to 8, which were similar to the peaks of the other species, it was included in the decision scheme.

Visual observations of the heights of comparable peaks with the same RRT were used to calculate the peak height ratios used in the empirical design of a flow chart decision scheme. Figure 2 shows the flow chart with only six peak height ratios. Identification of some species such as *M. szulgai* and *M. kansasii* required the calculation of all six peak height ratios. Identification of *M. tuberculosis*, usually the most frequently encountered clinical species, and *M. marinum* required the determination of only two of the ratios. Identifications were correctly made for all 319 strains of *M. tuberculosis*.

A total of 799 cultures representing nine species in the evaluation sets were analyzed by CDC and TDH. Additional cultures of *M. africanum* and *M. microti* were not available for testing.

All 129 cultures in the CDC evaluation set were identified correctly. In the Texas evaluation set, 661 of 670 (98.6%) strains were identified correctly. The nine strains that were
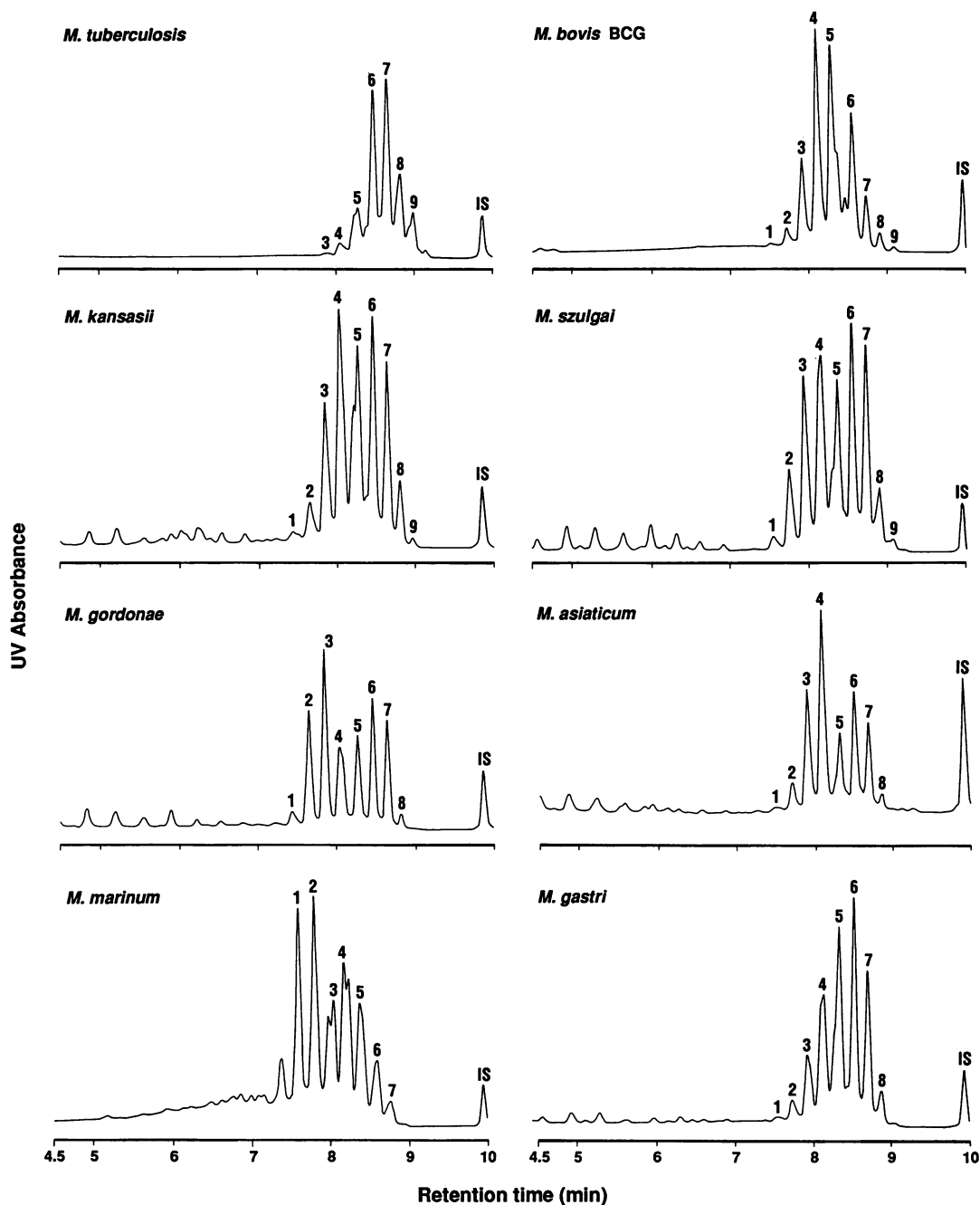
FIG. 1. Representative patterns of bromophenacyl esters of mycolic acids determined by HPLC. See text for an explanation of the *M. tuberculosis* pattern. IS, internal standard.

