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Selective Procedures for Detecting Femtomole Quantities of Tuberculostearic Acid in Serum and Cerebrospinal Fluid by Frequency-Pulsed Electron Capture Gas-Liquid Chromatography

J. B. BROOKS,[1]* M. I. DANESHVAR,[1] D. M. FAST,[2] AND R. C. GOOD[1]

Division of Bacterial Diseases, Center for Infectious Diseases,[1] and Division of Environmental Health Laboratory Sciences, Centers for Environmental Health,[2] Centers for Disease Control, Atlanta, Georgia 30333

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Conditions are described for the detection of tuberculostearic acid (10-methyloctadecanoate; $C_{18} \cdot CH_3$) in cerebrospinal fluid and serum of patients with tuberculous meningitis. $C_{18} \cdot CH_3$ was found in both the cerebrospinal fluid and serum of patients with tuberculous meningitis at concentrations of 25 to 50 fmol (10^{-15} mol). The necessary specificity and sensitivity for detection of $C_{18} \cdot CH_3$ were obtained by extraction under acid conditions with organic solvent, specific functional group esterification with trichloroethanol, cleanup with disposable reverse-phase sorption chromatography columns, analysis on high-resolution polar and nonpolar capillary columns, and detection by a frequency-pulsed electron capture detector. Use of an IBM 9000 computer equipped with CAP software significantly aided comparison between known $C_{18} \cdot CH_3$ standards and $C_{18} \cdot CH_3$ in clinical specimens. Scale expansion and attenuation changes were the major contributions obtained by use of the computer. The data indicate that detection of $C_{18} \cdot CH_3$ by frequency-pulsed electron capture gas-liquid chromatography may be a valuable aid for early detection of tuberculous meningitis.

There are a variety of infectious and noninfectious diseases that give clinical symptoms similar to those of tuberculous meningitis (12). Some of these diseases are cancer, herpesvirus encephalitis, cryptococcal meningitis, *Nocardia* meningitis, and some forms of aseptic meningitis. Other similarities among these diseases are that diagnosis is slow and many of the infectious agents are difficult to culture so that an organism often is not isolated (12). Rapid diagnosis of tuberculous meningitis is important because it permits specific therapy, which significantly reduces mortality and suffering among patients.

Frequency-pulsed electron capture gas-liquid chromatography (FPEC-GLC) metabolic profiles obtained from cerebrospinal fluid (CSF) and serum (SR) have been used for some time to aid in the rapid diagnosis of tuberculous meningitis (6, 11). If FPEC-GLC tests were developed to detect a specific component reliably, tuberculostearic acid (10-methyloctadecanoate; $C_{18} \cdot CH_3$) in the CSF and SR associated with tuberculous meningitis, it could simplify data interpretation and increase the specificity of the FPEC-GLC test for detection of tuberculous meningitis. Other workers (P. A. Mardh, L. Larsson, N. Hiby, H. C. Engbaek, and G. Odham, Letter, Lancet i:367, 1983) have reported detection of $C_{18} \cdot CH_3$ in CSF by mass spectrometry; however, only one case was studied and no controls were reported. Routine use of mass spectrometry, however, is impractical and expensive. We reasoned that, since there are about 5,000 organisms present in all of the CSF in a typical case of tuberculous meningitis, we would need a test that would detect low femtomole quantities of $C_{18} \cdot CH_3$ and one that was specific enough to prevent misidentification of $C_{18} \cdot CH_3$. The purpose of this study was to develop a test to detect femtomoles of $C_{18} \cdot CH_3$ in CSF and SR through extraction, specific functional group derivatization, sample cleanup by disposable-column reverse-phase chromatography (RPC), and analysis by FPEC-GLC with high-resolution polar and nonpolar capillary columns.

* Corresponding author.

