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MINIREVIEW

Review of Frequency-Pulsed Electron-Capture Gas-Liquid Chromatography Studies of Diarrheal Diseases Caused by Members of the Family *Enterobacteriaceae*, *Clostridium difficile*, and Rotavirus

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INTRODUCTION

Frequency-pulsed electron-capture-gas-liquid chromatography (FPEC-GLC) studies of diarrheal diseases have produced some interesting findings (3, 4, 8; J. B. Brooks, M. T. Basta, and A. M. El Kholy, unpublished results) which could be useful to improve diagnosis, to study changes in metabolic patterns that occur in the gut during the diarrheal state, and to detect specific compounds that are potentially physiologically active or carcinogenic.

At the present stage of development, potential pathogens have been identified by previously described FPEC-GLC techniques (8, 11, 14a, 19), and in some instances a characteristic FPEC-GLC profile was detected in the presence of other potential pathogens. The FPEC-GLC technique involves extraction of organic acids, alcohols, and amines with organic solvents, functional group derivatization, and analysis on high-resolution columns. The FPEC detector, which measures the frequency of the applied pulses required to maintain a constant current when an electron-absorbing compound passes through it, is essential, primarily because of the selectivity and sensitivity it offers. The samples can be automatically or manually injected. The instrument can be set up to accommodate two analyses at a time. Datum interpretation is accomplished by a trained person. A computer can be used to advantage in making retention time comparisons of known and unknown components, in the integration of peak areas, in the labeling and overlaying of chromatograms, in the subtraction of chromatograms, and to expand specific areas of the chromatogram for more detailed observation. The purpose of this review is to summarize information obtained thus far in FPEC-GLC studies of diarrheal disease associated with members of the family *Enterobacteriaceae*, *Clostridium difficile*, and rotavirus.

TYPES OF COMPOUNDS DETECTED AND DERIVATIZATION

Extraction at different pHs and derivatization with specific functional group reagents increases the chances for the correct labeling of chemical compounds detected by GLC analysis. It is possible with a high degree of accuracy to identify functional groups, boiling-point relationships, and unsaturation, through derivatization, hydrogenation, or bromination and subsequent GLC analysis. In addition, derivatization makes the compound electron capturing and thus permits analysis by the selective, extremely sensitive FPEC detector. The types of compounds detected during the study (3, 4, 8) were carboxylic acids, hydroxy acids, alcohols, and amines. The carboxylic acids were extracted as previously described (4) with nanograde chloroform from acidified watery diarrheal stools that had first been centrifuged to remove particulate matter. Formed stools were first made watery by the addition of 9 ml of distilled water to 1 ml of stool; then they were processed in the same way as watery diarrheal stools. The carboxylic acids were derivatized as previously described (1, 4) with trichloroethanol (TCE) and heptafluorobutyric anhydride (HFBA). TCE derivatizes only the carboxylic groups; HFBA, which provides the catalyst for the reaction, also derivatizes and makes electron capturing alcohols and neutral amines that are occasionally extracted in the first extraction. We seldom encountered alcohols and neutral amines in our analysis of carboxylic acids, but when alcohols or neutral amines were suspected, they were confirmed by derivatization of a similar extract with HFBA without TCE and then by analysis of the derivative under the same conditions as the TCE derivative and comparing chromatograms. Amines were obtained in the basic chloroform extractions. They were analyzed by FPEC-GLC after derivatization with HFBA as previously described (2, 4). Occasionally an alcohol, such as acetyl methyl carbinol, was encountered along with amines in the basic extraction. These alcohols were differentiated from amines by comparison of chromatograms obtained from HFBA-derivatized extracts made under both acidic and basic conditions. The hydroxy acids were not derivatized with TCE; they were extracted with diethylether from the reacidified supernatant of the basic extraction. The two functional groups were esterified and made electron capturing in a single operation with HFBA and ethanol as previously described (2, 4). The presence of hydroxy acids can be confirmed by making a similar analysis in which the reagent is substituted for ethanol with a longer- or shorter-chain alcohol and then observing a retention shift in the peak or peaks of interest upon reanalysis. Verification of unsaturation and functional groups is only occasionally necessary and is not a routine part of the analysis.

