Utility of High-Performance Liquid Chromatography for Identification of Mycobacterial Species Rarely Encountered in Clinical Laboratories

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High-performance liquid chromatography (HPLC) has been demonstrated to be a suitable technique for determining the species of mycobacteria on the basis of their mycolic acid pattern. Representative HPLC profiles, which are needed for the visual recognition of chromatograms, have been published for the most frequently encountered mycobacterial species. No extensive study has been reported for less common species, and only a few, scattered chromatographic patterns are available in literature. This study evaluates the utility of this technique for the identification of several rare species. *Mycobacterium celatum*, *Mycobacterium genavense* and *Mycobacterium simiae* chromatographic profiles have been verified, and previously unreported profiles of other species investigated. The chromatographic pattern of *Mycobacterium malmoense* is presented for the first time.

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With the exception of the few species for which commercial genetic probes (Gen-Probe, USA) are available, the identification of nontuberculous mycobacteria still relies on the use of cumbersome conventional tests, which take from two to six weeks. Analysis of mycobacterial fatty acids reveals a variety of high molecular-weight α-alkyl, β-hydroxy acids (mycolic acids), whose distribution in the cell wall is species-related; their characterization has been used for the identification to the species level of such organisms (1–4). The potential of high-performance liquid chromatography (HPLC) for elucidating the distribution of mycolic acids, after they have been converted to bromophenacyl esters, has been well documented. This system has been demonstrated as adequate for identification of the most frequently encountered mycobacteria, for whom the representative patterns have been published, by means of visual or computer-aided comparison of relevant profiles (5–11). As the method is at present well standardized, we tested its usefulness for the identification of the less frequently encountered mycobacteria, which at times constitute a challenge even for the experts.

Materials and Methods. Part of our laboratory collection of nontuberculous mycobacteria, seven reference strains and several recent clinical isolates (Table 1), were subjected to the HPLC procedure developed at the Centers for Disease Control (CDC) (7). The identification of all the strains was achieved using conventional methods (12) and was confirmed using nucleic acid hybridization for all species for which genetic probes are commercially available. For ambiguously identified strains, the nucleotide sequence of 16S rRNA was also determined (13). For each strain, a 10-μl loopful of colonies recently grown on Middlebrook 7H11 medium was saponified in ethanolic potassium hydroxide (25 % KOH in 50 % ethanol) in an autoclave for 1 h at 121°C. After acidification with 18.5 % HCl, mycolic

acids were extracted with chloroform and derivatized to their UV-adsorbing p-bromophenacyl esters (14); after a further acidification a chloroform extraction was finally performed.

One hundred µl of the extract was spiked with 5 µl of a high molecular-weight standard (Ribi; ImmunoChem, USA), diluted to 0.5 mg/ml and 5 µl were injected into the reverse phase C-18 ultrasphere-XL cartridge column of an HPLC System Gold model (Beckman, USA), equipped with a 166 model detector set at 254 nm. The elution conditions changed linearly, from a 98 % methanol, 2 % dichloromethane mixture to an 80 %, 20 % mixture in 1 min and to a 35 %, 65 % mixture over the next 9 min (flow rate = 2.5 ml/min). At the end of the run the column was reequilibrated for 2 min to the initial conditions.

Peaks were identified on the basis of their retention times, relative to that of the internal standard (RRT) and were labelled as suggested by the CDC (11).

The bromophenacyl ester profiles were matched visually with those of all the species whose representative patterns had been published (5–9, 17). For rare mycobacteria, whose HPLC patterns are not available in the literature, the comparison with published chromatograms was used to check the uniqueness of their profiles. In the latter cases, the profiles obtained from reference strains or from genotypically identified isolates were taken as references for further comparisons; when multiple isolates were available the intraspecies variability of the pattern was assessed.

Results and Discussion. HPLC analysis was performed on 109 isolates representing 16 nontuberculous mycobacterial species (Table 1).

The visual match with published chromatograms, assisted on occasion by the comparison of the peaks' RRTs, allowed a correct identification of all the isolates belonging to the species *Mycobacterium asiaticum*, *Mycobacterium kansasii*, *Mycobacterium gordonae*, *Mycobacterium szulgai*, *Mycobacterium xenopi* and *Mycobacterium marinum*.

On the contrary, the bromophenacyl patterns of *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium paratuberculosis* and *Mycobacterium scrofulaceum* were very similar and did not always allow a distinction between species. Five isolates conventionally identified as belonging to the *Mycobacterium avium* complex but unable to hybridize with commercial DNA probes for either *Mycobacterium avium* or *Mycobacterium intracellulare* (AccuProbe; GenProbe, USA) displayed a profile indistinguishable from those of other strains of *Mycobacterium avium* or *Mycobacterium intracellulare*. The 16S rRNA sequences, determined in Hannover by E.C. Böttger, attributed all five such isolates to the species *Mycobacterium intracellulare*.

Our five strains of *Mycobacterium simiae* yielded very characteristic chromatograms, with three easily recognizable clusters of peaks (Figure 1). Peaks arranged in three clusters were also presented by the newly described species *Mycobacterium genavense* (15, 16), which however could be recognized mainly by the lack of a terminal peak (Figure 1), as already reported (17). The identification of *Mycobacterium malmoense* was very easy, thanks to its uninterrupted sequence of peaks (Figure 1). Our two strains of the new species *Mycobacterium celatum* showed bromophenacyl ester profiles identical to the '*Mycobacterium xenopi*-like' profiles already reported for a group of unidentified strains (9) which later became the basis for the description of the new species *Mycobacterium celatum*. A closer inspection revealed that the coincidence of patterns with *Mycobacterium xenopi* is limited to the first cluster of peaks, as those of the second one, although similar, can be differentiated on the basis of their RRTs (Figure 1). Unique profiles were also presented by rarely encountered species like: *Mycobacterium haemophilum*, *Mycobacterium shimoidei* and *Mycobacterium thermoresistibile*.