MATERIALS AND METHODS

Both the CSF and the SR used in the study were from patients subsequently found to be culture positive for tuberculous meningitis and gave an FPEC-GLC profile of hydroxy acids and amines typical of tuberculous meningitis when analyzed by FPEC-GLC as previously described (6, 11). Five control CSF samples were taken from patients undergoing myelogram. The CSF samples were not centrifuged, no reagents were added, and they were either analyzed by FPEC-GLC or frozen immediately for future use. Once the samples were acidified for the FPEC-GLC procedure, they were extracted within 30 min and processed.

A sample (2 ml) was placed in a clean 50-ml centrifuge tube closed with Teflon-lined caps and extracted with high-purity solvents (nanograde chloroform [Mallinckrodt, Inc.] and reagent-grade ethyl ether [Fisher Scientific Co.]) at different pH values as previously described (1, 9). Internal standards (heptanoic acid, 2-hydroxyisovaleric acid, and di-*n*-butylamine) were added before extraction as previously described (1). For carboxylic acids, the acidic chloroform extract was derivatized with trichloroethanol (TCE) as previously described (1). The samples were also analyzed for amines and hydroxy acids as previously described (2, 6–8). Extracts of known concentrations of $C_{18} \cdot CH_3$ and acidified extracts of body fluids from patients with culture-proven disease due to *Mycobacterium tuberculosis* were derivatized with bromomethyldimethyl chlorosilane as previously described (9) to further confirm the presence of $C_{18} \cdot CH_3$ in body fluids. TCE derivatives of acidified body fluid extracts were also brominated to test for the presence of unsaturated carboxylic acids. Bromination adds bromine atoms to unsaturated carbon groups and produces a change in the boiling point of the unsaturate which affects a change in the retention time of the unsaturated compound. This change in retention time can then be used to detect unsaturation. The

bromination procedure is also useful for removal of compounds (such as $C_{18:1}$) from the vicinity of a saturated carbon compound (such as $C_{18} \cdot CH_3$) which has a retention time close to that of the unsaturate. The bromination procedure was done as follows. (i) The TCE-derivatized sample in xylene-ethanol (50:50) was evaporated with clean, dry air (1, 7) to about 25 μl, and 100 μl of a 50:50 $CHCl_3$-CCl_4 solution was added. (ii) A 25-μl portion of a solution of 5% bromine in CCl_4 was added, and the reaction mixture was permitted to sit for 5 min. (iii) Step ii was repeated, and the sample was permitted to sit for an additional 30 min. (iv) The sample was reanalyzed by FPEC-GLC, and peaks that changed retention time or were removed because of their increased boiling points after addition of bromine to the unsaturated bond were noted as unsaturates.

We also tested several CSF samples from patients with culture-proven tuberculous meningitis for increased release of $C_{18} \cdot CH_3$ by saponification of the sample as follows. (i) An internal standard of heptanoic acid (C_7) was added to 2 ml of CSF as previously described (1). (ii) Four milliliters of 15% NaOH in distilled water was added to the sample, and it was heated in a boiling water bath for 30 min. (iii) The sample was then cooled, acidified, extracted with nanograde chloroform (Mallinckrodt), derivatized with TCE, and analyzed as described below.

To reduce or remove unreacted TCE reagent and make the analysis for carboxylic acids more specific, the TCE-derivatized samples were passed through disposable C_2 RPC columns (Analytichem International) as follows. (i) The TCE derivatives were prepared and dissolved in xylene-ethanol (50:50) as the final solvent as previously described (1, 7). (ii) The RPC column was conditioned as recommended by the manufacturer with 2 column volumes (2 × 3 ml) of methanol and 1 column volume (3 ml) of distilled water. The TCE derivative in 0.1 ml of xylene-ethanol was then added. (iii) The xylene-ethanol and water were removed from the column by vacuum and discarded. Next, the TCE derivatives were eluted from the column with 3 ml of methanol-chloroform (20:80). (iv) The methanol-chloroform eluent containing the TCE derivative was evaporated to about 25 μl with clean, dry air, 100 μl of xylene-ethanol (50:50) was added, and the sample was analyzed by FPEC-GLC.

