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High-Performance Liquid Chromatography of Mycolic Acids as a Tool in the Identification of *Corynebacterium*, *Nocardia*, *Rhodococcus*, and *Mycobacterium* Species

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High-performance liquid chromatography of bromophenacyl esters of mycolic acid was used as an aid to assign a particular organism to one of four mycolic acid-containing genera. A gradient elution system, with methanol and chloroform, was used to distinguish representative mycolic acid patterns for the genera *Corynebacterium*, *Rhodococcus*, *Nocardia*, and *Mycobacterium*.

Mycolic acids are high-molecular-weight α-branched, β-hydroxy fatty acids found in species of *Corynebacterium*, *Nocardia*, *Rhodococcus*, and *Mycobacterium* (2, 5, 7, 11). The number of carbon atoms which make up the mycolic acids varies from C_{20} to C_{36} in the genus *Corynebacterium* (12) to C_{60} to C_{90} in the genus *Mycobacterium* (11). Mycolic acids of the *Nocardia* and *Rhodococcus* species have chain lengths ranging from C_{36} to C_{66} (1). The insolubility of the long-chain mycobacterial mycolic acids in ethanol-diethyl ether mixtures has been used for the separation of the closely related bacteria (9). The production of multispot mycolic acid patterns by two-dimensional thin-layer chromatography is an additional technique for the differentiation of mycolic acid-containing bacteria (13). Cleavage products of mycolic acids detected by gas chromatography (10, 18) and gas chromatography mass spectrometry (3, 6) provide characteristic profiles of mycolic acid composition useful in chemotaxonomic identification schemes. High-performance liquid chromatography (HPLC) has been used to separate the various classes of mycolic acids in mycobacteria (15, 16). This separation is based on the chain lengths, the degree of unsaturation, and other functional groups found in these fatty acids (17).

Clinical and reference laboratories can experience difficulty when using growth characteristics, biochemical tests, and acid-fast staining to assign certain organisms to one of the four related genera. Thus, the purpose of this study was to determine whether HPLC could be used to separate one genus from another based on their characteristic mycolic acid profiles or retention times.

The following mycobacteria and other mycolic acid-containing bacteria (27 isolates) were obtained from laboratories of the Centers for Disease Control, except as otherwise indicated: *Corynebacterium ulcerans* (KC 217, KC 219), *Corynebacterium pseudotuberculosis* (KC 1365, E 1195), *Corynebacterium diphtheria* (F 5171), *Corynebacterium renale* (KC 1288), *Rhodococcus equi* (F 5849), *Rhodococcus rhodochrous* (ATCC 4276), *Rhodococcus rubropertinctus* (1054), *Nocardia asteroides* (B1042, 84-041085, 84-041075, 84-041618), *Nocardia brasiliensis* (84-032988, 84-008478, 77-020474, 84-041618), *Nocardia caviae* (76-073661A, 83-013735, 77-075177, 77-044065, 84-0477101), *Mycobacterium smegmatis* (derived from ATCC 607), *Mycobacterium fortuitum* biovar *peregrinum* (TMC 1547), *Mycobacterium chelonae* subsp. *abscessus* (TMC 1543), *Mycobacterium bovis* (83-3005), and *Mycobacterium tuberculosis* (83-4021). The mycobacteria were grown in 100 ml of Middlebrook 7H9 broth with 0.2% glycerol–0.05% Polysorbate 80. The medium was supplemented with 10% albumin-dextrose-catalase enrichment (Difco Laboratories, Detroit, Mich.). The corynebacteria, rhodococci, and nocardiae were grown in 0.5% yeast extract broth containing 1% glycerol and 1% tryptone. All cultures were grown at 35°C with continuous shaking to an A_{650} of 0.6. The cells were harvested by centrifuging at 2,500 × *g* for 15 min. Harvested cells (30 mg [wet weight]) were saponified overnight at 85°C in 10 ml of 50% ethanol containing 5% potassium hydroxide. The saponification mixture was acidified (pH 2.0) with 6 N hydrochloric acid followed by the addition of 20 ml of chloroform (15). After mixing and centrifuging at 2,500 × *g* for 5 min, the chloroform layer containing the fatty acids was carefully removed and added to 25-ml round-bottomed flasks. The solvent was evaporated, and the residue was suspended in 3 ml of chloroform and derivatized.

