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GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY OF PHOSPHOLIPID MIXTURES AFTER ENZYMIC HYDROLYSIS

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SUMMARY

Gas–liquid chromatography–mass spectrometry (GLC–MS), employing an open-tubular Silanox-type glass column, has been applied to the products of phospholipase C hydrolysis of natural and synthetic phospholipid mixtures. The materials studied were egg lysolecithin, synthetic L-α-1-stearoyl-2-oleoyl lecithin, bovine brain sphingomyelin, and phospholipids derived from human arterial tissue. 1-Monoglycerides and ceramides were analysed as methaneboronates, and 1,2-diglycerides as trimethylsilyl ethers. The results indicate the potential value of open-tubular GLC–MS in a rapid procedure for the concurrent analysis of the major classes of polar lipids after enzymic dephosphorylation.

INTRODUCTION

Techniques of enzymic hydrolysis are widely applied in the study of phospholipids. Thus, for example, phospholipase A selectively removes the acyl substituent from the 2-position of phosphatidylcholines (*e.g.* ref. 1). Particular value attaches to hydrolysis procedures employing phospholipase C which, by virtue of removal of the phosphorus-containing moiety, afford products amenable (after suitable derivatization) to gas-phase analysis[2,3]. Furthermore, the mild conditions of phospholipase C hydrolysis generally eliminate the problems of artifact formation which may be encountered in comparable chemical procedures[4,5]. The effectiveness of phospholipase C in the hydrolysis of several classes of phospholipids[4] (such as phosphatidylcholines, lysophosphatidylcholines and sphingomyelins) suggests the possibility of a rapid and simple procedure for the preliminary analysis of complex phospholipid mixtures, in which hydrolysis is followed by derivatization and gas–liquid chromatography–mass spectrometry (GLC–MS). Such analyses may indicate the desirability of more detailed studies, as exemplified by the elegant examination of phosphatidylcholines reported by Curstedt and Sjövall[6]. In this paper we report details of the GLC–MS analyses of the products of phospholipase C hydrolysis of several

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phospholipid mixtures, including a total phospholipid fraction derived from human arterial tissue.

EXPERIMENTAL

Materials

Sources of phospholipids, enzyme and reagents were as follows: egg lysolecithin and L-α-1-stearoyl-2-oleoyl lecithin (Applied Science Labs./Field Instruments, Richmond, Great Britain); bovine brain sphingomyelin (Calbiochem, San Diego, Calif., U.S.A.); phospholipase C, from *Clostridium welchii (perfringens)* (Sigma London, Kingston-upon-Thames, Great Britain); methaneboronic acid (Alfa Inorganics, Ventron-Hicol, Rotterdam, The Netherlands); hexamethyldisilazane and trimethylchlorosilane (Pierce and Warriner, Chester, Great Britain).

Phospholipase C hydrolysis[4,6,7]

Each phospholipase hydrolysis employed a solution of *ca.* 50 μg of phospholipase C dissolved in 1 ml of a 0.05 *M* Tris buffer (pH 7.3) containing 0.03 *M* calcium chloride. The enzyme solution was twice extracted with diethyl ether (2 ml) before mixing with a solution of the phospholipid (1–15 mg) in diethyl ether (1 ml). The hydrolysis mixture was subjected to thorough vortex mixing and allowed to stand at room temperature for 3 h, with occasional further mixing. The ether layer was removed and the aqueous layer twice extracted with diethyl ether (2 ml). The combined extracts were evaporated to dryness under nitrogen, redissolved in ethyl acetate and washed with water. The total products of each phospholipase C hydrolysis were analysed by micro thin-layer chromatography (TLC) (chloroform–ethyl acetate, 2:1). Such an analysis of the reaction products of pure phosphatidylethanolamine (Koch-Light, Colnbrook, Great Britain) indicated that phospholipase C was ineffective in this instance. (Phosphatidylethanolamine has, however, been reported to be hydrolysed by phospholipase C when other phospholipids are present[4].)

Preparation of derivatives

Products of phospholipase C hydrolysis of lysolecithin and sphingomyelin were converted to methaneboronate derivatives by solution in ethyl acetate and addition of methaneboronic acid (1.5 molar proportion)[8,9]. The reaction mixture was allowed to stand at room temperature for 30 min before GLC and GLC–MS analyses.