TCE derivatives of carboxylic acids were analyzed by FPEC-GLC on the same type of instrument equipped with a large-bore, nonpolar fused-silica capillary column (0.5 mm [inside diameter; i.d.] by 10 m) coated with 4.4-μm-thick film of OV-101 and on a similar OV-101 capillary column (0.5 mm [i.d.] by 25 m). Carboxylic acid derivatives were also analyzed on a polar fused-silica capillary column (0.32 mm [i.d.] by 25 m) coated with a 0.25-μm film of OV-225. Helium was used as a carrier gas in both capillary columns and was set at a flow rate of 5 ml/min. Makeup gas for the capillary columns was 95% argon–5% methane. The combined flow rate of the carrier gas and makeup gas through the detector was 70 ml/min. For analysis of the TCE-derivatized acids on the 10-m nonpolar column, two temperature programs were used. (i) The instrument was programmed for a 3-min hold at 90°C and then for a linear increase of 6°C/min to 275°C, or (ii) the instrument was programmed from 175 to 275°C at a linear increase of 2°C/min. The 25-m OV-101 nonpolar capillary column was kept isothermal at 90°C for 2 min and then programmed for a linear increase of 4°C/min to 275°C. When using the polar column, we kept the instrument at 100°C for 3 min and then programmed it to 220°C at a linear increase of 2°C/min. A 1-μl sample was used for each analysis. An IBM 9000 computer equipped with CAP 1.4 software was used to integrate the peaks, expand sections of the chromatogram for ease of comparison, or adjust peak attenuation.

A Finnigan TSQ46 gas chromatograph-mass spectrometry data system equipped with a splitless injector and a fused-silica, 0.25-μm-thick film DB-5 bonded-phase capillary column 30 m long by 0.25 mm (i.d.; J and W Scientific) was used. The carrier gas was helium at a flow rate of 1 ml/min. For analysis, 4 μl of the derivatized sample was injected, and the instrument was kept at 90°C for 3 min. After 3 min, the injector, which had a temperature of 180°C, was vented, and the instrument was programmed for a linear increase of 6°C/min to 275°C and kept isothermal for 26 min. The TCE ester of $C_{18} \cdot CH_3$ (TCE-$C_{18} \cdot CH_3$) eluted at 43.5 min. Various parameters for optional detection of TCE-$C_{18} \cdot CH_3$ by gas chromatography-mass spectrometry data system with electron impact and chemical ionization were investigated. The conditions finally used were the chemical-ionization mode with methane as the ionization gas and monitoring of the fragments at *m/z* 393, 395, 429, and 431. These ions were chosen by determining the most intense positive chemical-ionization ions during a full scan over the entire peak of the known TCE-$C_{18} \cdot CH_3$ standard.

RESULTS

Figure 1 shows an analysis of 500 ng (dry weight) of saponified cells of *M. fortuitum* (C), a standard acid mixture with $C_{18} \cdot CH_3$ (B), and separation of acid mixtures at different temperature programs (A and B). Beginning the program at 175°C and increasing the temperatures at 2°C/min to 275°C produced good separation between the C_{18} acid, which was present in all samples, and $C_{18} \cdot CH_3$ on the large-bore, 10-m capillary column; however, the more volatile acids (C_1 to C_5) coeluted with the reagent peak (Fig. 1B). Programming from 90 to 275°C at 6°C/min after an initial hold of 3 min gave resolution of the standard acid mixture in about 42 min. $C_{18} \cdot CH_3$ was resolved from stearic acid ($C_{18:1}$) under these conditions (Fig. 1A), but either faster chart speed or use of an IBM 9000 computer with CAP 1.4 software to expand this section of the chromatogram (Fig. 2B) was necessary. Both expansion of sections and the ability to change sensitivity (Fig. 2C) made comparison between the retention time of the $C_{18} \cdot CH_3$ standard and that of the suspected $C_{18} \cdot CH_3$ in CSF and SR more accurate, less time consuming, and easier to perform, as illustrated in chromatograms A and B (Fig. 2). When a computer is unavailable, similar results can be obtained by slower temperature programming, increased chart speed, and reanalysis of the sample at different concentrations. Through analysis of culture-positive CSF samples and comparison of the results against known standards, it was determined that 25 to 50 fmol of $C_{18} \cdot CH_3$ must be present to identify tuberculous meningitis (Fig. 2A, B, and C).

