Separation and identification of ceramides derived from human plasma sphingomyelins

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ABSTRACT Sphingomyelins from human blood plasma have been converted into ceramides by enzymatic hydrolysis with phospholipase C. After acetylation the ceramides were fractionated by thin-layer chromatography on silica gel containing silver nitrate. Four main fractions obtained by this method were subsequently converted to di-*O*-trimethylsilyl ether derivatives and separated by gas–liquid chromatography on 1% OV-1. 2–11 components could be distinguished in each of the four fractions. The major fractions emerging from the gas chromatograph were analyzed by mass spectrometry and their main molecular species were identified. Two of the gas chromatographic fractions contained essentially pure molecular species, namely *N*-tetracosenoyl sphingosine and *N*-tetracosenoylsphinga-4,14-dienine.

SUPPLEMENTARY KEY WORDS phospholipase C · ceramide acetates · argentation chromatography · trimethylsilyl ethers · gas–liquid chromatography · mass spectrometry

PREVIOUS STUDIES have shown that sphingomyelins, when subjected to thin-layer chromatography (TLC), give rise to two spots with preferentially long-chain fatty acids in the faster-moving fraction (1–4). Similar fractionations have been observed in chromatography on silicic acid (5). It has also been demonstrated recently that ceramide acetates derived from human serum sphingomyelins can be separated. Three fractions so obtained were analyzed by gas–liquid chromatography (GLC) after hydrolysis and were shown to differ with respect to the fatty acid pattern (6).

Analyses of the long-chain bases (LCB) obtained on hydrolysis of sphingomyelins from human plasma have shown the occurrence of several structurally related compounds (7–11). If each of these bases were combined with each of the fatty acids known to occur in sphingomyelins (12–16) it is evident that a large number of molecular species of ceramides could exist. These considerations indicate that efficient methods are required for the separation and identification of individual molecules of sphingomyelins.

The accompanying paper (17) shows that 1,3-di-*O*-trimethylsilyl ether derivatives of ceramides can be separated by GLC and analyzed by mass spectrometry. The method provides unequivocal evidence for the structures of the LCB and the fatty acids of ceramides. The present report describes the fractionation of ceramide acetates derived from human plasma sphingomyelins by TLC and further separation and identification of the 1,3-di-*O*-trimethylsilyl ether derivatives by GLC–mass spectrometry.

EXPERIMENTAL PROCEDURE

Extraction of Plasma (*18*)

9 ml of human plasma (from heparinized blood, obtained from an apparently healthy 33-yr-old male after an overnight fast) was added dropwise to 135 ml of chloroform–methanol 1:1. The extract was left for 15 min at room temperature, and subsequently filtered. To the filtrate was added 144 ml of redistilled water, and the mixture was stirred vigorously and then centrifuged at 2500 RPM for 15 min. The supernatant solution was discarded and the chloroform layer was evaporated to dryness under reduced pressure.

Abbreviations: LCB, long-chain base(s); TGCU, triglyceride carbon units; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; LCB 18:1–14:0, *N*-myristoyl sphingosine; LCB 16:1–16:0, *N*-palmitoyl hexadecasphing-4-enine; etc. Fatty acids are designated by chain length:number of double bonds.

Isolation of Sphingomyelin (13)

The lipid extract obtained as described above was subjected to mild alkaline methanolysis by the addition of 15 ml of chloroform and 15 ml of 0.6 N sodium hydroxide in methanol. After 1 hr at room temperature 0.78 ml of 12 N hydrochloric acid, 15 ml of chloroform, and 12 ml of water were added. The chloroform layer was evaporated to dryness. The residue was separated on a 2 g silicic acid column (Mallinckrodt 100 mesh, activated at 150°C) by elution first with 150 ml of chloroform–methanol 4:1 and then 150 ml of chloroform–methanol 1:4. The latter fraction, which contained the sphingomyelin, was evaporated to dryness.

Enzymatic Hydrolysis

To the sphingomyelin obtained from 9 ml of plasma was added 1.5 ml of 0.1 M Tris buffer pH 7.4, (0.03 M with respect to $CaCl_2$) (6). After sonication (Branson sonifier model 125) for about 10 sec, 3 mg of phospholipase C from *Clostridium welchii* (Pierce Chemical Corp., Rockford, Ill.) and 1.5 ml of ether was added. After the mixture had been stirred for 1 hr, the ceramides were isolated by extraction twice with 3 ml of ether. The extracts were washed with water and evaporated to dryness.