Products of phospholipase C hydrolysis of lecithin were dissolved in pyridine–hexamethyldisilazane–trimethylchlorosilane (5:4:3, v/v/v; 100 μl). Reaction at room temperature for 30 min afforded the trimethylsilyl (TMS) ethers[6]. Solvent was removed under nitrogen and the products were dissolved in ethyl acetate.

Phospholipid constituents of human arterial tissue

Severely atherosclerotic human arterial tissue, from a 54-year-old woman, was obtained within 24 h *post mortem*. The intima was removed and washed thoroughly with saline solution. Total lipids (2.49 g) were obtained by chloroform–methanol (2:1) extraction and were separated on a silica gel column. A phospholipid fraction (130 mg) was obtained by elution with methanol after elution of neutral lipids with

chloroform. Micro-TLC (chloroform–ethyl acetate, 2:1) of an aliquot of the phospholipid fraction indicated that major components remained at the origin with only minor amounts of more mobile material. An aliquot (10%) of the phospholipid fraction was subjected to phospholipase C hydrolysis as described above, except that reaction was continued in this instance for 15 h. Micro-TLC, using two different solvent systems (chloroform–ethyl acetate, 2:1, chloroform–methanol, 95:5) of a portion of the products indicated the major products to be ceramides, with lesser amounts of diglycerides. Only small amounts of polar material remained. An aliquot (40%) of the hydrolysis products was dissolved in ethyl acetate and to this was added a solution (70 μl; 15 mg/ml) of methaneboronic acid in ethyl acetate. After standing at room temperature for 30 min the solvent was removed under nitrogen and the mixture trimethylsilylated as described above.

Gas–liquid chromatography

Prior to GLC–MS, derivatized products of phospholipase C hydrolysis were analysed by GLC using a glass column (1 m × 3.5 mm, I.D.) packed with 1% OV-1 on Gas-Chrom Q (100–120 mesh) with nitrogen (40 ml/min) as carrier gas. Analyses of monoglyceride methaneboronates were performed at 230° and of ceramide methaneboronates and diglyceride TMS ethers at 300°.

Gas–liquid chromatography–mass spectrometry

GLC–MS, employing an open-tubular Silanox-type glass column (30 m × 0.5 mm, I.D.) coated with OV-1 liquid phase, was carried out using an LKB 9000 instrument. Preparation and coating of the glass spiral (according to the procedures of Desty *et al.*[10] and of German and Horning[11], respectively) were performed as previ-

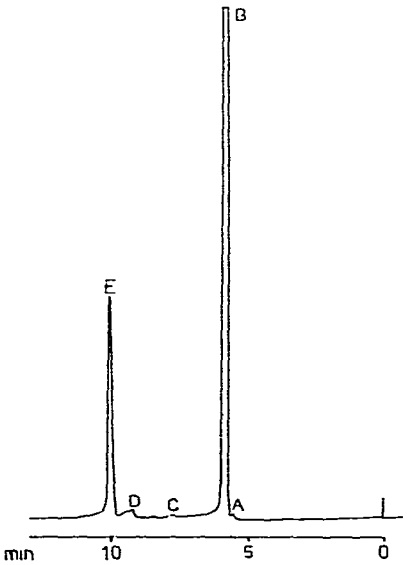

Fig. 1. TIC trace obtained during GLC–MS of the products of phospholipase C hydrolysis of egg lysolecithin, as methaneboronate derivatives. Conditions: open-tubular glass column (30 m × 0.5 mm, I.D.) of OV-1/Silanox at 230°, with a helium flow-rate, at room temperature, of 5 ml/min.

