Paper Chromatography as an Adjunct in the Identification of Anaerobic Bacteria

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Received for publication 16 November 1973

Modified paper chromatography procedures for the analysis of fatty acids produced by anaerobic bacteria are described. Both ethylamine and hydroxyamine derivatives of fatty acids were prepared from inoculated anaerobic culture broth. The derivatives were spotted on chromatography paper and developed with appropriate solvents. Paper chromatography is a valuable alternative to gas liquid chromatography as an ancillary procedure in the identification of anaerobic bacteria in the clinical bacteriology laboratory.

Clinical studies indicate the need for specific identification of anaerobic bacteria in infectious processes (15). The progress of the diagnosis, treatment, and epidemiological evaluation of anaerobic bacterial infections depends on the correct and rapid identification of these organisms. Clinical bacteriologists are intimately concerned with the characterization and taxonomy of anaerobes.

It is established that the identification of lower-chain fatty acids produced by the anaerobic bacteria is a significant adjunct in the classification of the anaerobic bacteria (17). Moore and his associates have standardized and simplified anaerobic identification, in part, through the application of gas liquid chromatography (GLC) for the detection of volatile, short-chain fatty acids produced by anaerobes in culture media (11). Each anaerobe generally produces a specific profile of short-chain fatty acids. Results from the GLC "fingerprints" of fatty acids, along with other biochemical characteristics of a particular anaerobe, can be compared with available reference information for the identification of an anaerobic isolate (11).

Many clinical microbiology laboratories, however, are not equipped with GLC units due to the lack of a serviceable area for GLC instrumentation and the initial cost required to purchase a GLC unit. Although clinical service laboratories may have at least one GLC unit, in most cases, they are utilized primarily for clinical chemistry services, including toxicology.

Paper chromatography (PC) appears to have the potential to determine the characteristic fatty acid metabolites of anaerobic bacteria (2, 8, 9). PC has at least three distinct features favorable to a clinical microbiology laboratory limited in space or funds, or both: (i) simplicity of operation, (ii) relatively small cost, and (iii) a relatively small laboratory space requirement.

Of the various methods for PC of simple volatile fatty acids described in the literature, our results indicate that our modification of two procedures are both required and appear to be the most effective for clinical application. In one procedure, volatile fatty acids, including lactic acid, are first converted to esters, then converted to hydroxamic acids, and detected as colored iron complexes (6, 21). In the second procedure, C3-C4 volatile fatty acids and succinic acid are separated as their ethylamine salts (10, 14, 19).

This investigation was conducted in order to evaluate the usefulness of PC as an alternative to GLC in the identification of anaerobic bacteria in the clinical laboratory.

MATERIALS AND METHODS

Bacteria. Anaerobic bacteria were obtained from clinical specimens, stock cultures, and the American Type Culture Collection. Peptostreptococcus productus (Virginia Polytechnic Institute [VPI] no. C35-42) and Peptostreptococcus intermedii (VPI no. 8462E) were obtained from Elizabeth P. Cato, VPI Anaerobic Laboratory.

Culture media for fatty acid analysis. Prereduced brain heart infusion broth (Scott Laboratories, Inc., Yonkers, N.Y.) or a Vacutainer culture tube with supplemented peptone broth (Becton-Dickinson, Rutherford, N.J.) was inoculated with the respective anaerobes and incubated at 35 C for 48 to 72 h. The lower-chain fatty acid contents of uninoculated and
inoculated media were examined by both PC and GLC analysis.