GAS CHROMATOGRAPHY ANALYSIS

In the gas chromatographic analysis of body fluids, it is essential to use high-resolution columns and to have a gas chromatograph with a reproducible temperature programmer and carrier gas flow controller. We used a Perkin-Elmer

(PE) 900 and two 3920B instruments in our studies. All of the instruments were equipped with FPEC detectors. The two 3920B instruments were fitted with dual 7-m glass columns (inner diameter, 2 mm) that were packed with OV-101 on Chromosorb W 80/100 mesh, dimethyldichlorosilane (DMDCS) treated. The packed columns used 95% argon–5% methane as the carrier gas, with a flow rate of 50 ml/min. All the instruments were fitted to use make-up gas which increased the flow through the detector to 70 ml/min or higher, depending on the need. The sensitivity of the detector was controlled by adjustment of the standing current from a range of 3 (new detector foil) to 0.5 (old detector foil), by adjustment of the attenuation (250, new detector foil; 500, old detector foil), and by adjustment of the flush gas flow.

The flush gas was increased, when necessary, to reduce sensitivity. The sensitivity was set so that a flat baseline was obtained from temperature program analysis on well-conditioned columns and the internal standards previously described (1, 6, 7) were above full scale. Newly packed OV-101 columns usually required conditioning overnight at 285°C to obtain a good baseline. The life of a packed column was about 1 year, and the life of a Ni^{63} detector foil ranged from 1 to 3 years. For analysis of carboxylic acid TCE esters, the instrument was programmed for a linear increase of 4°C/min from 100 to 265°C and held isothermal for 16 to 32 min. For analysis of HFBA-ethanol derivatives of hydroxy acids and HFBA derivatives of alcohol and amines, the instrument was held isothermal for 8 min at 90°C, programmed for a linear increase of 4°C/min to 265°C, and held isothermal at 265°C for 16 min. The PE recorders were set on 1 mV with a chart speed of 10 mm/min. Switches were installed that turned the recorder off when the door raised at the end of a programmed run. Two analyses were made at a time by injection of both columns with two needles held in a plastic retainer. The amount of sample injected and sample dilution factors are described elsewhere (3, 4, 8; Brooks et al., unpublished results). The PE 900 gas chromatograph was fitted with a PE splitless injector and a 50-mm fused silica OV-101 capillary column (inside diameter, 0.2 mm). The carrier gas was the same as that used in the packed column, with a flow rate of 3 ml/min, and make-up gas was added to make the flow through the detector 70 ml/min or greater. The capillary column was used as previously described (4) to help confirm the identification of new peaks. When deemed necessary for identification of new compounds, cochromatography, a polar column, and mass spectra were used. The polar column was glass, 7 m in length (inner diameter, 2 mm), packed with 3% OV-225 on Gas Chrom Q. The polar column was programmed like the OV-101 packed column. The unsaturated compounds shifted retention time position on the polar column and eluted later than the saturated hydrocarbons of similar chain length.

AIDS TO DATUM INTERPRETATION AND AUTOMATION

Interpretation of the FPEC-GLC data can be accomplished by a skilled chromatographer. The FPEC-GLC chromatogram is first studied for its general appearance. The questions that should be answered first are as follows. (i) Is the internal standard observable and present in the expected concentration? (ii) Are the peaks on the chromatogram resolved and in about the right concentration when compared with published findings? (iii) Has excess reagent been removed? Next, the peaks in the chromatogram are labeled by comparing them with known standards. It is always helpful to have FPEC-GLC profiles from diarrheal disease of known etiology obtained from current analysis. Comparisons for differentiation purposes are then made of an FPEC-GLC-defined group. FPEC-GLC profiles, in general, are first compared; then, qualitative and large quantitative differences are compared. A computer, such as the IBM 9000, can be used with available software to aid in making the above comparisons. Chromatograms analyzed on the same column can be overlaid for comparison, peaks can be integrated, peak names can be established, control analysis can be subtracted from analysis made of diseased stools, and individual areas of chromatograms can be expanded and enlarged for better comparison.

