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FREQUENCY-PULSED ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHIC STUDIES OF CHEMICAL CHANGES IN SERA OF PATIENTS WITH SCHISTOSOMIASIS

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SUMMARY

Sera from well documented cases of *Schistosoma mansoni* and *S. haematobium* infections as well as controls, were studied by frequency-pulsed electron-capture gas—liquid chromatography (FPEL-GLC) and mass spectrometry for detection of carboxylic acids and amines. Many carboxylic acids and unidentified peaks were detected. In a few serum specimens from infected patients, putrescine and cadaverine were detected. Indications are that in these few patients with high egg counts enough diamines were present to possibly produce amine toxicity. Following the initial investigation, the basic chloroform extractions, which contained amines, were further studied by FPEC-GLC with the aid of splitless injection and a capillary column. Several amines were detected which seemed to be related to schistosomiasis. Mass spectra were obtained on an unidentified schistosamine peak. The possible significance of the data is discussed.

INTRODUCTION

Schistosomiasis is a disease that affects a significant proportion of the world's population, especially in certain developing nations. The disease is endemic and chronic in some countries, and much of the population is infected repeatedly and for long periods of time. Eradication of the disease has relied mainly on snail elimination and other measures which in many instances are cost-inhibitory and slow; and many citizens of these countries live most of their lives infected with the organism. While eradication of the disease is not practical in many instances, improved diagnostic techniques, drug treatment, and investigation of physiologic changes in metabolites that occur in the host during the diseased state are of immediate concern. For example, it is known that people with some type of schistosomiasis have a higher incidence of cancer [1], and it is possible that a metabolite could be responsible. Other physiologic effects have been observed such as an opiate-like effect the infection has on the host. If these physiologic changes were due to metabolites, treatments might be devised that could counteract some of the toxic product build-up that may occur during the course of the disease.

Frequency-pulsed electron-capture gas—liquid chromatography (FPEC-GLC) has been demonstrated to be an effective tool for selective and sensitive detection of chemical changes that occur in body fluids during some diseased states [2—7]. It has been used both to detect metabolic products in vitro and carcinogenic nitrosoamines produced in vivo by *Proteus mirabilis* during urinary tract infections [5, 8, 9]. The method has also been employed as a diagnostic tool to aid in the rapid identification of tuberculous meningitis [3, 6], viral meningitis, cryptococcal meningitis, Rocky Mountain spotted fever [5], and several other diseases [7] by the analysis of serum and cerebrospinal fluid. The purpose of this research was to study sera taken from patients at the stage of the disease when the eggs were detectable, to study derivatized extracts of the sera by FPEC-GLC for changes in these sera, to study reproducibility of the FPEC-GLC patterns from patient to patient, to determine if peaks associated with infection were related to egg count, and to attempt physicochemical identification of some of the important peaks.

MATERIALS* AND METHODS

Serum specimens

A total of 57 serum samples from 19 controls, 34 persons with *Schistosoma haematobium* infection, and 4 persons with *S. mansoni* infection were analyzed by FPEC-GLC. The serum samples were obtained from residents of Kerdasa and Tamooh villages near Cairo, Egypt. The control specimens were obtained from the employees at the Biomedical Research Center for Infectious Diseases in Cairo and from the villages. *S. mansoni* and *S. haematobium* infections were confirmed by fecal and urine egg counts. The 1-ml volume of serum used for FPEC-GLC tests was that remaining after routine laboratory tests had been

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

performed. Sera were collected and stored at −20°C without addition of chemicals.

