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Analysis of Short-Chain Acids from Anaerobic Bacteria by High-Performance Liquid Chromatography

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A standard mixture of 25 short-chain fatty acids was resolved by high-performance liquid chromatography, using an Aminex HPX-87 column. The acids produced in culture media by anaerobic bacteria were analyzed by high-performance liquid chromatography after extraction with ether and reextraction into a small volume of 0.1 N NaOH. The presence of fumaric acid in culture extracts of *Peptostreptococcus anaerobius* was confirmed by gas chromatography-mass spectrometry analysis of the trapped eluent fractions from the high-performance liquid chromatography column.

The determination of short-chain fatty acids in culture media after growth of anaerobic bacteria is useful for the identification of these bacteria (4, 6, 13–15). Also, the detection of these acids in clinical specimens has been used for presumptive identifications of anaerobic infections (5, 11, 12). The method used to detect these acids is gas-liquid chromatography (GLC) preceded by chemical processing of the sample. Procedures for such analyses are found in the Wadsworth (14), the Virginia Polytechnic Institute (6), and the Centers for Disease Control (4) laboratory manuals and the text by Willis (15) and the paper by Rizzo (13).

In the Wadsworth procedure, the volatile fatty acids (VFA) are analyzed directly by GLC, using the aqueous supernatant from culture media; formic acid is analyzed separately after conversion to dimethylformamide (2). The nonvolatile dicarboxylic and keto acids (NVFA) are analyzed after formation of methyl esters and subsequent extraction from the reaction mixture with chloroform. This procedure uses a gas chromatograph equipped with two different columns and detectors, one for analysis of VFA and the other for analysis of NVFA. Using the procedures of Willis (15) and Rizzo (13) and the methods described in the Virginia Polytechnic Institute (6) and Centers for Disease Control (4) manuals, the VFA are analyzed by GLC after extraction from culture media with ether and the NVFA after methylation and extraction with chloroform. Although different chromatographic parameters are used with each of these four procedures, the VFA and NVFA are analyzed separately on a single chromatograph, using one column and one detector.

All of these GLC procedures require at least two sample preparation steps, one for the analysis of VFA and another, including methylation, for the analysis of NVFA. Interpretation of the chromatograms is sometimes difficult because of interferences from a large solvent peak and from unresolved acid peaks. The present investigation was undertaken to evaluate high-performance liquid chromatography (HPLC) for determination of short-chain fatty acids from bacteria. The potential advantages of HPLC over GLC were a single sample preparation step not requiring methylation, the simultaneous determination of both VFA and NVFA in a single chromatographic run, and increased sensitivity for detection of NVFA.

MATERIALS AND METHODS

Standard acids. Short-chain fatty acids that are solids at ambient temperature were weighed and dissolved in 0.007 N H_2SO_4 to prepare standard stock solutions. Final composite standards were prepared from samples of these stock solutions; these were combined with liquid acids by using a microsyringe. The final molarities of the composite standards were as follows: VFA, 0.02 M; nonaromatic NVFA, 0.0001 to 0.01 M; and aromatic NVFA, 0.0001 M. Standards in solution (except oxalacetic acid) were stable at ambient temperatures.

Bacterial cultures. Stock cultures of *Peptostreptococcus anaerobius*, *Bacteroides fragilis*, and *Clostridium difficile* were obtained from the Centers for Disease Control Anaerobe Laboratory after growth for 24 to 48 h in peptone-yeast extract-glucose (PYG) broth (4).