More efficient use can be made of the gas chromatograph if an automatic injector system is attached to the instrument. Automatic injection of the sample permits unassisted analysis to be made during and after normal working hours. In our work, we found a high-boiling-point solvent mixture which was suitable for automatic injection of TCE and HFBA derivative. The solvent was 50% xylene-ethanol. This xylene-ethanol solvent mixture performed better than did lower-boiling-point solvents of similar polarity, because the sample volume remained constant over a much longer period.

In general, automatic injection injects a more consistent volume of sample onto the column than is generally accomplished by hand injection and starts the computer clock more reproducibly than can be accomplished manually.

SUMMARY OF IMPORTANT DIFFERENTIAL CHARACTERISTICS OF MEMBERS OF THE FAMILY *ENTEROBACTERIACEAE*, *C. DIFFICILE*, AND ROTAVIRUS

The results of this and other studies (3, 4, 8) indicate that there are several reproducible differences in the FPEC-GLC profiles obtained from the stools of patients with diarrhea. The data indicate that FPEC-GLC analysis of stools might be a rapid way to distinguish the most commonly encountered members of the *Enterobacteriaceae*, *C. difficile*, and rotavirus associated with acute diarrhea in infants.

Improved tests to differentiate between *Escherichia coli* heat-stable toxin (ST) and heat-labile toxin (LT), as well as other causative agents of diarrhea, are needed (12–15, 18). Gene probes may meet several of these needs (17); however, they are not yet generally commercially available. They require culture growth, and they would not give data on changes of metabolites in the gut. Based on the results of a recent study (4), which contained a representative number of cases and controls, the feasibility of distinguishing *E. coli* ST- and LT-producing strains by FPEC-GLC examination of derivatized stool specimens is clearly indicated. The findings (4) that *E. coli* ST-positive stools had small amounts of short-chain fatty acids C-3 to C-5 compared with control stools, contained 6-methoxy-2-hydroxyhexanoic acid, unidentified peaks I, II, and III, and large amounts of lactic acid when compared with controls, and did not contain the unidentified amine peaks 4, 5, and 6 found in *E. coli* LT-positive stool gave strong indication that these two toxin-producing strains can be distinguished by FPEC-GLC analysis of stool specimens (Table 1; 4). Metabolites detected by FPEC GLC in diarrheal stools were also useful as diagnostic criteria not only for differentiation between *E. coli* ST, *E. coli* LT, and controls, but also for differentiation between *E. coli* ST, *E. coli* LT, and many other etiologic agents associated with acute diarrhea (Table 1).

TABLE 1. Key differential characteristics detected through FPEC-GLC analysis of diarrheal stool specimens[a]

