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DETERMINATION OF ENDOTOXINS BY GAS CHROMATOGRAPHY: EVALUATION OF ELECTRON-CAPTURE AND NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRIC DETECTION OF HALOGENATED DERIVATIVES OF β-HYDROXYMYRISTIC ACID

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SUMMARY

The sensitivity and selectivity of gas chromatography for analysing several halogenated ester derivatives of β-hydroxymyristic acid were studied using both selected-ion monitoring detection with negative-ion chemical-ionization mass spectrometry and electron-capture detection. Six different derivatization methods were compared with respect to yield, chemical stability and formation of by-products. Procedures for removal of excess reagents using disposable silica columns and thin-layer chromatography were elaborated. The 3-O-pentafluorobenzoyl-methyl ester was the preferred derivative since it provided high sensitivity and had the molecular ion as the base peak in the mass spectrum. The detection limit was 0.5 pg with electron-capture detection and 0.3 pg with the mass spectrometric system. Using β-hydroxymyristic acid as a chemical marker it was possible to detect *Escherichia coli* endotoxin in aqueous solution at a level of 1 ng/ml.

INTRODUCTION

Endotoxins (lipopolysaccharides, LPS) constitute a part of the outer membrane in the cell wall of Gram-negative bacteria [1], and can induce a variety of acute pathophysiological reactions in humans [2]. Endotoxins have been shown

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to cause problems in work environments, for instance the humidifier disease, an illness caused by inhalation of aerosols containing microbe-contaminated humidifier water [3,4], and byssinosis, caused by inhalation of airborne LPS among workers in cotton mills [5]. At present, the *Limulus* amebocyte lysate (LAL) test is the most commonly used method to determine endotoxins; however, the test has a limited specificity [6] and is sometimes difficult to use in complex matrices such as sera [7]. Clearly, there is a profound demand for a sensitive and specific alternative method for determining endotoxins.

Endotoxins are composed of a lipid moiety called lipid A, a core polysaccharide and often a O-specific side-chain [8]. Lipid A consists largely of D-glucosamine, phosphate and long-chain fatty acids [9]. The most abundant acids are 3-hydroxy-substituted [10], principally 3-hydroxytetradecanoic (3-OH 14:0, β-hydroxymyristic) acid [11].

3-OH 14:0 has been suggested for use as a marker substance for endotoxins using gas chromatography (GC) with flame-ionization detection (FID) [12], electron-capture detection (ECD) [13] and selected-ion monitoring (SIM) detection employing electron-ionization (EI) mass spectrometry (MS) [14]. Recently, negative-ion (NI) chemical-ionization (CI) SIM of halogenated fatty acid derivatives was reported as providing a hitherto unmatched degree of sensitivity and selectivity [15].

In the present study we compared the performance of ECD with that of NICI MS–SIM for determining several halogenated derivatives of 3-OH 14:0. The findings led to a proposed method for the determination of endotoxins in water samples.

EXPERIMENTAL

Chemicals and glassware

Solvents were of glass-distilled reagent grade and were not redistilled before use. Heptafluorobutyric anhydride (HFBA), p.a. grade, and tetrabutylammonium hydrogen sulphate (TBA), puriss grade, were from Fluka (Buchs, Switzerland); trichloroacetyl chloride (TCAO-Cl), purity >97%, trichloroethanol (TCE-OH), purity >96%, acetyl chloride, p.a. grade, and methyl nonadecanoate, GC grade, from Merck (Darmstadt, F.R.G.); 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br), purity >99%, 2,3,4,5,6-pentafluorobenzoyl chloride (PFBO-Cl), purity >98%, and 2,3,4,5,6-pentafluorobenzyl alcohol (PFB-OH), purity >96%, from Janssen Chimica (Beerse, Belgium); N-heptafluorobutyrylimidazole (HFBI), p.a. grade, from Pierce (Rockford, IL, U.S.A.) and the bacterial fatty acid mixture from Supelco (Bellefonte, CA, U.S.A.). The hydroxy fatty acid standards 3*R*-hydroxynonanoic acid (3-OH 9:0) and racemic 3-hydroxytetradecanoic acid (3-OH 14:0), purity >98%, were from our laboratory collection. Phenol–water-extracted LPS of *Escherichia coli* 055:B5 came from Sigma (St. Louis, MO, U.S.A.).

All glassware was washed with 5% Deconex, rinsed several times with hot tap water and distilled water, soaked overnight in 5 *M* hydrochloric acid and rinsed

with water and ethanol (95%) before being heated for 10 h at 400°C. The test-tubes used were equipped with PTPE-lined screw caps.

Derivatization procedures

Six different halogen-containing 3-OH 14:0 derivatives (starting material 5 μg) were prepared. The yields were calculated using 100 ng/μl methyl 19:0 ester as internal standard. The evaporations were made using dry nitrogen, and all derivatives were dissolved in heptane prior to analysis.

Methyl esters were prepared by heating LPS or free acid overnight at 90°C in 0.3 ml of 1 *M* methanolic hydrogen chloride [16]. After cooling, 0.7 ml of water and 2 ml of hexane were added and the tubes were vigorously shaken and centrifuged (ca. 1000 *g*). The organic phase was transferred to another tube and evaporated. Free fatty acids were prepared from methyl esters by saponification. Typically, ca. 50 μg of ester was heated in 1 ml of methanol–water solution (1:1, v/v) containing sodium hydroxide (15%, w/w) at 90°C for 30 min [15]. After cooling, 2 ml of water and 1 ml of hexane were added and the tubes were shaken and centrifuged. The hexane phase was then removed. Dilute aqueous hydrochloric acid was added to the aqueous phase until the pH was less than 2, and the free acids were then extracted with two 1-ml portions of methylene chloride. The combined phases were washed with water and evaporated to dryness.

