Separation of molecular species of sphingomyelin by reversed-phase high-performance liquid chromatography

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Abstract A convenient method for the separation of molecular species of sphingomyelin by reversed-phase high-performance liquid chromatography (HPLC) is described. Sphingomyelin species from bovine brain and sheep and pig erythrocytes were resolved into 10–12 separate peaks on a μ-BondaPak C_{18} or Nucleosil-5-C_{18} reversed-phase column with methanol–5 mM potassium phosphate buffer, pH 7.4, 9:1 (v/v) as the solvent. Detection was at 203–205 nm. The sphingomyelin species were primarily resolved due to specific hydrophobic interaction of their fatty acid and sphingoid chains with the alkyl ligand of the stationary phase. The retention time of the sphingomyelin species increased progressively as the number of carbon atoms in the hydrophobic chains increased in the homologous series. The presence of one double bond in the molecule reduced the retention time significantly. Introduction of a second double bond in the fatty acid side chain did not reduce the retention time to the same extent as the first double bond. The presence of a *trans* double bond in the sphingoid moiety increased the retention time of sphingomyelin more than did a *cis* double bond in the fatty acid side chain. The differential hydrophobic interaction observed between the ligand of the stationary phase and different alkyl chains of the sphingomyelin species illustrates that reversed-phase HPLC technique can be conveniently used to study the extent of relative hydrophobicity of different types of alkyl chains.—**Jungalwala, F. B., V. Hayssen, J. M. Pasquini, and R. H. McCluer.** Separation of molecular species of sphingomyelin by reversed-phase high-performance liquid chromatography. *J. Lipid Res.* 1979. **20:** 579–587.

Supplementary key words fatty acid and sphingoid base composition · hydrophobic interaction · effective methylene number · bovine brain and pig and sheep erythrocyte sphingomyelins

Recently, it has become increasingly clear that the biological properties of membranes such as fluidity, enzymic activity, and membrane integrity are controlled by the acyl group composition of lipids in the membrane bilayers (1). Great interest has been generated in the analysis and preparation of different molecular species of individual phospholipids. As a first step towards the goal of molecular species separation of phospholipids, we have attempted the separation of sphingomyelin species by reversed-phase high-performance liquid chromatographic (HPLC) technique. The composition of sphingomyelin hydrophobic chains, in nature, is relatively simple as compared to other phospholipids. Most of the sphingomyelins in nature have C_{18}-sphingenine as sphingoid base, while the fatty acid portion of the molecule varies.

Previously, we described HPLC methods for the quantitative analysis of phospholipids and glycolipids after derivatization (2–8). We also described the HPLC analysis of phosphatidylcholine and sphingomyelin with detection in the region of 200 nm without derivatization (9). Here we describe the HPLC separation of individual molecular species of sphingomyelin from various sources on a reversed-phase HPLC column with detection in the region of 200 nm. The method is also applicable for the large-scale preparation (mg–g quantities) of individual molecular species of sphingomyelin.

The physico-chemical phenomena underlying the reversed-phase chromatographic process with nonpolar stationary phases has been interpreted in the light of the "solvophobic theory" (10). The fundamental feature of the hydrophobic interaction is that the magnitude of the nonpolar contact area appears to play a paramount role in determining the interaction energy between the solute and the ligand of the stationary phase. Here we have applied this

Abbreviations: HPLC, high-performance liquid chromatography; EMN, effective methylene number; GLC, gas–liquid chromatography.

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basic theory to interpret the order of elution of various sphingomyelin species in relation to molecular structure and conformation and have suggested how reversed-phase HPLC methodology could be useful for studying hydrophobic interactions of biological substances. Part of this work has been reported previously in an abstract (11).

EXPERIMENTAL METHODS

Materials

Sphingomyelin from beef and pig brain as well as pig and sheep erythrocytes was purchased from either Supelco, Bellefonte, PA or Research Products International Corp., Elk Grove Village, IL. Each sample gave a single spot on thin-layer chromatography and charring the plate. All solvents used for HPLC, obtained from either Fisher Chemical Co. (Fairlawn, NJ) or Burdick and Jackson, Inc. (Muskegan, MI) were of HPLC grade quality and were degassed by boiling briefly before use.