Whereas the sensitivity necessary for detection of $C_{18} \cdot CH_3$ is dependent on the FPEC detector, the specificity depends on the FPEC detector, extraction with organic solvents at different pH values, specific functional-group derivatization, sample cleanup with RPC columns, and analysis on high-resolution polar and nonpolar capillary columns (Fig. 3A and B). The standard mixture eluted on the OV-225 polar column (Fig. 3B) at a lower temperature (100 to 220°C) than it did on the OV-101 nonpolar column (90 to 275°C). In addition, the unsaturated acids eluted later than did the saturated acids of the same chain length. On the OV-101 column, $C_{18:1}$ eluted before C1 (Fig. 3A), and $C_{18:1}$ eluted after C_{18} on the OV-225 column (Fig. 3B). Benzene-

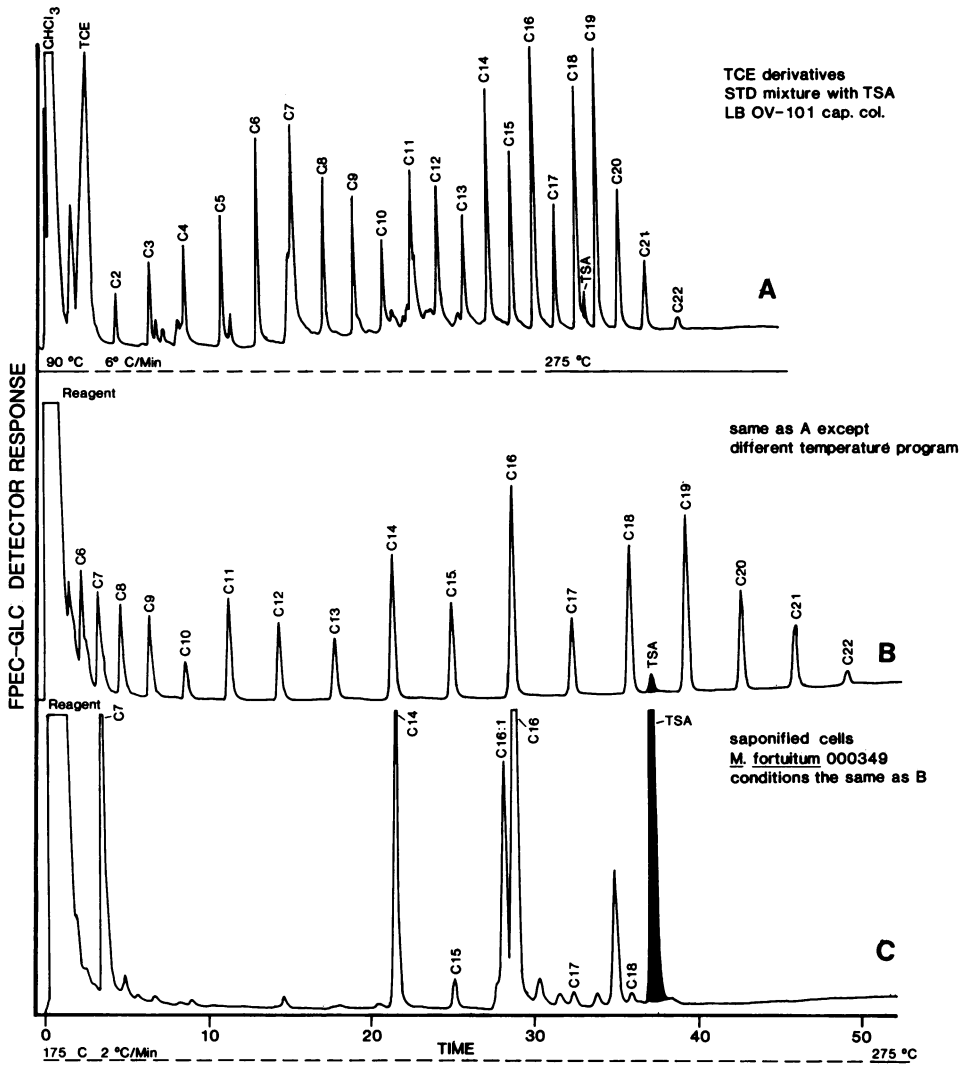

FIG. 1. FPEC-GLC of TCE derivatives. (A) Standard acid mixtures were programmed isothermally for 3 min at 90°C and then programmed for a linear increase of 6°C/min to 275°C. (B) Standard mixture of acids programmed at a linear increase of 2°C/min to 275°C. (C) Saponified cells of *M. fortuitum* (50 ng [dry weight]). Conditions were the same as for panel B. The analyses were made on a 10 m by 0.5 m (i.d.) OV-101 capillary column (cap. col.). The letter C followed by a number indicates a saturated carboxylic acid with the number of carbon atoms indicated by the number. A colon between two numbers indicates unsaturation. Abbreviations: TSA, tuberculostearic acid (10-methyloctadecanoate); STD, standard.

containing acids (data not shown) also changed positions on the OV-225 column, eluting much later than the straight-chain acids with similar carbon numbers.