TABLE I

GLC–MS OF THE PRODUCTS OF PHOSPHOLIPASE C HYDROLYSIS OF EGG LYSOLECITHIN, AS METHANEBORONATE DERIVATIVES

*Constituent**	*Assignment*	*Retention index***	*Mass spectrum (electron impact: 20 eV)****												
			$M^{+\cdot}$ (m/e)	*Fragment ions (m/e)*											
				Base peak (a) (m/e)	*b*	*c*	*d*	*e*	*f*	*Other§*					
A	Glyceryl 1-hexadecenoate	2355	352 (3)	98	237 (16)	236 (28)	–	158 (10)	171 (5)	123 (28) 110 (24)	99 (28) 55 (24)	97 (26)	96 (26)		
B	Glyceryl 1-hexadecanoate	2373	354 (2)	98	239 (24)	238 (7)	257 (12)	158 (36)	171 (10)	97 (20)	84 (20)				
C	Glyceryl 1-heptadecanoate	2478	–	98	253 (13)	252 (8)	271 (9)	158 (28)	171 (10)	84 (45)	97 (33)	99 (32)			
D	Glyceryl 1-octadecenoate	2552	380 (9)	98	265 (23)	264 (57)	–	158 (15)	171 (15)	96 (43) 110 (24)	99 (34) 97 (23)	84 (34) 82 (22)	112 (27) 123 (21)	111 (24)	
E	Glyceryl 1-octadecanoate	2572	382 (2)	98	267 (13)	266 (7)	285 (6)	158 (33)	171 (10)	84 (23)					

* See Fig. 1.

** Determined during GLC–MS on a glass open-tubular column (30 m × 0.5 mm, I.D.) of OV-1/Silanox at 230° with helium as carrier gas.

*** Fragment ions, a-f, designated as in Fig. 2; relative intensities in parentheses.

§ Other fragment ions with relative intensities ⩾ 20%.

ously described[8,12]. The LKB 9000 was equipped with a "falling needle"-type dry injector device[13]. Helium was added to the effluent of the column to afford optimum flow-rate (*ca.* 30 ml/min) to the two-stage jet separator. Chromatographic conditions for each analysis are cited in the appropriate figure legend. Mass spectra were recorded at 20 eV, with separator and ion source temperatures of 270°. The LKB 9000 was on-line to a VG 2035 data system; spectra were also obtained by conventional oscillographic recording.

RESULTS AND DISCUSSION

Phospholipase C hydrolysis of egg lysolecithin

Micro-TLC of the products of phospholipase C hydrolysis of egg lysolecithin indicated a single spot corresponding in R_F to 1-monoglycerides. Fig. 1 shows the total ion current (TIC) trace obtained during GLC–MS of the hydrolysis products after preparation of methaneboronate derivatives. GLC–MS data for components A–E are summarized in Table I. Each constituent was identified as a 1-monoglyceride methaneboronate; the mass spectra of 1-monoglyceride methaneboronates are distinguished from those of the 2-isomers by the abundance of *m/e* 99 in the latter[14]. Components B, C and E were identified as saturated acyl glycerides; their spectra compared closely with previously reported spectra of synthetic 1-monoglyceride methaneboronates[14]. No synthetic standards were available for comparison with components A and D. Their assignment as monounsaturated acyl glycerides, however, may be confidently made by interpretation of the mass spectra. The acyl substituent is indicated by fragment ions b and c (Table I, Fig. 2), the latter ion being the more intense in the spectra of the unsaturated acyl glyceride methaneboronates, in contrast with the saturated acyl analogues. The mass spectrum of component D is shown in Fig. 3. The compound eluting on the trailing edge of peak D (Fig. 1) was not identified.

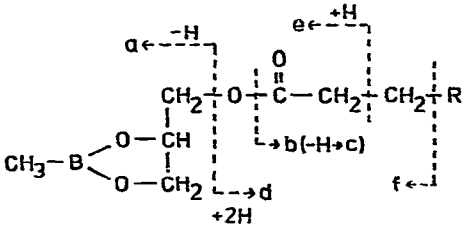

Fig. 2. Fragmentation of 1-monoglyceride methaneboronates under electron impact.

Phospholipase C hydrolysis of bovine brain sphingomyelin

Micro-TLC of the products of phospholipase C hydrolysis of bovine brain sphingomyelin indicated a major component with an R_F value corresponding to those of ceramides, with only traces of unhydrolysed material. The total products were converted to the methaneboronate derivatives and analysed by GLC–MS. The TIC trace is shown in Fig. 4; in this example, analysis time was reduced by increasing the carrier gas flow-rate to *ca.* 10 ml/min [well above the optimum flow-rate (*ca.* 5 ml/min) for the column], with a consequent sacrifice in separation efficiency.