Analysis procedure. One milliliter of the broth culture was pipetted into a screw-capped culture tube (13 by 100 mm) fitted with a Teflon-lined cap. The following reagents were added: 0.2 ml of 18 N H_2SO_4, 0.6 g of NaCl, 5 ml of diethyl ether, and 25 μl of acetonitrile; the mixture was blended with a Vortex mixer for 1 min to mix the ether and aqueous phases. The tube was centrifuged at 1,000 × *g* for 1 min to remove any

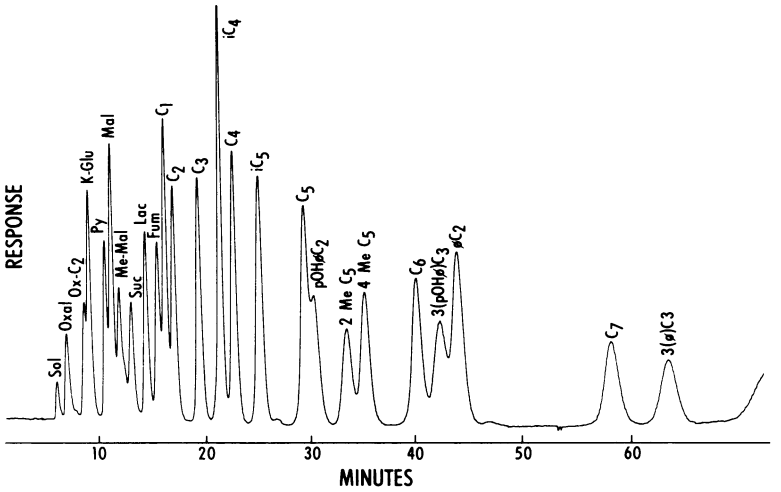

FIG. 1. HPLC chromatogram of a standard mixture of 24 acids (and their molar concentrations): Sol, solvent and unadsorbed components; Oxal, oxalic acid (0.002); OX-C_2, oxalacetic acid (0.0001); K-Glu, α-ketoglutaric acid (0.0005); Py, pyruvic acid (0.004); Mal, malonic acid (0.006); Me-mal, methylmalonic acid (0.002); Suc, succinic acid (0.001); Lac, lactic acid (0.01); Fum, fumaric acid (0.001); C_1, formic acid (0.02); C_2, acetic acid (0.02); C_3, propionic acid (0.02); iC_4, isobutyric acid (0.02); C_4, butyric acid (0.02); iC_5, isovaleric acid (0.02); C_5, valeric acid (0.02); pOHφC_2, *p*-hydroxyphenylacetic acid (0.0001); 2 MeC_5, 2-methylvaleric acid (0.005); 4 MeC_5, 4-methylvaleric acid (0.02); C_6, caproic acid (0.02); 3(pOHφ)C_3, 3-(*p*-hydroxyphenyl)propionic acid (0.0001); φC_2, phenylacetic acid (0.002); C_7, heptanoic acid (0.02); and 3(φ)C_3, 3-phenylpropionic acid (0.0001). The eluent was 0.007 M H_2SO_4–10.8% acetonitrile.

dispersed aqueous droplets from the ether phase. The ether layer was transferred to another tube with a Pasteur pipette, and approximately 2 mm of ether was left at the solvent interface to ensure that none of the aqueous phase was transferred. To the ether, 0.2 ml of 0.1 N NaOH was added, and the tube was gently shaken. With a Pasteur pipette, a small portion of the lower NaOH phase was removed and tested with pH paper. If necessary, 1 N NaOH was added, 1 drop at a time, to obtain a pH of 9 or greater. The tube was blended with a Vortex mixer for 1 min and centrifuged, and the ether phase was removed and discarded. Twenty-five microliters of acetonitrile was then added, and the tube was left uncapped for about 5 min to allow residual ether to evaporate. The tube was blended with a Vortex mixer to mix the contents, and 20 μl of sample was introduced into the chromatograph for analysis. Uninoculated PYG medium was processed and analyzed in the same manner to determine the acids present; the concentration of acids produced by the bacteria was obtained by subtracting the concentration found in the uninoculated medium.