3-O-Heptafluorobutyryl (HFBO)-methyl ester. To the methyl ester of 3-OH 14:0, dissolved in 50 μl of hexane, were added 30 μl of HFBA, and the sample was heated at 100°C for 15 min. After cooling, 0.5 ml of hexane and 2 ml of 1 *M* phosphate buffer solution (pH 7.0) were added [17]. The tube was shaken and centrifuged (ca. 1000 *g*), and the organic phase evaporated to dryness.

This reaction was also performed at room temperature for 30 min using 10 μl of pyridine as a catalyst [18].

3-O-Pentafluorobenzoyl (PFBO)-methyl ester. Two derivatization methods were compared. In the first, 10 μl each of pyridine and PFBO-Cl were added to the methyl ester dissolved in 30 μl of methylene chloride. The sample was heated at 80°C for 5 min. After cooling, 0.5 ml of methylene chloride and 2 ml of the buffer solution were added. The tube was shaken and centrifuged and the organic phase evaporated to dryness. This reaction was also studied at room temperature with a 20-min reaction time according to the method of Zlatkis and Pettitt [19]. The results obtained using triethylamine (TEA) as catalyst instead of pyridine were also studied at both temperatures.

In the second method, 10 μl of PFBO-Cl were added to the methyl ester dissolved in 60 μl of acetonitrile, after which the sample was heated at 150°C for 60 min . After cooling, 0.5 ml of methylene chloride were added; the preparation was then purified using the buffer extraction procedure as described above.

3-O-Trichloroacetyl (TCAO)-methyl ester. This reaction was carried out as described for the first method for the PFBO-methyl ester, except that TCAO-Cl was used as reagent and the reaction was performed at 80°C for 30 min.

3-Hydroxypentafluorobenzyl (OH-PFB) ester. Two derivatization methods were compared. In the extractive alkylation method (method I), 0.2 ml of 1 *M* aqueous sodium hydroxide solution, 0.4 ml of 0.1 *M* aqueous TBA and 1 ml of

methylene chloride were added to the free acid. The tube was shaken for 5 min and centrifuged, after which the organic phase was transferred to another vial and evaporated to dryness. PFB-Br (0.1%) in methylene chloride (0.1 ml) was added and the tube was heated at 80°C for 30 min. The sample was then evaporated.

In the room temperature method (method II), the acid, dissolved in 30 μl of acetonitrile, was supplemented with 10 μl of 35% PFB-Br (in acetonitrile) and 10 μl of TEA. After 15 min at room temperature, 0.4 ml of hexane were added. The sample was shaken and centrifuged (ca. 1000 *g*) and the organic layer evaporated.

3-O-HFBO-PFB ester. Three methods were compared. In method I, extractive alkylation followed by HFB derivatization was conducted as described above.

In method II, PFB-esters were first prepared at room temperature, then after 15 min 30 μl of HFBA were added. This was followed by the addition of 0.4 ml of hexane after a further 15 min. The tube was then shaken and centrifuged and the hexane phase evaporated. The reaction was also performed at 80°C (with pyridine) with HFBI instead of HFBA and with pyridine instead of TEA.

In method III, the acid was dissolved in 30 μl of 10% PFB-OH in methylene chloride, 30 μl of HFBA were added and the tube was heated at 100°C for 30 min. After cooling, 0.5 ml of methylene chloride and 2 ml of the buffer solution were added. The tube was shaken and centrifuged and the organic phase evaporated.

3-O-HFBO-trichloroethyl (TCE) ester. Two methods were used. In method I HFBA (30 μl) and 20 μl of TCE-OH (10%, in hexane) were added to the acid, dissolved in 30 μl of hexane. The tube was heated at 100°C for 30 min and, after cooling, 0.5 ml of hexane and 2 ml of the buffer solution were added. After extraction and centrifugation the organic phase was transferred to another tube and evaporated. The reaction was also carried out with chloroform as solvent.

In method II, the acid was dissolved in 30 μl of chloroform, after which 10 μl of 10% TCE-OH (in chloroform) and 20 μl of HFBA were added. The tube was left at room temperature for 32 min following the procedure described by Alley et al. [20]. Chloroform (0.2 ml) and 0.1 *M* aqueous hydrochloric acid (0.3 ml) were added and the tube was shaken. After 3 min the aqueous phase was removed and 0.3 ml of 0.1 *M* aqueous sodium hydroxide was added. The organic phase was washed for 3 min, transferred to a dry tube and evaporated.

Endotoxin samples

The LPS preparation was dissolved in sterile water, and dilutions were made to known amounts in 1-ml fractions. The samples were freeze-dried, and 3.6 ng of 3-OH 9:0 were added to serve as an internal standard. Methanolysis and derivatization with PFBO-Cl (method II) was carried out as described above. The final product was dissolved in 20 μl of hexane.

Clean-up of derivatives

Before GC–ECD analyses, excess reagents were removed from the PFB- and TCE-containing preparations using either thin-layer chromatography (TLC) or disposable silica columns.