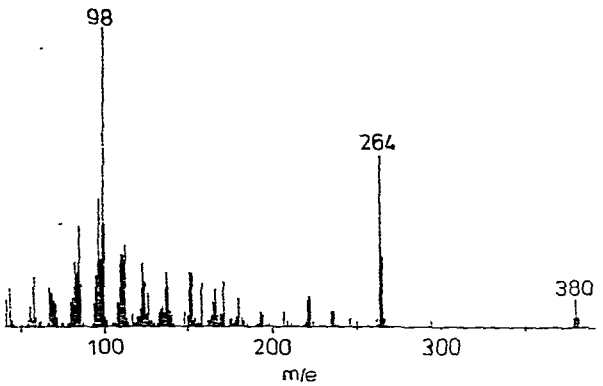

Fig. 3. Electron impact (20 eV) mass spectrum of component D of the products of phospholipase C hydrolysis of egg lysolecithin, analysed as methaneboronate derivatives (Fig. 1). Component identified as glyceryl 1-octadecenoate methaneboronate.

Components corresponding to the peaks F–K were identified on the bases of retention indices and mass spectra[8]. Salient GLC–MS data are recorded in Table II. Peaks F and H were each found to contain two components. Interpretation of the mass spectra of mixtures of ceramide methaneboronates is facilitated by the presence of intense

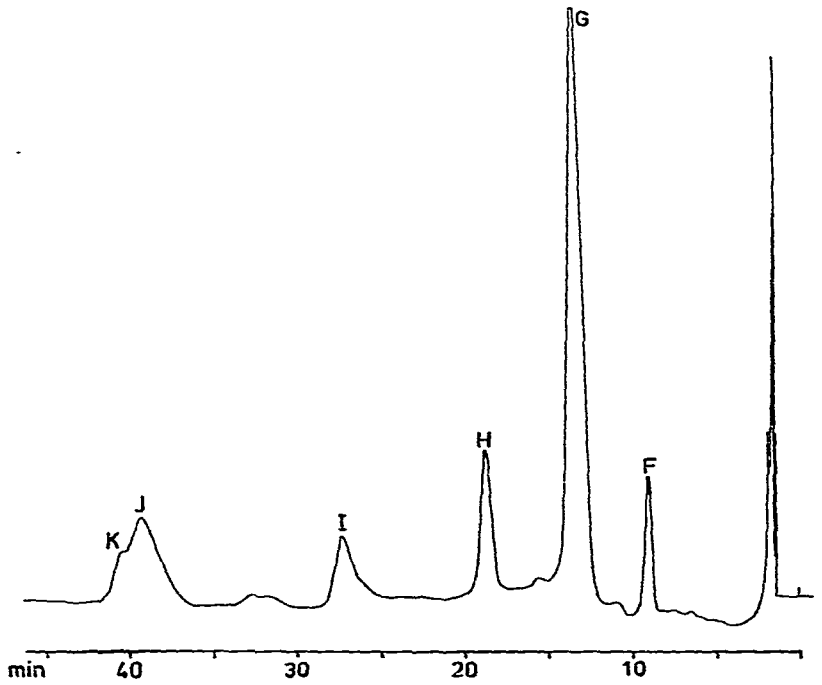

Fig. 4. TIC trace obtained during GLC–MS of the products of phospholipase C hydrolysis of bovine brain sphingomyelin, as methaneboronate derivatives. Conditions: open-tubular glass column (30 m × 0.5 mm, I.D.) of OV-1/Silanox at 300° with a helium flow-rate, at room temperature, of *ca.* 10 ml/min. For assignments of constituents F–K, see Table II.

