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Note

Detection of tuberculostearic acid in biological specimens by means of glass capillary gas chromatography—electron and chemical ionization mass spectrometry, utilizing selected ion monitoring

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Tuberculostearic acid [(*R*)-10-methyloctadecanoic acid] is regarded a characteristic constituent of microorganisms of the order *Actinomycetales*. By using selected ion monitoring (SIM), the acid has been demonstrated to be present in several mycobacterial and nocardial species [1—3], and in sputum specimens from patients with pulmonary tuberculosis [2, 3]. The latter observation implies a possible way of using SIM for the diagnosis of such infections.

When using electron ionization (EI) mass spectrometry (MS), SIM allows the methyl ester of tuberculostearic acid to be determined quantitatively down to approximately 20 pg, monitoring at *m/z* 312 (= M) [1]. In earlier studies [3] it was sometimes found that sputum specimens from patients with pulmonary tuberculosis contained tuberculostearic acid in amounts close to the detection limit of the instrument. When such small amounts of the acid are present in clinical specimens, there is no proof of identity other than the chromatographic retention time. Therefore it is important that the gas chromatographic (GC) system used should possess optimal separation qualities.

In the present investigation we assessed the gain in sensitivity in the detection of tuberculostearic acid by using EI—SIM analysis of the *tert.*-butyldimethylsilyl (t-BDMS) ester [4], as compared with the corresponding methyl ester [1—3]. The former derivative yields spectra having ions of a very high intensity at *m/z* $(M - 57)^+$ upon electron impact [4]. Furthermore, we investigated the chemical ionization (CI) mass spectra of methyl tuberculostearate, using methane and isobutane as reactant gases, and compared the sensitivity

for its detection with that of EI. The GC system developed uses glass capillary columns to optimize the separation of various methyl-branched C_{18} positional isomers possibly present in clinical specimens.

EXPERIMENTAL

Organisms

One strain of *Mycobacterium tuberculosis*, H37Rv, was tested.

Sputum samples

Sputum specimens (2—4 ml) were collected from eight patients with pulmonary tuberculosis. All sputa contained acid-fast rods detectable by microscopic studies of Ziehl-Nelseen stained smears. For comparison, sputum samples from six patients with non-tuberculous pneumonia were also used.

Standards and reagents

The solvents and reagents used were analytical grade. Three per cent methanolic hydrochloric acid was prepared by adding 5 ml of acetyl chloride to 100 ml of dry methanol. 2- and 10-methyloctadecanoic acids were synthesized [3], while 16- and 17-methyloctadecanoic acids were purchased from Larodan Lipids (Malmö, Sweden). Imidazole, N,N-dimethylformamide and t-BDMS chloride were obtained from Merck (Darmstadt, G.F.R.), Mallinckrodt (St. Louis, MO, U.S.A.) and Fluka (Buchs, Switzerland), respectively.

Preparation of organisms and sputum specimens

The strain of *M. tuberculosis* studied was cultured on Proskauer-Beck medium, autoclaved, washed and lyophilized as previously described [5].

The sputum specimens were digested with 5 ml of aqueous sodium hydroxide (4% v/v) at 37°C for 15 min with occasional shaking. The pH was then adjusted to 7.0 with diluted hydrochloric acid and the preparations autoclaved and lyophilized.

Extraction and derivatization procedures

The reference acids were esterified using 3% methanolic hydrochloric acid at 80°C for 20 h. Also, 10-methyloctadecanoic acid (tuberculostearic acid) was derivatized to its t-BDMS ester as previously reported [4].

The lyophilized sputum specimens and mycobacterial cells were extracted using 2 ml of chloroform—methanol (2:1, v/v) at room temperature overnight. The organic solutions were then evaporated to dryness and the residues esterified using 2 ml of methanolic hydrochloric acid as described above. The methanolic phases were evaporated to dryness, 100 μl of *n*-hexane were added and the solution used for GC—mass spectrometric (MS) analysis.

In some studies, 4 μg of lyophilized cells of *M. tuberculosis* were added to each of two sputum specimens from patients with non-tuberculous pneumonia before being lyophilized and derivatized.

