Composition of human serum sphingomyelins

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ABSTRACT Serum sphingomyelins were analyzed by argentation chromatography of the corresponding ceramide diacetates. Six subfractions were obtained. Three of them contained 4-sphingenines in combination with saturated, *trans*-, or *cis*-monoenoic fatty acids; the remaining three contained sphingadienine, also in combination with saturated, *trans*-, or *cis*-monoenoic fatty acids. Palmitic acid was the principal fatty acid combined with 4-sphingenines, while nervonic acid was the principal fatty acid combined with sphingadienine. About 4% of the total fatty acids of sphingomyelin were *trans*-monoenoic. They were comprised of many positional isomers of straight-chain C_{22-24} compounds. The *cis*-monoenoic acids made up 33% of the total acids and consisted of almost pure nervonic acid. The rest of the acids were saturated. The 4-sphingenines contained small amounts of iso-C_{18} and anteiso-C_{19} compounds in addition to the straight-chain C_{16-18} bases.

SUPPLEMENTARY KEY WORDS *trans*-monoenoic fatty acids · positional isomers · branched-chain 4-sphingenines · ceramide diacetates · argentation TLC · diet · chain elongation · nonrandom combinations of long-chain bases and fatty acids

CHROMATOGRAPHY OF CERAMIDES has been applied to analysis of spingomyelin species (1) in different ways. Renkonen (2) carried out argentation TLC of ceramide diacetates; Karlsson and Steen (3) separated free ceramides by adsorption TLC, whereas Morrison (4) used TLC on borate-containing plates; Samuelsson and Samuelsson (5) and Casparrini, Horning, and Horning (6) have fractionated ceramides by GLC.

The present report describes argentation TLC of ceramide diacetates derived from human serum sphingomyelins. Better resolution was obtained than before (2, 5), and some novel components were detected and identified.

Some of the experiments presented are taken from the doctoral thesis of E. L. Hirvisalo, University of Helsinki, 1969 (*Ann. Acad. Sci. Fenn. Ser. A II Chem.* 146.).

Abbreviations: LCB, long-chain base(s); GLC, gas–liquid chromatography; TLC, thin-layer chromatography; D, dicarboxylic acid.

MATERIALS AND METHODS

Isolation of the Sphingomyelins

The serum (8 liters) was obtained from 64 young men in the military service. The blood was drawn in the morning 2 hr after a light breakfast consisting of tea, sugar, and cheese sandwiches. The lipids were extracted as described elsewhere (7). After mild alkaline methanolysis (2) the lipids were chromatographed on aluminum oxide (8). The isolated sphingomyelin appeared pure when analyzed by TLC. The estimated yield was better than 90% as judged from the elution pattern and TLC of the fractions other than sphingomyelin. This means that the sample can be regarded as a representative mixture of different molecular species. GLC results on the component fatty acids and LCB (see below) were similar to those described by Svennerholm, Ställberg-Stenhagen, and Svennerholm (9), and Karlsson (10), respectively.

Preparation and Study of Ceramide Diacetates

In a typical experiment the sphingomyelins (1.00 g) were hydrolyzed with phospholipase C of *Clostridium welchii* (2). TLC showed that the reaction was complete. Extraction of the reaction mixture gave 0.72 g of ceramides which corresponds to a yield of about 94%. The ceramides were then acetylated with pyridine and acetic anhydride at 20°C. TLC on Silica Gel G with chloroform–methanol 995:5 showed that a pure product was obtained.

Argentation TLC of ceramide diacetates was carried out on plates (200 × 200 mm) with 0.25-mm thick layers of Silica Gel G (E. Merck A. G., Darmstadt, Germany) containing 20% (w/w) silver nitrate. The plates were activated for 16 hr at 140°C (11). Analytical plates were stained with ammonium hydrogen sulfate

(12). Preparative separations were carried out essentially as described elsewhere (2). In a typical experiment 90 mg of ceramide diacetates was separated on 25 TLC plates; the combined recovery of the different subfractions was 84 mg.

