Determination of Fatty Acids and Carbohydrate Monomers in Micro-organisms by means of Glass Capillary Gas Chromatography: Analysis of *Mycobacterium gordonae* and *Mycobacterium scrofulaceum*

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Trifluoroacetylated whole-cell methanolysates of four strains each of *Mycobacterium gordonae* and *Mycobacterium scrofulaceum* were analysed by gas chromatography, using a glass capillary column. The major chromatographic peaks were identified by mass spectrometry as derivatives of fatty acids and carbohydrates. In addition, two predominant peaks, present in chromatograms representing *M. scrofulaceum*, were identified as 2-octadecanol and 2-eicosanol. These secondary alcohols were not found in any of the strains of *M. gordonae* studied. The amount of tuberculostearic acid in the latter species was less than 1% of that in *M. scrofulaceum*.

INTRODUCTION

In recent years, a number of gas chromatographic (GC) methods for the characterization and identification of micro-organisms have been elaborated. Profiling of derivatives of cellular constituents such as fatty acids, carbohydrates and amino acids from pure cultures has been employed. Furthermore, extracellular microbial metabolites, particularly of anaerobic bacteria, have been studied by GC. The resulting chromatographic patterns are usually specific as to genus or sometimes as to species (Larsson & Mårdh, 1977).

One approach to the GC study of microbial constituents includes mild fragmentation of lyophilized organisms with methanolic hydrogen chloride, followed by trimethylsilylation or trifluoroacetylation. In this way, free and bound fatty acids are converted to corresponding methyl esters, and carbohydrates, including polysaccharides, to silylated or trifluoroacetylated monomeric methyl glycosides. Provided that the correct GC conditions are chosen, these two classes of compounds can be detected in one and the same GC analysis (Jantzen *et al.*, 1972).

The development of diagnostic techniques for the rapid identification of mycobacteria ought to receive high priority, since the methods used today are both laborious and time consuming. GC analysis of cellular fatty acids as used, e.g. by Tisdall *et al.* (1979) and Guerrant *et al.* (1981), made it possible to distinguish various mycobacterial species encountered in human infections. Larsson & Mårdh (1976) and Larsson *et al.* (1979a) employed both analysis of fatty acids and of trifluoroacetylated whole-cell methanolysates. In these latter studies, no attempts (other than those based on GC retention data) were made to establish the identities of the chromatographed compounds.

The present investigation aimed to optimize the chromatographic conditions for the simultaneous GC registration of both microbial carbohydrates and fatty acids, analysed as trifluoroacetyl (TFA) derivatized methyl glycosides and methyl esters, respectively. A glass

capillary column was used in order to obtain the best possible resolution. Two scotochromogenic mycobacterial species were selected as test organisms, viz. *Mycobacterium gordonae* and *M. scrofulaceum*. Mass spectrometry (MS) was employed to establish the identity of the chromatographic peaks.

METHODS

Organisms. The mycobacteria studied comprised *M. gordonae* (MNC 1226, MNC 1227, MNC 1230 and MNC 1233) and *M. scrofulaceum* (MNC 1225 and MNC 1232), isolated from clinical specimens at the Department of Bacteriology, University Hospital, Lund, Sweden. Furthermore, two strains of *M. scrofulaceum* (MNC 96 and MNC 98, obtained from Dr A. M. Masson, Montreal, Canada) were tested. All strains were identified by biochemical and immunological methods.

Each strain had been cultured on two slants of Löwenstein-Jensen medium. Bacteria were collected from each slant and inoculated into Proskauer-Beck medium (Difco) at 37 °C for 28 d. The organisms were autoclaved, washed, lyophilized and stored in a vacuum desiccator at reduced pressure until use (Larsson & Mårdh, 1976).

Chemicals. All chemicals used were of analytical grade and used without further purification. Solvents were purchased from Merck and trifluoroacetic anhydride from Fluka (Buchs, Switzerland). Methyl tuberculostearate, used as a reference, was synthesized (Odham *et al.*, 1979).

Derivatization. Lyophilized cells (4–6 mg) were stored overnight in an excess (about 1 ml) of 1 M-methanolic HCl in sealed glass ampoules at 80 °C as previously described (Larsson *et al.*, 1979a). After centrifugation of the samples the supernatants were transferred to 2 ml glass tubes and evaporated to dryness at room temperature under a stream of dry nitrogen. Ethyl acetate (20 µl) and trifluoroacetic anhydride (30 µl) was added and the tubes sealed with Teflon-lined screw caps before being heated at 80 °C for 10 min. Then, after 10 to 15 min at room temperature, the samples were used directly for GC analysis.

