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Separation of *Mycobacterium bovis* BCG from *Mycobacterium tuberculosis* and *Mycobacterium bovis* by Using High-Performance Liquid Chromatography of Mycolic Acids

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Profile analysis of mycolic acid ester patterns of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium bovis* bacillus Calmette-Güerin (BCG) using high-performance liquid chromatography indicated that separation of BCG from *M. tuberculosis* and *M. bovis* by elution and relative retention times is possible. Mycolic acid patterns of BCG eluted from the column 0.5 min before *M. tuberculosis* or *M. bovis*, resulting in relative retention times for two peaks not seen in the pattern of *M. tuberculosis* or *M. bovis*. Identification was confirmed by phage typing, which has been the standard procedure for confirmation of BCG strains. These results showed that high-performance liquid chromatographic analysis of mycolic acid esters can be used in the mycobacterial reference laboratory for separation of BCG from *M. tuberculosis* and *M. bovis*.

The *Mycobacterium tuberculosis* complex contains subspecies such as *Mycobacterium bovis* bacillus Calmette-Güerin (BCG) that are difficult to differentiate by conventional biochemical methods. DNA restriction fragment analysis has shown that BCG strains are most closely related to virulent strains of the *M. tuberculosis* complex (5). BCG is used in the treatment of tumors in cancer patients and is the preferred treatment for superficial stages of bladder cancer (7). It is therefore important to distinguish it reliably from virulent strains of the *M. tuberculosis* complex (1, 6, 9, 12). Until recently, the standard procedure for identifying BCG at the Centers for Disease Control (CDC) was phage typing, which required at least 3 weeks for completion. Since phage typing is no longer performed routinely at CDC, an alternative and rapid method of identifying BCG using high-performance liquid chromatography (HPLC) of mycolic acid esters was investigated (4).

Bacterial strains. A total of 84 strains were tested: (i) 37 clinical laboratory strains identified as *M. tuberculosis* (15 strains), *M. bovis* (4 strains), BCG (16 strains), or *M. tuberculosis* complex (2 strains) by conventional biochemical methods (11); (ii) 15 strains of *M. bovis* originally isolated from animal tissues from U.S. Department of Agriculture, Ames, Iowa, TB nos. 1349, 1700, 1877, 1892, 2428, 2662, 3283, 3409, 3591, 4928, 6111, 6119, 6146, 6751, and 6752; (iii) 2 clinical strains identified as *M. tuberculosis* complex by HPLC only (4); (iv) and the remaining 30 mycobacterial strains, used as controls, from the American Type Culture Collection and the Trudeau Mycobacterial Culture Collection (which is now maintained at the American Type Culture Collection), which included 10 *M. tuberculosis* strains (TMC 108, TMC 109, TMC 117, TMC 203, TMC 302, TMC 314, TMC 320, TMC 327, TMC 331, and ATCC 27294), 10 *M. bovis* strains (TMC 403, TMC 404, TMC 405, TMC 409, TMC 410, TMC 412, TMC 602, TMC 605, TMC 606, and TMC 609), and 10 BCG strains (TMC 1010 [Daniel], TMC 1013 [Brazilian], TMC 1020 [Mexican], TMC 1021 [Australian], TMC 1022 [Russian], TMC 1025 [Prague], TMC 1029 [Phipps], TMC 1030 [Connaught], TMC 1103 [Montreal], and TMC 1028 [Tice]).

Tests. Mycobacteria were grown on Lowenstein-Jensen medium (Carr-Scarborough Microbiologicals, Atlanta, Ga.) at 37°C for 14 to 21 days or until sufficient growth for derivatization was obtained. Growth scraped from slants was saponified in 2 ml of 25% potassium hydroxide in 50% ethanol and extracted with 2 ml of chloroform. The mycolic acids were derivatized to bromophenacyl esters by the procedure first described by Durst et al. (8) and modified by Butler et al. (2).