In some argentation experiments the amounts of the ceramide diacetates were estimated by adding known amounts of methyl heneicosanoate (21:0) (as internal standard) to each zone. The scrapings were then subjected to acid-catalyzed methanolysis (as described below), and the resulting methyl esters were analyzed by GLC. Quantifications were made by comparing the summed areas of all the sample peaks to that of 21:0. Two binary model mixtures of ceramide diacetates were analyzed by this procedure; the relative errors were less than 10% for all components.

Analysis of Component Fatty Acids of Sphingomyelins and Ceramide Diacetates

The fatty acids of the original sphingomyelins were converted into methyl esters by treating the intact sphingolipids with 6% concentrated sulfuric acid in absolute methanol at 70°C for 16 hr. Sulfuric acid was removed by subsequent partition of the reaction mixture between chloroform and water. This procedure resulted in complete transesterification. A highly purified sample of serum sphingomyelin (21.9 μmoles of P) gave 21.8 μmoles of methyl esters, which was assayed by hydroxylaminolysis as described previously (2). The ceramide diacetates (from the quantitative TLC experiments) and the free fatty acids obtained after alkaline hydrolysis of ceramide diacetates (see below) were converted into methyl esters by the same procedure.

GLC of original and hydrogenated methyl esters was carried out as previously described (2). TLC plates for argentation chromatography of the fatty acid methyl esters were developed with hexane–ether 92:8. The model substances used were methyl palmitate (saturated), methyl 13-*trans*-docosaenoate (*trans*-monoenoic), and methyl 15-*cis*-tetracosaenoate (*cis*-monoenoic).

Rudloff oxidation of the methyl esters was carried out by a modification (13) of the procedure of Davidoff and Korn (14). The dicarboxylic acids which were formed were converted to dimethyl esters, purified by preparative TLC, and finally identified by GLC on a 6 ft column of EGSS-X at 170°C. Pure methyl 13-*trans*-docosaenoate gave a high yield (98%) of the expected C_{13}-dicarboxylic acid.

IR spectra of the methyl esters were recorded with a Perkin–Elmer instrument type 137, using potassium bromide windows.

Analysis of Component LCB of Ceramide Diacetates

The LCB were obtained by alkaline hydrolysis of ceramide diacetates (15). The hydrolysate was chromatographed on silicic acid to separate the fatty acids and the LCB (16). The LCB were then acetylated with pyridine and acetic anhydride and separated into two fractions by preparative argentation TLC (13). The slower moving fraction contained mainly triacetyl sphingadienine. The faster fraction was a mixture of triacetyl sphinganines (2%) and triacetyl 4-sphingenines (98%). This latter fraction was deacetylated with 1 N KOH in 90% methanol (15), and the free 4-sphingenines and sphinganines were separated by TLC on regular Silica Gel G (17). The free bases were oxidized with $NaIO_4$, as described by Sweeley and Moscatelli (18).

Rudloff oxidation of triacetyl 4-sphingenines was carried out in the same way as that of the methyl esters. The fatty acids which were formed were treated with ethereal diazomethane at 20°C, and the resulting methyl esters were purified by preparative TLC. GLC identification of the purified methyl esters was performed on a column (2.2% SE-30 on Gas Chrom S 100–120 mesh; 2 m × 3.5 mm) which could resolve iso and anteiso isomers; the carbon numbers were (n − 1) +0.64 and (n − 1) +0.72, respectively. The standards of branched-chain methyl esters were obtained from Applied Science Laboratories, Inc. (State College, Pa.). The procedure gave very pure myristic acid in model experiments with pure 4-sphingenine (13).

RESULTS

Fractionation of ceramide diacetates was carried out on argentation TLC plates with chloroform–methanol 99:1 as solvent. Fig. 1 shows that six subfractions (I–VI) were obtained. They all had similar IR spectra and identical R_f's on Silica Gel G TLC plates (without silver nitrate). This shows that all six subfractions were ceramide diacetates. The composition of the ceramide diacetates is shown in Table 1. The three faster moving fractions contained monoenoic LCB (4-sphingenines) in

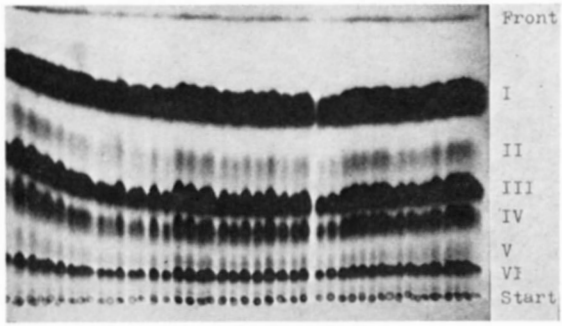

FIG. 1. Argentation TLC of ceramide diacetates derived from serum sphingomyelins. The plate was developed three times with chloroform–methanol 99:1, and the zones were visualized by charring.

