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PRELIMINARY RESULTS ON THE DETECTION OF MYCOLIC ACIDS FROM *MYCOBACTERIUM LEPRAE* BY GEL PERMEATION CHROMATOGRAPHY AND PROTON NUCLEAR RESONANCE SPECTROSCOPY

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Summary

The presence of mycobacteria can be detected by identifying the mycolic acids from its cell wall. The acidic components of human biopsy material can be extracted and esterified with p-bromo-phenacyl bromide. The success of the esterification process may be verified by Nuclear Magnetic Resonance (NMR). Since the molecular weights of mycolate esters are relatively higher than those of the other components, they could be identified by gel permeation chromatography technique using a high resolution column. Samples from lepromatous leprosy patients were shown to contain high molecular weight fractions while those of the control samples did not have any component with molecular weight larger than 1,000. The techniques described in this paper could be developed as a quick method for screening large numbers of patients suspected of having leprosy, although at this stage it is not possible to differentiate the several strains of mycobacteria.

Keywords: Mycolic acid, mycobacteria, chromatography, spectroscopy.

INTRODUCTION

Mycolic acids are high molecular weight β-hydroxy, a-alkyl-branched fatty acids which are characteristic constituents of acid-fast bacteria of the genera *Mycobacterium, Norcardia, Rhodococcus,* and *Corynebacterium.*[1] Since the different structures of these mycolic acids have been a valuable criterion in the classification of acid-fast bacteria, they have become the subject of numerous reviews.[1-3]

As leprosy is widely believed to be related to a complex cell-mediated immunity against *Mycobacterium leprae,* studies of mycolic acids are inevitably of interest in leprosy research. The isolation and identification of mycolic acids from *mycobacteria* have attracted much attention over the years.[4-12] Studies by the techniques of Thin Layer Chromatography (TLC)[5,7,12], Gas Chromatography (GC)[11,13], High Pressure Liquid Chromatography (HPLC) and Mass Spectroscopy (MD)'[4] have been reported recently.

Undoubtly TLC is a convenient technique in the preliminary qualitative identification of mycobacteria. However, it suffers the set-back of being not in itself specific and diagnostic for leprosy. GC has not been successful in the separation of the high molecular weight mycolic acid species which are unstable at high temperature. On the other hand, the method involving HPLC coupled with MS[14] claimed to be able to differentiate the mycolic acids from a range of mycobacteria and thus offer a more definite identification of *M. leprae.* The procedure involves extraction and esterification followed by three stages of HPLC separation before final analysis by mass spectroscopy.

This paper describes the preliminary results of our attempt to develop a simple and rapid method for the detection and identification of the mycolic acids from human skin biopsy materials.

MATERIALS AND METHODS

Skin biopsies

Skin biopsies of active lesions were takkn from 10 lepromatous leprosy patients. For control, skin biopsies from 2 atopic eczema (non-mycobacterial) patients were taken. In each case about 1.0 g of sample was used.

Extraction and Esterification

The mycolic acids were extracted and esterified with p-bromo-phenacyl bromide by the same procedure as described by Kusaka

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et al.[14] However the resultant esters were dissolved in chloroform ($CHCl_3$) instead of tetrahydrofuran (THF).

Gel permeation chromatography (GPC)

GPC was run at room temperature on a Waters Associates HPLC model 400 instrument equiped with a universal injector fixed with a 20 μl sample loop, a model 6000A solvent delivery system and a model 440 UV detector set to λ 254 nm. The GPC column measures 7.5 mm in internal diameter and 500 mm in length and was packed with high resolution styrene-divinyl benzene gels developed by Tanaka *et al.*[15] The theoretical plate number of the column was found to be higher than 22,300. Isocratic elution was carried out using $CHCl_3$ as the solvent at a fixed flow rate of 0.8 ml/min. and a pressure of 75 bar. The column was first calibrated with a 2% solution of styrene oligomers in $CHCl_3$ to obtain the relation between molecular weight and elution volume.

10 μl of samples containing about 2 mg/ml of dissolved solids were introduced into the injector with a microsyringe. The esterified derivatives contain a strongly W absorbing aromatic group[16] which greatly enhanced the detectability by W detector which is known to be much more sensitive than a differential refractometer detector.