The derivatization procedure for the fatty acids was as described by Durst et al. (4) and modified by Pei et al. (14). The fatty acids were converted to their *p*-bromophenacyl esters by reacting with *p*-α-dibromoacetophenone and dicyclohexyl-18-crown-6 ether, under reflux. These reagents and directions for use were available in kit form from Alltech Associates, Inc., Applied Science Div., State College, Pa. Authentic bromophenacyl esters of mycolic acids derived from *M. smegmatis*, kindly provided by Kuni Takayama, Veterans Administration Hospital, Madison, Wis., were used as standards for comparison of retention times to the derivatized fatty acids.

HPLC was conducted on a Beckman model 330 liquid chromatograph (Beckman Instruments, Inc., Berkeley, Calif.) equipped with an Altex/Hitachi model 155 UV detector for measuring A_{254}, two Beckman model 110A solvent pumps, and a model 420 pump controller.

The mycolic acid *p*-bromophenacyl esters (5-μl samples) were applied to a 5-μ particle size, C_{18} reverse-phase column (4.6 mm by 25 cm) equilibrated in 91% methanol–9% chloroform. After injection of the sample, the gradient was

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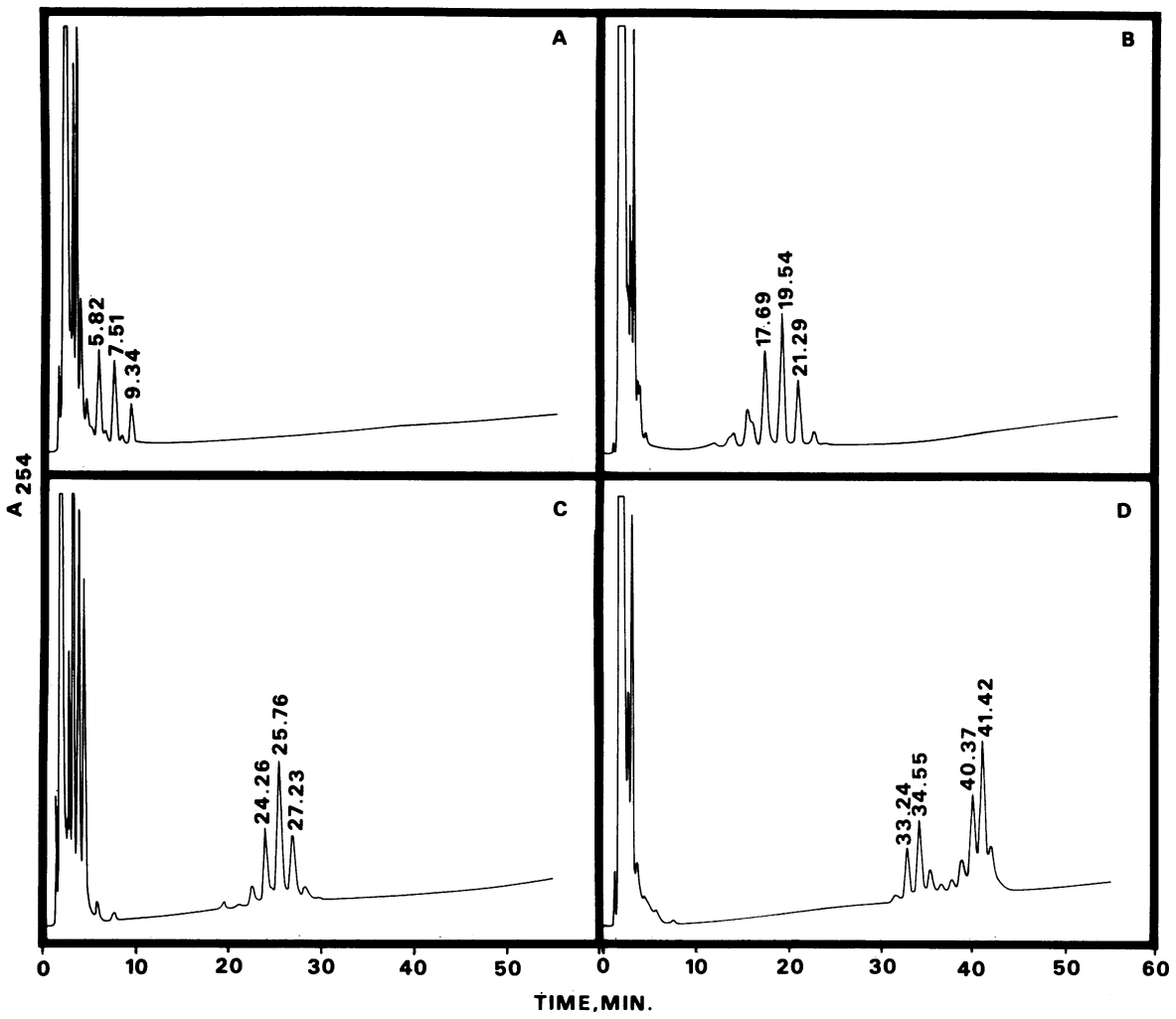