Chromatograms were produced using System Gold chromatography software (version 3.1) (Beckman Instruments, Inc., San Ramon, Calif.). HPLC detection of mycolic acid esters was performed using a Milton Roy UV monitor with 254-nm fixed wavelength (Milton Roy Co., Riviera Beach, Fla.). High Molecular Weight Internal Standard for HPLC (Ribi ImmunoChem Research, Inc., Hamilton, Mont.) was used as a reference standard for peak location and relative retention time determinations.

Samples were injected into Hewlett Packard 1050 Series Autosampler (model 79855A; Hewlett Packard Co., Avondale, Pa.). Two solvent gradient methods controlled by a 406 Analog Interface (Beckman) and two 110B pumps (Beckman) were used.

Method A. Method A was developed at the CDC using a reverse-phase C-18 column (22 cm by 2.1 mm) (Beckman). The initial solvent conditions were 10% chloroform–90% methanol, with a flow rate of 0.6 ml/min. The chloroform concentration increased linearly to 25% in 1.0 min and to 70% over 20 min and then equilibrated to 10% in 1.0 min. At 22.1 min, the flow rate changed to 0.8 ml/min for 17.9 min before returning to the initial flow rate for the final 2.0 min.

Method B. Method B was a modified version of a method developed at the Texas State Health Department Laboratory, Austin, Tex., using an ultrasphere reverse-phase C-18 column (4.6 mm by 70 mm) (Beckman). The initial solvent conditions were 20% methylene chloride–80% methanol. The methylene chloride concentration increased linearly to

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TABLE 1. Relative retention times of major peaks produced by chromatograms of control strains of BCG, *M. tuberculosis*, and *M. bovis*

Peak[a]	Mean relative retention time (min) ± SD (n = 10)		
	BCG	*M. tuberculosis*	*M. bovis*
b	0.717 ± 0.002		
c	0.741 ± 0.002		
d	0.765 ± 0.002	0.768 ± 0.002	0.768 ± 0.0006
e	0.799 ± 0.001	0.799 ± 0.001	0.801 ± 0.001
f	0.823 ± 0.001	0.822 ± 0.001	0.824 ± 0.001
g		0.846 ± 0.001	0.846 ± 0.001

[a] See text for explanation of major peaks.

40% in 1.0 min and to 60% over 4.5 min and then held at 60% for 0.5 min before equilibration. A flow rate of 3.0 ml/min remained constant for a total run time of 8.0 min.

Phage typing was performed at CDC Mycobacterial Phage Laboratory using mycobacteriophages 33D(Warsaw) and DS6A as described by Jones (9, 10). Phage 33D(Warsaw) lysed all strains of *M. tuberculosis* and *M. bovis* but failed to lyse BCG strains. Phage DS6A lysed all strains of *M. tuberculosis*, *M. bovis*, and BCG and was used as a control to ensure that the cultures being tested belonged to the *M. tuberculosis-M. bovis*-BCG complex.

Chromatograms were produced using methods A and B. Both methods produced comparable results; however, the data and illustrations shown were obtained with method B. The peak produced by the high-molecular-weight standard was used as a reference peak to adjust and calculate the relative retention time differences between peaks from run to run. Peaks were selected and labeled by the HPLC program on the basis of a peak identification table established by our laboratory from multiple runs of mycobacterial cultures. Minimum peak height, peak width, and peak threshold were determined from data of multiple runs and incorporated in the program to eliminate peak readings generated by trace mycolic acids or background noise. Major peaks for a species were those with a height greater than 2% of the total for all peak heights in the chromatogram. The major peaks for each species were labeled according to their relative retention time. Relative retention time was calculated by dividing the retention time of each peak by the retention time of the reference peak for each chromatogram. Daily changes in ambient laboratory conditions were detected by examination of a chromatogram from a *Mycobacterium avium* strain routinely tested in our laboratory for 3 years. This strain was confirmed positive for *M. avium* with a species-specific RNA-DNA probe (GenProbe, Inc., San Diego, Calif.). Retention times calculated for this strain indicated that peaks were labeled correctly. Any peak shift that occurred from changes in conditions was shown to be relative to the reference standard.