TABLE II

GLC–MS OF THE PRODUCTS OF PHOSPHOLIPASE C HYDROLYSIS OF BOVINE BRAIN SPHINGOMYELIN, AS METHANEBORONATE DERIVATIVES

*Constituent**	*Assignment***		*Retention index****	*Mass spectrum*§				
	LCB	*acyl*		$M^{+\cdot}$	$[M-15]^{+}$	*g*	*h*	*i*
F_1	18:1	16:0	3909	561 (3)	546 (13)	256 (100)	323 (45)	306 (51)
F_2	16:1	18:0				284 (29)	351 (10)	278 (13)
G	18:1	18:0	4113	589 (2)	574 (7)	284 (100)	351 (35)	306 (46)
H_1	20:1	18:0	4309	617 (3)	602 (7)	284 (100)	351 (35)	334 (39)
H_2	18:1	20:0				312 (18)	379 (4)	306 (12)
I	18:1	22:0	4506	645 (7)	630 (17)	340 (100)	407 (24)	306 (57)
J	18:1	24:1	4688	671 (63)	656 (12)	366 (100)	433 (34)	306 (65)
K	18:1	24:0	4708	673 (11)	658 (15)	368 (100)	435 (34)	306 (48)

* See Fig. 3.

** LCB = constituent long-chain base; acyl = N-acyl substituent. Both are designated as carbon number:degree of unsaturation.

*** Determined during GLC–MS on a glass open-tubular column (30 m × 0.5 mm, I.D.) of OV-1/Silanox at 300° with helium as carrier gas.

§ Ions, cited as: *m/e* (relative intensity), are designated as in Fig. 4.

fragment ions which indicate the long-chain base and N-acyl constituents (Fig. 5)[8,15]. Thus in the spectrum recorded during elution of peak H (Fig. 6), ions at *m/e* 284 and *m/e* 312 are attributable to fragmentation g (Fig. 5) in ceramides containing C_{18} and C_{20} N-acyl substituents, respectively. Ions at *m/e* 351 and *m/e* 379 (fragmentation h, Fig. 5) provide confirmation. The long-chain base constituents of the two ceramide methaneboronates of peak H are indicated as $C_{20:1}$ and $C_{18:1}$ by ions at *m/e* 334 and *m/e* 306, respectively (fragmentation i, Fig. 5).

Phospholipase C hydrolysis of synthetic L-α-*1-stearoyl-2-oleoyl lecithin*

After conversion to the TMS derivative under mild conditions (see Experi-

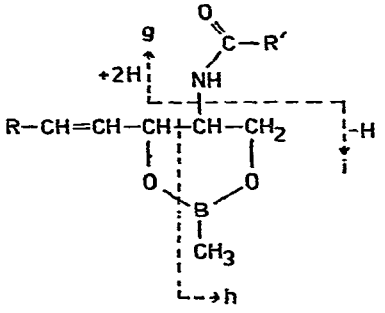

Fig. 5. Fragmentation of ceramide methaneboronates under electron impact.

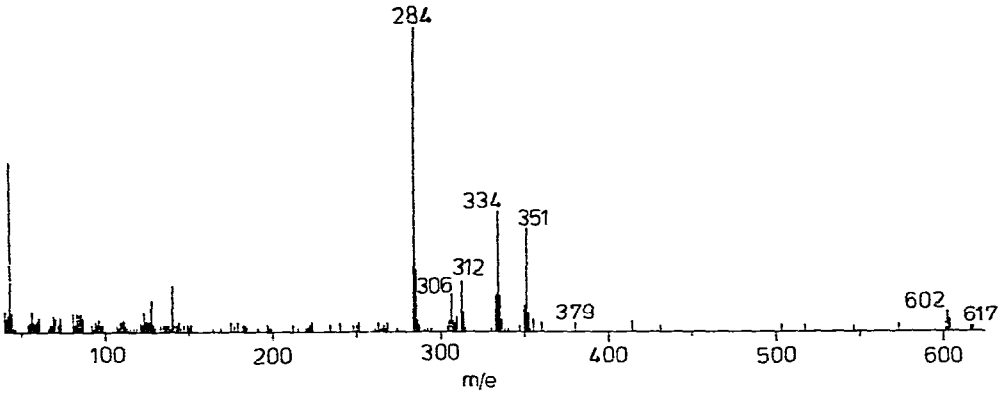

Fig. 6. Electron impact (20 eV) mass spectrum recorded during elution of peak H of the products of phospholipase C hydrolysis of bovine brain sphingomyelin analysed as methaneboronate derivatives (Fig. 4). Spectrum attributed to a mixture of N-dodecanoyl-4-sphingenine methaneboronate and N-octadecanoyl-4-dodecasphingenine methaneboronate.

mental), the product of phospholipase C hydrolysis of L-α-1-stearoyl-2-oleoyl lecithin was analysed by GLC–MS (open-tubular column of OV-1/Silanox at 300°). A single peak was observed with retention index 4113, confirming that isomerization to the 1,3-diglyceride had not occurred during enzymic hydrolysis or derivatization.