Gas chromatography—mass spectrometry

A Varian MAT Model 112 GC—MS combination, equipped with a glass capil-

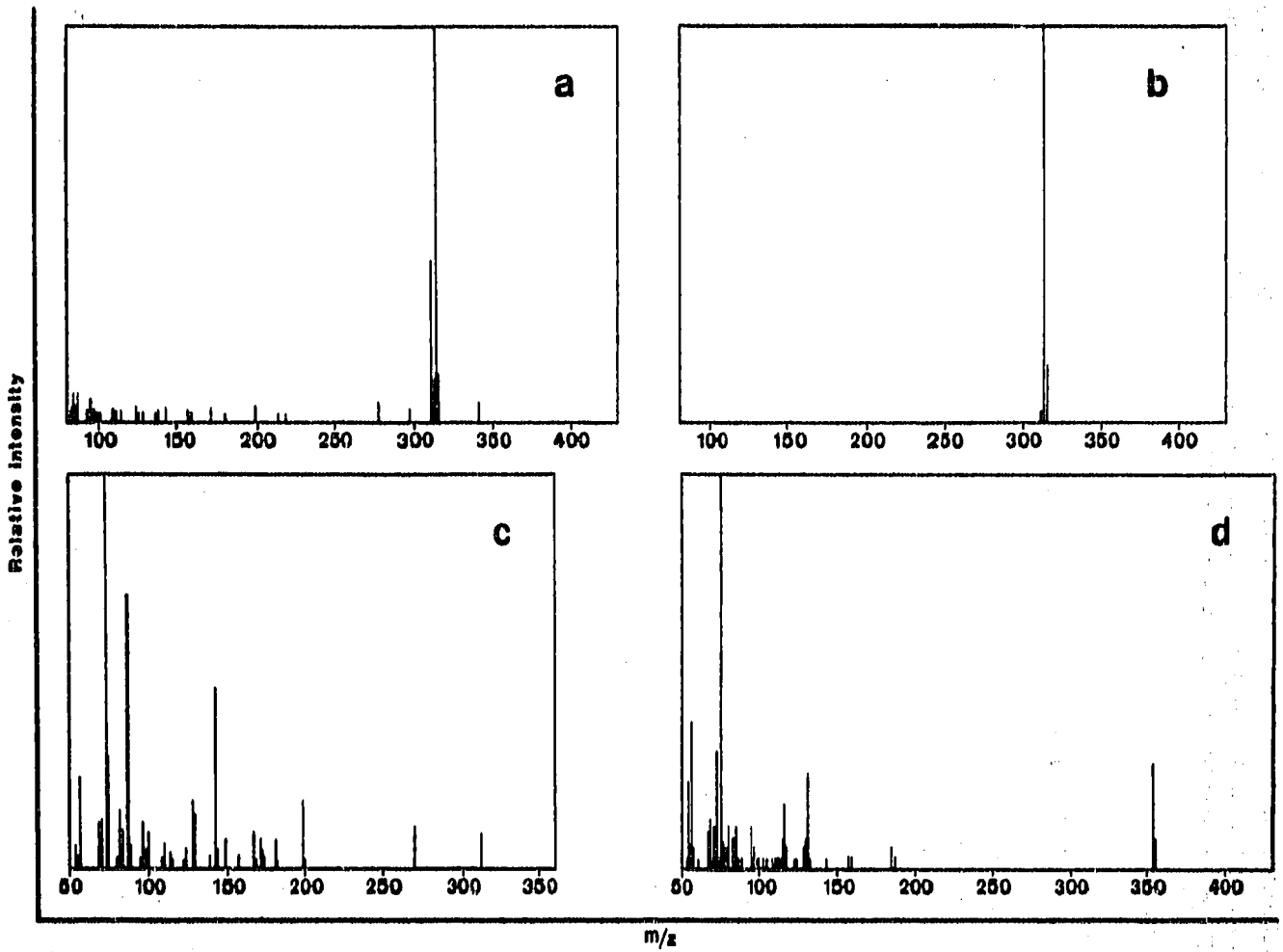

Fig. 1. Mass spectra of derivatized tuberculostearic acid. (a) CI methane spectrum of methyl ester; (b) CI isobutane spectrum of methyl ester; (c) EI spectrum of methyl ester; (d) EI spectrum of t-BDMS ester. The esters were prepared from the corresponding free acids as described in the text. See also text for specifications of GC–MS conditions.

lary column (25 m × 0.25 mm I.D.) coated with OV-17, was used in all EI studies. The analyses were performed using both split (split ratio 1:20) and splitless injections. The nitrogen carrier gas flow-rate was 2 ml/min through the column. The column temperatures were 210 and 240°C for the analysis of methyl and t-BDMS esters, respectively. The electron energy was 70 eV and the ion source temperature 250°C.

Studies using CI were carried out on a Finnigan Model 4021 GC—MS system, equipped with a 20-m glass capillary column coated with SE-30. Injections were performed at a split ratio of 1:25 and the temperature of the column was programmed from 80 to 230°C using a temperature increase of 20°/min. The carrier gas flow-rate (nitrogen) was 2 ml/min and the electron energy 150 eV at 0.3 torr of reactant gas. The ion source temperature was 250°C.

The SIM analyses were carried out using single ion detection monitoring at *m/z* 312 (EI) and *m/z* 313 (CI) when studying the methyl esters, and at *m/z* 355 (EI) for the t-BDMS ester.