The peaks for BCG eluted from the column 0.5 min before those for *M. tuberculosis* and *M. bovis*. The reproducibility of the relative retention times for major peaks was calculated for each species tested (Table 1). The major peaks for BCG were designated b, c, d, e, and f (Fig. 1A). The major peaks

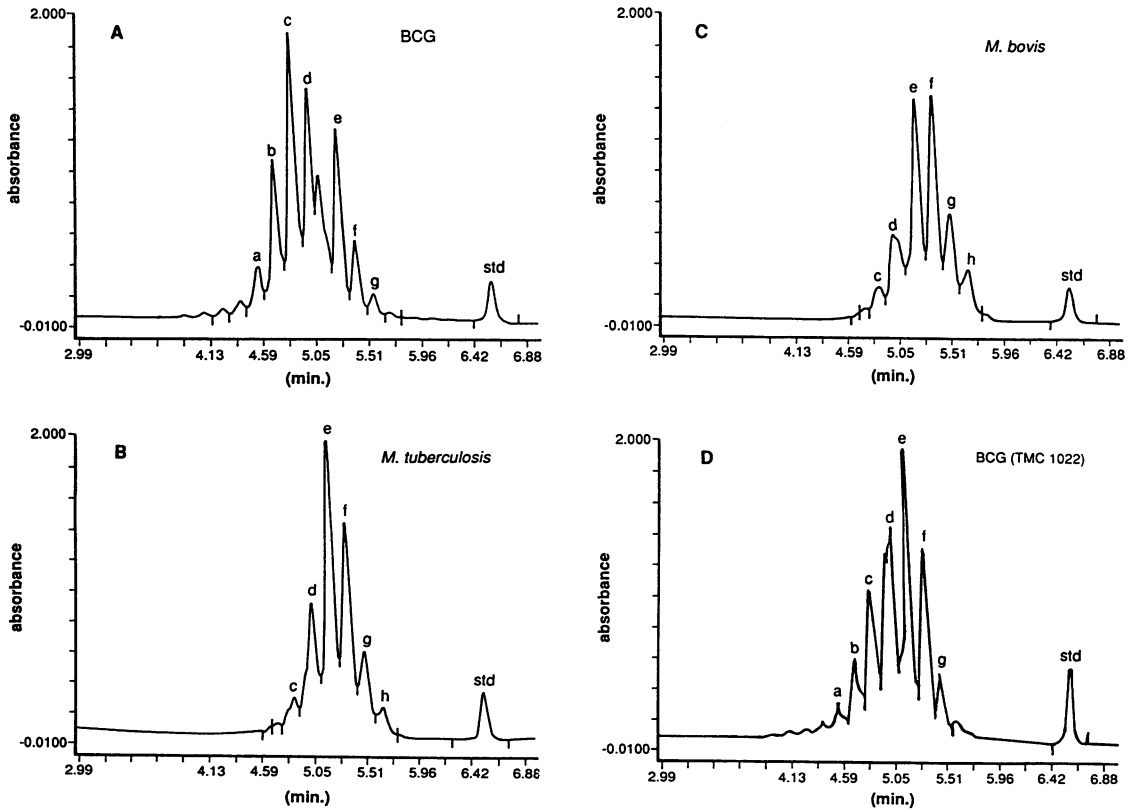

FIG. 1. Chromatograms of clinical strains of BCG, *M. tuberculosis*, *M. bovis*, and BCG (TMC 1022). std, high-molecular-weight standard.