**PC procedures.** A modification of the procedure described by Thompson was used for the preparation of hydroxylamine derivatives of fatty acids (20). A 2-ml amount of culture broth was pipetted into a screw-capped tube with Teflon liner (16 by 125 mm) to which 4 ml of n-propanol and 0.8 ml of 50% aqueous H$_2$SO$_4$ was added. This was incubated at 55 C for 60 min in a temperature block. Then, 4.5 ml of iso-octane was added, and the tube was gently shaken for 30 min. After complete separation, the iso-octane phase, containing the propyl esters, was transferred to a new tube (16 × 75 mm) containing 0.5 ml of freshly prepared hydroxylamine reagent (21). The tube was gently inverted for 30 min, and the upper iso-octane phase was discarded. Then, 100 µl of glacial acetic acid was added to the lower methanol phase, containing the hydroxamic acids, which was subsequently analyzed by PC. A culture broth containing 10 meq of formic, acetic, propionic, butyric, valeric, caproic, lactic, and succinic acids per liter (Analabs, Inc., North Haven, Conn.; J. T. Baker Chemical Co., Phillipsburg, N.J.) was used as a reference standard. In other experiments, methyl or ethyl esters were used.

Five-microliter samples were spotted on Whatman no. 4 chromatography paper, along with a standard reference sample and a media control. The spots were made approximately 2.5 cm from the bottom edge of the sheets at 1.5-cm intervals, air-dried, and then developed in a Whatman no. 3 paper-lined tank (Thomas-Kolb; Arthur H. Thomas Co., Philadelphia, Pa.) humidified with acidified, amyl alcohol-saturated water (21). The chromatogram was immersed 1 cm into a trough containing approximately 45 ml of acidified, water-saturated amyl alcohol (21). Development to approximately 180 mm took approximately 5 to 6 h at 35 C. The chromatograms were then air-dried and lightly sprayed with a 10% aqueous ferric chloride solution in order to visualize the respective hydroxylamine derivatives (hydroxamic acids) of the fatty acids as purple spots.

For the ethylamine PC procedure (19), 16 ml of culture broth was transferred to a 50-ml Erlenmeyer flask. The broth was titrated to pH 8.5 with 33.3% aqueous ethylamine. Whatman no. 1 filter paper was spotted with 5 µl of each specimen, including the standard reference and the uninoculated medium control. The spots were air-dried and developed at 35 C in a Whatman no. 3 paper-lined tank (Thomas-Kolb; Arthur H. Thomas Co., Philadelphia, Pa.). Butanol-saturated water containing 0.1 N ethylamine was placed in the bottom of the tank (18), and the solvent, a water-saturated butanol (19), was placed in a solvent trough. After development for 5 to 6 h (approximately 180 mm), the paper was air-dried and lightly sprayed with a bromophenol blue-citric acid indicator until the background was a light yellow and blue spots were evident (12).

Succinic acid was identified by preparing an ethylamine derivative. The derivative was placed on Whatman no. 1 filter paper and developed in a methyl alcohol-iso-octane (19:1, vol/vol) solution at approximately 22 C (plus or minus two degrees) for 1 h in a Whatman no. 3 paper-lined tank (Thomas-Kolb). The bromophenol blue-citric acid indicator was also applied for visualization of this dicarboxylic acid.

**Other chromatographic procedures.** A thin-layer procedure (3, 4, 20), using an azo-dye synthesized in our laboratory, was examined. The method involved the coupling of the dye with the acids. The ensuing esters were separated on Silica Gel G plates in a tank containing 20:1 (vol/vol) benzene and ethyl acetate.

Gas chromatographic analysis of volatile fatty acids were made with a Beckman G-C-2A gas chromatograph (Richmond, Calif.) equipped with a thermal conductivity detector and aluminum columns packed with Resoflex (Burrell Corp., Pittsburgh, Pa.). The GLC technique and conditions for gas chromatography were those described by the VPI (11).

**RESULTS**

A standard fatty acid profile was examined in order to determine the range of fatty acids detectable by the PC procedures utilized in this study. The chromatographic separation of the ethylamine derivatives of the fatty acids resulted in well-defined spots for the C$_2$-C$_6$ volatile fatty acids (Fig. 1A). Formic, acetic, and lactic acids were indistinguishable from their normal forms. Although succinic acid was not demonstrable with the butanol solvent, it was clearly identifiable when methyl alcohol-iso-octane (19:1, vol/vol) was used as the solvent system. Sensitivity was such that 5 meq of fatty acid per liter was detectable by PC.