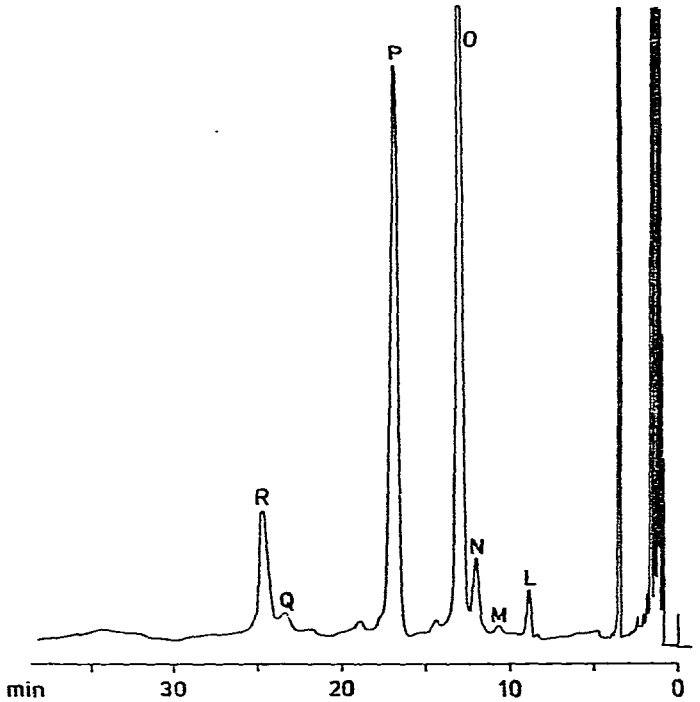

Fig. 7. TIC trace obtained during GLC–MS of the products of phospholipase C hydrolysis of a phospholipid mixture from human arterial tissue as methaneboronate and TMS derivatives. Conditions: open-tubular glass column (30 m × 0.5 mm, I.D.) of OV-1/Silanox at 300°, with a helium flow-rate, at room temperature, of 5 ml/min. For assignments of constituents L–R, see Table III.

The electron impact (20 eV) mass spectrum comprised a base peak of *m/e* 413 ($M-C_{17}H_{33}COO$) and prominent ions of *m/e* 679 (27%; $M-CH_3$), *m/e* 604 (16%; *M*—TMSOH), *m/e* 410 (36%; $M-C_{17}H_{35}COOH$), *m/e* 145 (76%) and *m/e* 129 (56%). The data are consistent with the assignment 1-stearoyl-2-oleoyl glycerol TMS ether[16].

Phospholipase C hydrolysis of a phospholipid mixture derived from human arterial tissue

Micro-TLC of the products of phospholipase C hydrolysis of a phospholipid mixture from human arterial tissue (see Experimental) indicated that ceramides (derived from sphingomyelin) and 1,2-diglycerides (derived from glycerophospholipids) were the major constituents. A TIC trace recorded during GLC–MS of the hydrolysis products, after sequential preparation of methaneboronates and TMS ethers, is shown in Fig. 7. Each of the major constituents was identified, from the mass spectra, as a ceramide methaneboronate or a 1,2-diglyceride TMS ether (Table III). The absence of ceramide TMS ethers indicated that the boronate derivatives were stable to the conditions of trimethylsilylation. A rapid indication of the compound type of each of the major constituents of the mixture was obtained by plotting the appropriate mass chromatogram, calculated from data obtained by repetitive scanning during GLC–MS. Fig. 8 shows mass chromatograms, generated by the GLC–MS data system, for *m/e* 129 and *m/e* 256. The former ion ($[CH_2=CH-CHOSi(CH_3)_3]^+$) appears at high intensity in the spectra of 1,2-diglyceride TMS ethers; the fragment is derived from the glycerol moiety and is consequently inde-