The preparations were dissolved in hexane, spotted on TLC silica gel plates (10×10 cm, layer thickness 0.2 mm; silica gel 60 F_{254} aluminium sheets, E. Merck) and developed with chloroform. The PFB derivatives were detected under UV light, the UV-absorbing zones being scraped off and extracted with diethyl ether.

Disposable silica columns containing ca. 100 mg of silica gel (<0.08 mm, E. Merck) were used to clean up the 3-O-PFBO-methyl ester preparations. The columns were washed with two 2-ml portions of hexane before the samples were applied (in hexane). The gel was again washed with two 2-ml portions of hexane, and the product was eluted with 1 ml of methylene chloride.

Gas chromatography

Two Carlo Erba Model 4160 instruments (Rodano, Italy) were used. One was equipped with an FID system, with an all-glass splitless injection system, and a fused-silica capillary column (16 m×0.2 mm I.D.) with cross-linked SE-30 as stationary phase. Helium, at a flow-rate of 0.9 ml/min, served as carrier gas. The temperature of the injector was 280°C and that of the detector 290°C; the temperature of the column was programmed (starting 1 min after injection) from 120 to 260°C at 15°C/min. The split valve was opened 1 min after injection.

The second instrument, equipped with a ^{63}Ni (10 mCi) electron-capture detector operating in the frequency-pulsed mode, had an on-column injector and a fused-silica capillary column (25 m×0.32 mm I.D.) with cross-linked SE-30. The helium carrier gas and the argon–methane (95:5) make-up gas were used at flow-rates of 1.7 and 50 ml/min, respectively. The temperature of the detector was 300°C in most experiments. The column temperature was initially 80°C but was increased directly after injection to 110°C and then programmed to 260°C at 10°C/min.

A Hewlett-Packard Model 3390A electronic integrator (Avondale, PA, U.S.A.) was used for the evaluations. The fatty acids of LPS were identified by retention time comparisons with the bacterial fatty acid standard mixture.

Mass spectrometry

A Ribermag R10-10c quadrupole GC–MS data acquisition system (Rueil-Malmaison, France) was used. The gas chromatograph was a Carlo Erba Model 4160, equipped with a fused-silica capillary column (25 m×0.2 mm I.D.) with cross-linked SE-54 as stationary phase and an all-glass splitless injector. Helium, at an inlet pressure of 0.8 kg/cm^3, served as carrier gas. The temperature of the injector was 270°C and that of the interface between the chromatograph and the spectrometer was 290°C. The initial column temperature was 120°C; after 1 min the temperature was increased to 260°C at 10°C/min. The split valve was opened 1 min after injection. The methane reagent gas in CI at 0.07 Torr (purity >99.95%) was ionized with electrons at an energy of 93 eV, and the ion-source temperature was 80°C (sometimes increased to 200°C). The manual integration facility in the standard software of the spectrometer system was used for peak integration.

TABLE I

RELATIVE RETENTION TIMES OF DIFFERENT ESTER DERIVATIVES OF 3-HYDROXY-MYRISTIC ACID, $CH_3(CH_2)_{10}CHORCH_2CO_2R'$

Determined on a fused-silica capillary column (16 m × 0.2 mm I.D.) immobilized phase SE-30.

Derivative★	R	R'	Relative retention time
3-O-HFBO-methyl	$CF_3CF_2CF_2CO$—	CH_3—	1.00
3-OH-methyl	H—	CH_3—	1.08
3-O-HFBO-TCE	$CF_3CF_2CF_2CO$—	CCl_3CH_2—	1.45
3-O-HFBO-PFB	$CF_3CF_2CF_2CO$—	$C_6F_5CH_2$—	1.51
3-O-TCAO-methyl	CCl_3CO—	CH_3—	1.60
3-O-PFBO-methyl	C_6F_5CO—	CH_3—	1.65
3-OH-PFB	H—	$C_6F_5CH_2$—	1.72

★Abbreviations: 3-O-HFBO-methyl = 3-O-heptafluorobutyryl-methyl ester; 3-OH-methyl = 3-hydroxy-methyl ester; 3-O-HFBO-TCE = 3-O-heptafluorobutyryl-trichloroethyl ester; 3-O-HFB-PFB = 3-O-heptafluorobutyryl-pentafluorobenzyl ester; 3-O-TCAO-metyl = 3-O-trichloroacetyl-methyl ester; 3-O-PFBO-methyl = 3-O-pentafluorobenzoyl-methyl ester; 3-OH-PFB = 3-hydroxy-pentafluorobenzyl ester.

RESULTS AND DISCUSSION

Gas chromatography

The relative retention times of the different derivatives are summarized in Table I. HFB acylation of the hydroxyl group resulted in a decreased retention time for the methyl and PFB esters. Notably, the retention time of the PFB ester was only ca. 1.7 times that of the methyl ester, despite the considerable difference in molecular weights.

Derivatization methods

The yields of the different derivatization methods are given in Table II.

3-O-HFBO-methyl ester. The 3-O-HFBO-methyl ester derivatization method was a modification of the procedure used by Sud and Feingold [21]. The alternative procedure, using pyridine as an acid scavenger, led to a lower yield; whether a longer reaction time would have improved the result was not investigated. The phosphate buffer wash was very efficient in removing excess HFBA and any interfering by-products. Halogenated anhydrides other than HFBA, e.g. trifluoroacetic and pentafluoropropionic anhydride, were not investigated because of the lower sensitivity of the electron-capture detector to the corresponding derivatives compared with HFBA [22].

