JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1986, p. 392–394
0095-1137/86/020392-03$02.00/0

Vol. 23, No. 2

Modified Extraction Procedure for Gas-Liquid Chromatography Applied to the Identification of Anaerobic Bacteria

WAYNE R. THOMANN[1]* AND GALE B. HILL[2]

Environmental Safety Office[1] and Departments of Obstetrics and Gynecology, Microbiology and Immunology, and Anaerobe Laboratory, Clinical Microbiology Laboratories,[2] Duke University Medical Center, Durham, North Carolina 27710

Received 12 July 1985/Accepted 1 November 1985

Chloroform and ether commonly are used as solvents to extract metabolic organic acids for analysis by gas-liquid chromatography in the identification of anaerobic bacteria. Because these solvents are potentially hazardous to personnel, modified extraction procedures involving the use of a safer solvent, methyl *tert*-butyl ether were developed which remained both simple to perform and effective for organism identification.

The identification of anaerobic bacteria is facilitated by gas-liquid chromatographic analysis of organic acids produced during growth in glucose-containing media (1, 4). Typically the organic acids are extracted from the growth medium by using the solvents chloroform or ethyl ether (4). More recently, other methods for analysis of these acids have been developed, including direct injection of centrifuged broth culture (8), temperature programming (7), and head-space gas chromatography (5).

The methods for gas-liquid chromatography described by Holdeman et al. (4) are probably the most commonly used techniques. The main advantages of these methods are the simplicity of the extraction procedures and their applicability to unsophisticated gas-chromatographic equipment. The major disadvantages are the potential hazards associated with the extraction solvents. Ether is highly flammable and may be explosive if peroxides form on exposure to air. In addition, chloroform is classified as a potential human carcinogen (6). Because of these safety factors, significant problems are associated with the storage, handling, and disposal of these solvents. This study was undertaken to determine whether ether or chloroform or both could be replaced by a solvent(s) which would be safer to use but would still be effective in the extraction of bacterial metabolic end products by a relatively simple procedure.

Testing protocol and performance of potential replacement solvents. Our plan for testing was to use the volatile (ether) and the nonvolatile (chloroform) extraction procedures of Holdeman et al. (4) as reference methods for comparison with the extraction efficiencies of each potential replacement solvent. Standard mixtures of volatile and nonvolatile acids (Clinical Analysis Products Co., Sunnyvale, Calif.) were used to provide chromatographic patterns for the purpose of comparing these extractions. The solvents evaluated were ethyl acetate (Fisher Scientific Co., Fairlawn, N.J.), methylene chloride (Fisher Scientific), trichlorotrifluorethane (Mallinckrodt, Inc., Paris, Ky.), and methyl *tert*-butyl ether (MTBE; Fisher Scientific) in addition to the reference solvents chloroform and ethyl ether (Mallinckrodt). Steps in the reference extraction procedures were modified for testing potential replacement solvents whenever improved extraction efficiency could be achieved, but the extraction procedures when used with the reference solvents were unaltered, since the goal was to evaluate whether a safer solvent(s) could function as effectively in organism identification as these commonly used reference solvents. Both flame ionization (FID) and thermal conductivity (TC) gas chromatograph systems were used: a Hewlett-Packard series 5880A gas chromatograph equipped with a 7672 automatic sampler (Hewlett-Packard, Avondale, Pa.) and a Dorhmann 15-3C gas chromatograph (Dorhmann Div., Envirotech Mountain View, Calif.), respectively. Relevant system components and operating parameters were used, as described by Holdeman et al. (4) and Sutter et al. (8).