Proton Nuclear Magnetic Resonance (NMR)

The ester solution in $CHCl_3$ was evaporated to dryness at room temperature under reduced pressure and redissolved in $CDCl_3$. TMS was added as the internal standard. Proton NMR was recorded on a JEOL JMN PS-100 spectrometer. The instrument was equiped with the PET-100FT system.

RESULTS

GPC column calibration

Fig. 1 shows the GPC curves of a mixture of styrene oligomers with degrees of polymerization ranging from 2 to 10 corresponding to molecular weights ranging from 266 to 1100. At a flow rate of 0.8 ml/min., the largest oligomer was eluted after 9 min. while the smallest oligomer after 11.8 min. In a separate run, benzene which is of molecular weight 78, was eluted after 14.9 min. under the same conditions.

Fig. 2 shows the relationship between the molecular weights and the elution volumes by plotting the log (Mol. Wt.) vs elution volumes. Linear regression analysis shows that the linear relation fits well with a coefficient of correlation of 0.9996 and a standard error of 0.012.

GPC curves of samples

Fig. 3(a) shows the chromatograph of a sample from a clinically corifirmed lepromatous leprosy patient. The principal peak on the left corresponds to a molecular weight of 1300. The high molecular weight fraction had been shown to contain mycolate esters by Kusaka *et al*[14] using the same extraction and esterification procedure. The other minor peaks could be due to the phenacyl esters of low molecular weight acids, and also excess reagents such as the crown ether and the p-bromo-phenacyl bromide since no prior separation was carried out before running the GPC.

Fig. 3(b) is the chromatograph of a sample from a skin biopsy of a patient suffering from atopic eczema. The most striking feature is the absence of the high molecular weight fraction.

Proton NMR

Fig. 4 summarises the results of the proton NMR studies. Spectrum (a) is that of p-bromophenacyl bromide in CDC1, The aromatic protons at δ 7.5-7.7 and the Br-O-CH2-CO- proton at δ 4.36 are in the ratio of 2:1. Spectrum (b) is that of a mixture of crown ether in $CDCl_3$ with some $CHCl_3$ added. It clearly established the crown ether peak at 3.68 ppm and that of $CHCl_3$ at 7.22 pprn. Spectrum (c) is that of the ester derivatives from the skin of a lepromatous leprosy patient. It is apparent that the two large peaks at δ 7.22 and 3.68 are due to $CHCl_3$ and unremoved crown ether respectively. Peak at a 4.36 is now hardly noticeable, indicating that only trace amounts of unreacted p-bromophenacyl bromide are present. Thus the aromatic protons at 7.5-7.7 ppm region must be those of the p-bromophenacyl ester. The protons of the ester linkage -CO-CH,-0-CO-resonance at a 527.

Comparing with the published NMR spectrum of the methyl mycolates of *M. tuberculosis*,[17] *we* assigned the signals centered at δ 1.0 to $-CH_3$, while that around a 1.4 (consisting of 2 large, broad and unsymmetrical peaks at δ 1.2 and δ 1.6) to -CH,-. The ratio of ethylene to methyl protons was only about 18:1 and was not in agreement with the mycolate structure. However, this is not totally

GPC column calibration

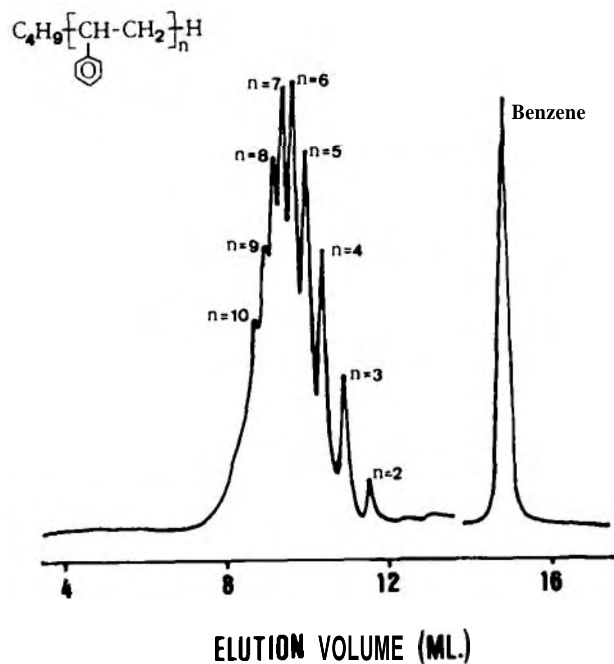

Fig. 1: GPC calibration curves of a mixture of styrene oligomers with degrees of polymerization ranging from 2 to 10, corresponding to molecular weights ranging from 266 to 1100.