The use of bromophenacyl esters of mycolic acids for the identification of mycobacteria is presently a well standardized technique which gives consistent results. The system is neither quantitative nor able to determine the nature of the mycolic acids extracted from the mycobacterial cells, however it is suitable for rapid species identifications.

Table 1: Strains subjected to HPLC analysis.

Species	No. of strains
Mycobacterium gordonae	30
Mycobacterium avium	22
Mycobacterium kansasii	18
Mycobacterium xenopi	10
Mycobacterium intracellulare	8
Mycobacterium simiae	6
Mycobacterium scrofulaceum	5
Mycobacterium malmoense	3
Mycobacterium genavense	2
Others*	7

*One strain each of the following species: *M. asiaticum*, *M. haemophilum*, *M. marinum*, *M. paratuberculosis*, *M. shimoidei*, *M. szulgai*, *M. thermoresistibile*.

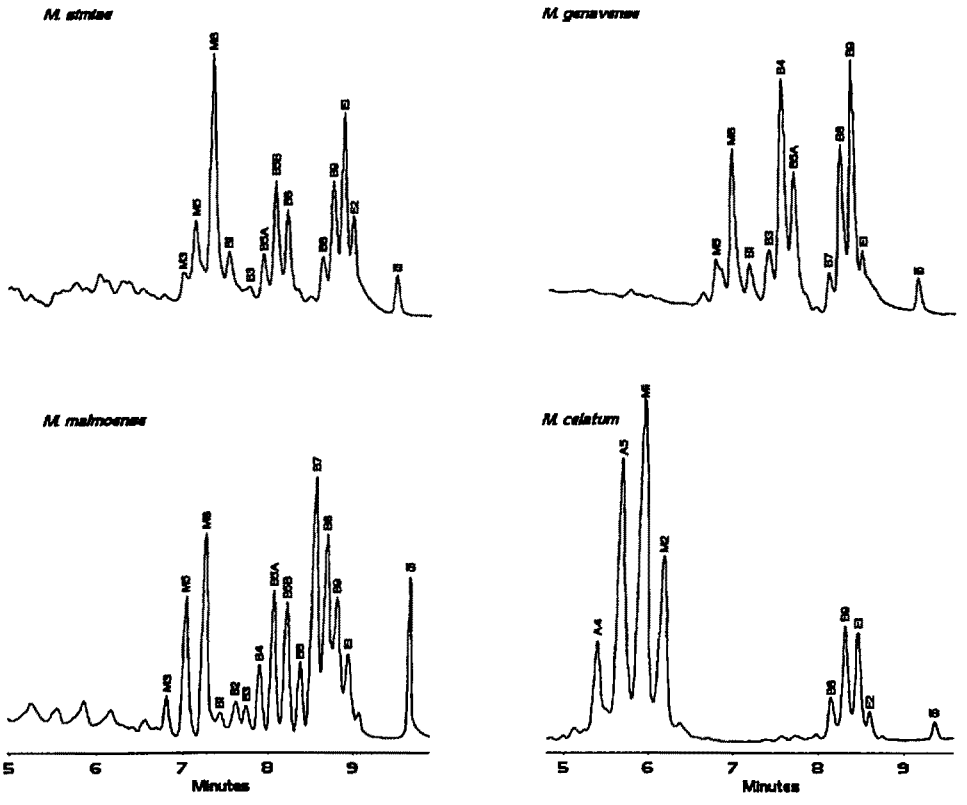

Figure 1: Representative HPLC pattern of the bromophenacyl esters of mycolic acids. (IS: internal standard).

Reference chromatograms are so far available only for the species more frequently encountered in clinical laboratories and are not available for the rare or fastidious mycobacteria.

Although the limited number of samples available imposes some caution, this study shows that the adoption of this technique for the identification of the less frequent mycobacterial species is extremely promising, especially in view of the fact that no commercial probes are likely ever to be developed for their identification.

A limitation in the accuracy of identification was found for isolates of the *Mycobacterium avium* complex and of *Mycobacterium scrofulaceum*, which presented patterns not consistently different. For these species, the study of supplemental features is highly recommended for a correct identification.

The patterns of other strains considered in this work were all easily distinguishable between species and consistently similar within species. The simple visual inspection of chromatograms, aided by peak identification on the basis of their RRT, allowed the identification of all the rare microorganisms tested.

Interestingly, HPLC seems less subject to variability than other characteristics. Such is the case with *Mycobacterium kansasii,* of which two biotypes have been recently reported, one of which fails to hybridize with commercial DNA probes (18); no difference in the two HPLC profiles has been observed. Similarly, the mycolic-acid pattern was uniform within a species, even when strains showed variable phenotypic traits (for example, niacin accumulation and photochromogenicity in *Mycobacterium simiae*).

In conclusion, we confirm the usefulness of HPLC profiles for the species identification of mycobacteria, and we endorse it for the less frequently encountered isolates. Even in the absence of a matching reference pattern, HPLC may help to avoid, on the basis of profile incompatibility, incorrect identifications that could spring from conventional procedures. The publication of further profiles will contribute to the diffusion of this rapid technique in mycobacteriological laboratories, as will the adoption of a

standard method for labelling the peaks; the CDC scheme (11), which we followed, seems adequate for this. The exploitation of automatic algorithms for the comparison of chromatograms will undoubtedly enhance the performance of this method (11).

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