Separation of Ceramides by TLC

The ceramides were dissolved in 2 ml of dry pyridine and 1.2 ml of acetic anhydride. After 16 hr at room temperature, aqueous pyridine and ice were added and the mixture was taken up in 35 ml of ether. The ether was washed with 2 N hydrochloric acid, water, 5% sodium bicarbonate, and finally with water to neutral reaction. After evaporation to dryness the residue was dissolved in 0.1 ml of chloroform. Thin-layer plates (200 mm × 200 mm) were coated (0.2 mm) with a slurry prepared from 30 g of Silica Gel G (E. Merck A.G., Darmstadt, West Germany) and 2 g of silver nitrate in 60 ml of water by means of the applicator designed by Stahl (C. Desaga, G.m.b.H., Heidelberg, West Germany). The plates were first dried at room temperature for 2 hr and then activated at 120°C for 30 min. The acetates were quantitatively applied as a band to the thin-layer plate, which was developed with chloroform–benzene–methanol 80:20:1. The plates were sprayed with 0.2% 2′,7′-dichlorofluorescein in ethanol. The zones were marked under UV light and scraped off the plate with a razor blade. The acetates were eluted with a solution of 9 ml of 0.6 N sodium hydroxide in methanol and 1 ml of chloroform. After addition of 8 ml of chloroform, the solution was kept at room temperature for 1 hr. This results in methanolysis of the *O*-acetyl esters, but not the amide bond. 18 ml of water was added and the mixture was shaken and centrifuged. The supernatant solution containing the dye was removed, and the chloroform phase was washed once with 5 ml of water and then evaporated to dryness.

GLC

The fractionated ceramides were treated with 100 μl of pyridine, 20 μl of hexamethyl disilazane, and 10 μl of trimethyl chlorosilane (11). After 15 min at room temperature the samples were evaporated to dryness via an oil pump and the residue was dissolved in 0.2 ml of carbon disulfide. An F&M gas chromatograph, model 400 equipped with hydrogen flame ionization detector, was used with a U-shaped glass column (1.2 × 3 mm I.D.) filled with 1% OV-1 (nonpolar silicone phase) on Gas-Chrom Q, 60–80 mesh (Applied Science Laboratories Inc., State College, Pa.). The column was conditioned at 350°C for 24 hr. Helium was used as carrier gas, inlet pressure 3.0 kg/cm². The column temperature was 270°C and the flash heater and detector temperatures were about 300°C.

Mass Spectrometry

Mass spectra were recorded in separate runs on an LKB model 9000 combined gas chromatograph–mass spectrometer. Column and temperatures were equivalent to those given above. The electron energy was 22.3 ev and the trap current, 120 μamp.

The mass spectrometric data given in Tables 2–5 (below) have been corrected by subtracting the isotope peak of the ion having an m/e value two units lower. The mass spectra of the reference ceramides were used for these corrections and the isotope peaks found were to be in good agreement with those calculated on the basis of the natural abundance of isotopes (19). It was found that, in the mass spectra of sphingosine (sphing-4-enine)[1] ceramides, an ion appeared at m/e 309, which interfered with the identification of traces of sphinga-4,14-dienine ceramides (base peak at m/e 309). In Tables 2–5 the contribution of the sphingosine ceramides to the ion at m/e 309 has been subtracted from the observed values.

Reference Ceramides

The ceramides were prepared (details to be published) with either DL-sphinganine or DL-sphingosine obtained from Miles Laboratories, Inc., Elkhart, Ind., and with the fatty acids 16:0, 18:0, 20:0, 22:0, 24:0, *cis*-18:1-(*n*–9), *trans*-18:1(*n*–9), 18:2, and *cis*-24:1(*n*–9) obtained from The Hormel Institute, Lipids Preparation Laboratory, Austin, Minn. (purity >99%) and *trans*-24:1-

[1] The nomenclature for sphingolipid bases is based on recommendations of the Commission on Biochemical Nomenclature of IUPAC and IUB (see 1967 *J. Lipid Res.* **8**: 523).

(*n*–9) (mp 65–66°C) prepared from nervonic acid (20). The ceramides were purified by silicic acid chromatography and the purity was established by TLC and GLC.

RESULTS

The sphingomyelins isolated by silicic acid chromatography after mild alkaline methanolysis of the lipid extract of human plasma were hydrolyzed enzymatically with phospholipase C. We established that the hydrolysis was complete (>98%) by TLC. The ceramides were acetylated in order to improve the separation by TLC on silica gel containing silver nitrate. The reference compounds *N*-stearoyl sphingosine diacetate, *N*-oleoyl sphingosine diacetate, and *N*-linoleoyl sphingosine diacetate had R_f values of 0.75, 0.44, and 0.23, respectively. There was no significant separation between corresponding sphinganine and sphingosine ceramide derivatives (Fig. 1).

In order to test the effect of the chain length of the fatty acids on the thin-layer chromatographic separation, we prepared and analyzed additional ceramide acetates. We found that with saturated fatty acid derivatives the chain length had little influence on the mobility (*N*-lignoceroyl sphingosine diacetate, R_f 0.78, and *N*-stearoyl sphingosine diacetate, R_f 0.75). On the other hand, the mobility of ceramide diacetates containing *cis* monounsaturated fatty acids was greatly influenced by the chain length of the fatty acid (Fig. 2) (*N-cis*-nervonoyl sphingosine diacetate, R_f 0.60, and *N*-oleoyl sphingosine diacetate, R_f 0.44). An intermediate effect of the chain length was observed with *trans* monounsaturated fatty acid derivatives (*N-trans*-nervonoyl sphingosine diacetate, R_f 0.68, and *N*-elaidoyl sphingosine diacetate, R_f 0.60).