misidentified were representative of two species, *M. kansasii* and *M. gordonae*. Misidentification of *M. kansasii* occurred with six strains; three were misidentified as *M. gastri*, two were misidentified as *M. szulgai*, and one was misidentified as *M. bovis* BCG. *M. gordonae* strains were misidentified three times: twice as *M. szulgai* and once as *M. asiaticum*. These three strains of *M. gordonae* and the two strains of *M. kansasii* initially misidentified as *M. szulgai* were reanalyzed by HPLC from fresh subcultures grown as described for the CDC training and evaluation sets. Upon reanalysis, all five strains were correctly identified. The

strains of *M. kansasii* that were tested from original referred cultures and misidentified as *M. bovis* BCG and *M. gastri* were not recovered for reanalysis by using standardized growth conditions. Strains in the Texas evaluation set that were misidentified yielded chromatograms with relatively low detector responses because of a small sample size, resulting in peak height ratio values that fell just outside the critical ratio cutoff value in the flow chart for the correct species. It was noted that some strains of *M. gordonae* that were grown at 22 and 35°C yielded unusual chromatographic patterns with two discrete multiple clusters of mycolic ester
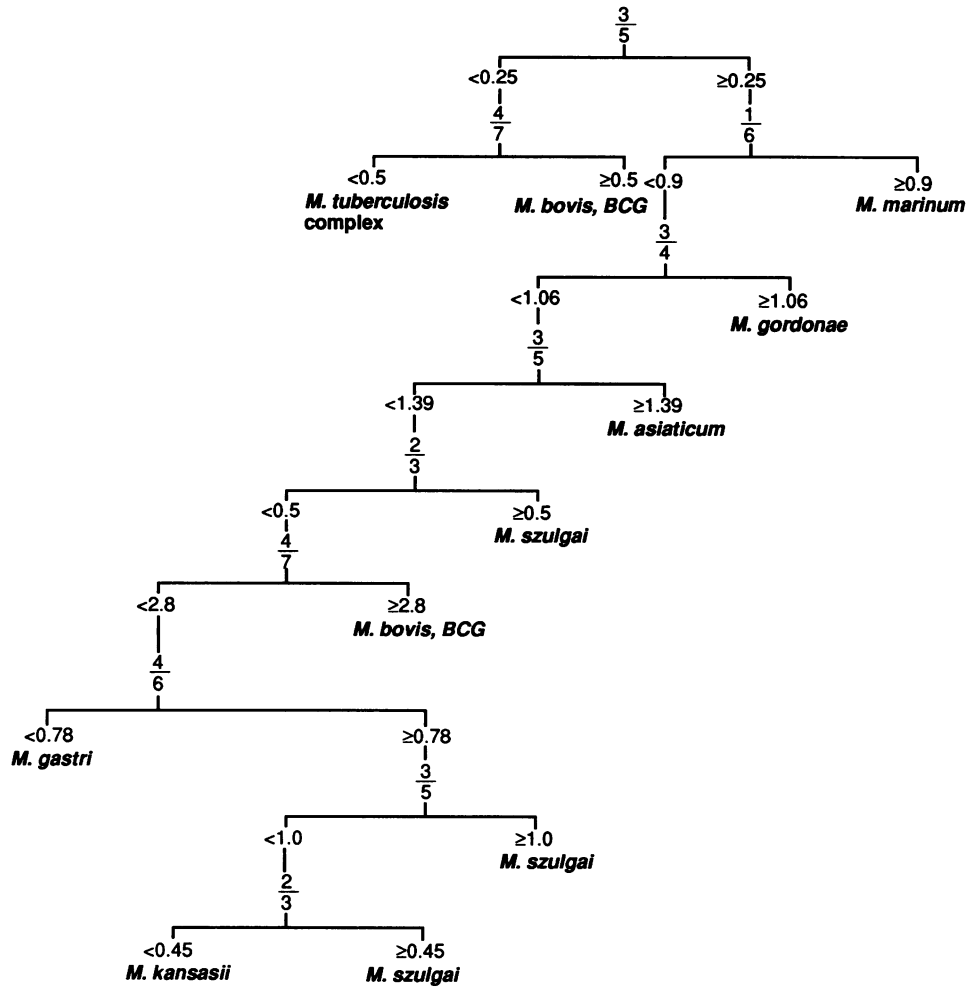
FIG. 2. Flow chart decision scheme for identification of *Mycobacterium* species on the basis of selected peak height ratios. See text for an explanation of the values.

