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TWO-DIMENSIONAL GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION USED IN THE DETERMINATION OF SPECIFIC PEPTIDOGLYCAN AND LIPOPOLYSACCHARIDE CONSTITUENTS OF GRAM-NEGATIVE BACTERIA IN INFECTED HUMAN URINE

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SUMMARY

Gas chromatography with electron-capture detection was used in the determination of diaminopimelic acid (as the N-heptafluorobutyryl isobutyl derivative) and 3-hydroxymyristic acid (as the O-pentafluorobenzoyl methyl derivative) in Gram-negative bacterial cells in infected human urine. Use of the column-switching (two-dimensional gas chromatography) technique greatly enhanced the selectivity of the detection and simplified the processing of samples. The system described should prove useful for trace detection of specific bacterial constituents in complex environments.

INTRODUCTION

Microorganisms can be detected directly in clinical specimens by demonstration of their specific constituents, the so-called chemical markers or biomarkers, using gas chromatographic (GC) analysis [1]. A highly sensitive and selective GC detector is usually required. Mass spectrometry (MS) operating in the selected-ion monitoring (SIM) mode has been used in several studies,

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but it is a resource not usually available in bacteriological laboratories. Electron-capture detection (ECD) may prove to be a useful, less expensive alternative. This detector is known to respond strongly to halogenated (preferentially fluorinated) derivatives of biomarkers [2–4]. A major drawback of ECD, however, is its limited selectivity, which may result in high 'background interference' from non-bacterial components in the specimens, preventing detection of trace amounts of the biomarkers being studied.

Here we report the use of two-dimensional GC (the column-switching technique) to enhance the selectivity of the GC–ECD system. With column-switching, sample components are first separated on a pre-column, after which selected fractions are switched to a cold-trap unit and then re-injected into the analytical column of the GC–ECD system. We describe the use of this method for detecting trace amounts of 3-hydroxymyristic acid (3-OH-14:0) and of diaminopimelic acid (DAP), derived from the lipopolysaccharide (LPS) and peptidoglycan (PG) constituents, respectively, of Gram-negative bacteria in infected human urine.

EXPERIMENTAL

Chemicals and glassware

Analytical-grade DAP was obtained from Sigma (St. Louis, MO, U.S.A.) and stock solutions were prepared in 0.1 *M* hydrochloric acid and stored at 4°C. Heptafluorobutyric anhydride (HFBA) and 2,3,4,5,6-pentafluorobenzoyl chloride (PFBO-Cl) were purchased from Fluka (Buchs, Switzerland) and acetyl chloride from Merck (Darmstadt, F.R.G.). The 3-hydroxy fatty acid standards 3*R*-hydroxynonanoic acid (3-OH-9:0) and *rac*-3-hydroxymyristic acid (3-OH-14:0) were synthesized [5]. Solvents and reagents were of analytical-reagent grade.

All glassware was heated for 10 h at 400°C before use. The test-tubes used had PTFE-lined screw caps.

Urine specimens

Urine samples from five patients with Gram-negative bacteriuria and five culture-negative controls were studied. The specimens (1 ml) were centrifuged at 10 000 *g* for 15 min, and the supernatants were discarded. Bacteriuria was confirmed by standard microbial cultivation techniques.

Lipopolysaccharide analysis

Each pellet was suspended in 1 ml of 4 *M* hydrochloric acid, transferred to a glass tube and heated at 110°C for 3 h [6]. After cooling, 1 ml of saturated sodium chloride solution (prepared in pyrogen-free water), 2 ml of chloroform and 50 ng of 3-OH-9:0 dissolved in chloroform (internal standard) were added. The tube was shaken and centrifuged (1000 *g*), and the organic phase was

transferred to a new tube and evaporated in a desiccator under reduced pressure. The residue was subjected to methanolysis with 1 ml of 2 *M* methanolic hydrochloric acid at 85 °C for 18 h followed by derivatization with PFBO-Cl as described in ref. 7. The final preparations [containing the O-pentafluorobenzoyl methyl (O-PFBO-Me) ester derivatives] were made up with heptane to a final volume of 50–1000 μl, and 0.5 μl was injected.