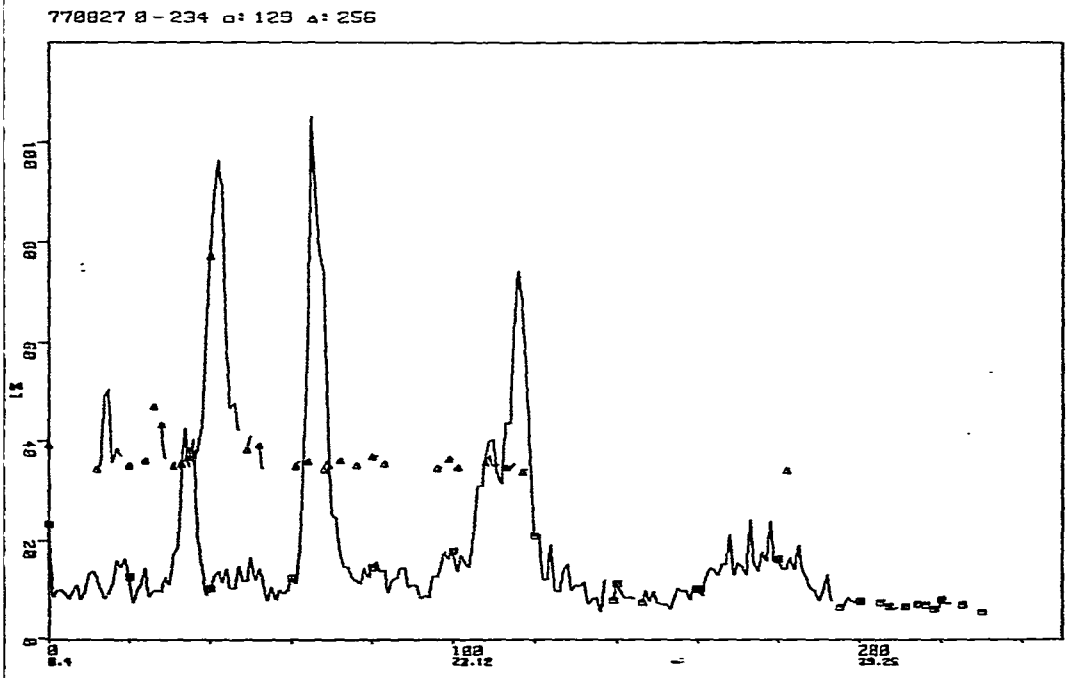

Fig. 8. Mass chromatogram (*m/e* 129, 256) output by the data system following analysis by repetitive scanning GLC–MS of the products of phospholipase C hydrolysis of phospholipids from human arterial tissue as methaneboronate and TMS derivatives. (see Fig. 7).

TABLE III

GLC-MS OF THE PRODUCTS OF PHOSPHOLIPASE C HYDROLYSIS OF PHOSPHOLIPIDS FROM HUMAN ARTERIAL TISSUE, AS METHANEBORONATE AND TMS DERIVATIVES

*Constituent**	*Assignment***	*Mass spectrum****									
		Ceramide methaneboronates					*Diglyceride TMS*				
		$M^{+\cdot}$	$[M-15]^+$	*g*	*h*	*i*	$[M-15]^+$	$[M-RCOO]^+$	$[M-RCOOH]^{+\cdot}$	*m/e* 129	*m/e* 145
L_1	Ceramide (LCB 16:1; 16:0)	533 (4)	518 (5)	256 (100)	323 (53)	278 (47)					
L_2	Ceramide (LCB 18:1; 14:0)			228 (60)	295 (23)	306 (28)					
M	Ceramide (LCB 17:1; 16:0)	547 (2)	532 (4)	256 (100)	323 (48)	292 (47)					
N	Diglyceride (16:0; 16:0)						625 (33)	385 (40)	384 (24)	33	100
O	Ceramide (LCB 18:1; 16:0)	561 (3)	546 (7)	256 (100)	323 (34)	306 (45)					
P_1	Diglyceride (16:0; 18:1)						651 (16)	409 (10)	410 (20)		
P_2	Diglyceride (16:0; 18:2)						649 (14)	385 (100) —	408 (21)	50	47
Q	Diglyceride (18:1; 18:2?)						675 (13)	411 (58)	409 (11) 410 (10)	408 (15) 100	16
R_1	Diglyceride (18:0; 18:2)						677 (12)	409 (13)	408 (25)		
R_2	Diglyceride (18:0; 18:1)						679 (10)	413 (100) 411 (5)	410 (17)	54	38

* See Fig. 7.