With the exception of MTBE, all of the solvents tested were unacceptably inefficient in extracting at least one of the organic acids (generally short-chain [C_2 or C_3] acids) that are important in the identification of anaerobic bacteria. Ether and MTBE were the most efficient solvents for the extraction of volatile acids, and there were essentially no differences in the chromatograms of standard volatile organic acid mixtures extracted in these solvents (Fig. 1). On the other hand, methyl esters of the nonvolatile acids were most efficiently extracted into chloroform and methylene chloride. Although MTBE was less efficient than these solvents, sufficient quantities of the methyl esters of the nonvolatile acids were extracted by the MTBE procedure to allow for their accurate identification and discrimination of major versus minor peaks (Fig. 2). Also, subsequent testing has documented that phenylacetic and hydrocinnamic acids can be effectively extracted in MTBE. Thus, MTBE was chosen as the potential replacement solvent for further testing because of safety considerations and because we thought that the option of using a single solvent for both the volatile and nonvolatile extractions would be a considerable added convenience. The procedures modified for the use of MTBE in lieu of ether and chloroform for extraction of volatile and nonvolatile acids, respectively, are adapted from Holdeman et al. (4) and are as follows. For preparation of the volatile acid extract, 1 ml of culture or standard is transferred into a tube (13 by 100 mm). Then 0.2 ml of 50% H_2SO_4, 0.4 g of NaCl, and 2.0 ml of MTBE are added, and the tube is stoppered. The contents are mixed by inverting the tube 18 times in 10 s. The tube is centrifuged at 250 × *g* for 30 s. The top layer is transferred into a tube (12 by 75 mm) containing 0.25 g of 3 nm molecular sieve (1/16-in. pellets, Union Carbide, South Plainfield, N.J.), and the tube is stoppered and allowed to stand for 10 min. For preparation and extraction of methyl derivatives, 1 ml of culture or standard is transferred into a tube (13 by 100 mm). Then 0.2 ml of 50%

* Corresponding author.

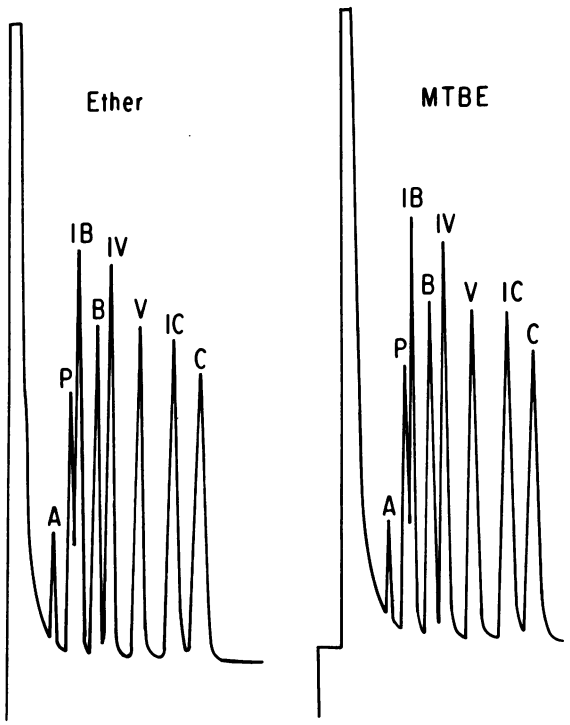

FIG. 1. Comparison of the volatile acids extracted from a standard mixture by the reference ethyl ether method and the modified MTBE method. Symbols: A, acetic acid; P, propionic acid; IB, isobutyric acid; B, butyric acid; IV, isovaleric acid; V, valeric acid; IC, isocaproic acid; C, caproic acid.

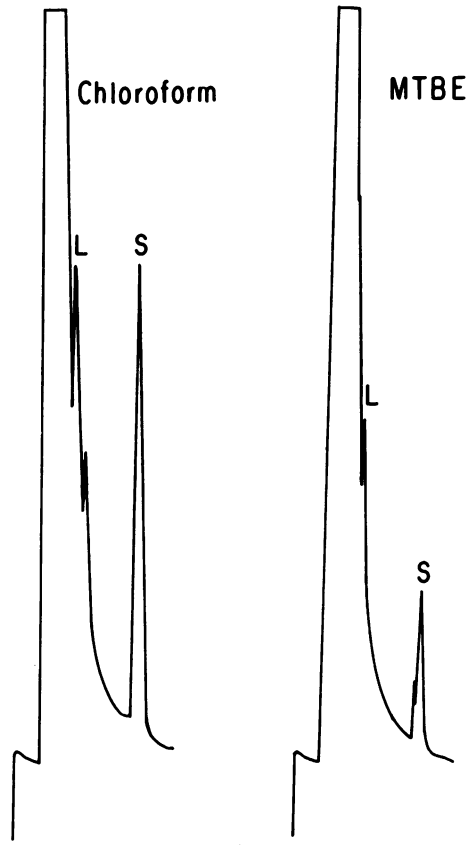

FIG. 2. Comparison of the methyl esters of nonvolatile acids extracted from a standard mixture by the reference chloroform method and the modified MTBE method. Symbols: L, lactic acid; S, succinic acid.