Peptidoglycan analysis

Each pellet was suspended in 1 ml of 6 *M* hydrochloric acid, transferred to a glass tube and heated at 150 °C for 6 h. The acid solution was removed in a lyophilizer and the residue transferred to a new tube with three 0.2-ml portions of 0.1 *M* hydrochloric acid. After removal of the acid solution under reduced pressure, the amino acids were derivatized with 3 *M* isobutanolic hydrochloric acid and HFBA as described previously [7]. The final preparations [containing the N-heptafluorobutyryl isobutyl (N-HFB-isoBu) derivatives] were made up with ethyl acetate to a final volume of 50–1000 μl, and 0.5 μl was injected.

Gas chromatography

A Varian Model 3700 (Los Altos, CA, U.S.A.) gas chromatograph equipped with a ^{63}Ni electron-capture detector operating in the frequency-pulsed mode

TABLE I

MEASUREMENT OF DIAMINOPIMELIC ACID AND 3-HYDROXYMYRISTIC ACID IN HUMAN URINE

Sample	Culture	DAP			3-OH-14:0	
		ng/ml	cells/ml[a]	LPS[b] (ng/ml)	ng/ml	LPS[c] (ng/ml)
1	*E. coli*	150	$4.3 \cdot 10^8$	1300	42	210
2	*K. pneumoniae*	51	$1.4 \cdot 10^8$	420	77	380
3	*E coli*	8.6	$2.5 \cdot 10^7$	75	34	170
4	*P. mirabilis*	6.0	$1.7 \cdot 10^7$	51	Traces	
5	*E coli*	–			Traces	
6	Negative	–			Traces	
7	Negative	–			Traces	
8	Negative	–			–	
9	Negative	–			–	
10	Negative	–			–	

[a]Calculated from the concentration of DAP, assuming that 1 ng of DAP corresponds to $2.9 \cdot 10^6$ cells [2].

[b]Calculated from the concentration of cells (according to the concentration of DAP), assuming that 1.0 10^7 cells contain ca. 6 ng of 3-OH-14 0 [9] and that the LPS studied contains 20 wt.-% 3-OH-14:0 [16].

[c]Calculated from the concentration of 3-OH-14:0, assuming that the LPS studied contains 20 wt.-% 3-OH-14·0 [16].

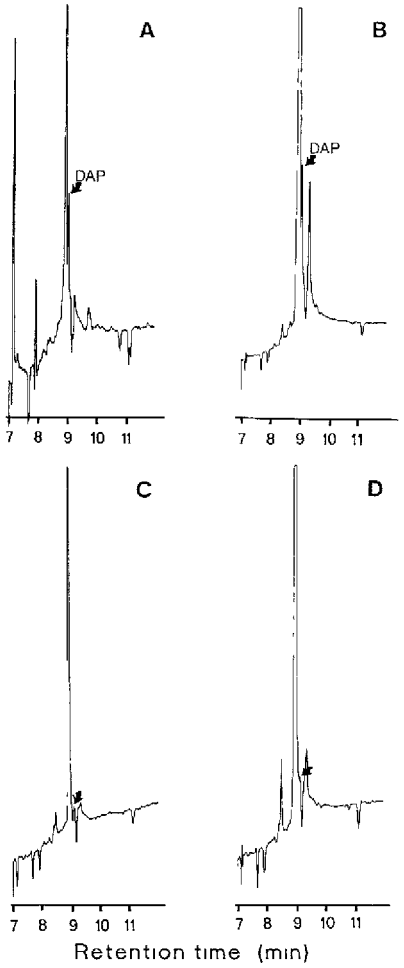

Fig. 1. Representative chromatograms from analyses of urine samples. (A) *E. coli* bacteriuria (sample 3), (B) *K. pneumoniae* bacteriuria, (C) culture-negative sample 6 and (D) culture-negative sample 7. Peaks representing the N-HFB-isoBu derivative of DAP are indicated with arrows.