Chromatographic results from bromination of the TCE-derivatized sample are shown in Fig. 3C. Note that $C_{18:1}$ (chromatogram B) has been removed, making $C_{18} \cdot CH_3$ more observable. New peaks appeared in the chromatogram (peaks 1 to 6) between 5 and 25 min. Since these peaks were not in the control reagent, the new peaks may be compounds that were undetected before bromination. The bromination test is convenient for testing of unsaturated compound and removal of $C_{18:1}$; however, it is not used routinely. Derivatization with bromomethyldimethyl chlorosilane to obtain additional evidence for the presence of $C_{18} \cdot CH_3$ in body fluid samples was successful in some cases in which there were high concentrations of $C_{18} \cdot CH_3$ (over 100 fmol). Use of bromomethyldimethyl chlorosilane derivatives for detection of $C_{18} \cdot CH_3$, while yielding additional proof for the presence of $C_{18} \cdot CH_3$ in body fluids, was inferior to TCE derivatives in specificity and sensitivity. Therefore, we recommend the use of TCE derivatives for routine use to detect $C_{18} \cdot CH_3$.

The use of disposable RPC columns to remove electron-capturing reagent (TCE) is shown in Fig. 4B and C. The pattern in chromatogram A is from TCE-derivatized CSF sample from a patient with tuberculous meningitis. The derivative was prepared and cleaned up by acid and base washes and air evaporation in xylene to remove TCE as previously described (1, 7). Chromatogram B shows the same derivative put through an RPC C_2 column to remove additional unreacted TCE. Chromatogram C shows that, with additional removal of TCE, more sample can be applied to the column without overloading the detector with TCE and that $C_{18} \cdot CH_3$, along with other acids, then becomes more detectable. The saponified CSF taken from five different culture-positive patients with tuberculous meningitis did not show an increase in $C_{18} \cdot CH_3$, and saponification of the CSF is not recommended. We also found that centrifu-