TABLE 1 COMPOSITION OF SUBFRACTIONS OF HUMAN SERUM SPHINGOMYELINS

Subfraction	Composition of Ceramide		Relative Amount	
	Fatty Acid	LCB	Gravimetric Analysis after Preparative TLC	GLC Analysis with Internal Standard
			%	%
I	Saturated	Sphingenines	58	52
II	*Trans*-monoenoic	Sphingenines	3.6	4.4
III	*Cis*-monoenoic	Sphingenines	26	26
IV	Saturated	Sphingadienine	5.4	8.9
V	*Trans*-monoenoic	Sphingadienine	0.2	*
VI	*Cis*-monoenoic	Sphingadienine	6.8	8.0

* Not determined.

combination with saturated (I), *trans*-monoenoic (II), and *cis*-monoenoic fatty acids (III), respectively. The three slower moving fractions contained dienoic LCB (sphingadienines), combined with saturated (IV), *trans*-monoenoic (V), and *cis*-monoenoic fatty acids (VI).

GLC analyses of the component fatty acids of the six fractions are shown in Table 2. The fatty acid compositions of fractions I and IV resembled each other, as did those of II and V, as well as those of III and VI. It is important to note that fraction II contained slightly shorter fatty acids than did fraction III, although it moved faster on silver nitrate TLC plates. This shows that the separation of fractions II and III was not based on differences in the chain length of the fatty acids. The same conclusion is also valid for the argentation separation of fractions V and VI.

The fatty acid methyl esters from the six fractions were also separated by argentation TLC. Fig. 2 is an example of a TLC plate on which methyl esters derived from serum sphingomyelin were separated. Methyl esters derived from fractions I and IV revealed only saturated components; those from fractions II and V contained mainly *trans*-monoenoic components; methyl esters from fractions III and VI consisted of *cis*-monoenoic components.

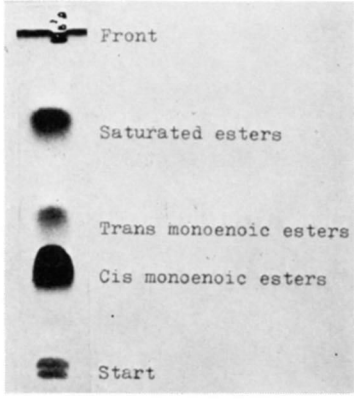

FIG. 2. Argentation TLC of methyl esters derived from (serum sphingomyelin) ceramide diacetates. The plate was developed with hexane–ether 92:8, and the spots were visualized by charring.

TABLE 2 FATTY ACID COMPOSITION OF CERAMIDE DIACETATE SUBFRACTIONS

Fatty Acid*	Fraction					
	I	II	III	IV	V	VI
				%		
14:0	1.5					
16:0	48	7.5	2.2	15	5.5	0.6
18:0	9.6	4.3	1.0	13	7.0	0.8
18:1		1.8	1.6	1.6	5.5	1.3
20:0	5.7			8.7	6.4	
20:1					3.5	
21:0				1.0		
22:0	16	1.9		24	5.0	
22:1		35	1.5	3.1	30	1.3
23:0	9.1			13		
23:1		14			7.0	
24:0	10	1.5		15	1.9	
24:1		34	94	6.0	27	96
Total saturated acids	100	15	3	90	26	3
Total monoenoic acids	0	85	97	10	73	97

* Number of C atoms: number of double bonds.

The LCB of the ceramide diacetates were also analyzed by GLC and TLC. Table 3 shows the results of GLC analyses of $NaIO_4$-generated aldehydes. Fractions I, II, and III had rather similar LCB mixtures with the C_{18} 4-sphingenine as the largest component. Fractions IV and VI contained almost pure sphingadienine as their LCB.