and a MUSIC (multiple switching intelligent controller) column-switching system (Chrompack, Middelburg, The Netherlands) was used. A fused-silica wide-bore column (10 m × 0.53 mm I.D.) with cross-linked CP-Sil-8 (Chrompack) as the stationary phase (film thickness 6.4 μm) was used as a pre-column attached to a flame-ionization detector, and a fused-silica capillary column (25 m × 0.22 mm I.D.) coated with SE-30 (film thickness 0.2 μm) (SGE, Ringwood, Australia) served as the analytical column attached to the electron-capture detector. The nitrogen carrier gas flow-rate was 4 ml/min through the pre-column and 1 ml/min through the analytical column. The make-up gas

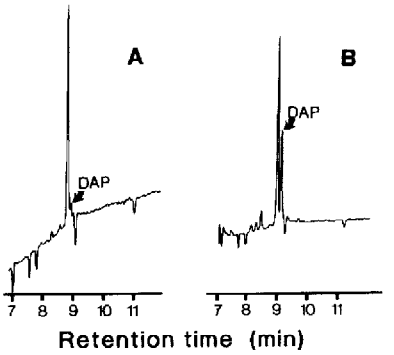

Fig. 2. Analysis of urine sample 3 (*E. coli* bacteriuria) (A) before and (B) after co-injection of 80 pg of authentic N-HFB-isoBu derivative of DAP.

(nitrogen) flow-rate through the electron-capture detector was 15 ml/min. Injections were made on-column. The temperature of the injector was 250°C and that of the detector 350°C. The initial temperature of the pre-column (120°C for 3-OH-14:0 analysis and 140°C for DAP analysis) was programmed (10°C/min) to a final temperature of 260°C. The cold trap, chilled to −70°C with liquid carbon dioxide, was heated to 250°C on re-injection of the trapped fractions into the analytical column (initial temperature 200°C, increased to 260°C at 10°C/min). Data handling was performed with the Chrompack control and integration system using an IBM PS/2 Model 30 and Chrompack BD 70 printer–plotter.

A Carlo Erba (Rodano, Italy) Model 4160 gas chromatograph equipped with a ^{63}Ni electron-capture detector (Carlo Erba HT 25) operating in the frequency-pulsed mode, an on-column injector and a fused-silica capillary column (25 m×0.32 mm I.D.) coated with DB-5 (film thickness 0.25 μm) (J & W, Folsom, CA, U.S.A.) were used in comparative studies. The helium carrier gas and the argon–methane (95:5) make-up gas flow-rates were 2.0 and 50 ml/min, respectively. The temperature of the column, initially 120°C, was programmed (10°C/min) to a final temperature of 280°C. The temperature of the detector was 300°C.

Peaks were identified by means of retention time comparisons with authentic standards and co-injection of samples with the standards. MS data of the derivatives studied have been published elsewhere [3,7].

RESULTS

Detection sensitivity

At a signal-to-noise ratio of 3:1 the detection limit, both for the DAP N-HFB-isoBu derivative and for the 3-OH-14:0 O-PFBO-Me derivative, was found to be ca. 5 pg. A calibration curve for DAP with the equation $y=1.5x+240$ ($r=0.98$) was linear over the range 1–1000 ng (10–600 pg injected amounts).

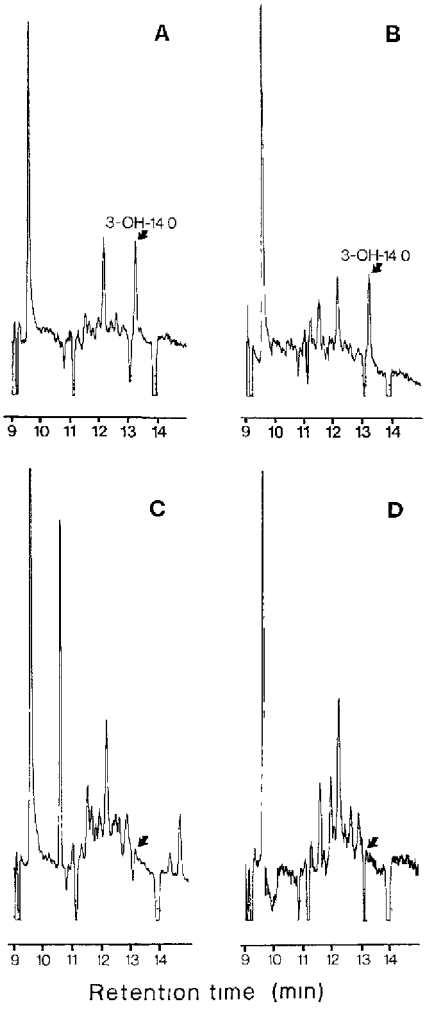

Fig. 3. Representative chromatograms of the same urine samples as in Fig. 1 analysed for the O-PFBO-Me derivative of 3-OH-14:0 (indicated with arrows).