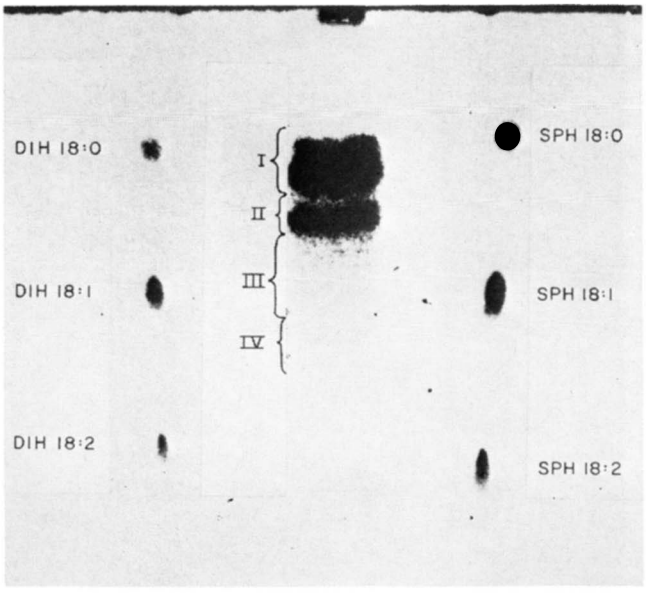


FIG. 1. TLC of acetylated ceramides derived from human plasma sphingomyelins. Adsorbent: Silica Gel G–silver nitrate 15:1. Solvent: chloroform–benzene–methanol 80:20:1. Reference compounds: *N*-stearoyl sphinganine diacetate (DIH 18:0), *N*-oleoyl sphinganine diacetate (DIH 18:1), *N*-linoleoyl sphinganine diacetate (DIH 18:2): *N*-stearoyl sphingosine diacetate (SPH 18:0), *N*-oleoyl sphingosine diacetate (SPH 18:1), and *N*-linoleoyl sphingosine diacetate (SPH 18:2).

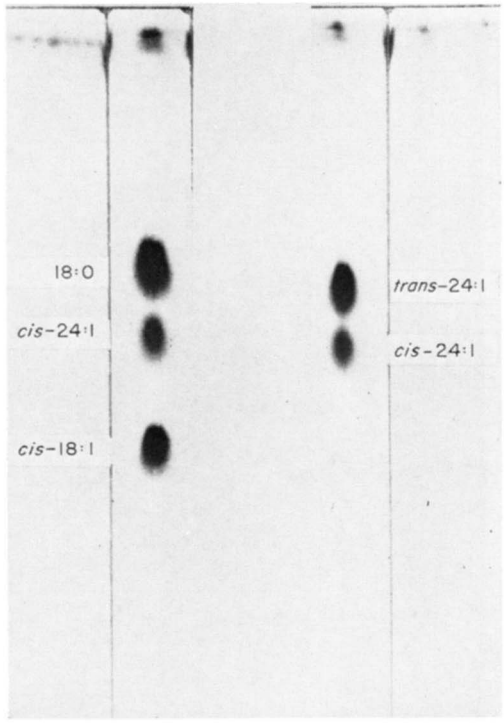


FIG. 2. TLC of diacetates of synthetic ceramides. *N*-stearoyl sphingosine diacetate (18:0), *N*-nervonoyl sphingosine diacetate (*cis*-24:1), *N-trans*-nervonoyl sphingosine diacetate (*trans*-24:1) *N*-oleoyl sphingosine diacetate (*cis*-18:1).

The TLC of the ceramide diacetates derived from human plasma is shown in Fig. 1. For practical purposes the separated material was divided into four fractions. Fraction *I* had a mobility corresponding to that observed for saturated fatty acid ceramide diacetates. Fraction *II* had approximately the same R_f value as *N-cis*-nervonoyl sphingosine diacetate, and fraction *III* appeared between that compound and *N*-oleoyl sphingosine diacetate. Fraction *IV* appeared as a well-defined band slightly below the last reference compounds.

The isolated fractions were subjected to methanolysis of the *O*-acetyl esters and converted into trimethylsilyl ether derivatives. These were further separated by GLC on 1% OV-1 at 270°C. The gas chromatogram of fraction *I* is shown in Fig. 3. At least eleven different peaks could be recognized. Peaks *I:3*, *I:5*, *I:7*, *I:9*, and *I:11* had retention times approximately equal to those obtained for the same derivative of sphingosine ceramides with the fatty acids 16:0, 18:0, 20:0, 22:0, and 24:0, respectively (Table 1).

In a separate run fraction *I* was analyzed by the com-

TABLE 1 RETENTION TIMES FOR DI-*O*-TRIMETHYLSILYL (TMS) ETHER DERIVATIVES OF SYNTHETIC CERAMIDES AND OF CERAMIDES DERIVED FROM PLASMA SPHINGOMYELINS

TMS Derivatives of Synthetic Ceramides		TMS Derivatives of Plasma Sphingomyelin Ceramides							
Molecular Species LCB FA	TGCU	GLC fraction	TGCU	GLC fraction	TGCU	GLC fraction	TGCU	GLC fraction	TGCU
LCB 18:1–14:0*	35.5†	*I:1*	35.4 (±0.2)			—	—		
LCB 18:1–16:0	37.5 (±0.1)‡	*I:3*	37.3 (±0.2)			*III:3*	37.5 (±0.2)		
LCB 18:1–18:0	39.4 (±0.2)	*I:5*	39.4 (±0.1)			*III:5*	39.5 (±0.1)		
LCB 18:1–20:0	41.4 (±0.2)	*I:7*	41.3 (±0.2)	*II:7*	41.3 (±0.1)	*III:7*	41.5 (±0.2)		
LCB 18:1–22:0	43.5 (±0.1)	*I:9*	43.3 (±0.1)	*II:9*	43.3 (±0.1)	*III:9*	43.5 (±0.1)	*IV:9*	43.4 (±0.1)
LCB 18:1–24:0	45.5 (±0.1)	*I:11*	45.3 (±0.1)	*II:11*	45.3 (±0.1)			*IV:11*	45.3 (±0.1)