** Ceramide structures cited as long-chain base:acyl substituent. Both are designated as carbon number:degree of unsaturation. Diglycerides cited as two acyl substituents, designated as above.

*** Ions listed as *m/e* (relative intensity), except *m/e* 129 and *m/e* 145 where relative intensities are indicated. Fragment ions g, h, i (ceramide methaneboronates) refer to Fig. 5. In diglyceride TMS spectra, RCOO refers to acyloxy substituents.

pendent of the acyl substituents[16]. The ion of *m/e* 256 ($[C_{15}H_{31}CONH_3]^+$) constitutes the base peak in the spectra of ceramide boronates containing N-palmitoyl substituents[8,15]; the chromatogram in Fig. 8 indicates two such components in this mixture.

A detailed examination of full mass spectra obtained during GLC–MS indicated the presence of four ceramide components, two of which were not resolved (peaks L, P and R; Fig. 7; Table III). The present results are consistent with previous observations on ceramides derived from human arterial sphingomyelin[8]. Identification of the diglyceride TMS ether constituents of the mixture was limited by a lack of reference MS data for diglycerides containing two different acyl groups. Moreover, the positions (*sn*-1 and *sn*-2) of the acyl substituents are not readily determined from the mass spectra of diglyceride derivatives. $[M-15]^+$ ions were prominent, with $[M-90]^{+\cdot}$ ions providing confirmatory evidence of the molecular weights. Prominent ions were observed corresponding to loss of acyloxy groups, though in some instances where unsaturated acyl residues were retained in the charged fragment, ions $[M-RCOOH]^{+\cdot}$ were of greater intensity. Spectra recorded during the elution of several peaks indicated unresolved mixtures. The spectrum of peak P, for example (Fig. 9), suggested a mixture of two diglyceride TMS ethers, probably 1-hexadecanoyl-2-octadecadienoyl glycerol TMS and 1-hexadecanoyl-2-octadecenoyl glycerol TMS. An improved gas chromatographic separation is clearly desirable and might be achieved by the use of a thermostable liquid phase (such as a polysulphone) selective for olefinic character.

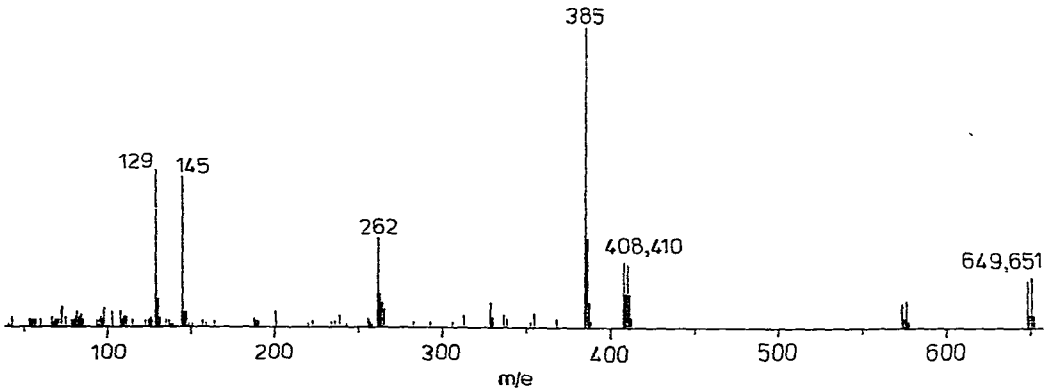

Fig. 9. Electron impact (20 eV) mass spectrum of component P of the products of phospholipase C hydrolysis of phospholipids from human arterial tissue, as derivatives (Fig. 7). Spectrum attributed to a mixture of components of probable structures 1-hexadecanoyl-2-octadecadienoyl glycerol TMS and 1-hexadecanoyl-2-octadecenoyl glycerol TMS.

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