The concentration of 3-OH-14:0 was calculated from standards (100 ng of 3-OH-14:0 derivatized amount).

Urine samples

Five urine samples were culture-positive for Gram-negative bacteria (*Escherichia coli* in three cases and *Klebsiella pneumoniae* and *Proteus mirabilis* in one sample each).

DAP was detected in four of the culture-positive samples and in none of the culture-negative samples. The quantitative results are summarized in Table I,

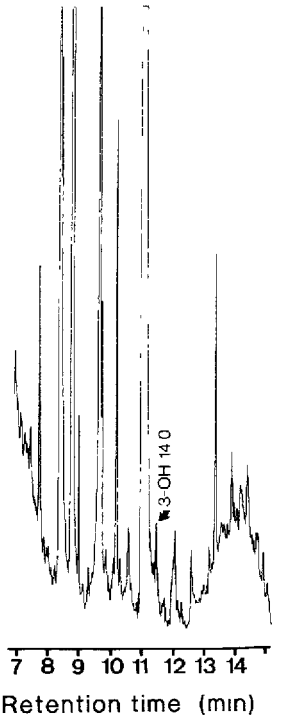

Fig. 4. Chromatogram of the same preparation as in Fig. 3A, obtained without the column-switching technique.

and representative chromatograms are shown in Fig. 1. An unknown substance was found to elute close to DAP under the GC conditions used; however, co-injection of the authentic N-HFB-isoBu DAP derivative confirmed the presence of DAP (Fig. 2).

Three of the culture-positive urine samples were found to contain easily detectable amounts of 3-OH-14:0 (Table I, Fig. 3). Only traces were found in samples 4 and 5 and in two of the culture-negative samples. The improved selectivity obtained with the column-switching technique was demonstrated by comparing analyses of the same sample with its use (Fig. 3A) and without (Fig. 4).

DISCUSSION

Demonstration of specific microbial constituents in body fluids has great potential as an aid in the diagnosis of infections. As the concentrations of such biomarkers in clinical samples are usually very low – in the picomole range or

lower [1] – highly sensitive means of detection are required. Maitra et al. [8] successfully used electron-impact GC–SIM for detecting the LPS-specific 3-hydroxy fatty acids, derivatized to O-trimethylsilyl ether methyl esters, in cerebrospinal fluid specimens from patients with meningitis caused by Gram-negative bacteria. The detection limit of the method corresponded to ca. 100 ng of *E. coli* LPS per ml of sample. Negative-ion chemical ionization (NICI) GC–SIM was found to provide a detection limit for the 3-O-PFBO-14:0 methyl ester corresponding to ca. 1 ng of *E. coli* LPS per ml [3]; the same technique was used to detect the pentafluorobenzyl ester derivative of 3-OH-14:0 in *E. coli*-infected human urine [9]. Sud and Feingold [10] examined a GC–ECD method for demonstrating *Neisseria gonorrhoeae* in cervical specimens by analysing for 3-hydroxylauric acid (O-HFBA-*n*-butyl ester derivative); however, interfering substances made it difficult to work at maximum sensitivity and complex purification procedures were required.

In the present work we tested the possibility of improving the selectivity of GC–ECD considerably by means of the column-switching technique. The detector is well protected from contamination due to overloading, as only carefully selected fractions of the total sample are introduced into the analytical column. The arrangement also allows simplified sample processing. In the present study we omitted cation-exchange column isolation of DAP, which is usually a standard step [2,7]. In addition, post-derivatization clean-up procedures, such as use of disposable columns [4,11], may not be necessary, and the lifetime of the analytical column should be greatly extended. Use of wide-bore columns as pre-columns allows injection of large amounts of sample, which should improve sensitivity.