The retention times have been expressed as triglyceride carbon units (TGCU), i.e. the total number of carbon atoms in a triglyceride with the same retention time. Only those fractions that have been analyzed by mass spectrometry (Table 2–5) have been recorded.

* Unlike the other ceramides listed, synthetic *N*-myristoyl sphingosine was not available.

† Calculated.

‡ SD, five determinations.

bined gas chromatograph–mass spectrometer which gave an equivalent gas chromatogram. Mass spectra were recorded on some of the peaks. The mass spectrometric fragmentation of trimethylsilyl derivatives of synthetically prepared ceramides of both the sphingosine and the sphinganine series is described in the accompanying report (17). These studies showed that some fragments ("LCB fragments") can be used for the identification of the LCB and other fragments ("fatty acid fragments") provide information on the fatty acid residue of ceramides. On the basis of this information and previous studies concerning the nature of the LCB (7–11) and fatty acids (12–16) of human plasma sphingomyelins, pertinent mass spectrometric data have been summarized in Table 2.

The mass spectrum of the gas chromatographic fraction *I:1* showed that the LCB fragments M–*d* appeared at m/e 283 (28%) and at m/e 311 (100%). These ions are due to hexadecasphing-4-enine and sphingosine, respectively (Table 2). The LCB fragments M–(*b* + 1), appearing at m/e 398 (3%) and at m/e 426 (2%), provided additional evidence. The fatty acid fragments M–*a* had m/e values of 342 (12%) and 370 (20%), which showed the presence of the fatty acid residues 14:0 and 16:0. Low intensity ions due to these acids were also observed at m/e 415 and 443 [M–(*a*–73)]. The retention time of *I:1* (TGCU 35.4) was essentially the same as that expected for the same derivative of *N*-myristoyl sphingosine (TGCU 35.5) (Table 1). On the basis of the GLC data and the mass spectrometric analysis we conclude that one of the molecular species is LCB 18:1–14:0.

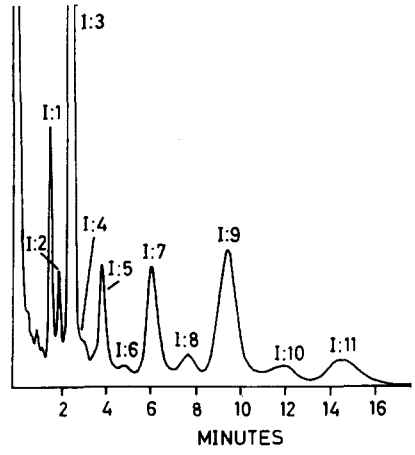


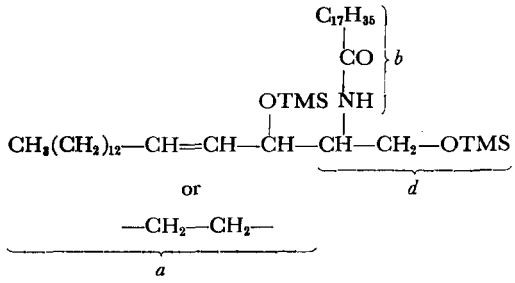
FIG. 3. GLC of ceramides in fraction *I* of Fig. 1. The acetates were hydrolyzed and converted to trimethylsilyl ether derivatives. Column: 1% OV-1. Temperatures: column 275°C, flash heater 300°C, detector 300°C. Carrier gas: helium.

However, the mass spectrometric data also showed the presence of the 16:1 LCB and the fatty acid residue 16:0. Since it is expected that the gas chromatographic separation on the column is primarily determined by the total number of carbon atoms in the ceramides, another constituent in this fraction must be LCB 16:1–16:0. Low intensity ions, which may be due to the LCB sphinganine, hexadecasphinganine, and heptadecasphing-4-enine were also observed. These LCB should be combined with the fatty acids 14:0, 16:0, and 15:0, respectively.

Other gas chromatographic fractions derived from material in TLC fraction *I* were analyzed in a similar

TABLE 2 MASS SPECTROMETRIC DATA FOR DI-*O*-TRIMETHYLSILYL (TMS) ETHER DERIVATIVES OF CERAMIDES FROM TLC FRACTION *I* (FIG. 2)

The designation of mass spectrographic fragments is shown below.



