Disposable Reversed-Phase Chromatography Columns for Improved Detection of Carboxylic Acids in Body Fluids by Electron-Capture Gas-Liquid Chromatography

M. I. DANESHVAR,* J. B. BROOKS, AND R. M. WINSTEAD

Analytical Chemistry Laboratory, Meningitis and Special Pathogens Branch, Division of Bacterial Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Received 21 November 1986/Accepted 6 April 1987

Disposable reversed-phase chromatography columns were tested for their effectiveness in removing unreacted trichloroethanol (TCE) from derivatized samples for gas-liquid chromatography analysis. Derivatized acidic chloroform extracts of saponified whole cells of Mycobacterium species, spent culture media, and derivatized acidic chloroform extracts of serum and cerebrospinal fluids from patients with tuberculous meningitis were tested. Samples were added to preconditioned reversed-phase chromatography columns, and various solvents and solvent mixtures were tested to determine maximum recovery of the TCE derivatives. With this procedure, we were able to quickly remove the TCE reagent and efficiently recover TCE-derivatized carboxylic acids. Use of these columns improved the reagent cleanup procedure, simplified the derivatization step, permitted increased detection of trace components, such as tuberculostearic acid, in body fluids, and improved the selectivity of the procedure for detection of carboxylic acids.

One problem associated with derivatization of sample extracts for analysis by frequency-pulsed electron-capture gas-liquid chromatography (FPEC-GLC) is the removal of excess electron-capturing reagent. Failure to remove excess reagent limits the amount of sample that can be analyzed and thus reduces detection of important metabolites that may be present in body fluids at femtomole (10^-15) or picomole (10^-12) quantities. Disposable reversed-phase chromatography (RPC) columns were tested for their effectiveness in the removal of unreacted trichloroethanol (TCE) from derivatized samples. The basis for reversed-phase sorbent extraction is that the solid phase has a greater attraction for the TCE-derivatized acid than for the solvent in which the derivative is dissolved (5). The RPC column makes use of a solid phase of silica to which is bonded packing material with hydrophobic functionality. Usually the RPC column material has a 2-, 8-, or 18-hydrocarbon chain functional group which is bonded to the solid silica. If it is necessary to remove unreacted TCE from the derivatized sample, an RPC column-packing material is chosen that is nonpolar, and the polar TCE reagent is bound to the column by hydrophilic interaction. The less polar acid TCE esters are subsequently eluted with a solvent of low polarity. Elution can be accomplished by choosing an appropriate nonpolar solvent (hexane) or by a combination of solvents such as methanol-chloroform (MeOH-CHCl_3) which selectively elute short-chain, more polar TCE esters (C_2 to C_6) or long-chain, less polar TCE esters (C_7 to C_22). The purpose of this investigation was to determine the effectiveness, practicality, and time-saving features of RPC columns for the removal of unreacted TCE reagent. A further goal was to simplify the derivatization procedure previously described (1) by eliminating certain time-consuming steps.

MATERIALS AND METHODS

Body fluids, spent culture media, or saponified cellular material (2 ml) was adjusted to pH 2 with 0.1 ml of 50% (vol/vol) sulfuric acid-distilled water. Internal standards were added (1), and the sample was extracted with nanograde chloroform (CHCl_3; Mallinckrodt, Inc.) to obtain carboxylic acids as previously described (1–3). The acidic CHCl_3 extracts were then derivatized with TCE to form highly electron-absorbing TCE esters of the carboxylic acids. The TCE derivatives were prepared by one of the following procedures.

Procedure 1. The acidic CHCl_3 extract (20 ml) was evaporated with clean dry air to 25 μl as previously described (1). Then, 25 μl of a freshly prepared mixture (1:9) of TCE-CHCl_3 was added to the sample. Next, 30 μl of heptafluorobutyric anhydride was added to catalyze the TCE esterification, and the reaction was permitted to stand for 30 min. CHCl_3 (200 μl) was added, and the derivatized sample was acid and base washed to remove excess heptafluorobutyric anhydride as previously described (1). Next, 100 μl of xylene was added, and the sample was transferred to a 3-ml conical test tube (Kimble, Div. Owens-Illinois) and evaporated in a 100°C sand bath with clean dry air to the 10-μl mark. Then, 100 μl of xylene-ethanol (50:50) was added as the final solvent for FPEC-GLC analysis.

Procedure 2. The TCE derivative was prepared as described for procedure 1 above by adding TCE and heptafluorobutyric anhydride reagents to the concentrated acidic CHCl_3 extract, and the sample was permitted to stand for 30 min. Next, 200 μl of CHCl_3 and 100 μl of xylene were added to the unwashed derivatized sample and evaporated with clean dry air in a 100°C sand bath to about 25 μl. Then, 400 μl of nanograde hexane (Mallinckrodt) was added as solvent for cleanup with an RPC C18 column (Analytichem International, Inc.). The C18 column was conditioned by placing it onto a VAC-ELUT vacuum manifold cover.