FIG. 1. Reverse-phase HPLC of *p*-bromophenacyl esters of mycolic acids from representative isolates: *C. diphtheria* (A), *R. rhodochrous* (B), *N. brasiliensis* (C), and *M. smegmatis* (D).

changed linearly to 30% methanol–70% chloroform over a period of 65 min at a total flow rate of 2 ml/min.

The shorter-chain mycolic acids of the six isolates of corynebacteria eluted first, with overall retention times ranging from 5.30 min for the first peak to 11.41 min for the last peak to emerge. A typical chromatographic profile of *C. diphtheria* is shown in Fig. 1A. Materials eluting before 5 min were derivatizing reagents and noncorynomycolate fatty acids. Mycolic acids extracted from *Rhodococcus* species exhibited retention times from 7.16 to 19.28 min for *R. equi* and 26.79 to 30.88 min for *R. rubropertinctus*. Strains of *R. rubropertinctus* have been reported to have relatively long chain fatty acids compared with *R. equi* (8). *R. rhodochrous* exhibited intermediate retention times of 13.70 to 23.03 min (Fig. 1B). There was some overlap of the mycolic acid profile of *R. equi* with the corynebacteria; approximately 8 min longer was required for the elution of the last mycolic acid peak from this species compared with the corynebacteria, thus affording easy separation of the two genera. The 13 *Nocardia* isolates had retention times from 21.07 to 29.75 min for complete resolution of the mycolic acids. Unlike the rhodococci studied, the three *Nocardia* species had similar overall retention times. The longer-chain mycolic acids of *R. rubropertinctus* overlapped the early emerging *Nocardia* peaks; however, a delay of approximately 4 min in the emergence of the first mycolic acid peak in *R. rubropertinctus* compared with the *Nocardia* isolates was used to separate these organisms. A representative HPLC chromatogram of the mycolic acids from *N. brasiliensis* is shown in Fig. 1C.

The use of HPLC to separate the bromophenacyl esters of mycobacterial mycolic acids was first described by Steck et al. (16) and Qureshi et al. (15). The time for emergence of the first mycolic acids of mycobacteria was longer than for the other three genera examined. There was a difference in retention times between the slowly growing *M. bovis* and *M. tuberculosis* (40.31 to 45.94 min) compared with the rapidly growing *M. smegmatis*, *M. fortuitum*, and *M. chelonei* (31.33 to 41.99 min). The mycolic acid profile of *M. smegmatis* is shown in Fig. 1D.