Argentation TLC of the triacetyl LCB confirmed that fractions I, II, and III contained 4-sphingenines (and sphinganines), whereas fractions IV, V, and VI contained sphingadienine.

Fractions I–III contained small amounts (2%) of sphinganine-ceramides, which apparently cannot be separated from the 4-sphingenine-ceramides by argentation TLC. However, Morrison has recently separated these ceramides on borate-containing plates (4).

TABLE 3 LCB Composition of Ceramide Diacetate Subfractions

Parent LCB	Carbon Number of Aldehyde*	Fraction Number I	II	III	IV	V†	VI
				%			
17:0	15.0		0.9				
16:1	15.8	5.4	12.8	2.2			
18:0	16.0	2.1	1.5	1.5			
17:1 branched	16.5	1.0		1.0			
17:1	16.8	6.1	6.8	4.6			1.8
18:1 branched	17.5	1.9	3.2	3.6			
18:1	17.8	82	65	78	4.9		2.5
18:2	18.5				94		96
19:1 branched	18.5	1.8	8.5	9.1			
Total sphinganines		2	2	2			
Total straight-chain 4-sphingenines		93	85	85			4
Total branched 4-sphingenines		5	13	13	5		
Total sphingadienines					94		96

* GLC on EGSS-X (at 140°C).
† Argentation TLC showed that the LCB was dienoic.

The fatty acid composition of 4-sphingenine-sphingomyelins and sphingadienine-sphingomyelins was calculated (Table 4). Striking differences are seen in the distribution of palmitic and nervonic acids. 30% of the 4-sphingenines, but only 8% of sphingadienines, were combined with palmitic acid; on the other hand, 29% of the 4-sphingenines, and 52% of sphingadienines, were combined with nervonic acid.

Characterization of the Trans-Monoenoic Fatty Acids

The *trans*-monoenoic fatty acids were isolated (from another serum pool) in the following manner. Fatty acid methyl esters were prepared from unfractionated ceramide diacetates with the sulfuric acid–methanol procedure. They were subjected to preparative argentation TLC as shown in Fig. 2. The yields of pure fractions were: saturated esters, 59%; *trans*-monoenoic esters, 4.6%; and *cis*-monoenoic esters, 37%. These figures are similar to the yields of the corresponding ceramide diacetates (Table 1).

The three fractions of methyl esters were analyzed by GLC. The *trans*-monoenoic esters were comprised of 22:1 (22%), 23:1 (20%), and 24:1 (58%), whereas the *cis*-monoenoic fraction contained only small amounts of 22:1 (3.7%) and 23:1 (1.0%) and consisted mainly of 24:1 (95%). These results confirm the observations on the ceramide diacetate subfractions II, III, V, and VI which are in Table 2. Catalytic hydrogenation of the *trans*-monoenoic methyl esters gave only straight chain C_{22-24} compounds, as shown by GLC.

IR spectra of all three methyl ester fractions revealed the expected peaks. The spectrum of the *trans*-monoenoic ester is shown in Fig. 3. The characteristic peak for *trans* double bonds at 970 cm^{-1} is quite clear.

The *trans*-monoenoic esters gave a complicated mixture of dicarboxylic acids (D) after Rudloff oxidation. The largest components were D-14:0 (33%), D-15:0 (21%), and D-17:0 (16%), but other C_{12-20} dicarboxylic acids were also found in significant amounts (2–7%). This diversity of oxidation products is not caused by the *trans* double bond per se since model experiments with methyl-*trans*-13-docosaenoate gave 98% of D-13:0. Therefore several positional isomers must have been present in the *trans*-monoenoic esters, with double bonds mainly in the tail portion of the chains, between the fourth and the 12 carbon atom from the methyl end of the chains.

TABLE 4 Fatty Acid Composition of Sphingenine- and Sphingadienine-Sphingomyelins of Serum*

Fatty Acid	Sphingenine-Sphingomyelins†	Sphingadienine-Sphingomyelins
	%	%
16:0	30	8
18:0	7	7
20:0	4	4
22:0	10	12
23:0	6	7
24:0	7	8
24:1	29	52

* *Trans* fatty acids are neglected.
† Including small amounts of sphinganine-sphingomyelins.