Acetic, propionic, and butyric acids were completely distinguishable as individual hydroxylamine derivatives (Fig. 1B). Succinic acid was not detectable as a hydroxylamine derivative. Valeric and caproic acids had similar $R_f$ values, as did lactic and formic acids and, thus, were not distinguishable from a mixture (Table 1). It was impossible to differentiate any of the isomers from their normal forms. Sensitivity was comparable to that of the ethylamine procedure.

A faint spot representing acetic acid was observed in all uninoculated culture media and standards prepared in culture media.

It was apparent from these results that both ethylamine and hydroxylamine derivatives were required for PC. The ethylamine procedure was primarily used for the identification of propionic, butyric, valeric, caproic, and succinic acids, whereas the hydroxylamine procedure was used to identify acetic acid. Because it was impossible to distinguish lactic acid from formic acid, a hydroxamic acid spot with the $R_f$ value of these acids was designated as "formic and/or lactic acid" spot.

Agreement between the determination of PC analysis of the fatty acids produced by the
was achieved with the combined application of both PC procedures, despite the inability to distinguish the isomers from the normal forms of fatty acids and the lack of distinction between lactic and formic acids. The fatty acid profiles obtained with both derivatives revealed all fatty acids detectable by the respective procedures from the bacteria used in this investigation. Succinic acid was also detectable in cultures producing that acid. The application of the various VPI criteria, including Gram stain reaction, morphological and biochemical characteristics, and the fatty acid profiles obtained by either PC or GLC, assisted in the identification of the anaerobes. A relative concentration of fatty acid by PC analysis was indicated by the intensity of the spots and was generally correlated with the results obtained by GLC analysis.

PC analysis of fatty acids correlated with results obtained by GLC analysis in the determination of the anaerobic genera examined in this investigation (Fig. 2, Table 2). Accordingly, both the acetic and propionic acids from *Propionibacterium* and *Veillonella* species were detectable by PC analysis. Furthermore, the determination of butyric acid assisted in the distinction of *Fusobacterium* from *Bacteroides*. Although lactic acid was not distinguishable from formic acid, the presence of a relatively large hydroxamic acid spot in the area of these acids, along with other diagnostic criteria, assisted in the identification of *Lactobacillus*.

**DISCUSSION**

The application of both ethylamine and hydroxylamine PC methods described in this investigation provides a practical and effective alternative to the use of GLC fatty acid analysis in the identification of anaerobic bacteria. However, isomers of the fatty acids could not be distinguished, and alcohols of fermentation were not detected by these PC procedures (11). Furthermore, lactic acid could not be distinguished from formic acid by PC. Thus, a “best fit” analysis was applied with some of the bacteria when it was necessary to interpret a PC spot as an isomer and/or a normal form of a fatty acid. Similarly, this technique was applied for interpretation of lactic-formic acid spots. However, it is emphasized that there was never any ambiguity as to the identification of an organism when this means of interpretation was required. The PC profiles were as useful as the fatty acid profiles obtained by GLC for the identification of the genera and speciation of our clinical and reference organisms.

**Table 1.** *R* values of hydroxylamine and ethylamine derivatives of various fatty acids

| Fatty acid | Hydroxylamine derivatives (22°C) | Ethylamine derivatives (33°C) |
|-----------|---------------------------------|------------------------------|
| Succinic  | Not detectable                  | 0.15*                        |
| Lactic    | 0.34                            | 0.17                         |
| Formic    | 0.34                            | 0.17                         |
| Acetic    | 0.45                            | 0.17                         |
| Propionic | 0.63                            | 0.25                         |
| Butyric   | 0.77                            | 0.37                         |
| Valeric   | 0.88                            | 0.54                         |
| Caproic   | 0.92                            | 0.68                         |

* Average values based on six determinations.
* Methyl alcohol-iso-octane solvent system, 22°C.