GLC Fraction*	LCB Fragments M–(*b* + 1)		LCB Fragments M–*d*		LCB	Fatty Acid Fragments M–*a*		Fatty Acid Fragments M–(*a*–73)		Fatty Acid	Main Constituent(s)
	m/e	%	*m/e*	%		*m/e*	%	*m/e*	%		
I:1	400	—	285	1	16:0	342	12	415	1	14:0	
	398	3	283	28	16:1	340	—	413	—	14:1	
	414	—	299	—	17:0	356	—	429	—	15:0	LCB 18:1–14:0
	412	—	297	2	17:1	354	1	427	—	15:1	LCB 16:1–16:0
	428	—	313	3	18:0	370	20	443	2	16:0	
	426	2	311	100	18:1	368	3	441	—	16:1	
	424	—	309	1	18:2						
I:3	400	1	285	—	16:0	370	100	443	7	16:0	
	398†	3	283	3	16:1	368	2	441	—	16:1	
	414	—	299	—	17:0	384	—	457	—	17:0	LCB 18:1–16:0
	412	—	297	1	17:1	382	—	455	—	17:1	LCB 16:1–18:0
	428	2	313	2	18:0	398†	3	471	—	18:0	
	426	1	311	89	18:1	396	—	469	—	18:1	
	424	1	309	—	18:2						
I:5	400	7	285	2	16:0	398‡	77	471	6	18:0	
	398‡	77	283	62	16:1	396	3	469	—	18:1	
	414	1	299	2	17:0	412	2	485	—	19:0	LCB 18:1–18:0
	412	1	297	3	17:1	410	—	483	—	19:1	LCB 16:1–20:0
	428	—	313	3	18:0	426‡	47	499	4	20:0	
	426‡	47	311	100	18:1	424‡	2	497	—	20:1	
	424	2	309	5	18:2						
I:7	400	2	285	4	16:0	426§	33	499	3	20:0	
	398	16	283	100	16:1	424§	2	497	—	20:1	
	414	2	299	1	17:0	440	2	513	—	21:0	
	412	4	297	5	17:1	438	5	511	—	21:1	LCB 18:1–20:0
	428	—	313	2	18:0	454	60	527	7	22:0	LCB 16:1–22:0
	426	33	311	93	18:1	452	11	525	2	22:1	LCB 16:1–22:1
	424§	2	309	5	18:2						
I:9	400	1	285	1	16:0	454	54	527	5	22:0	
	398	3	283	8	16:1	452	21	525	4	22:1	
	414	1	299	2	17:0	468	1	541	—	23:0	LCB 18:1–22:0
	412	3	297	4	17:1	466	1	539	—	23:1	LCB 18:1–22:1
	428	—	313	—	18:0	482	5	555	1	24:0	LCB 16:1–24:0
	426	15	311	100	18:1	480	3	553	1	24:1	LCB 16:1–24:1
	424	1	309	2	18:2						
I:11	400	5	285	—	16:0	482	21	555	—	24:0	
	398	—	283	2	16:1	480	33	553	—	24:1	LCB 18:1–24:0
	414	2	299	5	17:0	496	—	579	—	25:0	LCB 18:1–24:1
	412	—	297	5	17:1	494	—	577	—	25:1	
	428	5	313	3	18:0	510	—	583	—	26:0	
	426	14	311	100	18:1	508	—	581	—	26:1	
	424	—	309	2	18:2						

*Designation of GLC fractions corresponds to that used in Fig. 3. Retention times are given in Table 1.
†398: LCB *and* fatty acid fragment.
‡398, 424, 426: LCB *and* fatty acid fragments.
§424 and 426: LCB *and* fatty acid fragments.

manner and the main constituents are given in Table 2. The LCB consisted mainly of sphingosine and hexadecasphing-4-enine combined in each fraction with saturated fatty acids differing in chain length by two carbon atoms. However, the gas chromatographic fractions *I:7*, *I:9*, and *I:11* also contained the fatty acid residues 22:1 and 24:1. In view of the mobility of fraction *I* on TLC (Fig. 1) compared with the reference ceramide LCB 18:1–24:1(*cis*) (Fig. 2) it seems unlikely that these ceramides contain (*n*–9) *cis* monounsaturated fatty acids. Further studies are required to establish the nature of the monounsaturated fatty acids in this fraction.

TLC fraction *II* (Fig. 1) contained mainly ceramides with long-chain fatty acids (for GLC separation see Fig. 4), namely 20:1, 22:1, and 24:1 and the LCB sphingosine and hexadecasphing-4-enine. Mass spectrometric data for the main constituents are given in Table 3. It is worth noting that the main peak (*II:11*) of fraction *II* is almost exclusively due to the molecular species LCB 18:1–24:1. For comparison, the mass spectra of *II:11* and synthetic LCB 18:1–24:1 are given in Fig. 5.

TLC fraction *III* (Fig. 1) was a minor component and consisted of a relatively diffuse band. The GLC separation of this material (Fig. 6) showed several peaks, of which *III:9* predominated; it had the same retention

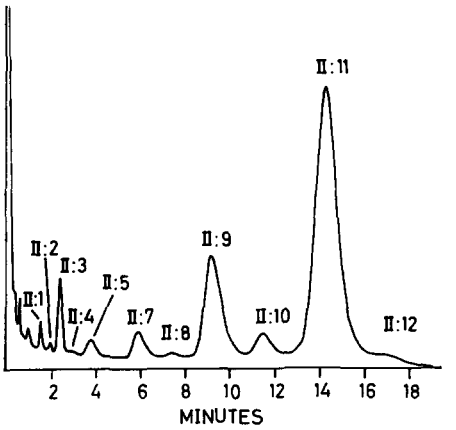


FIG. 4. GLC of ceramides in fraction *II* of Fig. 1. For details see Fig. 3.

time (TGCU 43.5) as that (TGCU 43.5) found for LCB 18:1–22:0 (Table 1). The GLC–mass spectrometric analyses are summarized in Table 4. All the peaks analyzed contained sphinga-4,14-dienine. The finding that TLC fraction *IV* (see below) consisted of sphinga-4,14-dienine ceramides with monounsaturated fatty acids indicates that the sphinga-4,14-dienine ceramides of fraction *III* contain mainly saturated fatty acids. The occurrence in fraction *III* of ceramides with the LCB sphingosine and hexadecasphing-4-enine and monounsaturated fatty acids is also evident from Table 4.