Extracted and derivatized mycolic acids from species representing each of the four genera studied were mixed and injected into the HPLC system to establish the effectiveness of this procedure in resolving a mixture of mycolic acids with short, intermediate, and long chain lengths. HPLC was capable of fractionating the mycolic acid ester mixture with

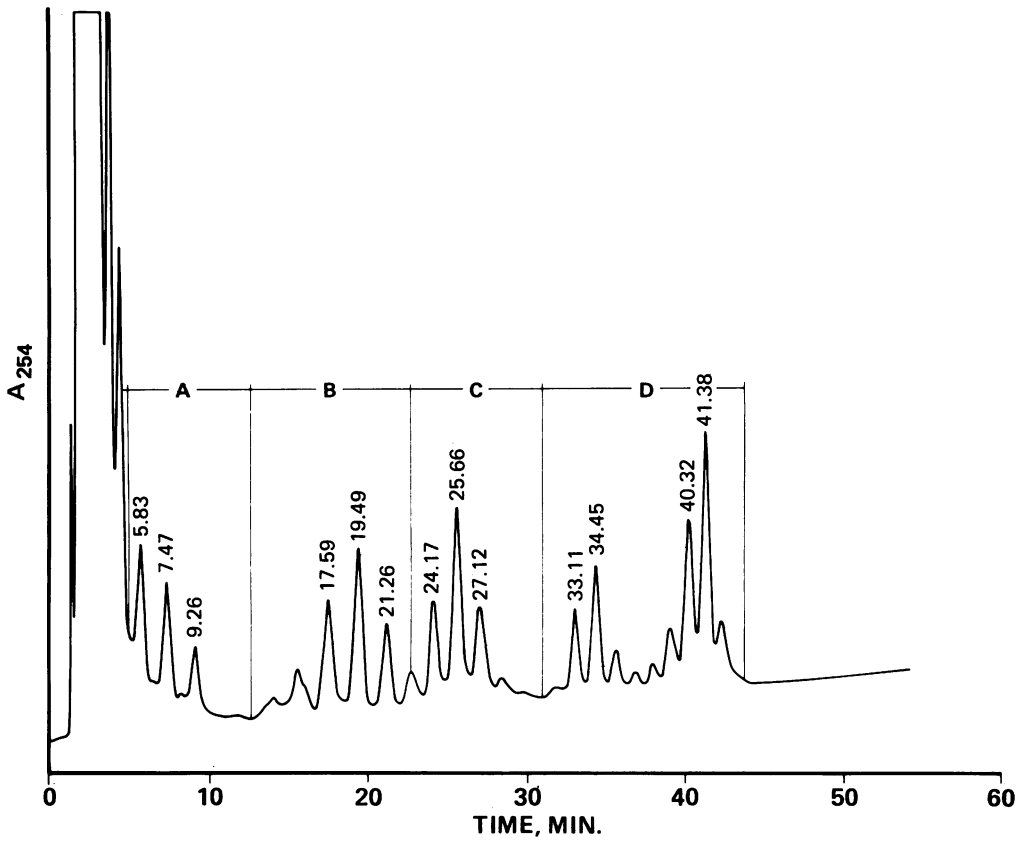

FIG. 2. Fractionation of a mixture of *p*-bromophenacyl esters of mycolic acids prepared from *C. diphtheria* (A), *R. rhodochrous* (B), *N. brasiliensis* (C), and *M. smegmatis* (D).

retention times comparable to that of individual injections (Fig. 2). The reproducibility of mycolic acid retention times was examined by making 10 repetitive injections of the mycolic acids isolated from *R. rhodochrous*. The standard error of the mean was less than 0.1 min. The age of the culture or the growth medium (Middlebrook 7H9 or yeast extract broth) did not affect the mycolic acid patterns or the retention times.

This report shows that HPLC analysis of mycolic acids may provide a rapid and specific procedure for the identification of mycolic acid-containing bacteria.

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