anaerobic bacteria and those expected by GLC was considered very acceptable for diagnostic application. The identification of genera and species of all anaerobic reference strains and clinical isolates examined in this investigation...
In many cases, various species of anaerobes were distinguishable according to their biochemical patterns, so that fatty acid profiles were not required. However, a relatively small number of anaerobic genera display many similar biochemical characteristics. These bacteria can be speciated by the determination of respective fatty acid profiles according to the VPI schema. For example, Clostridium pasteurianum and Clostridium sticklandii with essentially identical biochemical characteristics could be differentiated by PC analysis (11). The fatty acid profiles of these two bacteria, as determined by GLC analysis, differ, in that iso-valeric acid and propionic acid are produced only by C. sticklandii. Accordingly, PC analysis detected “valeric-isovaleric” and “propionic-isopropionic” acids from C. sticklandii.

Identification of succinic acid was achieved with an ethylamine derivative procedure developed in this laboratory. This procedure uses iso-octane and methyl alcohol as the solvent system. Although the presence of succinic acid in the culture broths of most clinical isolates is not expected (1), its determination was particularly useful for the differentiation of Bacteroides species.

The relative concentration of the fatty acids, as determined by PC, generally agreed with those obtained by GLC. There was no significant loss of fatty acids by preparing nonvolatile ethylamine and hydroxylamine derivatives (10). In only a few instances, inaccurate semi-quantitative estimations by PC occurred. When small amounts of both the isomer and normal form of a fatty acid were present in a bacterial culture, a spot more intense than expected would develop in the area at that acid. However, this rare occurrence did not interfere with correct identification of the organism.

The development time of approximately 5 h with PC analysis is contrasted to 15 to 40 min per organism by GLC determination (1, 10, 11).

The results of biochemical tests were usually not available until the day after the GLC or PC fatty acid chromatograms were obtained. The longer length of time required by PC, including the time required to form derivatives, did not appear to be a serious disadvantage in regard to obtaining identification of any clinical anaerobic isolate. Furthermore, 8 to 16 specimens can be spotted on a single sheet of chromatography paper, and development can be allowed to proceed unattended. Calculation of Rf values of spots was not required, because the identity of each fatty acid was easily determined by visually comparing its location to a respective spot on a reference fatty acid chromatogram. The chromatogram also allowed semiquantitation of the acid spots.

The PC methods described herein are uncomplicated and can be performed in most clinical bacteriology laboratories. There was no difficulty encountered in training medical technologists in the performance of these PC procedures or in the interpretation of the results. Only a minimal amount of equipment is required for the techniques, and the reagents are readily available from scientific supply houses.

Cellulose thin-layer sheets were used in an attempt to increase separation of derivatives by both PC procedures (unpublished data). No measurable difference could be shown for the hydroxamic acid derivatives, and the separation of the spots of the ethylamine derivatives were inferior to those obtained by PC. It was found that separation of the spots on Whatman filter paper, especially those of the higher acids,
Table 2. Comparison of fatty acid profiles of various anaerobic bacteria by GLC and PC