Extraction and derivatization procedures

Each serum specimen was placed in a 50-ml round-bottomed centrifuge tube with a PTFE-lined screw cap; then heptanoic acid (3.15 nmol in 0.1 ml of distilled water, made basic with sodium hydroxide to obtain solubility) and di-*n*-butylamine (1.19 nmol in 0.4 ml of distilled water made acidic to increase solubility) were added to each sample as internal standards. Next, the samples were acidified to about pH 2 with 0.1 ml of 50% sulfuric acid, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt) by shaking them for 5 min on a Burrell wrist action shaker at a setting of 10. To obtain the amines the residual aqueous phase was made basic (about pH 10) with 0.3 ml of 8 *M* sodium hydroxide and reextracted with 20 ml of chloroform, as described for the acidic extraction. The acidic chloroform extracts were derivatized with trichloroethanol—heptafluorobutyric anhydride (TCE—HFBA) to form TCE esters of carboxylic acids and HFBA esters of alcohols as described [9, 10]. The basic chloroform extracts containing amines were derivatized with HFBA pyridine—ethanol to form amides as described [6, 11]. Selected samples were extracted with chloroform at pH 2, derivatized with HFBA, and analyzed by FPEC-GLC on the same temperature program as the basic extraction to detect compounds that were neutral and to verify the basic extractability of the amines. After the TCE and HFBA derivatives of carboxylic acids, alcohols, and amines were prepared, they were dissolved in 0.1 ml of xylene—ethanol (1:1). A 2-μl injection was used for analysis. The techniques for filling and cleaning the syringe have been described [9].

Apparatus

The derivatives were analyzed on a Perkin-Elmer Model 3920 gas chromatograph equipped with dual 10-mCi ^{63}Ni frequency-pulsed electron-capture detectors. Two glass columns (7.3 m × 0.2 cm I.D.) packed with 3% OV-101 on 80—100 mesh Chromosorb WH P (AW-DMCS-treated) were used under conditions previously described [6, 10].

A Perkin-Elmer programmable processor (PEP-2) equipped with a Modular Software System (MS 16 revision B) accumulated data from the gas chromatograph, analyzed the data according to a stored method, and prepared a report. An internal standard analysis was performed on the data by using heptanoic acid, di-*n*-butylamine, and 2-hydroxyisovaleric acid, which were added as internal standards [6]. The latter part of the study on amines was carried out on a 50-m fused-silica OV-101 capillary column programmed as described below.

Identification of unknowns was made by comparing known standards on OV-101-packed columns as described above, and on a 50-m OV-101 fused-silica capillary column programmed as follows: for carboxylic acids, isothermal at 100°C for 12 min; then 6°C/min to 265°C; for amines, isothermal at 90°C for 8 min then 6°C/min to 265°C. The capillary column was used with a splitless injector. We used a 30-sec solvent vent, and helium as the carrier gas at a flow-rate of 3 ml/min. Analysis of unknowns was performed on a Finnigan

mass spectrometer 4023 (GC—MS—data system) equipped with a 25-m fused-silica OV-101 capillary column. Chemical ionization (positive and negative) and electron impact were used to obtain fragmentation patterns of unknown and known standards. Helium was used as the carrier gas for the GLC, and methane—ammonia was the reagent gas for chemical ionization.

RESULTS

Fig. 1 shows the types of acids detected by splitless injection capillary column (trace) analysis of sera from controls and patients infected with *S. haematobium* and *S. mansoni*. There were reproducible differences detected in the FPEC-GLC patterns in some groups of *S. haematobium* and *S. mansoni* patients' sera as indicated (Fig. 1A and B) by the blackened peaks 7, 8, 9, 10, 11, 16, and 20. However, these differences were not consistent enough