All of the bacterial species isolated in this study are known to contain 3-OH-14:0 as the only 3-hydroxy fatty acid in the LPS constituents [12] and DAP in the PG constituents [13]. According to Nachum [14], 200–2150 ng LPS per ml urine (as determined with the quantitative chromogenic *Limulus* assay) are usually found during Gram-negative bacteriuria (i.e., more than 10^5 colony-forming units per ml), and the concentrations found in the culture-positive urine samples studied here were within that range (Table I). In samples 1 and 4, the concentrations of LPS were much lower when calculated according to the amounts of 3-OH-14:0 than when estimated from the amounts of DAP; probably a portion of the LPS in these samples, instead of being cell wall-associated, was present in the (discarded) supernatant [15]. Including both intact cells and debris, it was possible to detect at least 6 ng DAP per ml urine (sample 4 in Table I) corresponding to ca. $1.7 \cdot 10^7$ cells/ml. The detection limit for LPS was calculated to be ca. 20 ng/ml (amount of 3-OH-14:0 in sample 3); however, samples containing LPS at lower concentrations should also be possible to determine by using larger volumes of urine than 1 ml.

It is well known that the performance of the electron-capture detector is highly dependent on the condition of the equipment (e.g. of the ^{63}Ni foil). The detection limit for the O-PFBO-Me ester derivative of 3-OH-14:0 was ca. ten

times higher in the present study than reported previously [3]. No internal standard was used for quantification of DAP, as no suitable substance was commercially available. However, by keeping the sample processing as simple as possible, a good linearity was achieved. In another recent study [7], we used D-lysine as an internal standard, but this requires the use of a chiral column. Since such a column has a low maximum temperature (e.g. 220°C) its use entails considerably increased analysis time as well as severe column bleeding, which reduce its compatibility with ECD.

Although the NICI–SIM system constitutes a much more selective and somewhat more sensitive GC detection mode than ECD [3], the GC–ECD column-switching technique may represent a useful and less expensive alternative. The MUSIC system is microprocessor-controlled and easy to operate. Further studies are in progress to evaluate the system as used in trace detection of bacteria in clinical samples and other complex environments.

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REFERENCES

1 L. Larsson, in J. Jorgensen (Editor), Automation in Clinical Microbiology, CRC Press, Boca Raton, FL, 1987, p. 153.
2 A. Tunlid and G. Odham, J. Microbiol. Methods, 1 (1983) 63.
3 A. Sonesson, L. Larsson, G. Westerdahl and G. Odham, J. Chromatogr., 417 (1987) 11.
4 A. Sonesson, L. Larsson and J. Jimenez, J. Chromatogr., 417 (1987) 366.
5 A. Tunlid, H. Ek, G. Westerdahl and G. Odham, J. Microbiol. Methods, 7 (1987) 77.
6 M. Nurminen, E.T. Rietschel and H. Brade, Infect. Immun., 48 (1985) 573.
7 A. Sonesson, L. Larsson, A. Fox, G. Westerdahl and G. Odham, J. Chromatogr., 431 (1988) 1.
8 S.K. Maitra, R. Nachum and F.C. Pearson, Appl. Environ. Microbiol., 52 (1986) 510.
9 G. Odham, A. Tunlid, G. Westerdahl, L. Larsson, J.B. Guckert and D.C. White, J. Microbiol. Methods, 3 (1985) 331.
10 I.J. Sud and D.S. Feingold, J. Invest. Dermatol., 73 (1979) 521.
11 M.I. Daneshvar, J.B. Brooks and R.M. Winstead. J. Clin. Microbiol, 25 (1987) 1216.
12 E. Jantzen and K. Bryn, in M. Goodfellow and D.E. Minnikin (Editors), Chemical Methods in Bacterial Systematics, The Society for Applied Bacteriology, Technical Series No. 20, Academic Press, London, 1985, p. 145.
13 K.H. Schleifer and O. Kandler, Bacteriol. Rev., 36 (1972) 407.
14 R. Nachum, Prog. Clin. Biol. Res , 231 (1987) 431.
15 A.J. Wicken and K.W. Knox, Biochim. Biophys. Acta, 604 (1980) 1.
16 P. Prehm, S. Stirm, B. Jann and K. Jann, Eur. J. Biochem., 56 (1975) 41.