* Corresponding author.
FIG. 1. FPEC-GLC chromatograms of TCE-derivatized samples from standard acid mixtures C2 to C22. TCE derivatives are shown after cleanup with a C2 RPC column and various eluting solvents and a solvent mixture. Analyses were done on a 25-m OV-225 capillary column. The letter C followed by a number indicates a saturated carboxylic acid with the number of carbon atoms indicated by the number. A, TCE derivative passed through C2 RPC column eluted with methanol (complete elution); B, TCE derivative passed through C2 RPC column eluted with chloroform; C, TCE derivative passed through C2 RPC column eluted with methanol-chloroform (30:70, vol/vol).

(Analytechm) and aspirating 6 ml of MeOH through the column. The MeOH level was maintained at the top of the sorbent, and 6 ml of hexane was added and aspirated through the column. The level of hexane was held at the top of the sorbent, and the derivatized sample, in hexane, was added to the column. Next, a partial vacuum of about 2 inches (ca. 6.77 kPa) of Hg was applied to the column, and hexane was aspirated until the sorbent was dry (about 2 min). The solvent was discarded, and a collection tube (10 by 75 mm; Corning Glass Works) was placed on the collector rack inside the VAC-ELUT vacuum manifold. The TCE esters were then eluted from the RPC column with 3 ml of hexane. The sample was evaporated to about 100 μl in a 100°C sand bath, and 100 μl of xylene-ethanol was added. Next, the sample was transferred to a 3-ml conical test tube, placed in the 100°C sand bath, and evaporated with clean dry air to the 10-μl mark, and 100 μl of xylene-ethanol (50:50) was added as the final solvent for FPEC-GLC analysis.

Derivatives prepared as described for procedure 1 were further tested for additional TCE cleanup and removal of short-chain acids with RPC columns as follows. C18, C8, and C2 RPC columns were conditioned with 2 column volumes of MeOH followed by 2 column volumes of acidified water. Then, the TCE-derivatized sample dissolved in 100 μl of xylene-ethanol (50:50) was added to the column, and the aqueous xylene-ethanol was aspirated through the column. The sample was then eluted from the columns with various ratios of CHCl3-MeOH ranging from 70:30 to 80:20. Finally, the sample was evaporated to 25 μl, and 100 μl of xylene-ethanol was added. The TCE esters were analyzed on a Perkin-Elmer 900 or 3920 gas chromatograph. The instruments were equipped with dual FPEC-GLC detectors. Two large-bore nonpolar fused-silica bonded-phase capillary columns, coated with a 4.4-μm-thick film of OV-101, were used. One of the OV-101 columns was 10 m, and the other column was 25 m long. Samples were also analyzed with a polar fused-silica bonded-phase capillary column (0.32 mm [inside diameter] by 25 m) coated with a 0.25-μm-thick film of OV-225. Helium was used as the carrier gas in all of the capillary columns at a flow rate of 5 ml/min. The makeup gas for each capillary column was a mixture of 95% argon and 5% methane. The combined flow rate of the carrier gas and the makeup gas through the 0.25 m, FPEC detector was 70 ml/min. For analysis of the TCE-derivatized acids on the 10-m OV-101 column, the instrument was held isothermal at 90°C for 3 min and then programmed to 275°C at a linear increase of 6°C/min. For the 25-m OV-101 column, the instrument was held at 90°C for 2 min and then programmed at 4°C/min to 275°C. For the polar OV-225 column, the instrument was held at 100°C for 3 min and then programmed to 220°C at a linear increase of 2°C/min. The derivatized sample (1 μl) was injected onto the columns for analyses. An IBM System 9000 computer equipped with CAP 1.4 software (IBM Corp.) was used to integrate the peaks, expand sections of the chromatograms for easy comparison, and evaluate various levels of attenuation.

RESULTS

TCE-derivatized standard mixtures of carboxylic acids (C2 to C22), along with derivatized body fluid and spent culture medium samples that had been derivatized and processed as described for procedure 1, were tested for further removal of excess TCE with C2, C8, and C18 RPC columns. In addition, various solvents and solvent mixtures were tested to determine the best recovery of the acids. Polar solvents, such as MeOH, eluted both the derivatized acids and the excess TCE reagent (Fig. 1A). Moderately polar solvents, such as CHC13, did not elute the excess TCE reagent and also reduced the recovery of shorter-chain acid derivatives (C2 to C10) on the C2 RPC column (Fig. 1B). Loss of these shorter-chain acids might be desirable if the major purpose were to test for long-chain (C10 to C20) acids, which are found in whole bacterial cells. Selective elution was tested for recovery of the shorter-chain acids by combining MeOH-CHCl3 in ratios of 30:70 (vol/vol) (Fig. 1C) or 25:75 to produce the desired polarity. MeOH-CHCl3 at a ratio of 25:75 proved to be the best eluting solvent with samples derivatized as in procedure 1, because the 30:70 mixture often eluted many of the TCE-derivatized short-
chain (C2 to C7) acids. For further studies of acid recoveries, samples were added to various preconditioned RPC columns with C18, C8, and C2 functional groups by using MeOH-CHCl₃ (25:75) as the eluting solvent. The following results were obtained. Unreacted TCE (Fig. 2A) and derivatized acids with chain lengths up to C10 were retained by the C18 RPC column, whereas acids with chain lengths longer than C10 were released (Fig. 2B). The C8 RPC column retained unreacted TCE and derivatized acids with chain lengths up to C8 and released acids with chain lengths longer than C8 (Fig. 2C). The C2 RPC column retained unreacted TCE and derivatized acids with chain lengths up to C6 and released acids with chain lengths longer than C6 (Fig. 2D). Because the C2 RPC column retained the unreacted TCE and released more of the shorter-chain acids, it was chosen for routine use to clean up derivatized samples of body fluids which had been derivatized previously by procedure 1. Once the cleanup of these samples was accomplished, we began research in the use of RPC columns to shorten the derivatization steps described for procedure 1. We found that the solvent system described for procedure 2 gave the minimum loss of the TCE esters and removed the most unreacted TCE. Figure 3A shows the results obtained under the best solvent conditions with no xylene-air evaporation before the TCE-derivatized sample was added to the RPC column. Observe that there is a noticeable tailing and overloading of the columns from the excess (unreacted) TCE. The results obtained when the xylene-air evaporation was omitted after the sample had been eluted from the column are shown in Fig. 3B. Figure 3C shows the results obtained when xylene-
be positive for tuberculous meningitis by a positive purified protein derivative skin test and response to drug therapy.