3-O-PFBO-methyl ester. Use of TEA instead of pyridine in the room temperature preparation of the 3-O-PFBO-methyl ester resulted in a somewhat higher yield, but also in the formation of several by-products. A quantitative yield was achieved when the reaction mixture was heated with pyridine; the yield was lower with TEA. A low yield was also obtained in the absence of an acid-scavenger (method II), even when the reaction time was up to 4 h.

3-O-TCAO-methyl ester. The 3-O-TCAO-methyl ester was unstable and

TABLE II

YIELDS OF INVESTIGATED DERIVATIZATION METHODS FOR 3-HYDROXYMYRISTIC ACID

Average of six samples (each representing 5 μg of 3-OH 14:0), with standard deviations indicated.

Derivative*	Method	Variation	Yield (%)
3-O-HFBO-methyl			90±4
		Pyridine, room temperature	82±2
3-O-PFBO-methyl	I		93±3
		TEA	84±4
		Room temperature	83±2
		TEA, room temperature	84±2
	II		65±5
3-O-TCAO-methyl			50±6
3-OH-PFB	I		88±3
	II		55±2
3-O-HFBO-PFB	I		81±4
	II		77±2
	II	Pyridine	35±2
	II	Pyridine, 80°C	72±4
	II	HFBI	55±2
	III		82±3
3-O-HFBO-TCE	I		76±6
	I	Chloroform	71±3
	II		25±3

*For abbreviations see Table I.

exhibited a considerably lower sensitivity than the 3-O-PFBO-methyl ester in ECD (see below).

3-OH-PFB ester. Use of extractive alkylation in the preparation of PFB esters was described by Greving et al. [23] and Gyllenhaal et al. [24]. This method (method I) resulted in a higher yield than did room temperature esterification (method II) [15]. Furthermore, method I gave no by-products since an excess of reagent was easily removed by evaporation, whereas method II gave several extra peaks detected by both FID and ECD.

3-O-HFBO-PFB ester. When (the time-consuming) method I was used to prepare 3-O-HFBO-PFB esters, slight dehydration occurred, leading to formation of ca. 3% of the α,β-unsaturated tetradecenoic PFB ester. No additional by-products were found. The more rapid room temperature method [25] (method II) gave much larger amounts (more than 15%) of the dehydration product and, in addition, several by-products (notably, no dehydration was observed when preparing 3-O-HFBO-TCE or 3-O-HFBO-methyl esters). With HFBA acting as a catalyst, PFB esters of the non-hydroxy fatty acids were formed quantitatively; thus, this method represents an improvement of the PFB esterification method II. Use of HFBI instead of HFBA resulted in lower yield and did not decrease the level of dehydration. The use of pyridine instead of TEA resulted in cleaner samples and less dehydration (ca. 7%), but also in a lower yield. The highest yield was obtained with method III (dehydration ca. 7%).

The use of pyridine and TEA led to both browning reactions and extraneous peaks in the chromatograms. Similar results have been previously reported [26]. The buffer extraction step efficiently made the organic phase colourless and removed the extra peaks when pyridine was used; this was not the case for TEA, however.

3-O-HFBO-TCE ester. The method developed by Alley et al. [20] for preparing TCE esters of carboxylic acids using HFBA as a catalyst can also be used for simultaneous esterification and acylation of hydroxy acids. We found that the yields of both unsubstituted and hydroxy acids were poor when the reaction was performed at room temperature. A much higher yield was obtained when the reaction mixture was heated to 100°C, and hexane rather than chloroform was used as solvent.

With the exception of the 3-O-TCAO-methyl ester, which completely decomposed after two weeks of storage, all investigated derivatives remained stable at room temperature for several months.

Halogen-containing silyl derivatives, e.g. flophemesyl and *tert.*-buflophemesyl, were not studied owing to their limited hydrolytic stability [27]. Furthermore, the 3-OH-TCE ester derivative was not included since preliminary results showed that the responses of both the GC–ECD and GC–NICI-MS–SIM detection modes were lower to TCE esters of fatty acids than to the corresponding PFB esters.

Sample clean-up

When using the TLC procedure for removal of excess of reagents the recovery of the 3-OH-PFB ester was $70 \pm 10\%$ and recoveries of the 3-O-HFBO-PFB and 3-O-PFBO-methyl esters were $75 \pm 5\%$. The 3-OH-PFB esters separated well from the unsubstituted PFB fatty acids. Such class separation between the acylated esters was also achieved when using *p*-xylene as mobile phase.

The silica columns were less tedious to use than TLC. Recovery of the 3-O-PFBO-methyl ester was practically quantitative.