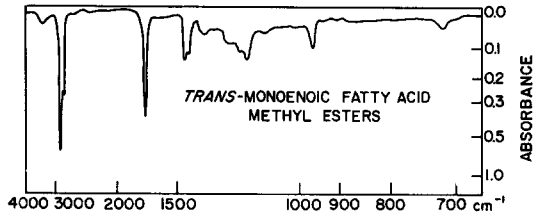

Fig. 3. IR spectrum of *trans*-monoenoic fatty acid methyl esters (smear on KBr window).

The *cis*-monoenoic esters, on the other hand, gave only small amounts of D-13:0 (3.0%) and D-14:0 (1.8%) and a very large amount of D-15:0 (95%) after Rudloff oxidation. These figures are similar to the distribution of chain length in this fraction (see above). Therefore it appears likely that practically all *cis*-monoenoic acids had their double bonds at the ninth carbon atom counted from the methyl end of the chain.

The above experiments reveal the presence of the *trans*-monoenoic fatty acids in sphingomyelins of two large serum pools. In addition sphingomyelin was isolated from 10 individual plasma samples (from healthy young men). The fatty acid methyl esters derived from these sphingomyelins were studied by argentation TLC as shown in Fig. 2. All 10 sphingomyelin samples revealed the spot characteristic to the *trans*-monoenoic esters.

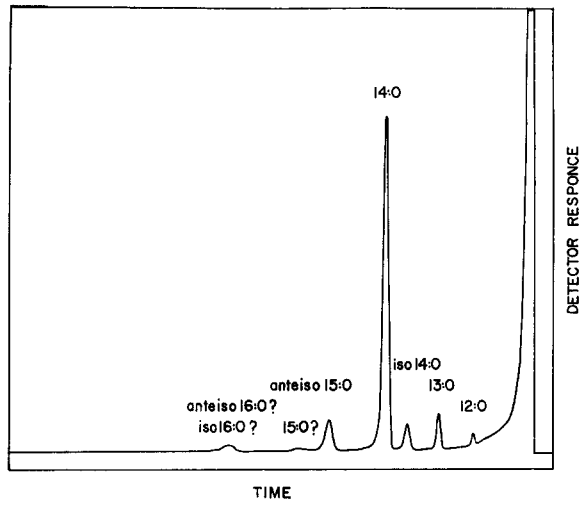

FIG. 4. GLC of fatty acid methyl esters derived by Rudloff oxidation of fraction III of ceramide diacetates. SE-30 column at 160°C.

Characterization of the Branched 4-Sphingenines

The fraction of triacetyl LCB which contained sphinganines and 4-sphingenines was hydrolyzed to the free bases. Pure 4-sphingenines were then isolated by preparative TLC on Silica Gel G (17). The double bond at C-4 appears to give a characteristic mobility (basicity) to the 4-sphingenines in this system. The molecules lacking this double bond (sphinganine and *cis*-14-sphingenine) are more basic and have a different (slower) mobility on Silica Gel G (13). The 4-sphingenines were then acetylated and subjected to Rudloff oxidation. GLC of the resulting fatty acids revealed the expected straight-chain compounds 12:0 (7%), 13:0 (5%), and 14:0 (84%) which were derived from the well-known 4-sphingenines of 16, 17, and 18 carbon atoms. In addition, three branched-chain acids were found, i.e. 13:0 br (traces), 14:0 br (2%), and 15:0 br (2%). No peaks were obtained in a "blank experiment" carried out with triacetyl 14-*cis*-sphingenine (13). Therefore the branched-chain acids were quite likely formed from branched-chain 4-sphingenines of 17, 18, and 19 carbon atoms.

The branched-chain 4-sphingenines were identified more definitely by direct Rudloff oxidation of ceramide diacetates of fraction III, in which the branched-chain LCB are particularly abundant (Table 3). Fraction III ceramides contain two olefinic centers: one at C-4 of the LCB, and the other at C_{n-9} of the fatty acid. Therefore Rudloff oxidation of the material should give C_{12-15}-monocarboxylic acids arising from the LCB and a noninterfering C_9-monocarboxylic acid arising from the fatty acid. Fig. 4 shows that the oxidation mixture contained isotetradecanoic acid and anteisopentadecanoic acid. Accordingly, the parent LCB were identified as isooctadeca-4-sphingenine and anteisononadeca-4-sphingenine. The same conclusion has been reached by Karlsson with mass spectrometric methods (personal communication).