H_2SO_4 and 1.0 ml of methanol are added, and the tube is stoppered and heated at 56°C for 60 min, after which 2.0 ml of water, 1.5 ml of MTBE, and 0.4 g of NaCl are added. The stopper is replaced, and the contents are mixed by inverting the tube 18 times in 10 s. The tube is centrifuged at 250 × *g* for 30 s. The top layer is transferred into a tube (12 by 75 mm) containing 0.25 g of 3 nm molecular sieve, and the tube is stoppered and allowed to stand for 10 min. The procedural changes were minor, and the time and effort required remained comparable to that of the reference procedures. Although it would have been desirable, we did not succeed in our attempts to design a single extraction procedure and chromatographic assay, e.g., by temperature programming, that would detect all the volatile and nonvolatile acids useful for identification.

Modified MTBE methods compared with reference methods for organism identification. A comparison of the modified MTBE procedures with the reference methods for gas-liquid chromatographic detection of the metabolic end products of 73 anaerobic bacterial strains selected to represent most of the clinically significant genera and species of anaerobes (Table 1) was performed to simulate standard laboratory usage. Most of these strains were recent clinical isolates, and all previously had been identified by recommended procedures (4). Pure cultures were inoculated into fluid thioglycolate medium without indicator-135C (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.2% yeast extract, 0.5 μg of vitamin K_1 per ml, 10 μg of hemin per ml, and 10% inactivated rabbit serum and incubated for 72 h in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) containing 85% N, 10% H, and 5% CO_2. Four 1-ml samples were transferred to four tubes, each of which was processed by one of the following four extraction methods: (i) reference volatile, (ii) reference nonvolatile, (iii) MTBE modified volatile, and (iv) MTBE modified nonvolatile. Uninoculated thioglycolate broth and the appropriate standard organic acid mixtures also were processed similarly. Each extract was analyzed in both the FID and TC systems.

We evaluated two aspects of the chromatographic patterns obtained from broth cultures when the modified MTBE extraction methods were used. First, the types and quantities (only whether a major or minor peak [4]) of volatile and methyl esters of nonvolatile acids extracted from the broth cultures by the modified MTBE procedures were determined on the basis of the profiles obtained with similar MTBE processing of the standard organic acid mixtures. These results were then compared with those obtained by using the reference methods for extracting the broth cultures and standard organic acid mixtures. The second aspect of the applicability of the MTBE modified methods to organism identification was evaluated by comparing the MTBE organic acid profiles detected from the 73 known anaerobic species with the reference chromatographic profiles published for these species in the reference manual of Holdeman et al. (4).

The identities and relative quantities (major or minor) of volatile and methyl esters of nonvolatile acids extracted from broth cultures by the modified MTBE and reference extraction methods were the same for all of the extracts

TABLE 1. Strains of anaerobic species used for the evaluation of MTBE modified extraction methods for detection of metabolic end products and identification of anaerobic bacteria

Organism	No. of strains	Organism	No. of strains
Bacteroides fragilis	4	*Veillonella parvula*	1
B. melaninogenicus subsp. *intermedius*	4	*Clostridium perfringens*	3
		C. innocuum	2
B. bivius	3	*C. ramosum*	1
B. thetaiotaomicron	2	*C. histolyticum*	1
B. melaninogenicus subsp. *melaninogenicus*	2	*C. difficile*	1
		Lactobacillus catenaforme	2
B. asaccharolyticus	2	*L. minutus*	1
B. ovatus	1	*L. ruminis*	1
B. disiens	1	*L. acidophilus*	1
B. distasonis	1	*L. casei* subsp. *alactosus*	1
Fusobacterium nucleatum	1	*Actinomyces viscosus*	3
F. mortiferum	1	*A. israelii*	2
Capnocytophaga sp.	1	*A. odontolyticus*	2
Vibrio succinogenes	1	*A. meyeri*	1
Peptostreptococcus anaerobius	3	*Eubacterium limosum*	2
Peptococcus magnus	3	*E. lentum*	1
P. prevotii	2	*E. aerofaciens*	1
P. asaccharolyticus	1	*E. brachy*	1
P. saccharolyticus	1	*Bifidobacterium breve*	1
Streptococcus intermedius	2	*B. adolescentis*	1
S. constellatus	2	*B. bifidum*	1
Gaffkya anaerobia	2	*Propionibacterium acnes*	2