Mass spectra

The NICI mass spectra are shown in Fig. 1. Only the 3-O-PFBO-methyl esters gave the molecular radical ion $[M]^-$ as the base peak. The 3-OH-PFB esters lost the PFB radical, producing ions of *m/z* 243 $[M-H_2CC_6F_5]^-$ to form the base peak. The second most abundant ion, *m/z* 225 $[M-(H_2CC_6F_5+18)]^-$, indicated the additional loss of a molecule of water. The most abundant peaks produced by the 3-O-TCAO-methyl esters, i.e. *m/z* 161 $[CCl_3CO_2]^-$ and *m/z* 127 $[CCl_3CO_2-Cl]^-$, derived from the derivative as indicated by the characteristic pattern of ions with chlorine isotopic clusters. However, peaks were also derived from the molecule itself through loss of the TCA carboxylate ion plus elimination of one proton producing a molecule-specific radical ion *m/z* 240 $[M-O_2CCCl_3-H]^-$. As previously reported [28], the 3-O-HFBO-methyl esters produced ions only from the heptafluorobutyryl group, *m/z* 213 $[CF_3F_2F_2CO_2]^-$, *m/z* 214 $[CF_3F_2F_2CO_2+H]^-$ and a radical ion resulting from a cleavage involving loss of an additional fluorine atom at *m/z* 194 $[CF_3F_2F_2CO_2-F]^-$ as major peaks. The same types of ion were observed for the 3-O-HFBO-TCE and 3-O-

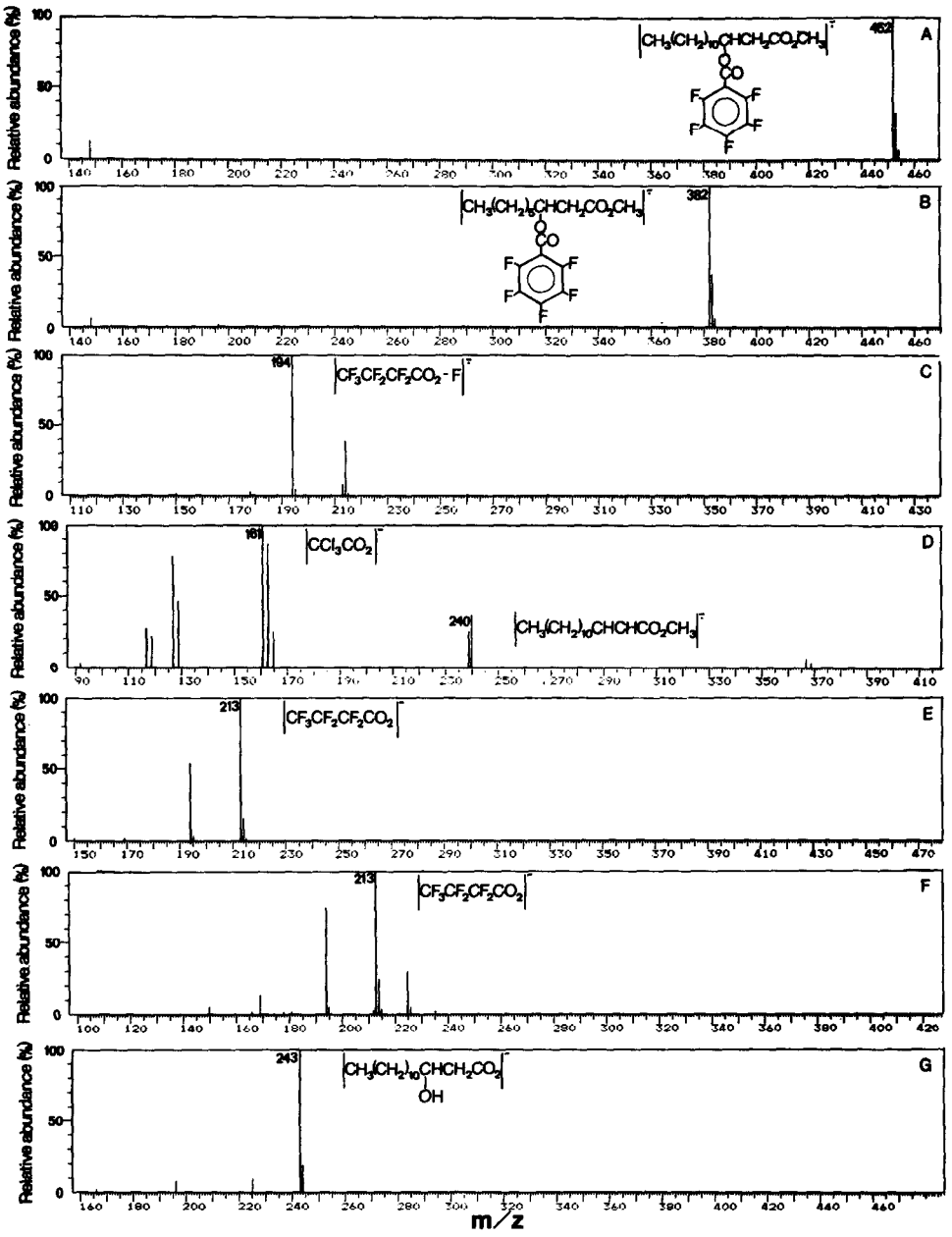

Fig. 1. NICI (methane) mass spectra of different halogenated derivatives; (A) 3-PFBO-methyl 14:0; (B) 3-PFBO-methyl 9:0; (C) 3-HFBO-methyl 14:0; (D) 3-TCAO-methyl 14:0; (E) 3-HFBO-TCE 14:0; (F) 3-HFBO-PFB 14:0; (G) 3-OH-PFB 14:0.

HFBO-PFB esters. However, the latter derivative also produced a molecule-specific ion, m/z 225, also obtained from the 3-OH-PFB esters. The other two HFB-containing derivatives produced no ions specific for the hydroxy acid.