Organism isolated from the stool	Characteristics in FPEC-GLC profiles of the stool			
	pH 2 chloroform extraction (TCE-HFBA derivative)	pH 2 ether extraction (HFBA-EtOH derivative)	pH 2 chloroform extraction (HFBA derivative)	pH 10 chloroform extraction (HFBA derivative)
Escherichia coli ST	Small amts of C-3 to C-5 acids compared with the control (Fig. 1 in reference 4)	MHH, peak III, and large amts of lactic acid (Fig. 3 in reference 4)	Peaks I[b] and II (Fig. 5 in reference 4)	Like control profile (Fig. 6 in reference 4)
Escherichia coli LT	50% of the samples had small amts of C-3 to C-5 acids compared with the control	Like control profile (Fig. 2 in reference 4)	Like control profile (Fig. 4 in reference 4)	Peaks 4[b], 5[b], and 6[b] (Fig. 7 in reference 4)
Shigella sonnei	Small amt of some C-3 to C-5 acids; C-6 acid (Fig. 1 in reference 3)	Peaks C[b], D[b], and H (Fig. 6 in reference 3)	Peaks B[b], E[b], F, and G (Fig. 7 in reference 3)	Like control profile
Shigella boydii	C-3 to C-5 acids; full-scale peaks like the control; peak 1 (Fig. 1 in reference 3)	Like control profile (Fig. 5 in reference 3)	Peaks I and II (Fig. 7 in reference 3)	Like control profile
Shigella flexneri	Small amt of some C-3 to C-5 acids (Fig. 1 in reference 3)	Like control profile (Fig. 5 in reference 3)	Peaks I and II (Fig. 7 in reference 3)	Like control profile
Klebsiella pneumoniae	Small amt of C-3 to C-5 acids compared with the control (Brooks et al., unpublished results)	Peak F[b] (Brooks et al., unpublished results)	AMC (Brooks et al., unpublished results)	Peak A (Brooks et al., unpublished results)
Serratia liquefaciens	50% of the samples had small amt of C-3 to C-5 acids	Like control profile (Brooks et al., unpublished results)	Like control profile	Small amt of peak A (Brooks et al., unpublished results)
Proteus mirabilis	C-3 to C-5 acids; full-scale peaks like control (Brooks et al., unpublished results)	Peaks b and d (Brooks et al., unpublished results)	Peak e (Brooks et al., unpublished results)	Peak 5 through 11 (Brooks et al. unpublished results)
Clostridium difficile	C-3 to iC-5 acids; full-scale peaks like control; iC-6 present (Fig. 1 in reference 8)			Tryptamine absent (Fig. 6 in reference 8)
Rotavirus	Small amt of iC-4, iC-5, and C-5 compared with the control; iC-4 increased (Fig. 4 in reference 8)			

[a] C-3 to C-5, propionic, isobutyric, butyric, isovaleric, and valeric acids; MHH, 6-methoxy-2-hydroxyhexanoic acid; AMC, acetyl methyl carbinol; EtOH, ethanol.
[b] Peaks detected in vivo and in vitro.

Shigella sp. is an important cause of diarrhea in infants and young children (10). The genus *Shigella* is subdivided into four species: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. It now seems likely that FPEC-GLC analysis of stools from patients with diarrhea can yield information that will permit the rapid differentiation of *S. sonnei*, *S. flexneri*, and *S. boydii* from other members of the family *Enterobacteriaceae* (3; Table 1). Some of the most important markers of stools positive for *S. sonnei* are caproic acid (C-6), hydroxy acids (peaks C and D), and alcohol peaks B, E, F, and G. These unidentified hydroxy acids and alcohol peaks are unique among the organisms studied thus far for *S. sonnei*-positive stools. One of the important markers of stools positive for *S. boydii* is the production of an unidentified carboxylic acid, which was designated as peak 2 (3).

We cannot categorically state that *Klebsiella pneumoniae*, *Serratia liquefaciens*, and *Proteus mirabilis* were causative agents of diarrhea in infants, but their FPEC-GLC patterns were different, and they were isolated in large numbers in the absence of other recognized pathogens. In addition, other workers have presented evidence that these organisms may be responsible for diarrhea in infants (9, 16). The FPEC-GLC markers of particular value for the differentiation of stools containing *K. pneumoniae*, *S. liquefaciens*, and *P. mirabilis* are shown in Table 1 and in unpublished results. Some of the most important markers of stools positive for *K. pneumoniae* were acetyl methyl carbinol, hydroxy acid peak F, and amine peak A. Acetyl methyl carbinol was a unique marker for *K. pneumoniae*-positive stools, and this compound was not detected in diarrheal stools obtained from patients with diarrhea caused by other members of the *Enterobacteriaceae* studied. The presence of large amounts of amine peaks 7 through 11 in *P. mirabilis*-positive stools adds support to previous findings (5) of amine production in vivo and in vitro by *P. mirabilis*.

