Modification of the Gas-Liquid Chromatography Procedure and Evaluation of a New Column Packing Material for the Identification of Anaerobic Bacteria

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Gas-liquid chromatography has become a useful aid for the identification of anaerobic bacteria in the clinical laboratory. When many extracted samples are to be analyzed, time is of prime importance. By altering the operating conditions of the chromatograph, we have decreased the total elution time of the volatile fatty acids examined to approximately 12 min. There is, however, a decrease in the ability to resolve propionic and isobutyric acids. A new column packing material, SP-1220 (Supelco), was found to give excellent separation of propionic and isobutyric acids as well as demonstrating the presence of formic acid which was not seen with the previously used Resoflex. Extracts of clinical isolates of anaerobic organisms were processed using the altered conditions and new column packing material with excellent resolution of all acids. The use of SP-1220 in the clinical laboratory will facilitate the identification of anaerobic bacteria.

The isolation and identification of anaerobic bacteria has achieved great importance in clinical microbiology. In this setting, gas-liquid chromatography has become widely used as an aid to identification by demonstrating the presence of the volatile fatty acids (VFA) produced by these organisms (1, 2, 5, 6, 8). In the busy laboratory, the elution times of the acids are of prime importance, especially when many extracted samples are to be analyzed by GLC.

By using Resoflex (Burrell Corp.) as a column packing and by altering the operating conditions of the chromatograph, we have been able to reduce the elution times of the individual acids. One problem created was that propionic acid formed a shoulder peak with isobutyric acid. We thus sought a column packing material which would separate these two acids. A new column packing provided by Supelco, Inc. (SP-1220) showed this separation. It also demonstrated the presence of formic acid which is not seen with Resoflex; the other volatile fatty acids could still be differentiated.

MATERIALS AND METHODS

Gas chromatograph. A Fisher (model no. 2400) gas chromatograph, equipped with a thermal conductivity detector and dual-column oven, was used throughout the study. A Fisher (model no. 5000) single pen strip chart recorder was used in conjunction with the chromatograph.

Column and packing. One stainless-steel column

(6 feet by 0.25 inch [182.48 by 0.63 cm outer diameter]) packed with Resoflex (20% diethylene glycol adipate) on 30/60 Chromosorb W, acid washed (Burrell) was used. The second glass column (6 feet [182.48 cm] by 4 mm internal diameter) (0.25 inch [0.65 cm] outer diameter) was packed with 15% SP-1220/1% H_3PO_4 on 100/120 Chromosorb W, acid washed. SP-1220 is a proprietary stationary phase developed by Supelco, Inc., Bellefonte, Pa. (7). Both columns were conditioned overnight in the oven at 180 C with the effluent end detached and a carrier gas flow rate of 60 cc/min.

Operating conditions. The following operating conditions were constant throughout the study: injector 150 C, detector 200 C at 200 mA and recorder speed of 0.5 inch per min with a 1 mV input response. The conditions which were varied during the study were the carrier gas (helium) flow rate and column oven temperature. A 14- μ l ether extract of the sample was injected using a Hamilton syringe. The column oven temperature was varied from 120 to 150 C and the carrier gas flow rate from 60 to 120 cc/min for the Resoflex column and 40 to 80 cc/min for the SP-1220 column. The attenuator was used to compensate for peak heights. Optimal operating conditions as determined by charted qualitative separation were established.

Cultures and extracts. The anaerobic bacteria used in this study were isolated from clinical specimens by the methods of Zabransky (9), Dowell and Hawkins (1), and Martin (4), and biochemically confirmed by the criteria of Dowell and Hawkins (1). They were transferred to a tube of peptone-yeast extract-glucose broth and incubated in a GasPak jar for 48 h at 35 C or until good growth was obtained.

The extraction procedure was that as described by Dowell and Hawkins (1).

RESULTS

Figure 1 shows three comparative chromatograms of the VFA standard solution. Chromatogram A is the VFA standard run on the Resoflex column at an oven temperature of 120 C and the carrier gas flow rate of 120 cc/min. The total elution time of the VFA is approximately 25 min. The results are similar to those illustrated in the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* using a different instrument (2). Propionic acid forms a shoulder peak with isobutyric acid and formic acid is not detected. The Virginia Polytechnic Institute procedure manual (2) describes the presence of formic acid by using other chromatographic systems with Resoflex. It was not discernible on our instrument even though the VFA standard included 1 meq of formic acid. Injection of a pure formic acid extract (10 meq/100 ml), however, resulted in a small peak with $2\times$ attenu-

ation. The inability to detect formic acid on Resoflex was verified by checking our VFA standard on the same type of chromatograph (Dohrmann) as described by Holdeman and Moore (2).