DISCUSSION

The use of electron-absorbing reagents such as heptafluorobutyric anhydride and TCE, although essential for specific functional group derivatization to produce highly detectable electron-capturing derivatives, presents problems when analyzed by FPEC detectors and capillary columns. The use of TCE esters offers an effective means for detection of metabolites in diseased body fluids (3, 4), but effective removal of these reagents is essential to prevent overloading of the FPEC detector and capillary columns. Removal of the excess TCE reagent without significant loss of the TCE esters permits the application of more sample onto the capillary columns, which results in increased sensitivity. The complete removal of unreacted TCE reagent without significant loss of the short-chain TCE esters proved to be difficult. We made several attempts to remove all or most of the unreacted TCE without prior cleanup by using C18, C8, C2, CN, and silicon extraction columns. We also tried different solvents and solvent combinations. When the only cleanup procedure used was the extraction column, the maximum removal of TCE reagent was obtained by use of

FIG. 4. FPEC-GLC chromatograms of TCE-derivatized samples from standard acid mixtures C2 to C22 shown to demonstrate reproducibility. The derivatives were not acid and base washed but were xylene-air evaporated, cleaned up by C18 RPC columns, and finally cleaned up by a second xylene-air evaporation. The FPEC-GLC conditions were the same as those described in the legend to Fig. 3. For definitions of abbreviations, see the legend to Fig. 1. A, B, and C, TCE derivative passed through C18 RPC column with pre- and postcolumn xylene-air evaporation cleanup.

air evaporation was done both before and after the sample was put through the RPC column. The xylene-air evaporation step with the sand bath (100°C) and clean dry air was easy to accomplish and reproducible. Figures 4A, B, and C show the degree of reproducibility obtained in three separate derivatizations with the C18 RPC column and the xylene-air cleanup described for procedure 2. Shown in Fig. 5A and B are chromatograms of cerebrospinal fluid from a patient with suspected tuberculous meningitis derivatized and processed through the C18 RPC column as described for procedure 2. Tuberculostearic acid, a known cellular constituent of Mycobacterium tuberculosis (P. A. Mardh, L. Larsson, N. Hibi, H. C. Engback, and G. Odham, Letter, Lancet i:367, 1983), was present in small amounts and was resolved on both columns. Subsequently, the patient was determined to

FIG. 5. FPEC-GLC chromatograms of TCE-derivatized acidic chloroform extract of cerebrospinal fluid from a patient with tuberculous meningitis. The cleanup procedure was the same as that described in the legend to Fig. 4. Use of a colon between two numbers indicates unsaturation. For definitions of abbreviations, see the legend to Fig. 1. A, TCE derivative, C18 RPC cleanup, CA2841 cerebrospinal fluid from a patient with tuberculous meningitis, OV-101 capillary column analysis; B, same as in chromatogram A except analyzed on an OV-225 capillary column.
the C18 column and hexane as both the sample solvent and the eluent. We also tried passing the sample through two consecutive columns, but we were unable to completely remove excess TCE reagent. Finally, we had to incorporate a xylene-air evaporation step as described for procedure 2. Two xylene-air evaporations were necessary to obtain the desired results; both were reproducible and easy to perform. The final xylene-air evaporation of procedure 2 must be done carefully to obtain reproducible results. Preparation of TCE esters of carboxylic acids as described for procedure 2 simplifies the derivatization steps by eliminating the acid and base washes. Moreover, six to eight derivatives can be conveniently prepared at the same time.

Although not totally selective for TCE esters, use of the C18 RPC column increased the selectivity and sensitivity of the FPEC-GLC analysis of TCE esters of carboxylic acids, and use of the improved derivatization procedure gives a cleaner analysis (less background) and increases sensitivity by permitting the injection of more sample.

LITERATURE CITED