Gas chromatography. A Varian, model 3700, gas chromatograph equipped with a 25 m wall-coated glass capillary column (i.d. 0.2 mm, OV-101), and connected to a Varian model CDS-101 electronic integrator was used. A 0.1 m glass pre-column (i.d. 3.5 mm) packed with 2% OV-1 on Chromosorb G, 45–60 mesh, was placed in the injector block and attached to the capillary column. The packing material in the pre-column was regularly renewed to remove non-volatile, carbonized compounds. An all-glass split device was inserted after the pre-column. The temperature of the injector was 220 °C and that of the flame ionization detector 240 °C. The column temperature was programmed from 80 to 230 °C, at a rate of 5 °C min⁻¹, and held at 230 °C for 10 min. The carrier gas (nitrogen) flow rate was 1.0 ml min⁻¹ through the column; the split ratio was 1:50. Make-up gas (nitrogen) was introduced before the detector at a flow rate of 25 ml min⁻¹.

Mass spectrometry. A Varian model MAT 112 GC/MS combination, was used. The ion source temperature was held at 240 °C and the electron energy was 70 eV. The chromatographic conditions were those described above. The identities of the major peaks in the chromatograms were established both by the recording of mass spectra and by comparative studies on GC retention data of reference compounds. In addition, selected ion monitoring (SIM), focusing at *m/z* 312 (M⁺), was used in studies on *M. gordonae* for the demonstration of the methyl ester of tuberculostearic acid as previously described (Larsson *et al.*, 1979b, 1980).

RESULTS

Identity of peaks

The chemical structure of the components corresponding to peaks 1 to 11 (Fig. 1) were deduced by GC and MS. The mass spectra of (1–4) proved identical with those of derivatized authentic arabinose, galactose, mannose, and glucose, respectively, whereas (5, 6, 8, 9 and 11) proved consistent with the methyl esters of palmitoleic, palmitic, oleic, stearic and tuberculostearic acids. When analysing a methanolysate of *M. scrofulaceum* which had not been subjected to trifluoroacetylation, it was observed that the compounds representing peaks (7) and (10) eluted approximately 2 min later, than when such derivatization was made, indicating that they contained hydroxyl groups. The mass spectra of these two underivatized components showed neither a molecular peak, nor peaks characteristic of fatty acid methyl esters. Peaks in the high mass range of component 10 were found at *m/z* 280, *m/z* 281 and *m/z* 283, while the base peak was found at *m/z* 45. It is known that long-chain aliphatic alcohols give spectra where the molecular ion peaks are exceedingly small, even absent, and with characteristic mass fragments at *m/z* M-18 and *m/z* M-15. The spectrum therefore appeared consistent with a C₂₀ saturated alcohol. Alpha-cleavage with respect to the carbon atom carrying the hydroxyl group, yielding the base peak at *m/z* 45 (CH₃CH=OH)⁺ and the comparatively large peak at *m/z* 283

Fig. 1. Chromatograms obtained by analysis of one strain of (a) *Mycobacterium scrofulaceum* and (b) *Mycobacterium gordonae*. See text for specifications of analytical test conditions. Symbols: 1, TFA methyl-arabinoside; 2, TFA methyl-galactoside; 3, TFA methyl-mannoside; 4, TFA methyl-glucoside; 5, methyl ester of $C_{16:1}$ fatty acid; 6, methyl ester of $C_{16:0}$; 7, TFA-2-octadecanol; 8, methyl ester of $C_{18:1}$; 9, methyl ester of $C_{18:0}$; 10, TFA-2-eicosanol; 11, methyl ester of $C_{19:0Br}$ (tuberculostearic acid).

$(CH_3(CH_2)_{17}CH=OH)^+$, tally with a proposed structure of 2-eicosanol for component (10). Analogously, the identity of peak (7) was established as 2-octadecanol.

Chromatographic patterns

The identities and relative areas of the peaks in the chromatograms of both of the species examined are given in Table 1.

Chromatograms representing *M. gordonae* exhibited a much larger peak of glucose than of other carbohydrates, e.g. galactose, arabinose and mannose. These chromatograms further differed from those of *M. scrofulaceum* by the absence of peaks representing 2-octadecanol, 2-eicosanol and tuberculostearic acid, as registered by flame ionization detection. However, analysis of *M. gordonae*, using SIM revealed a peak having a retention time identical with that of the synthesized sample of methyl tuberculostearate, in an amount corresponding to less than 1% of that in *M. scrofulaceum* (Larsson *et al.*, 1981).