TABLE 3 MASS SPECTROMETRIC DATA FOR DI-*O*-TRIMETHYLSILYL ETHER DERIVATIVES OF CERAMIDES FROM TLC FRACTION *II* (FIG. 2)
Designation of mass spectrographic fragments is shown in Table 2.

GLC Fraction*	LCB Fragments				LCB	Fatty Acid Fragments				Fatty Acid	Main Constituent(s)
	M–(*b* + 1)		M–*d*			M–*a*		M–(*a*–73)			
	m/e	%	*m/e*	%		*m/e*	%	*m/e*	%		
II:7	400	3	285	6	16:0	426†	4	499	—	20:0	
	398	5	283	100	16:1	424†	5	497	—	20:1	LCB 18:1–20:1
	414	—	299	1	17:0	440	—	513	—	21:0	LCB 16:1–22:1
	412	—	297	3	17:1	438	—	511	—	21:1	
	428	—	313	5	18:0	454	3	527	—	22:0	
	426†	4	311	58	18:1	452	20	525	2	22:1	
	424†	5	309	9	18:2						
II:9	400	1	285	—	16:0	454	4	527	—	22:0	
	398	4	283	28	16:1	452	15	525	1	22:1	
	414	—	299	—	17:0	468	—	541	—	23:0	LCB 18:1–22:1
	412	—	297	3	17:1	466	—	539	—	23:1	LCB 16:1–24:1
	428	—	313	1	18:0	482	1	555	—	24:0	
	426	3	311	100	18:1	480	19	553	3	24:1	
	424	—	309	6	18:2						
II:11	400	—	285	—	16:0	482	—	555	—	24:0	
	398	—	283	—	16:1	480	86	553	10	24:1	
	414	—	299	—	17:0	496	—	579	—	25:0	LCB 18:1–24:1
	412	—	297	—	17:1	494	—	577	—	25:1	
	428	—	313	—	18:0	510	—	583	—	26:0	
	426	13	311	100	18:1	508	—	581	—	26:1	
	424	1	309	2	18:2						

* Designation of GLC fractions corresponds to that used in Fig. 4. Retention times are given in Table 1.
† 424 and 426: LCB *and* fatty acid fragments.