Equipment. The HPLC equipment used was a model ILC305 modular system (Laboratory Data Control, Division of Milton Roy Co., Riviera Beach, Fla.), equipped with an Aminex HPX-87H cation-exchange column (300 by 7.8 mm) for organic acids (no. 125-0140; Bio-Rad Laboratories, Richmond, Calif.), a Micro-Guard column (no. 125-0129; Bio-Rad), and a holder (no. 125-0131; Bio-Rad). A 20-μl bypass sampling loop was used, and the Spectromonitor II was adjusted to a wavelength of 210 nm and to a sensitivity of 0.08 absorbance units (full scale). Other accessories included a model 56 strip chart recorder (The Perkin-Elmer Corp., Norwalk, Conn.), a 25-μl injector syringe (model 702SNR; The Hamilton Co., Reno, Nev.), and a model 7302 column inlet filter (Rheodyne, Inc., Berkeley, Calif.). Ambient temperature was used, and the flow was 0.5 ml/min, using column eluent A, composed of 10.8% acetonitrile (HPLC grade) in 0.007 N H_2SO_4 (0.19 ml of H_2SO_4 per liter), or column eluent B, composed of 5% acetonitrile in 0.012 N H_2SO_4 (0.33 ml of H_2SO_4 per liter). During chromatographic separations, the eluents were degassed with helium. The presence of fumaric acid was confirmed, using a model 21-491B (E. I. du Pont de Nemours & Co., Wilmington, Del.) gas chromatograph-mass spectrometer, with chemical ionization. Solvents were mixed for extractions, using a Vortex-Genie mixer (American Scientific Products, Division of American Hospital Supply Corp., McGaw Park, Ill.). Deionized water was redistilled in an all-glass system before use. All glassware was rinsed with HPLC eluent and acetonitrile before use.

RESULTS

The separation of a standard mixture of 24 short-chain fatty acids, using eluent A, is shown in Fig. 1. The molar concentrations ranged from 0.02 M for VFA to 0.0001 M for aromatic NVFA. The retention time (t_r) and capacity factor (k') of these short-chain acids when eluted with solvents A and B are shown in Table 1; the t_r and k' of late-eluting compounds, *p*-cresol and indoleacetic acid, are also included.

Separation of the acids on the Aminex HPX-87 analytical column (Fig. 1) was optimized empirically to resolve fumaric acid from formic

TABLE 1. Times of elution (t_r)[a] and capacity factor (k')[b] of standard acids separated by HPLC and eluted by sulfuric acid-acetonitrile

Acid	Eluent A[c]		Eluent B[d]	
	t_r	k'	t_r	k'
Oxalic	7.5	0.172	7.4	0.122
Oxalacetic	9.1	0.422	9.4	0.403
α-Ketoglutaric	9.5	0.484	10.0	0.493
Pyruvic	10.9	0.703	11.2	0.672
Malonic	11.6	0.813	12.2	0.821
Methylmalonic	12.3	0.922	13.2	0.970
Succinic	13.5	1.109	14.4	1.149
Lactic	14.6	1.281	15.0	1.231
Fumaric	15.7	1.453	18.4	1.746
Formic	16.4	1.563	16.6	1.470
Acetic	17.2	1.688	17.9	1.679
Propionic	19.4	2.031	20.8	2.112
Isobutyric	20.5	2.203	23.3	2.470
Butyric	22.7	2.547	25.3	2.769
Isovaleric	25.1	2.922	28.6	3.261
Valeric	29.0	3.531	34.5	4.149
p-Hydroxyphenylacetic	30.1	3.703	41.7	5.516
Isocaproic (2-methylvaleric)	33.0	4.156	40.3	5.105
Isocaproic (4-methylvaleric)	34.7	4.422	42.2	5.293
Caproic	40.1	5.266	50.9	6.597
3-(*p*-Hydroxyphenyl)propionic	42.3	5.609	47.0	6.015
Phenylacetic	43.9	5.859	58.8	7.776
Heptanoic	58.0	8.063	84.4	11.60
3-Phenylpropionic	63.2	8.875	93.5	12.96
p-Cresol	76.0	10.88	110.0	15.92
Indoleacetic	105.6	15.50	271.0	39.49
M (PYG medium component)	32.7	4.109	44.2	5.597