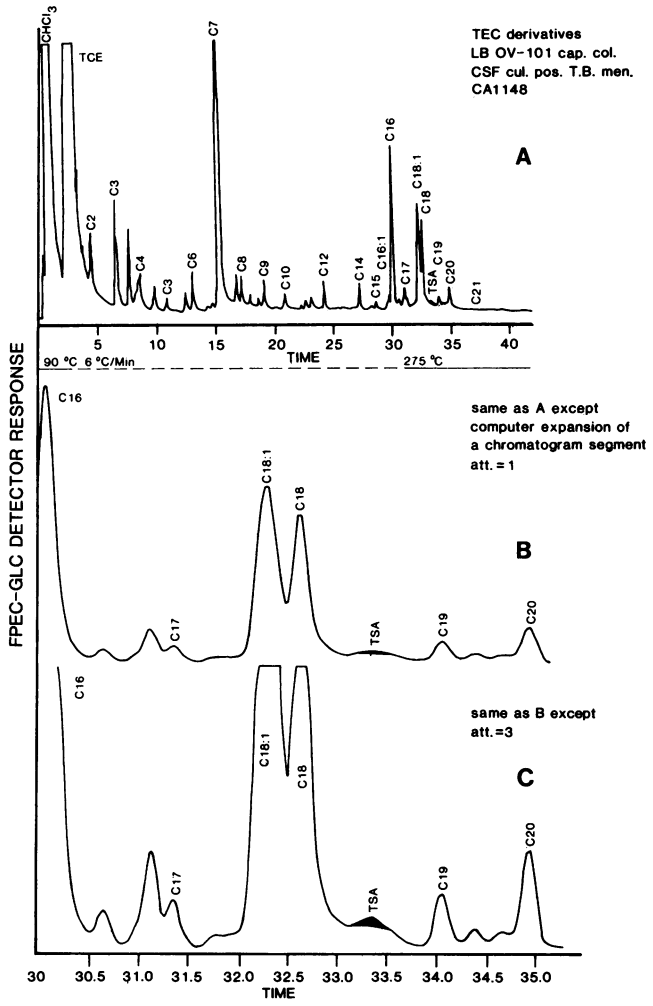

FIG. 2. FPEC-GLC of TCE (TEC) derivatives. (A) Acidic chloroform extracts of CSF from a patient with culture-positive (cul. pos.) tuberculous meningitis (T.B. men.). (B) A computer-expanded portion of chromatogram A between 30 and 35 min. (C) Computer-expanded and decreased attenuation (att.) of chromatogram A between 30 and 35 min. For additional definitions of abbreviations, see the legend to Fig. 1.

gation of CSF from patients with culture-positive tuberculous meningitis at 5,000 rpm (Sorvall Superspeed RC2-B) for 20 min decreased the amount of $C_{18} \cdot CH_3$ detected (data not shown). Since FPEC-GLC analysis of noncentrifuged saline spiked with 10^6 *M. tuberculosis* cells did not detect $C_{18} \cdot CH_3$, and since less than 0.1 of that number of cells is found in culture-positive CSF, it was concluded that $C_{18} \cdot CH_3$ did not come directly from *M. tuberculosis* cells and that loss of $C_{18} \cdot CH_3$ through centrifugation of CSF was not due to removal of *M. tuberculosis* cells from the CSF. Further, because it is known that removal of drying agents, such as sodium sulfate, through centrifugation can cause reduction of acids known to be present in CSF, it was concluded that diminished amounts of nonbound $C_{18} \cdot CH_3$ after centrifugation may have been due to adherence of $C_{18} \cdot CH_3$ to the particulate matter being removed. In limited studies with FPEC-GLC analysis of SR (data not shown) from patients with culture-positive meningitis, we also detected $C_{18} \cdot CH_3$ in concentrations equivalent to those in the CSF. $C_{18} \cdot CH_3$ was not detected in any of the five control samples.

DISCUSSION

At the beginning of the study, we knew that the key to successful detection of $C_{18} \cdot CH_3$ in CSF and SR lay in the sensitivity and specificity of the test. Other considerations were practicality, cost, and ease of data interpretation. It has been known for years that the cellular constituents of *M. tuberculosis* contained $C_{18} \cdot CH_3$, and recently (13) it has been shown that $C_{18} \cdot CH_3$ is probably membrane associated. We easily detected $C_{18} \cdot CH_3$ by FPEC-GLC as the TCE ester in 5 ng (dry weight) of saponified cells. We further thought that detectability of $C_{18} \cdot CH_3$ might be increased through saponification of the body fluid; however, this was