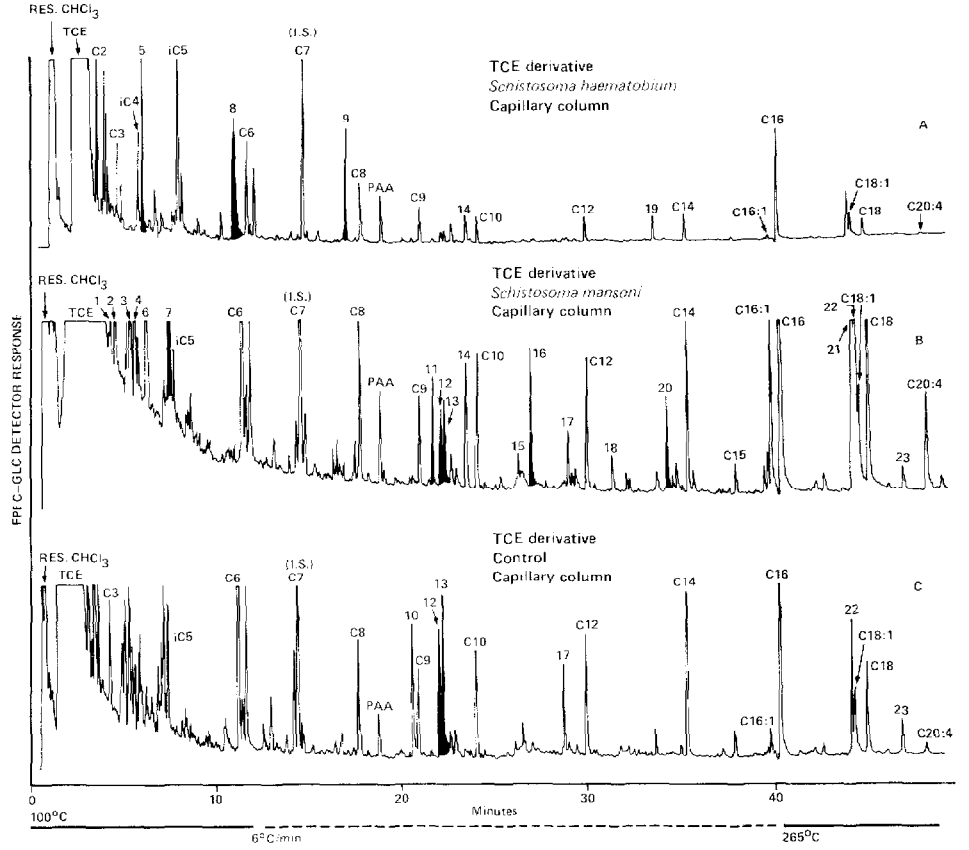

Fig. 1. FPEC-GLC profiles of TCE-derivatized acidic chloroform extracts of sera taken from patients with the indicated disease or control. Analyses were made on an OV-101 fused-silica capillary column. The letter C followed by a number indicates a saturated straight-chain carboxylic acid with the number of carbon atoms indicated by the number. The letter "i" indicates "iso", and the use of a colon between two numbers indicates unsaturation. I.S. is internal standard and PAA is phenylacetic acid. A number, or a number and a letter, over a peak indicates an unidentified peak; TCE is trichloroethanol; RES. $CHCl_3$ is residual chloroform.

throughout the entire study to be used as diagnostic markers to distinguish *S. haematobium* from *S. mansoni*. The FPEC-GLC patterns detected were different from the normal serum pattern and were unlike FPEC-GLC patterns detected in studies of certain types of bacterial, viral, and fungal infections

