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Short communication

Gas chromatographic–mass spectrometric study of lipids and rapid diagnosis of *Mycobacterium tuberculosis*

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Abstract

The method suggested is based on the identification of tuberculostearic acid as a *Mycobacterium tuberculosis* marker. Bacterial culture and sputum of patients with pulmonary tuberculosis and lung diseases of non-tuberculous etiology were investigated. A complete coincidence of the results of bacteriological and gas chromatographic–mass spectrometric (GC–MS) methods used in examining a group of patients with pulmonary tuberculosis was found. GC–MS ion monitoring provides a highly sensitive and rapid technique for the diagnosis of pulmonary tuberculosis.

1. Introduction

Analytical methods have an advantage over routine procedures because they do not need the isolation of pure cultures for the chemical identification of bacteria. The high sensitivity of physico-chemical methods (nano- and picograms) makes it possible to detect metabolites in biosubstrates without preculturing and to greatly shorten the diagnosis time of pathogenic mycobacterial species. During the last few years, gas chromatographic–mass spectrometric (GC–MS) detection of bacterial biomarkers has become a widely accepted and rather promising technique in clinical microbiology. One particular GC–MS method, ion-selective mass fragmentography, provides continuous selective detection of one or more ions with given masses by a mass spectrometer. If one or more ions typical of the compound under investigation are already known, then selected-ion monitoring (SIM) makes it possible to identify the maximum amount of this component in the chromatogram. SIM is especially efficient with complex mixtures and performs well even in cases where the substance cannot be separated on a chromatographic column. This technique offers a considerably higher sensitivity than that observed in recording the total mass spectrum. The SIM detection limit ranges from 10^{-12} to 10^{-15} g, depending on the type of compound and the character of analysis.

GC–MS also presents a powerful tool for tuberculosis diagnosis. Tuberculostearic (10-*R*-methyloctadecanoic) acid, TSA, is a unique cell component for a number of *Actinomycetales* including *Mycobacterium*. C_{32}-Mycocerosic (2,4,6,8-tetramethyloctacosanoic) acid, found only in *M. bovis, M. kansasii, M. africanum* and *M. tuberculosis* lipids, serves as a more specific

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metabolite. Mycocerosic acid (MCA) detection is indicative of a limited number of mycobacterial species including *Bacillus tuberculosis*. However, because the MCA/TSA ratio ranges from 0.1 to 0.04, MCA may remain unidentified due to its low content [1]. To verify *M. tuberculosis*, most scientists use selected-ion monitoring (SIM) at *m/z* 312 as a specific TSA ion [2–7].

In the investigation of a lipid fraction isolated from *M. tuberculosis* culture, we have proposed a modified GC–SIM-MS analysis of TSA in clinical material.

2. Experimental

The *M. tuberculosis* culture was grown on Löwenstein–Jensen medium from the inoculated sputum of a patient with active pulmonary tuberculosis for 4 weeks at an incubation temperature of 37°C. Bacterial cells were washed off with physiological salt solution and subjected to lyophilization.

For the extraction of lipids from bacterial cultures and clinical materials, a chloroform–methanol mixture (2:1, v/v) was used. Extraction was carried out for 24 h [3]. TSA methylation in the bacterial culture extract and sputum was performed according to a modified scheme. To 2 ml of nonlyophilized *M. tuberculosis* culture or a sputum specimen, 10 ml of extraction solvent was added; after 24 h the chloroform–methanol extract was filtered, dried and evaporated under vacuum. Extract TSA esterification was performed with a 3% solution of hydrochloric acid in methanol obtained by the addition of 5 ml of acetyl chloride to 10 ml of predried methanol. The mixture was evaporated under vacuum, dissolved in chloroform (200 μg of the dry residue per 200 μl of the solvent) and analysed by GC–SIM-MS. The GC–MS analysis was performed on an LKB-2091/152 unit equipped with a PDP-1134 computer. Electron-impact ionization, E = 70 eV; source temperature, 320°C; ion accelerating energy, 2300 V; column 40 m × 0.3 mm I.D.; helium carrier gas flow, 2 ml/min; injector temperature, 350°C; column temperature from 170 to 320°C, 4°C/min. The SIM analysis was carried out by monitoring *m/z* 312, which corresponded to the TSA methyl ether molecular ion.