RESULTS AND DISCUSSION

Mass spectra

Mass spectra of derivatized tuberculostearic acid, using CI and EI, are shown in Fig. 1. In the high mass range, the spectra of the methyl ester obtained by CI are dominated by the intense ion of *m/z* 313 $(M + 1)^+$ (Fig. 1a, b). Their relative abundance is considerably larger than at *m/z* 312 $(M)^+$, using EI (Fig. 1c). Variations of the reactant gas in the CI experiments caused only minor changes in the corresponding spectra (Fig. 1a, b). The spectrum representing the t-BDMS ester exhibited an intense ion at *m/z* 355 (Fig. 1d).

Estimation of detection limit

When the EI—SIM technique was employed, the detection limit of the methyl ester of tuberculostearic acid (monitored at *m/z* 312) was 5 to 10 times higher than that of the t-BDMS ester (monitored at *m/z* 355). Roughly the same increase in sensitivity was gained when focusing at *m/z* 313 $(M + 1)^+$, employing isobutane as reactant gas in the CI—SIM mode. The signal-to-noise ratio when using isobutane was approximately twice that found when employing methane. The detection limit was estimated to approximately 1 pg both for the methyl ester as detected by CI—SIM (isobutane) and for the t-BDMS ester, using EI.

Separation of C_{19} isomeric fatty acids

Of the isomeric C_{19} acids present in bacterial cells, the 2-, 16-, and 17-methyl-substituted C_{18} acids, and the straight-chain C_{19} acid, can be expected to be by far the most common. Fig. 2 shows a fragmentogram obtained by single ion detection, monitored at *m/z* 312 (EI) of the corresponding methyl esters, including methyl tuberculostearate. The analysis was carried out using a wall-coated glass capillary column with split injection, employing OV-17 as stationary phase. No difficulties were encountered in establishing the identity of methyl tuberculostearate by the GC retention time.

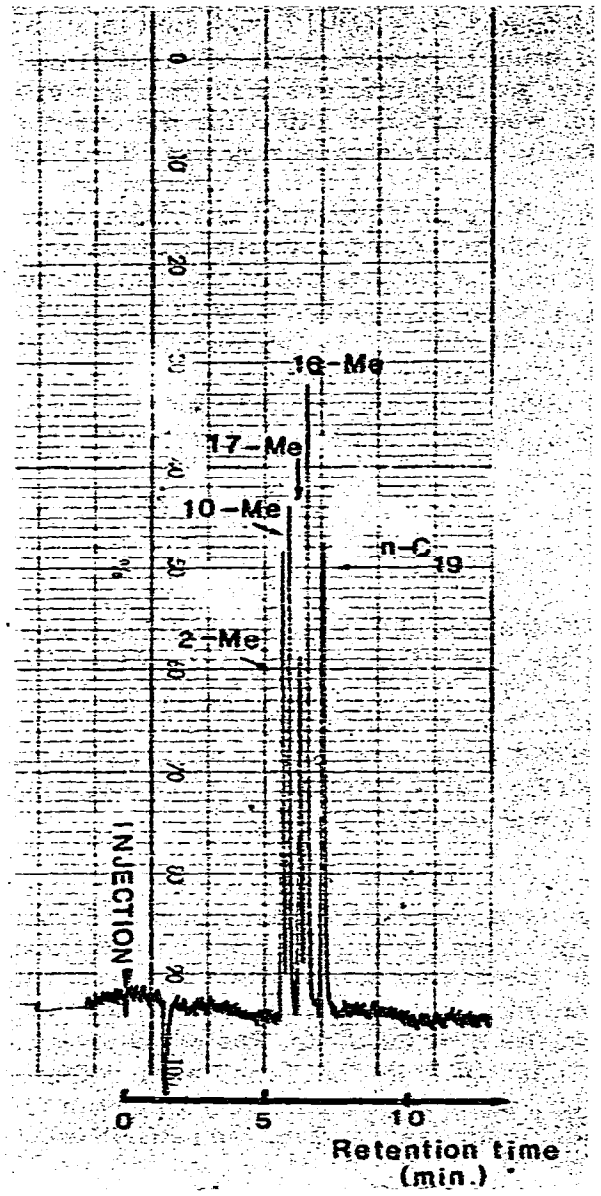

Fig. 2. EI mass fragmentogram of a mixture of equal amounts of the methyl esters of 2-, 10-, 16-, and 17-methyloctadecanoic acid and *n*-nonadecanoic acid monitored at *m/z* 312. A 25-m glass capillary column with OV-17 as stationary phase was used at an isothermal column temperature of 210°C. Each peak represents about 300 pg of methyl ester.