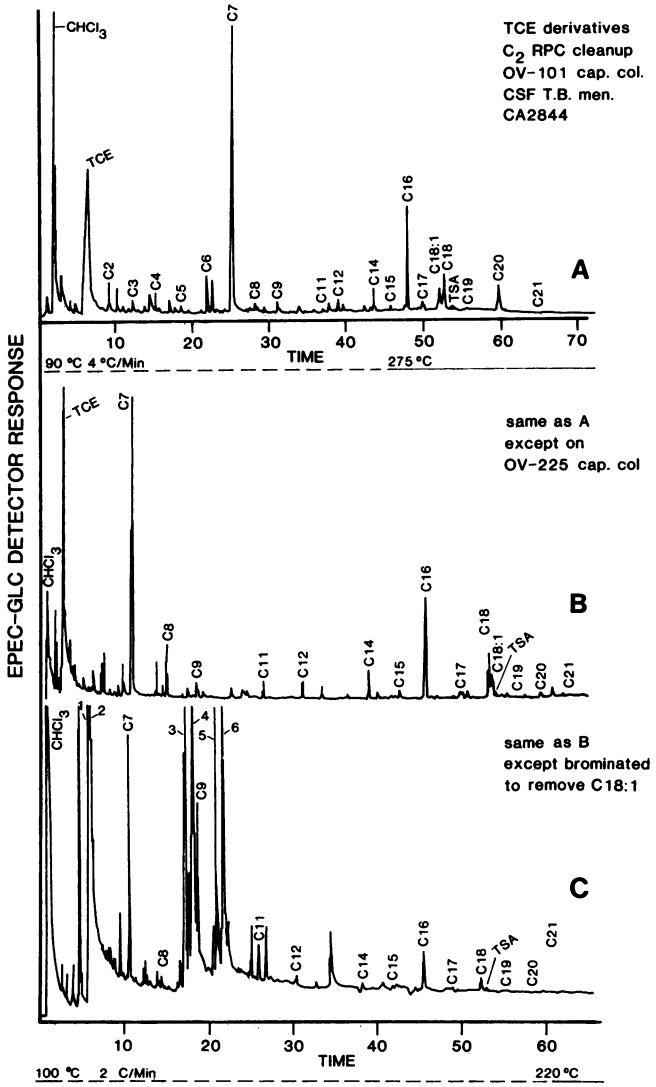

FIG. 3. FPEC-GLC of TCE derivatives. (A) The derivatives were put through a C_2 RPC column for cleanup and analyzed on a 25 m by 0.5 mm (i.d.) OV-101 capillary column. (B) The same derivative as in panel A analyzed on a 25 m by 0.38 mm (i.d.) polar OV-225 column. Note that $C_{18:1}$ eluted on the polar column after C_{18}. (C) The same derivative as panels A and B after bromination and reanalysis. Note that $C_{18:1}$ was missing and that new peaks 1 to 6 appeared. For definitions of abbreviations, see the legends to Fig. 1 and 2.