The detection limit for stearic acid following derivatization was 10 ng. For thin-layer chromatography of the TSA methylation products, Silufol plates were used with diethyl ether–hexane (1:1, v/v) as running solution [8]. Bromophenol blue alkaline solution, the developer for fatty acids and their esters, was prepared by mixing 40 mg of bromophenol blue with 100 ml of 0.01 *M* NaOH. After heating the plate at 70–90°C for 15 min, yellow spots of acids and dark-blue spots of esters appear against the blue background [9].

At the Laboratory of Express-Diagnosis and Drug Therapy, Research Institute of Surgery, a GC–MS technique for the identification of tuberculosis pathogenic species has been elaborated and tested on the bacterial culture and sputum specimens collected from 29 patients with pulmonary tuberculosis. The sputum specimens were analyzed by bacteriological and physicochemical methods. In the control group, 11 patients with bacterial lung destruction of non-tuberculous etiology were examined.

3. Results and discussion

A GC–MS study of the lipid extract of a *M. tuberculosis* culture has been carried out. The results are presented in the chromatogram of Fig. 1a. For each chromatographic maximum a mass spectrum was recorded, and the compound was identified using mass spectral reference data. As a result, the following 13 compounds were identified in the order of appearance of their maximums in the chromatogram: hexadeca-11-enic acid as well as palmitic, linolic, oleic, stearic, 10-methyloctadecanoic (tuberculostearic) acids, a furanoid compound of M_r 318, methyleicosanoic acid, a furanoid compound of M_r 315, etracosanoic acid, squalene, hexacosanoic acid and cholesterol.

We have found that the prolonged lyophilization of cell culture and, consequently, of sputum

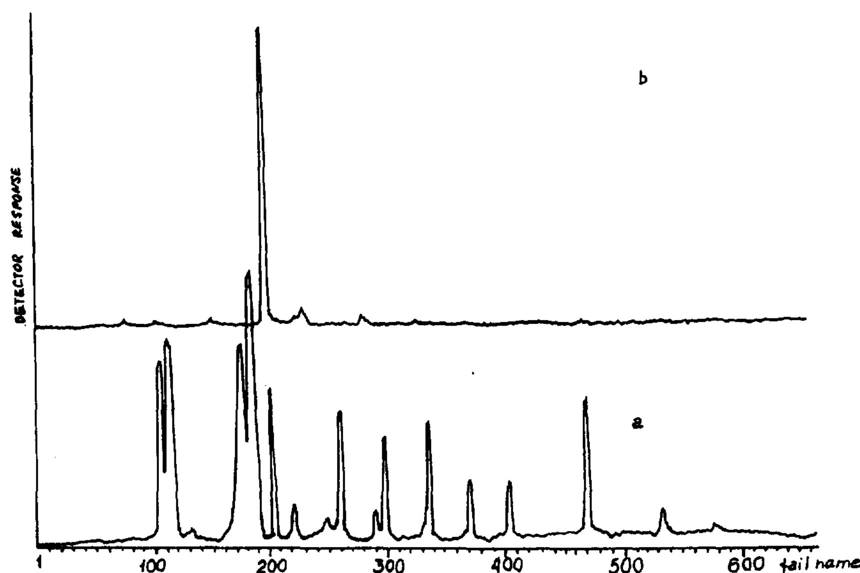

Fig. 1. Chromatogram and mass fragmentogram of the hydrolyzed and methylated *M. tuberculosis* lipid extract culture and the mass spectrum of TSA methylate from this extract: (a) chromatogram of the extract; (b) mass fragmentogram of the culture extract.