[a] Time in minutes.
[b] Number of column volumes required to elute each acid.
[c] Eluent A, 0.007 N H_2SO_4–10.8% acetonitrile.
[d] Eluent B, 0.012 N H_2SO_4–5% acetonitrile.

and acetic acids by adjusting the concentrations of H_2SO_4 and acetonitrile in the eluent. With eluent A, fumaric acid (t_r, 15.7) eluted before formic (t_r, 16.4) and acetic (t_r, 17.2) acids and with better resolution at 0.007 N H_2SO_4 than at a higher concentration (0.012 N) of H_2SO_4. As the concentration of acetonitrile was decreased, the t_r of fumaric and the aromatic acids increased more than the t_r of other VFA and NVFA. With eluent B, which contained 5% acetonitrile, fumaric acid (t_r, 18.4) eluted after formic (t_r, 16.6) and acetic (t_r, 17.9) acids. Also, *p*-hydroxyphenylacetic acid eluted before isocaproic acid (2-methylvaleric acid) with eluent A and after this acid with eluent B.

The free acids present in a culture extract from *P. anaerobius* were analyzed by HPLC (Fig. 2). The detector response value for each acid was determined, and molar concentrations were obtained by multiplying peak heights by response factors (Table 2). Thirteen acids were identified by comparing their t_r values with those of known standards. Several unidentified components were found, including a component (M) which was also present in the control PYG medium. With eluent A, M eluted with a t_r of 32.7 and was unresolved from 2-methylvaleric acid; however, with eluent B, M eluted after 2-methylvaleric acid. The identity of this compound has not been established. In addition to M, the control PYG medium contained fumaric, formic, and acetic acids; the concentrations of these acids found in culture broth from *P. anaerobius* were corrected by subtracting the amounts present in the control medium.

Culture extracts from *B. fragilis* and *C. difficile* analyzed by HPLC contained acids that were not reported in previous GLC studies. These included α-ketoglutaric, *p*-hydroxyphenylacetic, and phenylacetic acids from *B. fragilis* and *p*-hydroxyphenylacetic, phenylacetic, 3-phenylpropionic, and indoleacetic acids plus *p*-cresol from *C. difficile*.

The efficiency of acid recovery by a single extraction with ether was determined after adding known concentrations of formic, propionic, fumaric, and lactic acids to 1 ml of PYG medium (Table 3). Recoveries after a single extraction from water and after double extraction from PYG medium are shown for comparison. Only

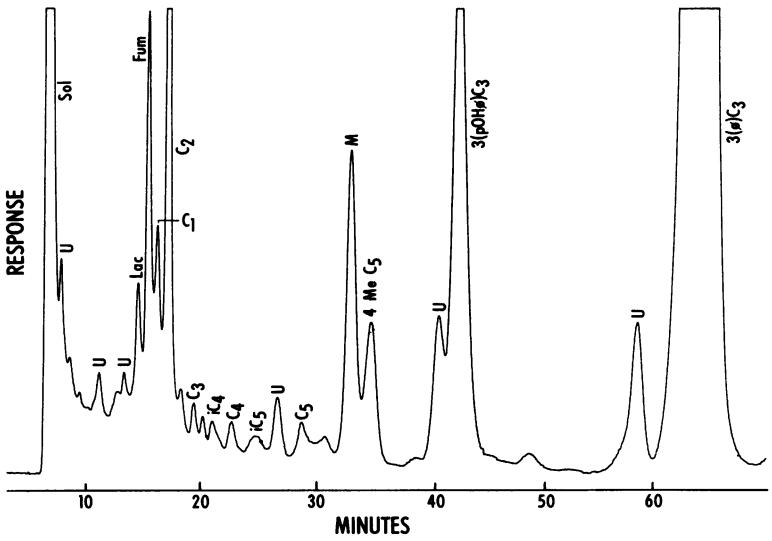

FIG. 2. HPLC chromatogram of the free acids of *P. anaerobius*. See legend to Fig. 1 for abbreviations. U, Unidentified components; M, PYG medium component.