Chromatogram B (Fig. 1) is the VFA standard processed through the Resoflex column but at an increased oven temperature of 140 C; the carrier gas flow rate was maintained at 120 cc/min. This higher temperature, which is not deleterious to Resoflex (maximum temperature for Resoflex is 190 C), decreased the elution time to 12 min. Propionic acid is still qualitatively recognizable and the formic acid peak again is not seen. Alternatively, maintaining the temperature at 120 C but increasing the gas flow rate will also shorten the total elution time of the VFA standard; however, the propionic acid and isobutyric acid peaks approach the same elution time. Under these conditions, recognition of the individual acids becomes very difficult. It would thus appear that an oven temperature of 140 C and a

FIG. 1. Chromatograms of volatile fatty acid standard solution. (A) Resoflex at 120 C and 120 cc/min gas flow rate; (B) Resoflex at 140 C and 120 cc/min gas flow rate; (C) SP-1220 at 145 C and 70 cc/min gas flow rate. All at $4\times$ attenuation. VFA: F, formic; A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; IC, isocaproic; C, caproic.

gas flow rate of 120 cc/min are the optimal conditions for practical resolution of the individual acids in the shortened period of time on Resoflex.

The VFA standard processed on the SP-1220 column packing material is illustrated in chromatogram C of Fig. 1. Because of the decreased diameter of the glass column used with the SP-1220, the gas flow rate had to be decreased. After repeated trials, an oven temperature of 145 C and carrier gas flow of 70 cc/min was chosen to provide the optimum separation of the acids in the shortest period of time. The total elution time is about 12 min; propionic acid and isobutyric acid form two distinct peaks. Another significant finding was the presence of formic acid which elutes before acetic acid, thus being recognizable for qualitative analyses. The Virginia Polytechnic Institute

procedure described formic acid as being detectable after acetic acid (2). The elution time of each acid was determined by injecting 14 μ l of a solution that contained approximately 1 meq of that acid per 100 ml on the SP-1220 column. In this manner the sequence of elutions of the individual acids were determined. This is in agreement with the data published by the manufacturer (7).

With the significant improvements in the elution of the VFA standard as described above, it seemed appropriate to test the SP-1220 column with extracts of anaerobic bacteria and compare these to the same extracts of the same organisms run on the Resoflex column. Representative strains of anaerobic organisms were selected for chromatographic analyses. These strains were chosen because they produce a variety of fatty acids (Fig. 2). The only major

FIG. 2. Comparative chromatograms of extracts of anaerobic bacteria on SP-1220 at 145 C and 70 cc/min and Resoflex at 140 C and 120 cc/min. A and A': *C. cadaveris*; B and B': *C. bifermentans*; C and C': *B. fragilis* subsp. *fragilis*; D and D': *C. ramosum*. VFA: F, formic; A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; IC, isocaproic; C, caproic.

difference in these comparative chromatograms is the demonstration of formic acid when it is produced by organisms such as *Clostridium cadaveris* (2A), *Clostridium bifermentans* (2B), and *Clostridium ramosum* (2D). The distinct separation of propionic acid and isobutyric acid by SP-1220 is demonstrated in Fig. 2B. Resoflex gives a shoulder peak of propionic acid on isobutyric acid with *C. bifermentans* (2B') although its presence is recognizable. The presence of two insignificant minor peaks of isoacids produced by *Bacteroides fragilis* on SP-1220 (2C) is shown. This is probably due to the increased sensitivity of the SP-1220 column packing material for minute amounts of the acid produced and not the sensitivity of the instrument since the same detector was used.

The SP-1220 column packing material was used to demonstrate the acids produced by numerous other anaerobic bacteria. Selected strains of the more common isolates are demonstrated in Fig. 3 and 4. The SP-1220 provides excellent chromatographic separation of the fatty acids. The elution times of the individual acids are identical to those of the VFA standard solution. On some organisms, *Clostridium hastiforme* (3B), *Fusobacterium necrophorum* (3C), *Propionibacterium acnes* (4B), and *Clostridium perfringens* (4D), the attenuation had to be increased because the major acid peaks produced by the organisms were very large. It can be seen that when formic acid is produced by the anaerobic bacteria as with *Clostridium sordellii* (3A), *C. ramosum* (3E), and *Peptococcus*

FIG. 3. Chromatograms of extracts of anaerobic bacteria on SP-1220 at 145 C and 70 cc/min gas flow rate. A: *C. sordellii*; B: *C. hastiforme*; C: *F. necrophorum*; D: *P. anaerobius*; E: *C. ramosum*; F: *B. fragilis* subsp. *thetaiotaomicron*. VFA: F, formic; A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; IC, isocaproic; C, caproic.