can be replaced by drying the organic phase of the sample after its chloroform–methanol extraction. In addition, with our procedure the TSA methylation time can be considerably shortened compared with that reported in the literature [1–7]. This was proved by the investigation of some kinetic aspects of the rate-limiting methylation reaction. The TSA methylation time was determined with stearic acid as a substrate, the methylating reagent being the same as before (hydrochloric methanol). The reaction products were analyzed by thin-layer chromatography and mass spectrometry after 6, 2, 1, 0.5 and 0.4 h. When the reaction products were placed on a Silufol plate at the above intervals, some spots with $R_F = 0.71$ corresponding to methylstearate were fixed after separation and developing; spots with $R_F = 0.08$ corresponding to the initial products were not observed. In the mass spectrum of the reaction products directly introduced into the mass spectrometer 15 min after the reaction started, no m/z 284 ions specific for stearic acid were present. Thus, it has been concluded that the process of derivation took 15 min. This was confirmed by derivation with Koch's bacillus culture. The GC–MS method for TSA detection was the subject of a patent application (No. 4934091/14 of May 5 1991) and obtained the required regulatory approval.

After extraction and derivation, the culture specimen was analyzed by mass fragmentography. Fig. 1 shows a chromatogram and mass fragmentogram of the methylated extract of the *M. tuberculosis* strain, and a mass spectrum of TSA methylate from this extract. The mass spectrum is characteristic of TSA methyl ester as evidenced from electron-impact fragmentation of the molecule (Fig. 2). As shown in Fig. 2, m/z 312 (M^+), 269, 199, 171, 167, 149 ions correspond to TSA methyl ester. The sputum specimens collected from patients with pulmonary tuberculosis were treated and analyzed in a similar manner.

Fig. 3 shows a chromatogram and mass fragmentogram of a methylated extract of the sputum of a patient with pulmonary tuberculosis and a mass spectrum of TSA methylate contained in the extract. It should be noted that, in spite of the difference in the chromatograms of the bacterial culture extracts and sputum (Figs. 1a and 3a), the mass fragmentogram (Fig. 3c) shows the same peak whose height, however,

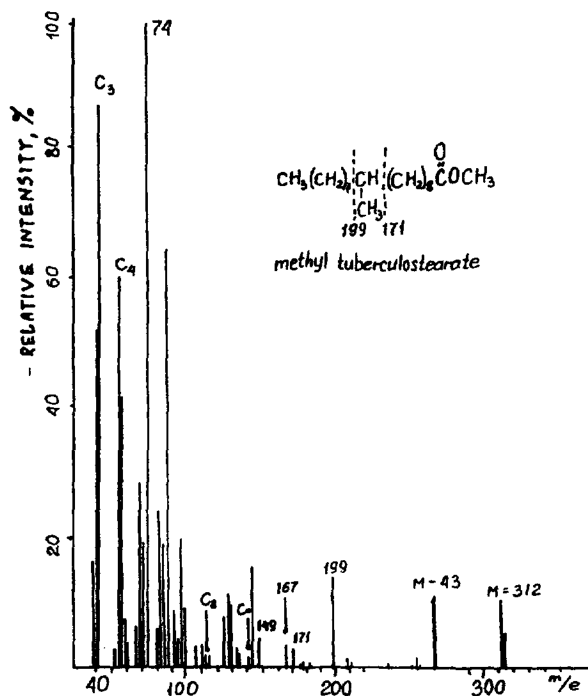

Fig. 2. Mass spectrum and the structural formula of TSA methylate. The ion current intensity, percentage of full ion current from m/z 74 ions, is plotted on the abscissa; the ordinate is the ion mass.

differs depending on the content of TSA methylate. If there is no TSA in the patient sputum, then this peak is absent in the fragmentogram. For a strict check of the presence of TSA, the mass spectrum of the peak due to TSA methylate was recorded (Fig. 3c). The identity of the mass spectra presented is evident from Figs. 2 and 3c. The spectra differ only in the intensity of the fragment ions, which can be explained by the content of the substance. The time necessary for GC–MS analysis of sputum is 2 days (quite short compared with routine culture methods taking 1.5 to 2 months), which is very important for timely therapy to be carried out. The method was tested in patients with pulmonary tuberculosis and in those with bacterial non-specific lung diseases, and the results of the analyses were compared. The sputum specimens of all 29 patients with pulmonary tuberculosis showed the presence of TSA. The results of GC–MS and the bacteriological method are fully coincident. Relying upon GC–MS data, 3 of the 11 examined patients with non-specific lung destruction proved to have TSA in their sputum. After a thorough X-ray and bacteriological examination,