peaks. Data on the occurrence of these strains are being accumulated (data not shown); however, the HPLC patterns that have been produced resemble those reported for *M. avium* (2). Additional studies to examine those mycobacteria which produced multiple clusters of mycolic acids are ongoing.

DISCUSSION

Considerable evidence has been reported showing that mycolic acids from mycobacteria are species specific. However, these analytical methods do not lend themselves to routine, practical use in a clinical situation. HPLC is a simple alternative to these methods. We previously used a 3-h HPLC method at CDC for preliminary species identification of rapidly growing mycobacteria (3). Simplification of this method now permits a large number of cultures to be examined in a normal 8-h workday. The HPLC pattern presentation with these conditions was almost identical to that which we reported with other conditions of analysis (2). Compared with conventional identification testing procedures, this test resulted in a greatly reduced identification time.

Analysis of strains in the Texas evaluation set was per-

formed to determine the reliability of the HPLC flow chart identifications under the conditions encountered in mycobacteria reference identification laboratories. Rapid results are desirable in such a setting, but knowledge of precise cultural growth, age, and transport conditions of the mycobacteria is not always available. Moreover, these strains represented fresh clinical isolates as opposed to strains which had been maintained in the laboratory for years. The importance of standardization of incubation temperature and medium on the HPLC patterns for species of rapidly growing mycobacteria has been reported (3). When five of the nine misidentified strains from subcultures were reanalyzed, they were correctly identified. Therefore, cultures with unspecified growth conditions that result in an identification of either *M. szulgai* or *M. asiaticum* should be repeated with standardized subcultures for final identification. The Texas evaluation set results indicated the utility of HPLC as a rapid, accurate, and cost-effective alternative to the identification of mycobacteria with genetic probes or conventional biochemical procedures.

M. tuberculosis formed the traditional complex with *M. africanum*, *M. bovis*, and *M. microti*. This was not surprising since DNA:DNA homology analysis does not demon-

strate a distinction in these species (1). Even relatively new genetic probes cannot separate these organisms (6). *M. bovis* BCG, an attenuated variety of *M. bovis* usually included in the complex, was distinguished by HPLC. Prior reports have noted that *M. bovis* BCG and *M. tuberculosis* contain different mycolic acid structural types. *M. tuberculosis* contains 77% α, 13% methoxy, and 10% keto mycolic acid structural types (14). Both *M. tuberculosis* and *M. bovis* have the same structural types of mycolic acids (10). *M. bovis* BCG does not contain methoxy mycolates (10, 13). Although not in agreement with DNA results, HPLC separation of *M. bovis* BCG strains from this complex was demonstrated (5).

The close phylogenetic relationship between *M. kansasii* and *M. gastri* determined on the basis of analysis of their 16S rRNA sequences has been described (12). It was noted by THD that the simple addition of a pigmentation criterion to the flow chart identification resulted in a specific test for these species. The erroneous identification of *M. kansasii* as *M. gastri* or *M. bovis* BCG could be avoided without additional subculturing.

A similar complexity of mycolic acids was demonstrated in chromatograms from different species. The chromatographic patterns examined were simple, demonstrating a single cluster of contiguous emerging peaks. Calculation of peak height ratios confirmed the gross visual observation that quantitative differences existed in the heights of peaks with the same RRT. The flow chart presented here correctly discriminated 790 of 799 (98.6%) of the strains examined by the peak height ratios. The scheme provides the clinical laboratory with a quick, reliable means of identification. However, sophisticated procedures for HPLC data analysis and pattern recognition are being examined (11).

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