The formation of negative ions in the CI mode for the β-hydroxy acid deriva-

TABLE III

RELATIVE SENSITIVITIES OF GC-ECD AND GC-NICI-MS-SIM FOR THE DERIVATIVES OF 3-HYDROXYMYRISTIC ACID

The ion-source temperature was 80°C for all NICI-MS-SIM measurements except for 3-O-TCAO-methyl 14:0, where it was 200°C. The temperature of the ECD was 300°C. Sample amounts were in the 50–300 pg range.

Shorthand designation of derivatives*	Molecular weight of derivative	Monitored ion in NICI-MS-SIM	*m/z*	Relative abundance	Relative sensitivity	
					ECD	NICI-MS-SIM
3-O-PFBO-methyl 14:0	452	$[CH_3(CH_2)_{10}CHO(C_7F_5O_2)CH_2CO_2CH_3]^-$	452	100	1.0	1.0
3-O-PFBO-methyl 9:0	382	$[CH_3(CH_2)_5CHO(C_7F_5O_2)CH_2CO_2CH_3]^-$	382	100	1.7	2.5
3-O-HFBO-methyl 14:0	454	$[CF_3CF_2CF_2COO-F]^-$	194	100	0.1	1.0
3-O-TCAO-methyl 14:0	404	$[CH_3(CH_2)_{10}CHCHCO_2CH_3]^-$	240	36	0.4	<0.1
3-OH-PFB 14:0	424	$[CH_3(CH_2)_{10}CHOHCH_2COO]^-$	243	100	0.8	0.8
3-O-HFBO-TCE 14:0	572	$[CF_3CF_2CF_2COO]^-$	213	100	0.8	0.5
3-O-HFBO-PFB 14:0	620	$[CF_3CF_2CF_2COO]^-$	213	100	2.0	2.0

*For abbreviations see Table I.

tives is highly dependent on the temperature of the ion source [25]. This dependence was reflected in the differences in the relative abundance of the fragments of the 3-O-TCAO-methyl ester at different temperatures. Increasing the temperature of the ion source from 80 to 200°C favoured the production of the radical ion *m/z* 240, resulting in an increase in the corresponding signal from 11 to 36% of the base peak. For the 3-OH-PFB esters a corresponding temperature increase favoured the loss of a water moleule in addition to the loss of the PFB radical.

Sensitivity and linearity

The relative responses of the GC–ECD and GC–NICI-MS–SIM systems to the various derivatives are given in Table III. All monitored ions formed the base peaks except for the 3-O-TCAO-methyl ester for which a molecule-specific ion (*m/z* 240) was used. The relative abundance of this ion was only 36% of the base peak and the sensitivity achieved was correspondingly lower. The sensitivity of both detection systems was highest for the 3-O-HFBO-PFB ester derivative. The addition of an HFB group to 3-OH-PFB 14:0 was found to lower the detection limit by a factor of ca. 2.5.

The 3-O-HFBO-methyl ester and 3-O-PFBO-methyl ester had similar detection limits in the NICI-MS–SIM system, but ECD was nine times more sensitive to the latter. The ion monitored for the 3-O-HFBO-TCE ester was the same as that monitored for the 3-O-HFBO-PFB ester but the former derivative had four times higher detection limit.

The response of the 3-O-PFBO-methyl 9:0 to NICI-MS–SIM was 2.5 times greater than that of the 3-O-PFBO-methyl 14:0; the corresponding difference for the ECD system was 1.7. We have previously noticed a considerably higher response for 3-O-HFBO butyric acid PFB ester than for 3-O-HFBO 14:0 PFB ester [25].

The ECD response often depends on the temperature [29]. The HFBO-TCE, HFBO-PFB and PFBO-methyl esters of 3-OH 9:0 and 3-OH 14:0 all showed maximum sensitivity at a detector temperature of 300°C (the temperature range investigated was 200–300°C). A high detector temperature is also advantageous when working with biological samples in order to minimize detector contamination [18].

Of the different derivatives investigated, the 3-O-PFBO-methyl ester turned out to be the most attractive for the following three reasons:

(1) The molecular ions produced in NICI-MS form the base peak, thus providing information on molecular weight.

(2) Sensitivity is excellent, corresponding to a detection limit of ca. 0.5 pg when using ECD and 0.3 pg when using NICI-MS–SIM, at a signal-to-noise ratio of 3:1.

(3) Owing to the selectivity of ECD for halogenated compounds, only derivatized hydroxy acids are detected. This is of great advantage since the presence of unsubstituted long-chain fatty acids in natural samples as well as in the chemicals used for sample preparation can be a major problem in trace analyses [23,24].

Dose–response curves for 3-O-PFB-methyl 14:0 were constructed for ranges of 1–100 and 50–2000 pg for the ECD and for the NICI-MS–SIM detection systems, respectively. The equations of the curves were $y=4.0x+3.8$ (ECD) and $y=0.5x+36$ (NICI-MS–SIM), and the correlation coefficients (r) of both curves were >0.99.

ECD and NICI-MS constitute highly sensitive techniques for the detection of compounds with high electron affinity [29,30]; the latter has been reported to be the more sensitive [30,31]. The GC–NICI-MS technique is also very specific, allowing determination of halogen-containing components in complex mixtures [32]. The electron-capture detector requires post-derivatization clean-up procedures for removal of excess reagents and by-products, etc. The NICI-MS–SIM technique should therefore be regarded as superior to ECD both in terms of selectivity and sensitivity, as shown in Fig. 2A and C.