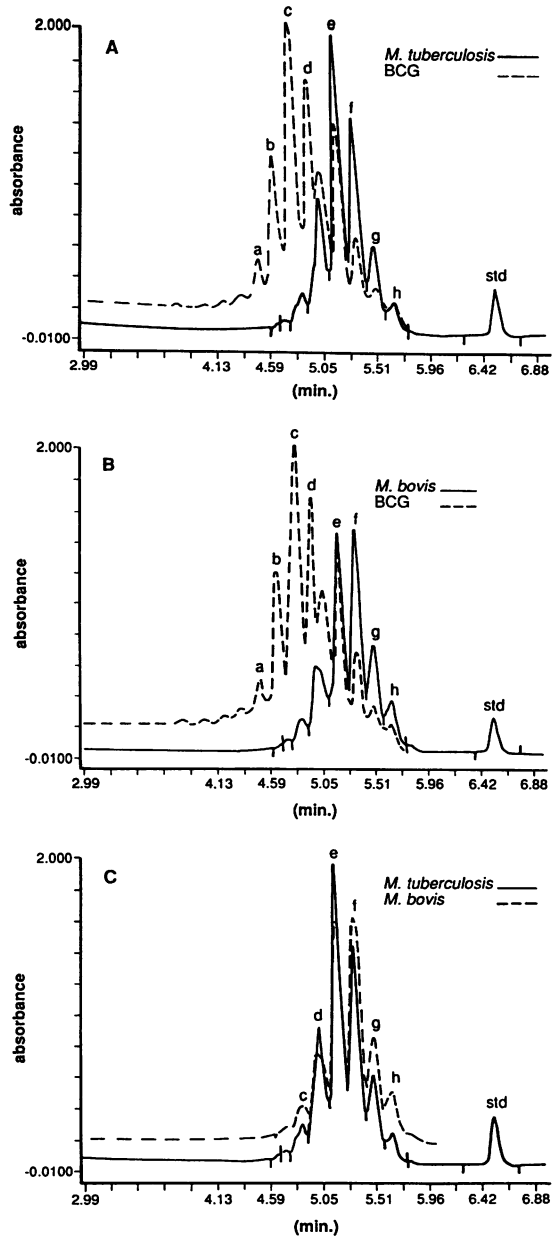

FIG. 2. Superimposed chromatograms of *M. tuberculosis* and BCG, *M. bovis* and BCG, and *M. tuberculosis* and *M. bovis*. std, high-molecular-weight standard.

for *M. tuberculosis* and *M. bovis* were designated d, e, f, and g (Fig. 1B and C).

Chromatograms of BCG produced distinct patterns that had at least two major peaks, b and c, not in common with *M. tuberculosis* or *M. bovis* (Fig. 2A and B). BCG patterns had one to three shoulder peaks between peaks d and e, which varied from strain to strain (Fig. 1A). The Russian strain of BCG (TMC 1022) produced an HPLC pattern that was different in appearance from those of the other BCG strains (Fig. 1D). However, the elution and relative retention times for BCG (TMC 1022) were consistent with BCG data.

Chromatograms of *M. tuberculosis* and *M. bovis* produced patterns that were similar in appearance with no differences in elution or relative retention times (Fig. 1B and C and 2C). Analysis of HPLC patterns of *M. tuberculosis* and *M. bovis* indicated that variation within the same species was not definitive enough to separate one strain from another.

The 84 strains tested were identified by HPLC as BCG or *M. tuberculosis* complex (not BCG). Butler et al. (3) reported that HPLC of *M. tuberculosis*, *M. bovis*, *Mycobacterium microti*, and *Mycobacterium africanum* produced similar patterns. Thus, HPLC by this method did not produce a unique pattern and could not be used to differentiate *M. tuberculosis* from *M. bovis*.

Analysis of HPLC profiles of mycolic acid esters demonstrated that pattern recognition by elution and relative retention times differentiated BCG from *M. tuberculosis* and *M. bovis*. The BCG peaks eluted earlier from the column than the peaks for *M. tuberculosis* and *M. bovis*. The late-emerging peaks of BCG and early-emerging peaks of *M. tuberculosis* and *M. bovis* shared common positions with the same relative retention times. The elution times and relative retention times for *M. tuberculosis* and *M. bovis* were the same.

Phage typing results confirmed HPLC identification of BCG strains and placed remaining strains in a group designated *M. tuberculosis-M. bovis*. Agreement between HPLC results and phage typing results was 100% for the 84 strains tested. Moreover, HPLC agreement with phage typing results confirmed that this procedure could be used to differentiate BCG from *M. tuberculosis* and *M. bovis*. Identification of BCG by HPLC was possible within 24 h compared with 3 weeks required for standard phage typing.

Currently, HPLC identification is used at CDC to separate BCG from other members of the *M. tuberculosis* complex without further tests. The procedure is reliable and reproducible and is recommended for use in the mycobacteriology reference laboratory.

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