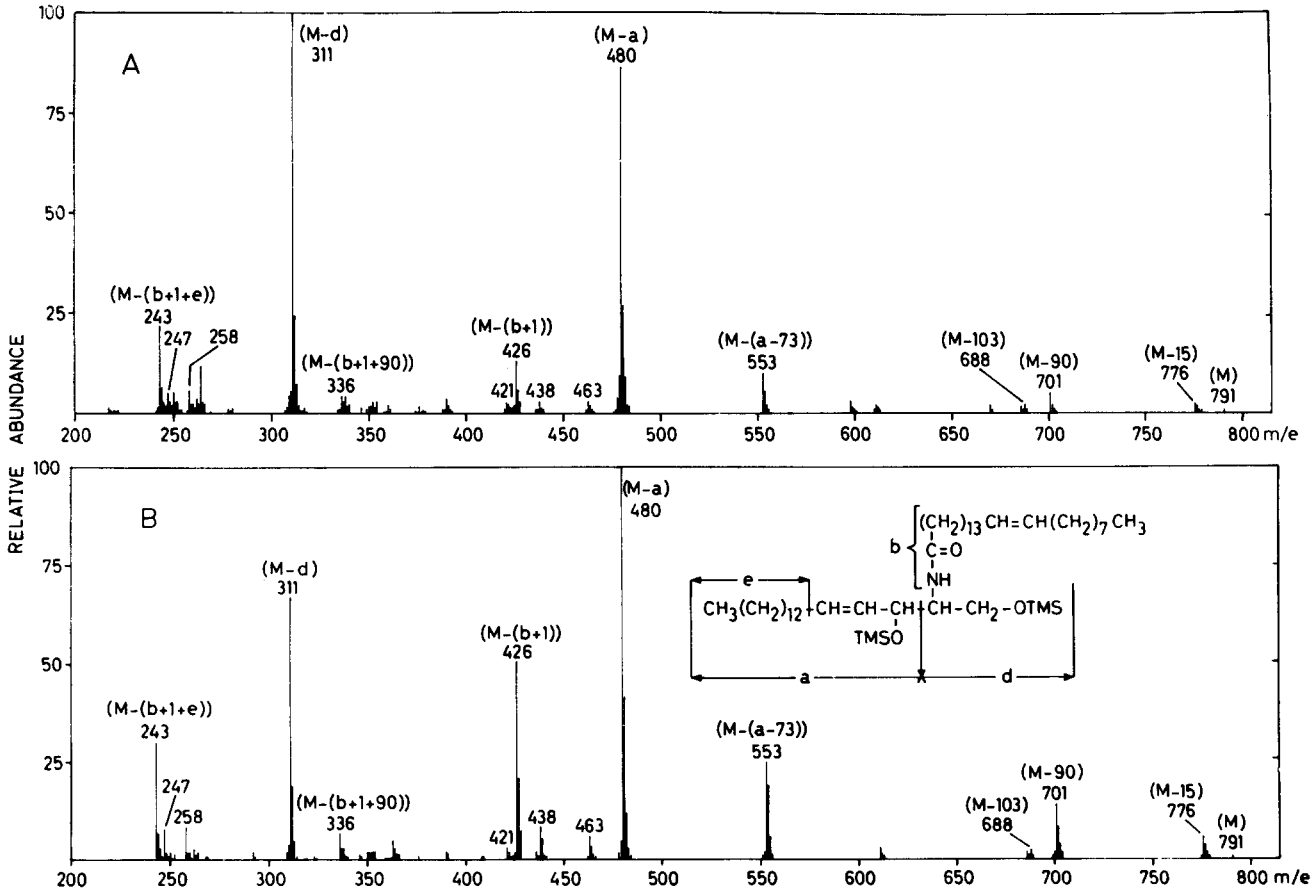


FIG. 5. Mass spectra of di-*O*-trimethylsilyl ether derivatives (*A*) of GLC fraction *II*:11 of Fig. 4 and (B) of *N*-nervonoyl sphingosine synthetic (LCB 18:1–24:1).

Some of these ceramides, i.e., LCB 81:1–22:1 and LCB 18:1–20:1, appear to be contaminants from fraction *II*.

TLC fraction *IV* appeared as a well-defined band which on GLC gave two main peaks (Fig. 7). These were almost exclusively due to the molecular species LCB 18:2–22:1 and LCB 18:2–24:1, respectively (Table 5).

DISCUSSION

This paper describes a preliminary approach to methods for analysis of sphingomyelins with respect to molecular species of derived ceramides. The procedure is based on fractionation of ceramide diacetates according to degree of unsaturation and also to some extent according to chain length. Ceramides are subsequently separated by GLC according to the number of carbon atoms after conversion to di-*O*-trimethylsilyl ether derivatives. Molecular species were identified by GLC–mass spectrometry, which as shown in an accompanying report (17) can be used to identify the components of a ceramide.

In early studies Sweeley and Moscatelli (7) analyzed the LCB of plasma sphingomyelins by periodate oxidation and GLC of the resulting aldehydes. This investiga-

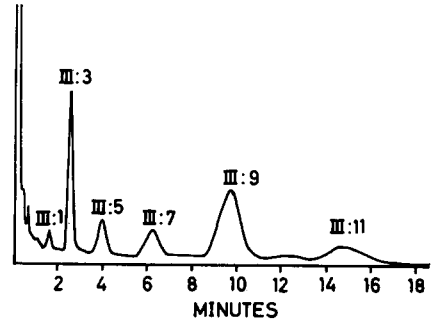


FIG. 6. GLC of ceramides in fraction *III* of Fig. 1. For details see Fig. 3.

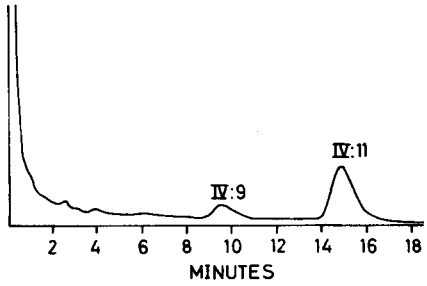


FIG. 7. GLC of ceramides in fraction *IV* of Fig. 1. For details see Fig. 3.

TABLE 4 MASS SPECTROMETRIC DATA FOR DI-*O*-TRIMETHYLSILYL ETHER DERIVATIVES OF CERAMIDES FROM TLC FRACTIONS III (FIG 2).
Designation of mass spectrographic fragments is shown in Table 2.