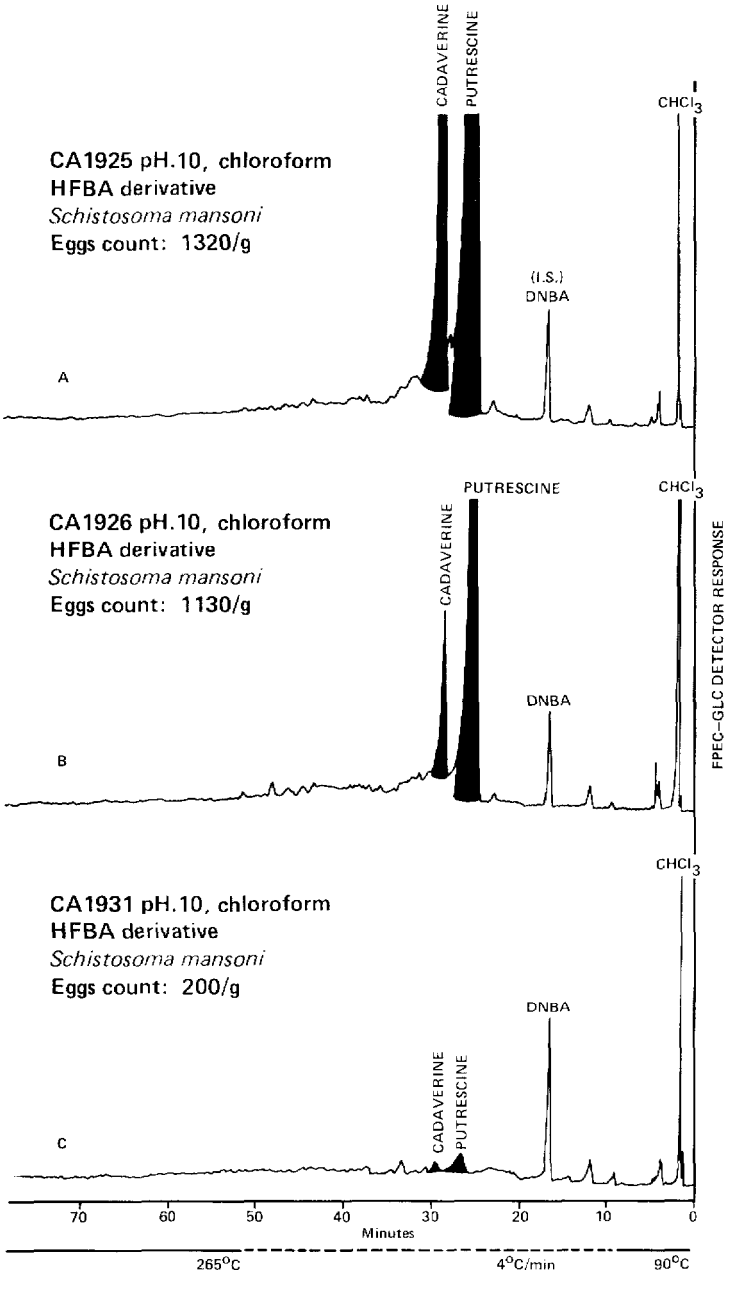

Fig. 2. FPEC-GLC profiles of basic HFBA-derivatized chloroform extracts of sera taken from patients with the indicated disease. Analyses were performed on an OV-101-packed column. I.S. is internal standard, DNBA is di-*n*-butylamine, $CHCl_3$ is residual chloroform, and HFBA is heptafluorobutyric anhydride. For other definitions see legend to Fig. 1.

[2—7]. Variation in certain metabolites found in the FPEC-GLC patterns could have been due to differences in the time of exposure to *Schistosoma*, number of schistosomes infecting the patient, as well as many other factors involved in the very complex life cycle of this organism.

Fig. 2 shows FPEC-GLC profiles obtained from packed-column analysis of the HFBA-derivatized basic extracts of sera. Putrescine and cadaverine were detected in sera from some patients infected with *S. mansoni*. Putrescine and cadaverine identification was verified by a comparison of the mass spectra obtained from the compounds present in the patient serum to known derivatized standards of putrescine and cadaverine. Fig. 2 also shows what appears to be a correlation with egg count. The amount of the diamines detected in these few patients was high. There was about 12.5 μM putrescine and about 6.3 μM cadaverine per ml of serum in the host with the highest egg count. Basic extracts of control sera did not contain putrescine or cadaverine and, except for the presence of the internal standard, were devoid of peaks.

Fig. 3 shows chromatograms obtained by trace analysis with splitless injection of the HFBA-derivatized basic chloroform extracts of sera from schistosomiasis patients and controls. Peaks were detected (Fig. 3A and B) that seem to be related to schistosomal infection. Peak 7 (referred to as "schistosamine") was found in full scale amounts in three patients. Two were infected with *S. haematobium* and one with *S. mansoni*. The three samples were from individuals residing in two different villages. Schistosamine which was found to be strictly basic extractable was further characterized using electron-impact (EI), negative-ion (NICI), and positive-ion chemical-ionization (PICI) mass spectrometry.