Reproducibility

The standard deviations of the peak areas are given in Table 1. Chromatograms from studies on the duplicate cultures, and on the different strains of the same species, were similar,

Table 1. *Relative peak areas of chromatograms representing mycobacterial cellular constituents as produced by a flame ionization detector and an electronic integrator*

Four strains of each species were used. The numbers in parentheses indicate the S.D.

Constituent	<i>M. gordonae</i>	<i>M. scrofulaceum</i>
Arabinose	14.6 (4.4)	20.2 (12.4)
Galactose	5.3 (1.0)	8.4 (1.8)
Mannose	11.2 (1.7)	17.0 (4.2)
Glucose	68.8 (6.6)	54.5 (12.9)
C _{16:1}	4.3 (0.9)	4.7 (2.0)
C _{16:0}	47.5 (4.4)	40.1 (3.3)
2-Octadecanol	0	5.0 (2.0)
C _{18:1}	36.3 (3.2)	18.8 (4.3)
C _{18:0}	9.4 (1.4)	18.3 (5.1)
2-Eicosanol	0	10.4 (1.2)
C _{19:0} Br	0	6.8 (0.8)

especially with respect to the fatty acid patterns, while the carbohydrate peaks showed somewhat larger variations.

No differences could be observed between chromatograms representing preparations derivatized 1 week or 1 year after the mycobacteria had been lyophilized. Derivatized samples could be stored for at least 1 week at -20°C without any change in their chromatographic patterns.

DISCUSSION

The analytic technique used in the present study, viz. acidic methanolysis of lyophilized bacterial cells followed by derivatization using trifluoroacetic anhydride prior to GC analysis, can be applied for characterization of bacteria in general, since all such organisms contain both carbohydrates and fatty acids. Alkaline saponification followed by esterification of cellular lipids has also been employed. This latter approach is rapid and easy to use, although not applicable for the study of both carbohydrates and fatty acids, since the former are degraded in the strongly alkaline environment employed during the saponification process.

The simultaneous determination by GC of bacterial carbohydrates and fatty acids can be achieved by using either trimethylsilyl or TFA derivatization of the methyl glycosides after acidic methanolysis (Jantzen *et al.*, 1972). The latter derivatives are more volatile and heat-stable than the former, but sensitive to moisture. Preferably, TFA derivatives should therefore be stored in an excess of reagent. The somewhat higher standard deviations of the peak areas of the TFA methyl glycosides than of the methyl esters found in the present study (Table 1) may be explained as resulting from incomplete TFA derivatization, or from degradation of the derivatives due to moisture in the atmosphere.

Larsson & Mårdh (1976), who analysed the fatty acid and carbohydrate composition of some species of *Mycobacterium* using, in principle, the same test procedure as described in the present study, found reproducible differences between chromatograms of *M. avium*, *M. kansasii*, *M. tuberculosis* and *M. bovis*, strain BCG. The latter two species produced virtually identical fatty acid GC profiles, and could only be distinguished when carbohydrates were also analysed. The 2 m packed column used in this and also in a preceding study (Larsson *et al.*, 1979a), could scarcely separate the three hexoses. Furthermore, peaks of tuberculostearic acid and 2-eicosanol could not be separated. The capillary column in the present study, which used the same stationary phase (viz. OV-101) as the two previous studies, enabled all these compounds to be separated. This stationary phase possesses a high thermal stability and is consequently suitable for analysis of the long-chain fatty acids of mycobacteria.

In initial studies, it was found that the excess of TFA reagent in the samples had a marked deleterious effect on the capillary column. This was overcome by using the pre-column described, the packing material of which was regularly renewed.

The finding of 2-octadecanol and 2-eicosanol in *M. scrofulaceum*, but not in *M. gordonae*, is of interest. The last-mentioned secondary alcohol has previously been found in the mycolic acids of a variety of mycobacterial species (Minnikin & Goodfellow, 1980; Asselineau, 1966). The detection of these alcohols by using capillary columns can add to the differential diagnostic capability of the GC method used. In contrast to previous findings (Julak *et al.*, 1980), we were able to detect tuberculostearic acid in *M. gordonae* by using SIM.

The GC technique described in the present paper appears to be useful for characterization of mycobacteria. Compared with the analysis of fatty acids alone, the method is more laborious and demanding, but elicits more information that might be of value in taxonomic studies on mycobacteria and possibly also on bacteria of other genera.

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