Analysis of sputum specimens

Mass fragmentograms representing sputa from patients with tuberculosis (Fig. 3a) and with pneumonia with no evidence of tuberculosis (Fig. 3c), employing EI—SIM of methylated samples, monitoring at *m/z* 312, are shown. For comparison, a mass fragmentogram of one of the latter specimens to which

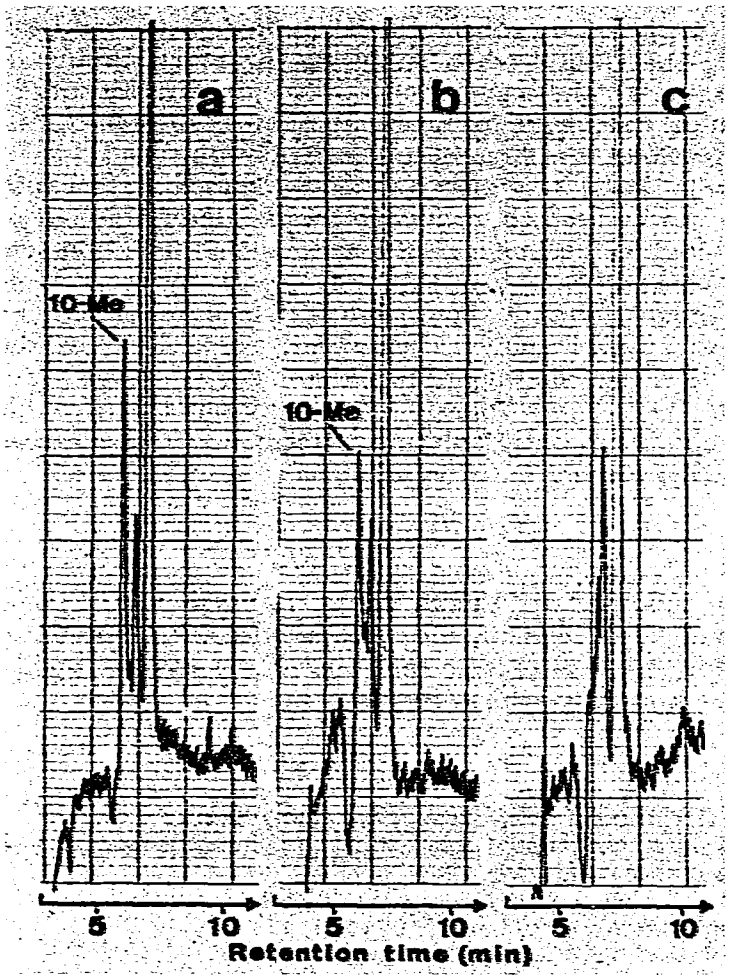

Fig. 3. EI mass fragmentograms of sputum specimens from a patient with tuberculous (a) and non-tuberculous pneumonia supplemented (b) and not supplemented (c) with 50 pg of authentic tuberculostearic acid, using splitless injections. The sputum specimens had been digested and derivatized as described in the text. The fragmentograms were recorded on a 25-m glass capillary column coated with OV-17 using single ion detection, monitoring at *m/z* 312.

50 pg of authentic tuberculostearic acid had been added is also shown (Fig. 3b). The fragmentograms obtained in CI–SIM analyses, focusing at *m/z* 313 $(M + 1)^+$, were similar, although the use of CI gave a far better sensitivity than EI. All analyses were carried out with splitless injection to avoid loss of material. Tuberculostearic acid was demonstrated in sputum samples from eight patients with pulmonary tuberculosis but not in sputa from six patients with non-tuberculous pneumonia.

The analytic procedure used seems well fitted for quantitative determination of tuberculostearic acid present in such a complex biological material as a sputum specimen. Thus it was found that the mass fragmentographic peak of methyl tuberculostearate in analyses of sputa from non-tuberculous patients

to which a given amount (4 μg) of mycobacteria (*M. tuberculosis*) had been added, was in size equal to that found when analysing the same amount of isolated bacteria of this species. The significance of the demonstration of tuberculostearic acid in clinical specimens for the rapid diagnosis of tuberculosis and other mycobacterial infections, is at present under evaluation.

The present investigation indicates that a glass capillary column system with OV-17 as stationary phase can be used to advantage for the determination of tuberculostearic acid in biological specimens. Furthermore, the detection sensitivity of the system can be enhanced by a factor of 5 to 10 by using EI of the t-BDMS ester or CI of the methyl ester, compared with analyses employing EI of the latter derivatives in SIM analyses. However, preparation of the t-BDMS ester is more laborious than methylation, and also seems to give lower yields. The almost exclusive formation of quasimolecular ions in the CI mode gives fragmentograms not complicated by additional fragments from cleavage of other ionized large molecules present in the sample.

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