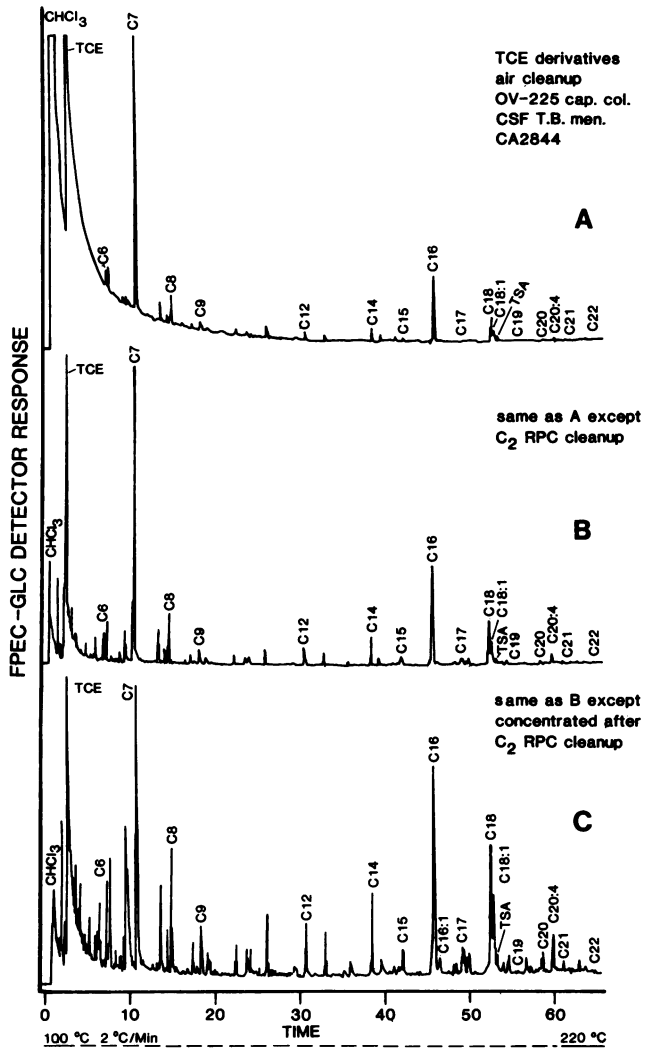

FIG. 4. FPEC-GLC of TCE derivatives from an acidic chloroform extract of CSF taken from a patient with culture-positive tuberculous meningitis. (A) Derivatives made with air cleanup of TCE as previously described (1). (B) Derivative A was passed through an RPC column. (C) The derivative was concentrated after RPC cleanup and reanalyzed. Note that all components were increased and TSA was more easily detected. For definitions of abbreviations, see the legends to Fig. 1 and 2.

not the case. Results from this study indicate that nonbound $C_{18} \cdot CH_3$ exists in CSF and SR during a state of infection, and we have limited data that suggest that it first appears in SR; however, since other species of *Mycobacterium*, *Corynebacterium*, and *Nocardia* contain $C_{18} \cdot CH_3$ and cause disease, detection of $C_{18} \cdot CH_3$ in CSF is probably more specific for the diagnosis of tuberculous meningitis. Computerized FPEC-GLC analysis of CSF and SR presents an effective means for detection of $C_{18} \cdot CH_3$ at low femtomole levels in CSF and SR. Further, through the use of the FPEC-GLC detector, TCE derivatives, RPC column cleanup, and analysis on polar and nonpolar columns, sufficient sensitivity and specificity can be obtained to make detection of $C_{18} \cdot CH_3$ in CSF and SR reliable. The IBM 9000 computer equipped with CAP software provides a convenient, fast way to make more accurate comparisons between known $C_{18} \cdot CH_3$ standards and $C_{18} \cdot CH_3$ in samples from patients. This is accomplished by scale expansion and attenuation changes.

RPC column cleanup was used to further remove TCE; however, the RPC column under conditions used in the study was selective for TCE esters of carboxylic acids and HFBA esters of alcohols. Long-chain TCE esters of carboxylic acids (C_{11} and higher) can be selected for by using C_{18} RPC columns or changing the polarity of the eluting solvent. It is possible to analyze for $C_{18} \cdot CH_3$ in a relatively short time by removing the short-chain carboxylic acids on RPC columns and then using a high starting temperature (above 175°C) on the gas chromatograph. This approach in the analysis of body fluids also works well for cellular fatty acids. A major drawback to this approach in the analysis of body fluids is loss of the FPEC-GLC profile of short-chain carboxylic acids. Detection of the entire spectrum of carboxylic acid from C_2 to C_{22} provides an additional diagnostic criterion that is potentially more useful than detection of $C_{18} \cdot CH_3$ alone (3, 4, 6, 10). Use of a 25-m OV-101 column is recommended over the 10-m OV-101 column for best resolution of both short- and long-chain acids.

Detection of $C_{18} \cdot CH_3$ in the CSF of a patient with supporting clinical data may offer a fast, reliable means for rapid diagnosis of tuberculous meningitis. Further, FPEC-GLC tests for carboxylic acids combined with tests for hydroxy acids and amines (8) yield additional data for diagnosis of tuberculous meningitis as well as for other diseases (5, 6, 11). It is possible that analysis of SR and pleural fluids for $C_{18} \cdot CH_3$ could provide diagnostic evidence for acute tuberculosis and pleural effusions caused by *M. tuberculosis* (unpublished data).

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