GLC Fraction*	LCB Fragments					Fatty Acid Fragments					
	M–(*b* + 1)		M–*d*		LCB	M–*a*		M–(*a*–73)		Fatty Acid	Main Constituent(s)
	m/e	%	*m/e*	%		*m/e*	%	*m/e*	%		
III:3	400	—	285	—	16:0	370	74	443	12	16:0	
	398	—	283	3	16:1	368	4	441	1	16:1	
	414	—	299	—	17:0	384	—	457	—	17:0	LCB 18:2–16:0
	412	—	297	—	17:1	382	—	455	—	17:1	LCB 18:1–16:1
	428	1	313	3	18:0	398	—	471	—	18:0	
	426	1	311	13	18:1	396	—	469	—	18:1	
	424	12	309	100	18:2						
III:5	400	—	285	—	16:0	398†	41	471	1	18:0	
	398†	41	283	27	16:1	396	4	469	3	18:1	
	414	—	299	—	17:0	412	—	485	—	19:0	LCB 18:2–18:0
	412	—	297	—	17:1	410	—	483	—	19:1	LCB 18:1–18:1
	428	1	313	3	18:0	426†	1	499	—	20:0	LCB 16:1–20:1
	426†	1	311	13	18:1	424†	10	497	—	20:1	
	424†	10	309	100	18:2						
III:7	400	—	285	—	16:0	426‡	9	499	1	20:0	
	398	—	283	16	16:1	424‡	3	497	—	20:1	
	414	—	299	—	17:0	440	—	513	—	21:0	LCB 18:2–20:0
	412	—	297	1	17:1	438	—	511	—	21:1	LCB 18:1–20:1
	428	—	313	—	18:0	454	—	527	—	22:0	LCB 16:1–22:1
	426‡	9	311	16	18:1	452	2	525	—	22:1	
	424‡	3	309	100	18:2						
III:9	400	1	285	—	16:0	454	45	527	5	22:0	
	398	3	283	12	16:1	452	23	525	3	22:1	
	414	—	299	—	17:0	468	1	541	—	23:0	LCB 18:2–22:0
	412	—	297	1	17:1	466	—	539	—	23:1	LCB 18:1–22:1
	428	—	313	2	18:0	482	1	555	—	24:0	LCB 16:1–24:1
	426	3	311	35	18:1	480	8	553	1	24:1	
	424	13	309	100	18:2						

* Designation of GLC fractions corresponds to that used in Fig. 6. Retention times are given in Table 1.
† 398, 424, and 426; LCB *and* fatty acid fragments.
‡ 424 and 426; LCB *and* fatty acid fragments.

TABLE 5 MASS SPECTROMETRIC DATA FOR DI-*O*-TRIMETHYLSILYL ETHER DERIVATIVES OF CERAMIDES FROM TLC FRACTION *IV* (FIG. 2)
Designation of mass spectrographic fragments is shown in Table 2.

GLC Fraction*	LCB Fragments					Fatty Acid Fragments					
	M–(*b* + 1)		M–*d*		LCB	M–*a*		M–(*a*–73)		Fatty Acid	Main Constituent(s)
	m/e	%	*m/e*	%		*m/e*	%	*m/e*	%		
IV:9	400	1	285	—	16:0	454	—	527	—	22:0	
	398	—	283	3	16:1	452	5	525	—	22:1	
	414	1	299	—	17:0	468	—	541	—	23:0	LCB 18:2–22:1
	412	—	297	2	17:1	466	—	539	—	23:1	
	428	2	313	—	18:0	482	—	555	—	24:0	
	426	—	311	1	18:1	480	—	553	—	24:1	
	424	—	309	100	18:2						
IV:11	400	2	285	1	16:0	482	—	555	—	24:0	
	398	1	283	2	16:1	480	69	553	10	24:1	
	414	1	299	1	17:0	496	—	579	—	25:0	LCB 18:2–24:1
	412	—	297	2	17:1	494	—	577	—	25:1	
	428	1	313	—	18:0	510	—	583	—	26:0	
	426	—	311	1	18:1	508	—	581	—	26:1	
	424	18	309	100	18:2						

* Designation of GLC fractions corresponds to that used in Fig. 7. Retention times are given in Table 1.

tion revealed the presence of a sphingadienine base in addition to sphing-4-enine and sphinganine. Later the sphingadienine was reported to consist of a mixture of sphinga-4,14-dienine and sphinga-4,12-dienine (8). More detailed chemical studies established that the doubly unsaturated base is sphinga-4,14-dienine (9, 10). Bases with shorter chain length, i.e., hexadecasphing-4-enine and heptadecasphing-4-enine (7, 8, 10, 11) were also identified, as well as trace amounts of hexadecasphinganine and heptadecasphinganine (8).

Analyses of fatty acids from human plasma sphingomyelins (12–16) have shown the presence of all even- and odd-carbon saturated fatty acids from C_{10} to C_{26}. Palmitic acid was the predominant component of these acids and 18:0, 20:0, 22:0, 23:0, and 24:0 were present in relatively large amounts. The monoenoic acid 24:1 was one of the major acids, whereas other monoenoic acids were present only in small amounts. Svennerholm and coworkers, however, have found a higher percentage of 22:1 and 24:1 than other investigators (15). Small amounts of polyunsaturated fatty acids have also been reported (12–14, 16).

This investigation resulted in the resolution of ceramides derived from sphingomyelins into four TLC fractions each of which could subsequently be separated by GLC into 2–11 components. TLC fraction *I* contained mainly sphingosine and hexadecasphing-4-enine combined with saturated fatty acids, whereas fraction *II* consisted primarily of the same LCB combined with monounsaturated fatty acids. The main GLC peak of this fraction was almost exclusively due to a single molecular species, *N*-tetracosenoyl sphingosine. Fraction *III*, a minor and rather heterogenous fraction, contained mainly ceramides with sphinga-4,14-dienine as base and saturated fatty acids or sphingosine combined with monounsaturated fatty acids. Fraction *IV* appeared as a well-separated single band and was shown by GLC–mass spectrometry to consist mainly of two components: *N*-docosenoyl sphinga-4,14-dienine and *N*-tetracosenoyl sphinga-4,14-dienine.

The structures of the observed LCB and fatty acids are in agreement with published data on the composition of human plasma sphingomyelins (7–16).

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