Several spectra suggest a molecular weight of 564. Ammonia PICI produced a relatively intense ion at *m/z* 582. The ion at *m/z* 582 obtained by PICI is produced as a result of the adduct of the molecular species with NH_4^+ (M + 18). The NICI spectra support the above data in that $(M - H)^-$ is observed at *m/z* 563. The NICI spectra had fragments at *m/z* 524, 504, 484, 366, and 197; all of these were from fragmentation of the HFBA derivative and while they showed that derivatization with HFBA occurred, they yielded little information about the structure of peak 7. The EI spectra produced a base peak at *m/z* 57, several fragments of HFBA, and a high-mass ion at *m/z* 387. The *m/z* 387 ion could have resulted from a loss of a neutral 177u. This ion was also observed in the chemical-ionization spectra. The structural significance of either *m/z* 387 or the neutral loss of 177u is not clear. Several structures are possible for the *m/z* 57 ion, but considering that the basic extractability of peak 7 indicates an amine, two likely candidates are C_3H_7N and C_2H_3NO. There were several major fragment ions observed in methane PICI. Two major fragments were observed at *m/z* 107 and 399. Other fragments ranging from 30% to 90% of full scale intensity were observed at *m/z* 123, 135, 149, 179, 191, and 387. The significance of these fragments in relation to the total structure of the compound is not clear.

As shown in Fig. 3A and B amines other than schistosamine were detected. These amines have not been identified, but they were strictly basic extractable, and even though the FPEC-GLC capillary patterns shown (Fig. 3A and B) differ from each other, in cases where schistosamine was low, as shown in Fig. 3B,