Analysis of endotoxins in aqueous environments

The 3-OH 14:0 acid in the *E. coli* endotoxin was converted into its 3-O-PFBO-methyl ester (method II) and analysed by SIM. Monitoring was performed at m/z 452.40 for the 3-OH 14:0 and at m/z 382.30 for the 3-OH 9:0 (internal standard). A calibration curve over the range 5–1000 ng was constructed by plotting the peak-area ratio of m/z 452.40 to m/z 382.30 against the amount of endotoxin added. The equation of the curve was $y=3.54\cdot10^{-3}x+75.7\cdot10^{-3}$ and the correlation factor was 0.9996. The detection limit of the endotoxin was found to be ca. 1 ng/ml.

CONCLUSION

This study demonstrates that halogenated esters of β-hydroxymyristic acid are useful as chemical markers for detecting minute amounts of endotoxin. By the reported approach we hope to analyse a variety of environmental and biological samples for endotoxins, i.e. filters from air samples, vaccines and pharmaceutical drugs, transfusion and dialysis solutions, and medical equipment. The approach should also have a great potential for analysing clinical samples, such as sera, provided that suitable steps for sample preparation are developed. Maitra et al. [14] were able to detect 60 pg of the trimethylsilylmethyl ester of 3-OH 14:0 with EI-MS–SIM in human serum spiked with endotoxins. Recently, the same method

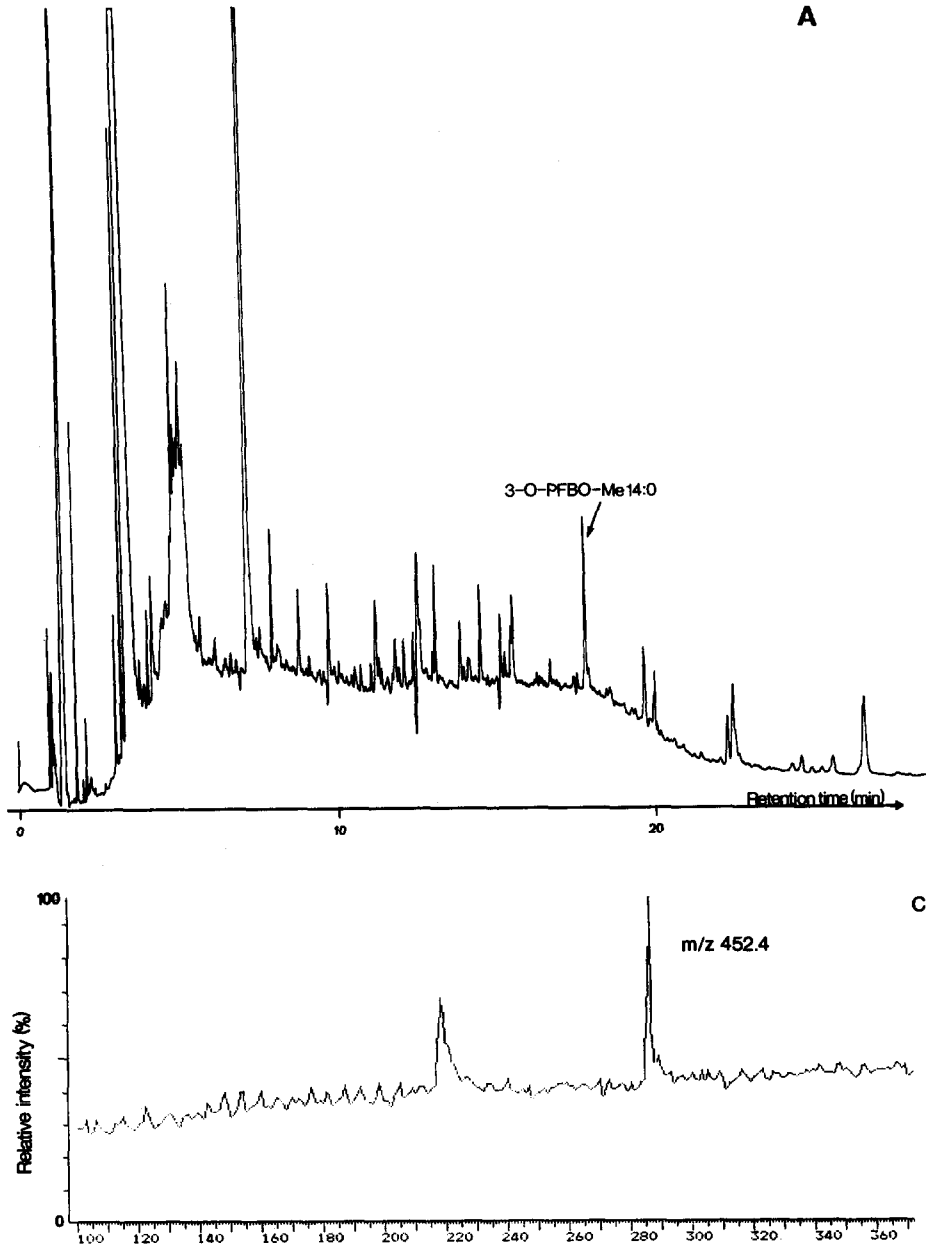
A
3-O-PFBO-Me14:0
Retention time (min)
0
10
20
C
m/z 452.4
Relative intensity (%)
100
0
100
120
140
160
180
200
220
240
260
280
300
320
340
360

Fig. 2.