FIG. 4. Chromatograms of extracts of anaerobic bacteria on SP-1220 at 145 C and 70 cc/min gas flow rate. (A) *E. limosum*; (B) *P. acnes*; (C) *V. parvula*; (D) *C. perfringens*; (E) *P. magnus*; (F) *E. alactolyticum*; (G) *B. eriksonii*. VFA: F, formic; A, acetic; P, propionic; IB, isobutyric; B, butyric; IV, isovaleric; V, valeric; IC, isocaproic; C, caproic.

magnus (4E), it is easily recognized as a distinct peak on the chromatogram. Furthermore, propionic acid and isobutyric acid are eluted as two separate peaks, thus making their qualitative identification easy. This is demonstrated with *C. hastiforme* (3B), *Peptostreptococcus anaerobius* (3D), and *B. fragilis* subsp. *thetaiotaomicron* (3F).

DISCUSSION

Moore's (6) description of the use of gas chromatography for the identification of anaerobic bacteria in 1966 probably represents the first

concrete and practical approach in using this technique for identifying organisms. The use of different instruments for chromatographic analyses should not affect the final results or conclusions. In addition, a particular column packing material should provide similar results on chromatographs within the limits of the sensitivity of the detector. The chromatographic separation of fatty acids is based upon several variables in addition to the above. These would include: temperature of the column, temperature of the injection port, nature of the carrier gas, the carrier gas flow rate,

and the nature of the active sites on the column packing material (3).

We have demonstrated that by altering certain operating conditions of the chromatograph, while using Resoflex as a column packing, the elution times of various fatty acids can be changed. By increasing the column oven temperature one is able to reduce the elution time of the VFA standard solution and thus enable the analysis of more organisms in less time. In a busy clinical laboratory this is of prime importance as long as the qualitative analysis of gas-liquid chromatography has not been affected. The altered conditions that we have described with Fisher model no. 2400 and Resoflex may not be the same conditions for other types of chromatographs; however, it is important to note that these conditions need but be permanently fixed and that the individual user should alter the conditions of operation to best suit his needs. If better resolution of specific acids is desired, such as differentiation of propionic and isobutyric, or if the demonstration of specific fatty acids such as formic is considered to be important, then alternative column packing materials should be used.

The new column packing material, SP-1220, provides excellent separation of propionic and isobutyric acid and also demonstrates the presence of formic acid. Furthermore, this particular column packing material is able to demonstrate the presence of all the VFA considered to be of importance in the identification of anaerobic bacteria. The results are comparable to those in the *Anaerobe Laboratory Manual* (2) but with the advantage of a shorter elution time. Furthermore, analysis on the SP-1220 of eight strains of anaerobic species extracted after 48 h and seven days of incubation showed no differences in the amount or type of VFA produced.

This material also has the flexibility that permits the oven temperature and gas flow rate to be adjusted so that the longer chain fatty acids such as caproic acid are detectable within 12 min after injection. It may be debated whether the presence of specific fatty acids, such as formic acid, are of value in identifying strains of anaerobic bacteria. Surprisingly, many organisms do produce formic acid in considerable amounts. If the presence of formic acid is important in identification of these organisms, then the use of a column packing material such as SP-1220 has a distinct advantage over the Resoflex.

We have used the SP-1220 packing material for more than 3 months and have processed approximately 600 extracts of anaerobic organisms. The material is yet quite stable and has

shown no difficulties in separation or variation in the elution times during this period. During this period identical patterns were obtained by analysis of extracts of many strains of the same species, especially *B. fragilis*, *Fusobacterium* species and *C. perfringens*.

The use of a glass column with this particular packing material, although not required, does have some advantages. With a glass column one can easily ascertain the uniformity of the packing within the column. Furthermore, it is established that glass is the most inert material to be used for gas-liquid chromatography (3). In our particular application the flow rate of helium carrier gas was approximately half of what would be used with the larger inside diameter stainless-steel columns and, therefore, one could realize a savings with this particular item. Of course, the major disadvantage of glass columns is their susceptibility to breakage. However, the newer columns have a thicker wall, are very resilient and should provide no difficulties with normal handling.

We have shown that the operating conditions, particularly, an increase in oven temperature, can decrease the elution time of the volatile fatty acids that would be important in the identification of anaerobic bacteria. We have also illustrated the use of a new column packing material which separates the same fatty acids in a shorter time than described by others, as well as providing better resolution of propionic and isobutyric acids and demonstrating the presence of formic acid. The procedures described here in conjunction with a shorter incubation period before extraction of the VFA illustrate an improvement in the presently described procedures, especially for use in the clinical laboratory. We believe that the SP-1220 will provide some distinct advantages; however, it should be evaluated on other chromatographic instruments and the conditions adjusted accordingly.

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