25% of lactic acid was recovered after the single ether extraction from PYG medium. The poor recoveries occurred at the initial ether extraction and not from reextraction into 0.1 N NaOH because the recoveries of standards directly extracted from ether with 0.1 N NaOH were greater than 95%.

With the exception of oxalacetic acid, all of the acids used for the standard were stable at ambient temperature. Oxalacetic acid decomposed to pyruvic acid in both acid and basic solutions. The amount of decomposition of this acid in 0.01 N H_2SO_4 was as follows: 25% after 1 h at 21°C, 75% after 20 h at 21°C, and 53% after 20 h at 4°C. Standards of oxalic acid were partially decomposed after several months of storage at ambient temperature.

TABLE 2. HPLC analysis of short-chain acids from *P. anaerobius* after a 36-h growth in PYG broth[a]

Acid	Mol%
Pyruvic	0.9
Succinic	0.3
Lactic	8.0
Fumaric	2.0
Formic	6.0
Acetic	35.0
Propionic	3.0
Butyric	2.0
Isovaleric	1.0
Valeric	4.0
4-Methylvaleric	24.0
3-(*p*-Hydroxyphenyl)propionic	0.8
3-Phenylpropionic	12.0

[a] Nine micromoles of total acid in 1 ml of culture broth.

Fumaric acid was tentatively identified in culture broth from *P. anaerobius* by observing the shift in t_r upon changing from eluent A to eluent B. With eluent A, fumaric acid elutes before formic and acetic acids, but with eluent B, it elutes after these two acids with the retention times shown in Table 1. For further confirmation of the presence of fumaric acid, a 5-ml sample of the culture broth was extracted and used for repeated analyses by HPLC. The eluent fractions containing the fumaric acid peak were collected; the composite fraction, containing approximately 1 μmol of fumaric acid, was extracted with ether, methylated, and analyzed by GLC on a polar Silar 10C column. In addition, the methylated fraction was analyzed by gas chromatography-mass spectrometry, and the acids were separated by GLC on a nonpolar OV-101 column and detected by mass spectrometry, using both electron impact and chemical ionization. In each instance, the presence of fumaric acid in the trapped HPLC eluent was confirmed by comparing the results by gas chromatography-mass spectrometry to those of a standard of fumaric acid which was methylated and analyzed under the same conditions.

DISCUSSION

The standard acids, used for the separations shown in Fig. 1, contained VFA at concentrations of 0.02 to 0.005 M and were within the same concentration range (0.01 M) recommended for analysis by GLC (4, 6, 14, 15). The concentrations of NVFA were 0.006 to 0.0001 M, except for oxalic acid (0.01 M), and were

TABLE 3. Recovery of standard acids from 1 ml of PYG medium or 1 ml of distilled water by extraction with 5 ml of diethyl ether

Acid	Amount (μg)	Amount recovered (%)		
		PYG medium		Water
Formic	400	51[a]	68[b]	60[a]
Propionic	600	85	86	100
Fumaric	8	66	—[c]	62
Lactic	400	25	53	38

[a] Single extraction.
[b] Double extraction.
[c] —, Not determined.

lower concentrations than those used for analysis by GLC.

The short-chain acids found in culture media after growth of *P. anaerobius*, as reported by the Virginia Polytechnic Institute (6) and Wadsworth (14) manuals, are succinic, lactic, acetic, propionic, isobutyric, butyric, isovaleric, and isocaproic acids. The presence of valeric acid was reported by Lambert and Armfield (8) and 3-(*p*-hydroxyphenyl)propionic or hydroxyhydrocinnamic acid and 3-phenylpropionic or hydrocinnamic acid were reported by Lambert and Moss (7). In addition to detecting all of these previously identified acids by HPLC, we also detected fumaric acid (a Krebs cycle acid) and formic acid from cultures of *P. anaerobius*.