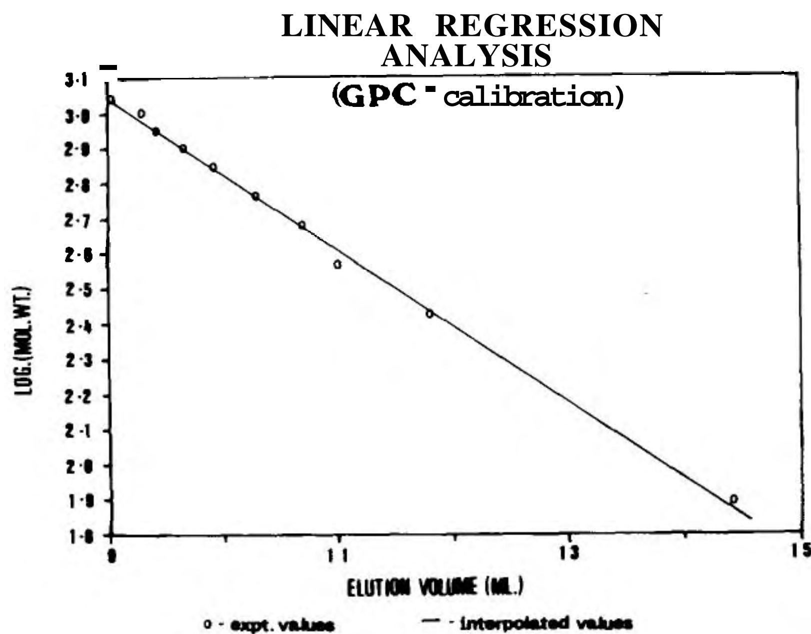

Fig. 2: Linear regression curve showing the relationship between molecular weight and elution volume.

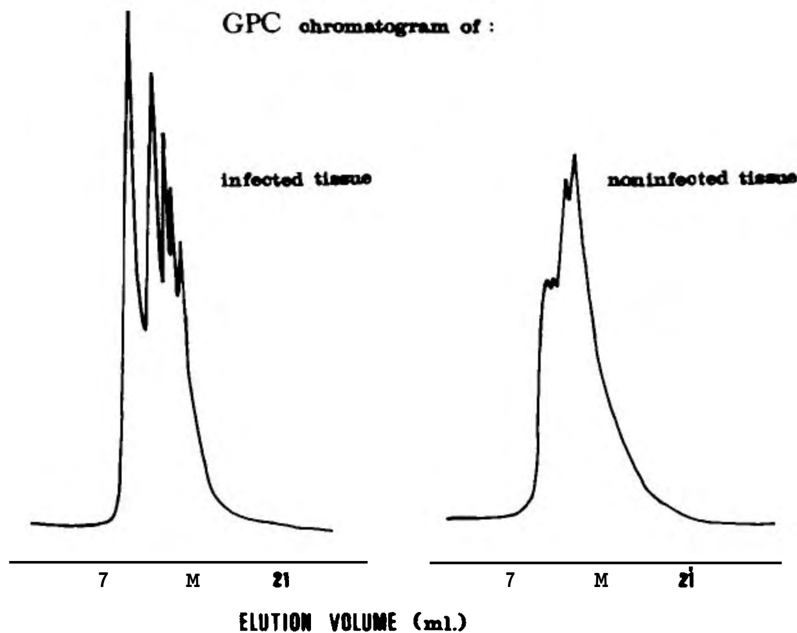

Fig. 3: GPC chromatogram of (a) an infected tissue, and (b) noninfected tissue.