Recent evidence (16, 19) indicates that rotaviruses are an

important cause of acute diarrhea in infants and young children in both developed and developing countries. In a recently conducted study (8), the FPEC-GLC profile of rotavirus-positive stools showed consistently smaller amounts of isobutyric (iC-4), isovaleric (iC-5), and valeric (C-5) acids and large amounts of butyric acid (C-4) compared with that in control stools. The rotavirus study, combined with the data reported for *C. difficile* (8) and in Table 1, indicate that the enteric organisms studied so far by FPEC GLC, along with rotavirus-associated diarrhea and diarrhea associated with *C. difficile*, might be distinguished by FPEC-GLC profiles obtained through the analysis of stools.

The just-concluded FPEC-GLC studies of diarrheal diseases (3, 4, 8; Brooks et al. unpublished results) provide several interesting observations which could have significant value for the rapid identification of the most frequently encountered etiologic agents associated with acute diarrheal diseases. First, short-chain fatty acids C-3 to C-5 were consistently detected in the control stools of infants 2 months of age and older. Second, normal stool profiles were changed by infection with a particular pathogen in a reproducible manner. Third, several chemical markers were reproducibly detected by FPEC-GLC analysis of stools when a particular organism was isolated. Fourth, some compounds that were detected in vivo were also produced by the organism in vitro (Table 1), and, finally, it may be possible, in many instances, to detect the major causative agent of diarrhea among several potential pathogens (unpublished data). Laboratory managers interested in establishing this FPEC-GLC technique should begin with the purchase of a quality gas chromatogram equipped with dual FPEC-GLC detectors and make-up gas. Next, the instrument should be equipped above with high-resolution columns as indicated. Then, standard mixtures of carboxylic acids, hydroxy acids, alcohols, and amines should be prepared by using high-purity solvents and reagent as indicated above. The beginner should lean to reproducible prepared derivatives, and then the instrument should be set to obtain the sensitivity and approximate retention time reported in published studies. Next, stools from patients with diarrheal disease of known origin should be analyzed, and FPEC-GLC results should be compared with published results and the results shown in Table 1. Before interpretation, the chromatogram should be examined to see that the internal standard, which was added before derivatization, is present in the expected concentration and that excess reagents have been sufficiently removed, as indicated by relatively low concentration in the chromatograms (1–8; Brooks et al., unpublished results).

The FPEC-GLC pattern obtained from derivatized extracts of diarrheal stools could be very useful for the following reasons. (i) In some instances they can be used for direct identification of the etiological agent. (ii) They are obtained rapidly and may, therefore, offer the physician information on the basis of which to prescribe fast, effective therapy. (iii) Some peaks in the patterns may represent compounds which are physiologically active in the patient and thus warrant identification and further studies. (iv) The patterns might supply clues to the identification of etiological agents responsible for certain types of disease for which no causative agent has been identified or for which several potential pathogens have been isolated. (v) The FPEC-GLC procedure could be automated and computerized. (vi) Simple chemical tests might be developed to rapidly differentiate between some of the causative agents by direct testing of stool extracts.

A limited number of organisms have been studied by FPEC GLC; therefore, comparisons for the purpose of identification among the *Enterobacteriaceae* must be made with organisms that were previously studied by FPEC GLC. The time for analysis with a two-column instrument is about 4 h. Automatic sample injection takes advantage of off-duty hours and reduces the cost of analysis. The cost of a good gas chromatograph with an automatic injector is about $19,000. Small computers, which can aid considerably in datum evaluation, can be purchased for $3,000 to $30,000. The FPEC-GLC system could also be used for the rapid determination of tuberculous meningitis (7) and perhaps other types of diseases by analysis of cerebrospinal fluid and serum. The time for most of these procedures could be shortened considerably through research.

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