Sphingomyelin LCB of 10 individual plasma samples (from healthy young men) were analyzed by GLC of the $NaIO_4$-generated aldehydes. All samples appeared to contain 2–3% of branched-chain octadeca-4-sphingenine. (The branched nonadeca-4-sphingenine could not be analyzed since in this system it behaves like the abundant octadecasphingadienine.)

DISCUSSION

Our observations on argentation TLC of ceramide diacetates confirm the recent findings of Samuelsson and Samuelsson (5). By argentation TLC they separated ceramide diacetates derived from plasma sphingomyelins into four fractions. Their preparations correspond to our fractions I, III, IV, and VI. The improved separation obtained in our experiments may be due to non-aromatic solvents, which are advantageous in argentation separation of dienoic lipids (1). Our more active plates may have also helped. It is interesting to note that fractions III and IV, which are clearly separated, contain dienoic molecules which have their *trans* double bonds in the same place of the molecules (13) and differ only in the localization of their *cis* double bonds. The delicate ceramide separations described in this and other laboratories (3–5) are clearly superior to those obtained with intact sphingomyelins (19). This is another example of improved fractionation through use of non-polar derivatives of polar lipids (1).

The presence of *trans*-monoenoic fatty acids has not heretofore been reported in any sphingolipids. It is unlikely that they are artifacts formed from the *cis*-monoenoic acids, since these two fractions differ strikingly in

the chain-length distribution. The following evidence also favors this view. Sphingomyelin was isolated in one case by direct chromatography of the serum lipid extract on silicic acid. When the sample was subjected to acid catalyzed methanolysis, the methanolysate revealed *trans*-monoenoic fatty acids in argentation TLC. This material had never been subjected to alkaline conditions. Secondly, argentation TLC of the ceramide diacetates also revealed the presence of *trans*-monoenoic fatty acids. These lipids in turn were never exposed to the rather severe conditions of acidic methanolysis. Finally, *cis*-monoenoic ceramide diacetates (fraction III) were not converted into the corresponding *trans*-compounds when subjected to renewed argentation TLC. This excludes the possible role of silver as a cause of isomerization.

We believe that "chain elongation" (20) (and α-oxidation) of dietary *trans*-monoenoic fatty acids is responsible for the formation of the *trans*-monoenoic acids found in the serum sphingomyelins. *Trans*-monoenoic C_{18} fatty acids are ingested in large amounts in the form of hydrogenated fats. These acids characteristically have the double bonds distributed along several positions between the fourth and the 12th carbon atom from the methyl end (21). The *trans* double bonds of the sphingomyelin acids were also found in this part of the chain.

The presence of branched-chain octadeca- and nonadeca-4-sphingenines in plasma sphingomyelins has been suggested by Karlsson (10). The present report confirms Karlsson's observations and extends them by the identification of isooctadeca- and anteisononadeca-4-sphingenines. Morrison (4) has recently shown that these compounds are the major branched-chain LCB in milk sphingomyelins. Milk and milk products are used in large amounts in the Finnish diet; therefore, the branched plasma sphingosines may arise either from the dietary LCB or dietary fatty acids.

The molecular species of serum sphingomyelin do not represent random pairing of the component LCB and fatty acids. The results of 4-sphingenine-bound palmitic acid and sphingadienine-bound nervonic acid prove this. Other examples of nonrandom pairing are also suggested in our results. Table 3 shows that the branched-chain sphingenines were more abundant in fraction II and III than in fraction I. In other words, the combination of branched LCB and monoenoic fatty acid is a favored one. A disproportionally high amount of hexadeca-4-sphingenine was in turn present in fraction II, i.e., combined with *trans*-monoenoic fatty acids (Table 3). Karlsson and Steen (3) reported a nonrandom pairing between fatty acids and dihydroxy or trihydroxy LCB in kidney medulla sphingomyelins. The nonrandom pairing may not be a general rule in sphingolipids, however. Morrison (4) has reported that identical fatty acid mixtures are combined with sphinganines and 4-sphingenines in bovine milk sphingomyelins.

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