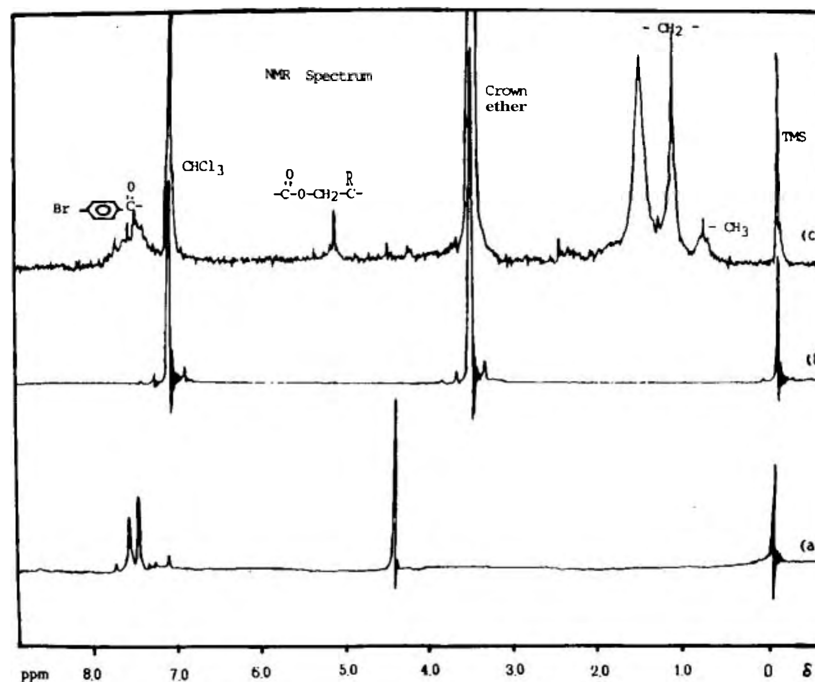

Fig. 4: NMR spectrum of (a) p-bromophenacyl bromide in $CDCl_3$ (b) a mixture of crown ether in $CDCl_3$ with some $CHCl_3$ added, and (c) ester derivatives from the skin biopsy of a lepromatous leprosy patient.

unexpected since GPC has already shown the presence of a considerable amount of esters of smaller fatty acids. The broad peak at δ 0.7 seemed to overshadow a smaller signal at δ 0.6 which belonged to cyclopropanoid groups. A weak unidentified signal at δ -0.1 was observed but none at δ 0.3. Peaks at around δ 0.6 and δ-0.3 have been reported to be characteristic of the cyclopropane rings of the α-mycolates.[6,14,17]

There was no evidence of the presence of glycolipids in the samples, especially in the regions of δ 2-3 and δ 4-5, by comparing them with published spectra of glycolipids.[18] A few minor peaks remained unidentified and there is also the possibility of signals around δ 3.6 being overshadowed by the presence of the unremoved crown ether.

There was a 0.2% $CHCl_3$ contaminant in $CDCl_3$ solvent (Sigma Chem. Co., St. Louise, U.S.A.). The peak area of the aromatic protons (4H), was roughly equal to the peak area of $CHCl_3$ (1H), thus as a rough estimation, the total concentration of phenacyl esters was in the region of 0.05%. Such low concentrations caused the high noise to signal ratio and all the peaks appeared broad and not well resolved.

DISCUSSION

NMR characterization of mycolic acids and their derivatives have been reported[2,4,6,9,17,19] while few spectra were actually published. The α-alkyl branch, except for length, is essentially invariant in any group of mycolic acids. Functional variations are always found in the mero portion. Thus cyclopropanoid, ethylenic, methoxyl, keto and lone methyl branch have been detected in mycolic acids of different sources.

Due to the small size of biopsy tissues, we were not able to isolate pure mycolates from any one sample for NMR. In this preliminary work, we report the NMR of unseparated samples with the view of throwing some light on the contents of the mixture as shown by the GPC.

It appears that the GPC method can be used as a quick test for patients suspected of mycobacterial infection. About 1 gm of skin biopsy material from each patient is sufficient. The extraction and esterification process is relatively simpler than that of the TLC techniques. Moreover, it takes a much shorter time and is more reliable for screening large numbers of specimens after setting up the right conditions for the instrument. The fraction at molecular weight around 1300 is probably indicative of the presence of mycolic acids. The relative peak areas can perhaps be used as a measure of the extent of infection.

It must be pointed out that at this stage, the GPC method is unable to differentiate mycolic acids from different mycobacterial species. Further work is currently under progress.

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