| Bacteria                        | No. of strains tested | VPI manual GLC profile* | PC profile* | GLC profile* |
|---------------------------------|-----------------------|-------------------------|-------------|--------------|
| Propionibacterium acidipropioni | 1                     | Pas (lfiv)              | PA          | PA           |
| Peptococcus asaccharolyticus    | 3                     | Ab (slf)                | Ab          | Ab           |
| P. magnus                       | 4                     | A (lsf)                 | A (LF)*     | A*           |
| P. prevotii                     | 3                     | Ab (Lspf)               | Ab          | Ab           |
| P. variabilis                   | 4                     | A (lspb)                | A           | A            |
| Peptostreptococcus intermedius  | 1                     | L (asf)                 | (LF)        | Lfas         |
| P. productus (VPI)              | 9                     | ap (f)                  | ap          | ap           |
| Veillonella alcalescens          | 4                     | ap (i)                  | ap          | ap           |
| F. parvula                      | 1                     | BA (Lf)                 | BA          | BA           |
| Fusobacterium russii            | 1                     | BAp (LF iv s)           | BAp         | BAp          |
| F. mortiferum                   | 1                     | BLAp (fs)               | BLAp        | BLAp         |
| F. necrophorum                  | 1                     | Bap (Lsf)               | Baps        | Baps         |
| F. varium                       | 1                     | SA (Lfib iv)            | SA (lf)     | SA           |
| Bacteroides fragilis ss. fragilis| 8                    | SAp (lib iv f)          | SAp         | SAp          |
| B. oralis                       | 1                     | SA (Lf ib iv)           | SA (lf)     | SA           |
| B. preacetobacter                | 1                     | AB iv (BP)              | AB (f)      | AB iv BP     |
| Clostridium barkeri ATCC 25849  | 1                     | BL (sp)                 | B (LF)      | BL           |
| C. bifermantans                 | 3                     | AFp ic ib iv c (bel)    | A (lf) pcvb | AP p ic ib iv c|
| C. haemolyticum ATCC 9650       | 1                     | PBA (la)                | PBA         | PBA          |
| C. lentoputrenescens            | 1                     | Bap (l)                 | Bap (lsf)   | Bap          |
| C. mangenotii ATCC 25761        | 1                     | iv ic ib a p s I (f)    | v c b a p s (f) | API BB IV VIC es |
| C. pasteurianum                 | 1                     | BA (lsf)                | BA          | BA           |
| C. scatologenes ATCC 25775      | 1                     | ABC iv v pib (fs)       | ABC vp (f)  | ABC iv v pib |
| C. sticklandii                  | 1                     | AB iv b lps             | ABV (lf) ps | ABP iv b ls  |
| C. sporogenes                   | 1                     | Ab iv ib (ic spc)       | Abv         | Ab iv ib s   |
| Lactobacillus sp.               | 14                    | L (afs)                 | La          | La           |

* Fatty acid profiles obtained directly from VPI Anaerobe Laboratory manual.
* Fatty acid profile derived from combination of ethylene and hydroxamate paper chromatography.
* Gas liquid chromatography analysis obtained in this investigation.
* Letter denotes fatty acid: A, acetic; B, butyric; C, caproic; F, formic; L, lactic; S, succinic; V, valeric. Size of letter denotes relative major and minor fatty acid concentrations; letter in parentheses denotes fatty acids produced by occasional strains in the species; the letter i preceding a letter denotes its isomer.
* (LF) denotes that lactic acid is not distinguishable from formic acid by PC analysis.

could be enhanced by development at a temperature higher than room temperature.

An azo dye technique was also investigated for the identification of the fatty acids. On initial introduction of the technique, it appeared to be sensitive and simple to perform. The azo dye was unavailable commercially. The considerable expenditure of time and money required to synthesize the reagent proved to be impractical for the clinical laboratory. In addition, we obtained many nonspecific spots with this azo dye, which interfered with the analysis of the fatty acid products of a bacterial culture. The reasons for the nonspecific spots are explained by Churaček, who showed that this azo dye may contain isomers (3). Based on this investigation, the application of PC as an ancillary procedure in the identification of anaerobic bacteria is an effective alternative to GLC in the clinical bacteriology laboratory. The simplicity and reproducibility of this technique and the relatively low cost of equipment should encourage its application as a means of identifying anaerobic bacteria. In those clinical bacteriology laboratories that have a GLC system, the PC analysis should be considered as an alternative method in instances when GLC instrumentation is temporarily unavailable.

ACKNOWLEDGMENTS

This investigation was supported, in part, by grant no. P-41 from the United Fund's Health and Research Services Foundation, Allegheny County, Pa.

We thank Dolores Freedel, Magee Women's Hospital, for her assistance in the GLC analysis of the cultures and R. J. Hartsco for his helpful comments on the manuscript.

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