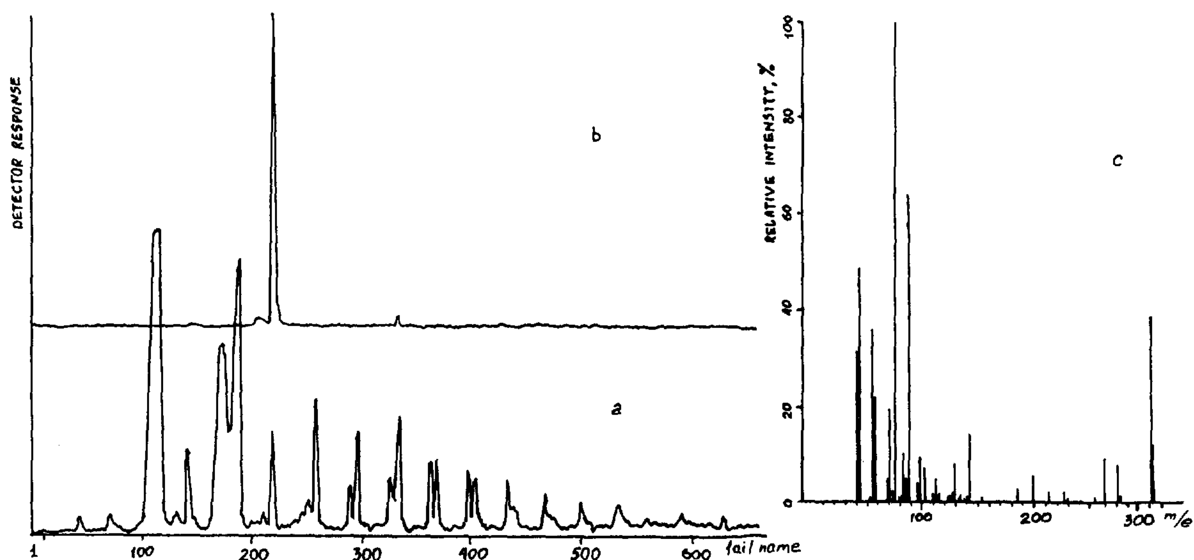

Fig. 3. Chromatogram and mass fragmentogram of the hydrolyzed and methylated lipid extract from the sputum of a patient with pulmonary tuberculosis and the mass spectrum of TSA methylate from this extract: (a) chromatogram of the extract from sputum; (b) mass fragmentogram of the above extract; (c) mass spectrum of the substance corresponding to the maximum in the chromatogram in file 220 position, which is the mass spectrum of TSA methylate.

these patients were admitted to a tuberculosis hospital. The other 8 patients with lung-destructive diseases of other etiology, with no GC–MS nor clinical signs of pulmonary tuberculosis, did not need bacteriological examination. In the third test group, the results of GC–MS and bacteriological analyses did not coincide in 5 of 8 patients. However, after a dynamic follow-up and thorough examination, pulmonary tuberculosis was confirmed in all 5. The initial disagreement in the data for this group of patients can be explained by the low sensitivity of the bacteriological method on the one hand, and by enhanced sensitivity of Koch's bacillus to a change in the growth conditions on the other hand. As is known, retarded growth of Koch's bacillus can be caused by a slight deviation in culturing conditions or by a low-quality culture medium.

Thus, GC–MS analysis of the *M. tuberculosis* lipid extract led to the identification of 13 compounds including tuberculostearic acid, a mycobacterial marker. A GC–MS procedure for rapid TSA detection comprising a modified technique for processing and derivation of the specimen prior to GC–MS analysis has been offered.

A complete (100%) coincidence of the results of bacteriological and GC–MS analyses in the examination of a large group of patients with pulmonary tuberculosis has been established.

The suggested technique is distinguished by its high sensitivity and speed and should therefore be widely accepted in clinical practice.

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