when analyzed in the FID system. Only minor discrepancies were noted when the extracts were analyzed in the TC system. For volatile acids, only two minor discrepancies occurred. For nonvolatile acids there were 20 instances in which a minor amount of lactic acid (methyl ester) was detected in the chromatograms from the modified MTBE extracts but not from the reference chloroform extracts. It was determined that in the TC system the very small peak of lactic acid (methyl ester) was being masked by the large chloroform solvent front. Thus, discrepancies in the organic acid profiles produced by the MTBE and reference methods were minor, and, as might be expected, no significant differences were encountered when the modified MTBE-derived profiles were compared with published reference profiles (4) for the 73 known species of anaerobic bacteria. All isolates could have been successfully identified by using the organic acid profiles generated by either the MTBE or the reference extraction method.

In conclusion, the modified methods and MTBE solvent described for extraction of volatile and nonvolatile organic acids meet the requirements posed for a replacement solvent(s) for ether and chloroform. That is, MTBE is a safer solvent than ether because it is considered less flammable, having a lower flash point and a higher auto-ignition temperature (2, 3). Oral toxicity, based on the 50% lethal dose in rats, also is lower, as is the tendency to form peroxides on exposure to air (2). Further, MTBE is not presently classified as a carcinogen. Our data indicate that the MTBE methods provide for accurate identification of anaerobic bacteria and can be conveniently substituted for use with either TC or FID chromatographs. The Anaerobic Microbiology Laboratory at Duke University Medical Center has been using the MTBE methods routinely for over 2 years, and personnel have found them very satisfactory. It is perceived that the useful life of the injection syringes and the analytical columns is longer than when the ether and chloroform extraction methods were used. The chromatographic columns may last longer because MTBE is less corrosive than chloroform or because of the very efficient drying produced by the molecular sieve, or both. We found no previously published reports of the use of molecular sieve for this purpose. The 1/16-in. pellets also may be responsible for the extended life of injection syringes by absorbing damaging contaminants or, since the molecular sieve pellets are essentially powder free, by preventing clogging of the syringe barrel, which can produce breakage in an automatic injection system.

(This research was conducted in partial fulfillment of the requirements for the Dr.Ph. degree of W. R. Thomann from the University of North Carolina, Chapel Hill, 1983.)

We are grateful to the entire staff of the Anaerobic Microbiology Laboratory, Duke University Medical Center, with special thanks to Freda Kohan, for encouragement and technical assistance during this study.

LITERATURE CITED

1. **Dowell, V. R., Jr., and A. C. Sonnenwirth.** 1974. Gram-positive, nonsporeforming anaerobic bacilli, p. 396–401. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
2. **Fisher Scientific Co.** 1981. Technical bulletin. Fisher methyl tert-butyl either (MTBE). Fisher Scientific Co., Pittsburgh, Pa.
3. **Hawley, G. G.** 1981. The condensed chemical dictionary, p. 435 and 671. Van Nostrand Reinhold Co., New York.
4. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed., p. 132. Virginia Polytechnic Institute and State University, Blacksburg.
5. **Larsson, L., P.-A. Marhand, and G. Odham.** 1978. Detection of alcohols and volatile fatty acids by head-space gas chromatography in identification of anaerobic bacteria. J. Clin. Microbiol. 7:23–27.
6. **National Institutes of Health.** 1982. Safety data sheet—chloroform. National Institutes of Health, Bethesda, Md.
7. **Rizzo, A. F.** 1980. Rapid gas-chromatographic method for identification of metabolic products of anaerobic bacteria. J. Clin. Microbiol. 11:418–421.
8. **Sutter, V., O. M. Citron, and S. M. Finegold.** 1980. Wadsworth anaerobic bacteriology manual, p. 131. C. V. Mosby Co., St. Louis, Mo.