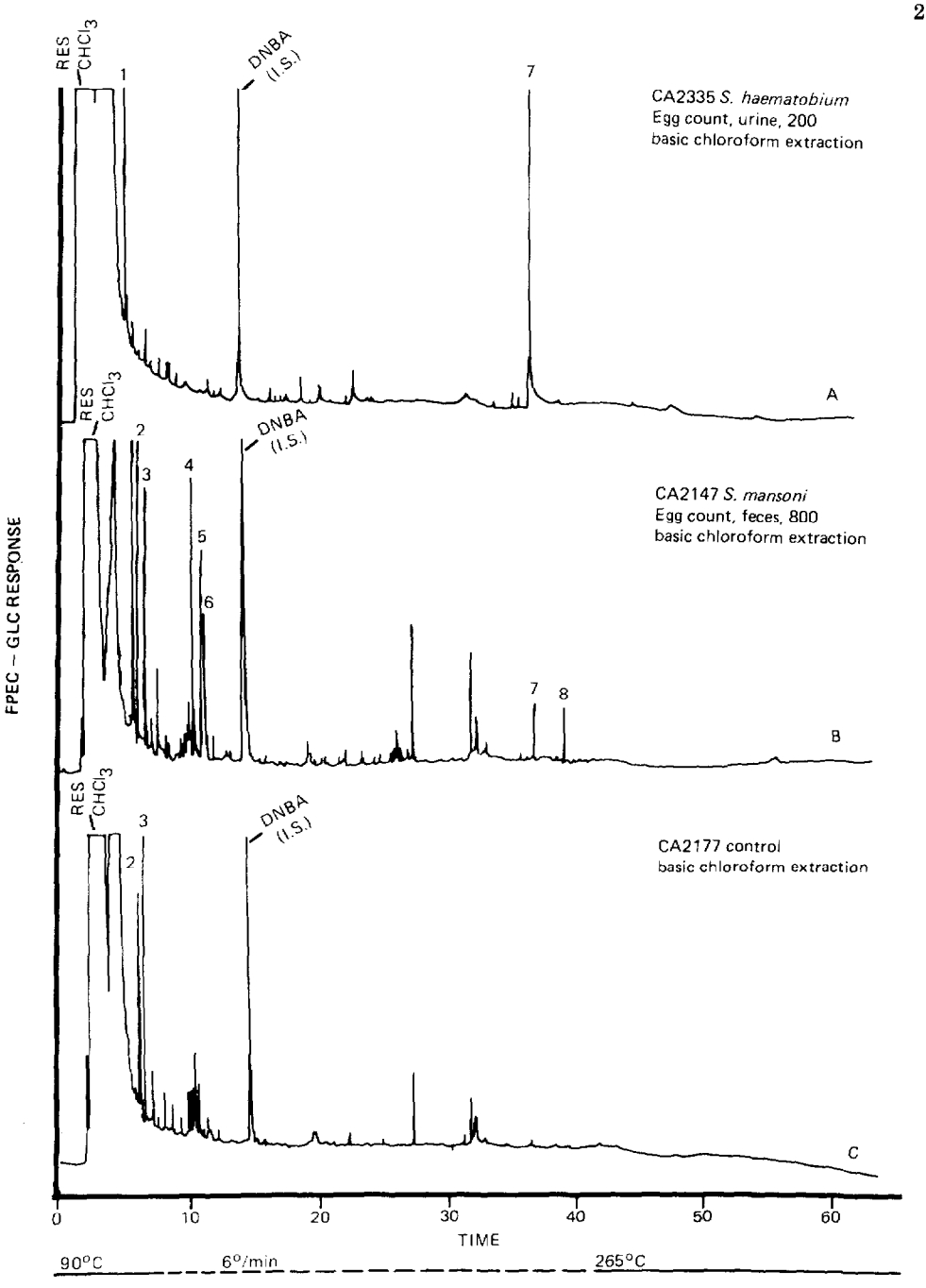

Fig. 3. FPEC-GLC profiles obtained from a 50-m OV-101 fused-silica capillary column. The chromatograms were obtained by analysis of HFBA-derivatized basic extractions of sera taken from patients with the indicated disease or controls. For definitions see legends to Figs. 1 and 2.

peak 7, the FPEC-GLC amine profiles of *S. haematobium* and *S. mansoni* were much alike. Peaks 4, 5, and 6 were generally much higher in the sera from patients with *S. mansoni* infection.

DISCUSSION

There have been relatively few studies made on the metabolites present in schistosomal disease [1]. Since these helminths live within large veins of the body, it stands to reason that their metabolic products might be detected in serum by a selective sensitive system such as FPEC-GLC. The early basic extractable peaks that eluted before 15 min seemed to have some relationship to egg count in that they were higher in the specimens with higher egg counts. Although these helminths have a much larger comparative body mass than other infectious agents the number of schistosomes is very small (10—20) in comparison to millions of bacteria or viruses involved in an infection. The organism also has a much longer life span, up to 30 years [1] and a much more complex life cycle. It is therefore not unreasonable to expect some variation in some types of metabolites detected by FPEC-GLC. The source of the acids and amines detected by FPEC-GLC could be a host response to the worms or a combination of both host response and worm metabolites. If the acids and amines detected by FPEC-GLC were a reflection of host response, again one would expect several factors such as duration of infection, number or organisms, and life cycle of the organism to affect the type metabolites detected. Animal model studies combined with human studies which could include chronic and new cases could help point out the source of some of the compounds detected in our study and perhaps find additional metabolites. We cannot rule out the possibility, even though both stool and urine were tested for eggs, that some of the patients we studied may have had dual infection with both *S. mansoni* and *S. haematobium*. Dual infections also could cause the FPEC-GLC profile to be different.

Since many of the peaks detected in the study are unidentified, we cannot determine their physiologic effects on the host. The physiology of diamines has been studied [12], and some, spermine and spermidine, are known growth factors. The affects of putrescine on cultures of *Anacystis nidulan* has been investigated by Guarine and Cohen [13]. They reported that in concentrations of 150 μM there was complete inhibition of protein synthesis. Th ribosomes were affected in an irreversible manner. Putrescine and cadaverine are known to have been produced in vitro by some microorganisms.

Except for the special cases of packed column analysis such as those shown for putrescine and cadaverine (Fig. 2), trace analysis with splitless injection and the OV-101 fused-silica capillary column produced more definitive differences between the schistosomiasis patient specimens and controls. More work is needed, however, to make FPEC-GLC analysis of serum a reliable tool for use in differentiation between *S. mansoni* and *S. haematobium* infection. FPEC-GLC analysis of a larger volume of serum, of feces from patients with *S. mansoni* infection, and of urine from patients with *S. haematobium* infection might improve chances for species differentiation. At the present in some developing nations diagnosis of the type of schistosomiasis is considered by some investigators to be less important than determining host response to schistosomal metabolites to which the host is constantly exposed. Elimination of the disease is very difficult, slow and expensive under present conditions, but counteractants for a toxic substance, once it is identified, is sometimes pharmacologically possible.

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