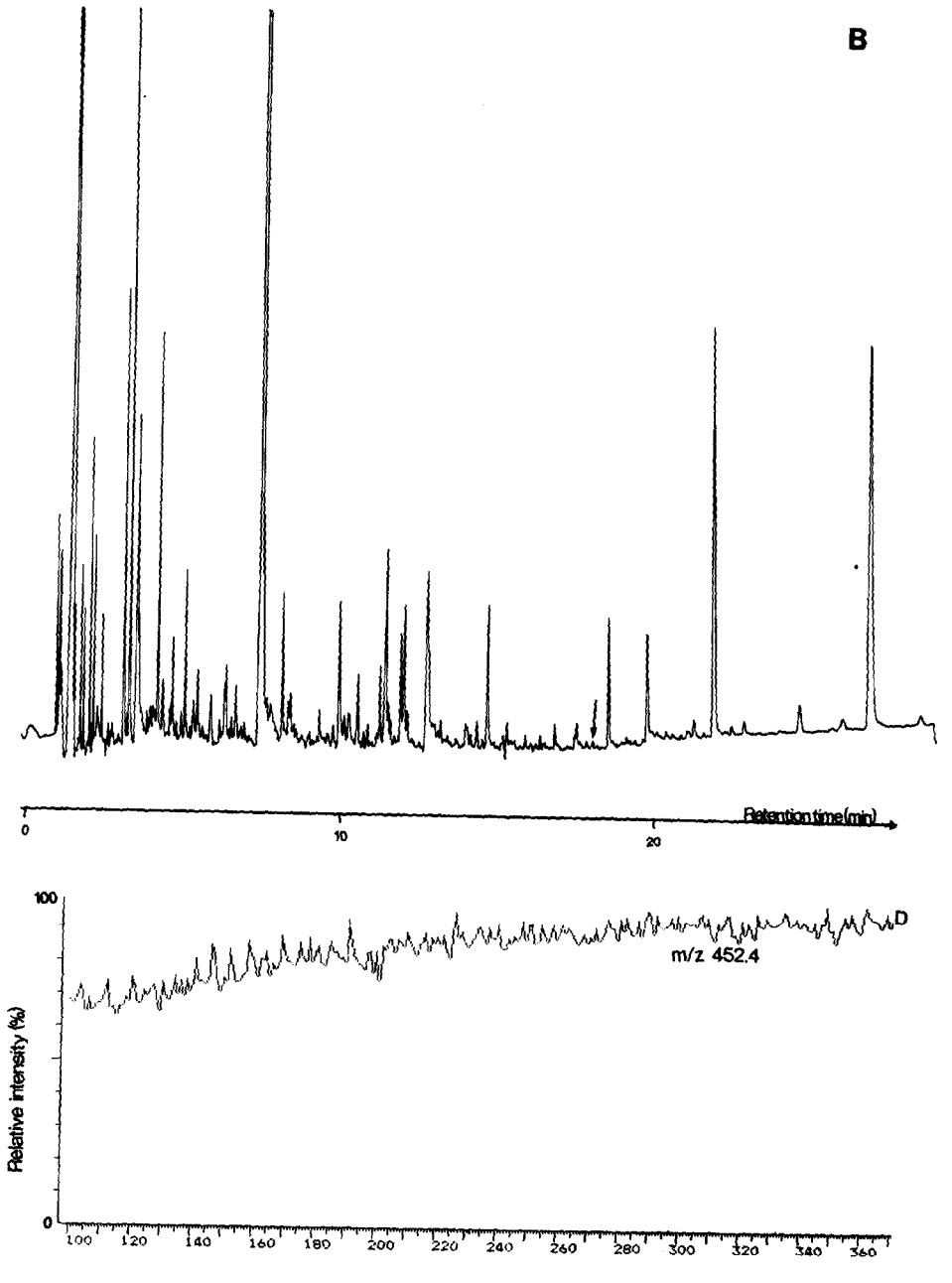

Fig. 2. (A) Gas chromatogram of endotoxin sample after hydrolysis, derivatization and purification as described using ECD. The peak corresponding to 3-O-PFBO-methyl 14:0 (3-O-PFBO-Me 14:0) represents ca. 12 pg. (B) Sterile water sample prepared and analysed as in A . (C) Selected-ion chromatogram using NICI (methane) from the same sample as that used in A, monitored at m/z 452.40; the same amount was injected in the gas chromatograph. (D) The same sample as in B analysed as in C.

was used to detect endotoxins in cerebrospinal fluid samples from patients with meningitis caused by Gram-negative bacteria. The method gave negative results for 3-OH 14:0 in studies on materials claimed to induce non-specific activation of the LAL test [33]. The detection limit was 100 ng of *E. coli* endotoxin, viz. ca. 100 times higher than that of our method utilizing NICI-MS–SIM.

GC analysis of β-hydroxymyristic acid is a direct measurement of endotoxin (and lipid A). The LAL test, on the other hand, measures the biological effect of gelation of LAL. This implies possible non-specific activation or inhibition, and a reduced value for quantitative analysis [7,33]. Accordingly, the chemical method presented here constitutes an interesting alternative technique of high potential for direct quantification of endotoxins in various materials.

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