When extracts of the culture media from *B. fragilis* and *C. difficile* were analyzed by HPLC, additional acids were detected that are not usually detected by gas chromatographic procedures (6, 14). α-Ketoglutaric acid, which was detected by HPLC in ether extracts from *B. fragilis*, has been reported as a metabolite of this organism by Allison et al. (1). The *p*-hydroxyphenylacetic, phenylacetic, 3-phenylpropionic, and indoleacetic acids and *p*-cresol, which were found in ether extracts from *C. difficile*, have been reported as metabolites of this organism (10) and other bacteria (3, 7, 9, 16).

The recoveries of known concentrations of acids after ether extraction from PYG media and from water were determined (Table 3) because NVFA were extracted as free acids, rather than methylated derivatives that are usually used for GLC analysis. A single extraction of the medium was preferred for simplicity of analysis, even though the recovery of lactic acid was only 25%. The recoveries of the two VFA, formic and propionic acids, after a single extraction from PYG medium with 5 ml of ether were 51 and 85%, respectively. It is evident that much lower recoveries are obtained by GLC procedures (4, 6, 13, 15) which use only 1 ml of ether for extraction. With the Wadsworth procedure (14), VFA are not extracted from the media. Instead, the PYG culture medium is centrifuged, and a sample of the supernatant is analyzed directly by GLC. Thus, components of the medium are introduced directly onto the column, and artifacts could be produced that would interfere with the identification of acids; components of the medium could also shorten the life of the analytical GLC column. It is possible that the supernatant from cultures could be analyzed directly by HPLC in a similar fashion; however, solvent extraction before analysis improves the reliability of the procedure by selectively removing the acids from potentially interfering compounds. The reextraction of the acids from the ether into 0.2 ml of 0.1 N NaOH provides additional purification and also concentrates the acids from the original 1-ml volume of the culture. The NaOH was added to convert the acids to water-soluble sodium salts; however, we avoided adding an excess of NaOH to minimize neutralization of the HPLC eluent as the sample is placed on the analytical column.

The response of the UV absorbance detector varies for different acids and is approximately 100-fold more sensitive for aromatic acids than for aliphatic acids. Thus, the chromatographic peaks shown in Fig. 2 for 3-(*p*-hydroxyphenyl)propionic and 3-phenylpropionic acids are large compared with those of the other acids and represent only 0.8 and 12%, respectively, of the total acids present (Table 2). The smaller acetic acid peak was the primary component from *P. anaerobius* and was present at 35% of the 9 μmol of total acid found in 1 ml of culture medium from this organism.

The results obtained using the Aminex HPX-87 column were reproducible, and the HPLC instrument was stable, with no apparent change in calibration over several months of use. However, to maintain the resolution of fumaric acid from formic acid, we occasionally adjusted the concentration of acetonitrile in eluent A by 0.1% increments. At an eluent flow of 0.5 ml/min, column pressures were 900 to 1,200 lb/in^2. Higher flow rates resulted in column blockage. However, if blockage occurred the column was simply reversed, and separations were resumed within 10 min, with essentially no change in retention times.

In a single analysis by HPLC we were able to separate 25 acids and *p*-cresol that may be produced in growth media by anaerobic and other bacteria. Although the 60-min time period required for the separation may be longer than the time required for the separation of both VFA and NVFA by GLC, the single-sample preparation step took only 15 min. A possible disadvantage of this HPLC procedure is that alcohols are not detected, whereas in some GLC methods

alcohols are detected. All of the acids usually detected by GLC methods (4, 6, 13–15) were also detected by HPLC, and the sensitivity was improved for the detection of formic acid (2) and NVFA. Additional components detected by HPLC were *p*-cresol and fumaric, α-ketoglutaric, phenylacetic, *p*-hydroxyphenylacetic, 3-phenylpropionic, 3-(*p*-hydroxyphenyl)propionic, and indoleacetic acids. The method has potential for routine use within the diagnostic microbiological laboratory.

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