Instrumentation

The HPLC analysis was performed with a Waters Associates (Milford, MA) Model 6000 solvent delivery system and a Model U-6K injector. The chromatographic column was a (30 cm × 4 mm) stainless tube prepacked with either μ-Bondapak-C_{18} material (Waters Assoc.) or slurry packed with Nucleosil-5-C_{18} 5-μm particles (Macherey-Nagel, Düren, Germany) with the aid of a Micromeritics Instrument Corp. (Norcross, GA) column packer. The detection was with a Laboratory Data Control (Riviera Beach, FL) variable wavelength spectromonitor.

Method

Sphingomyelin samples (50 μg–2 mg) dissolved in 10–100 μl of dichloromethane–methanol 1:1 (v/v) were injected on the reversed-phase column. The HPLC solvent was methanol–5 mM potassium phosphate buffer, pH 7.4, in a ratio varying from 9:1 to 9.8:0.2 (v/v) depending upon the required resolution of the individual peaks. The solvent was pumped isocratically at a flow rate of 1–2 ml/min. The detection was at 203–205 nm.

The individual molecular species that were resolved were collected and the purity of the major molecular species was checked by reinjecting the lipid under the same conditions. A portion of the material associated with the peak was subjected to methanolysis with anhydrous HCl according to the method of Kishimoto and Hoshi (12). The fatty acid methyl esters were analyzed by gas–liquid chromatography on either 3% OV-1 (on 80–100 mesh Suplecoport) or 10% SP-2340 (on 100–120 mesh Chromosorb W) packed in a glass coiled column (180 × 0.4 cm) using a Hewlett-Packard 7620-A gas chromatograph with a flame ionization detector (30 ml/min hydrogen, 295 ml/min air) and helium as carrier gas (60 ml/min).

The sphingoid analysis was done after aqueous methanolic hydrochloric acid hydrolysis of sphingomyelin essentially as described by Gaver and Sweeley (13). After methanolysis, the reaction mixture was neutralized with silver carbonate. The precipitated material was washed twice with 2-ml portions of chloroform:methanol 2:1 (v/v). The combined organic phases were then dried under nitrogen, the residue was redissolved in 200 μl of chloroform and chromatographed on a Unisil (Clarkson Chem. Co., Williamsport, PA) column (0.4 × 4 cm). The column was eluted with 6 ml of chloroform followed by 6 ml of methanol. The methanol fraction was collected and dried under nitrogen. The sphingoids were then analyzed either by GLC after trimethylsilylation (14) or by the newly developed HPLC method which involves the formation of the biphenylcarbonyl derivatives of the sphingoids (5) and chromatography on a "Fatty Acid Analysis" column (30 cm × 4 mm) from Waters Associates (15). Some of the biphenylcarbonyl sphingoids were silylated and analyzed by electron-impact mass spectrometry with a Finnegan-4000 mass spectrometer equipped with a model 6115 data system.

RESULTS AND DISCUSSION

Bovine brain sphingomyelin

The bovine brain sphingomyelin was resolved by HPLC into ten major separate peaks (**Fig. 1*A***). The GLC analysis of the fatty acids and the HPLC analysis of the sphingoids of the total bovine brain sphingomyelin are given in Fig. 1*A*, inset, and Fig. 1*B*, respectively. The fatty acid and sphingoid compositions of the individual sphingomyelin peaks are listed in **Table 1.**

The detection of sphingomyelin in the HPLC analysis results primarily from the absorbance of double bonds at 203 nm (9). Thus the peak areas do not represent true quantity of sphingomyelin present in the sample. Nevertheless the major fatty acid species in the bovine brain sphingomyelin detected were 18:0, 24:1, and 24:0. These results agreed with the results obtained by GLC analysis of the fatty acids after acid hydrolysis (Fig. 1*A* inset). The major sphingoid was C_{18}-sphingenine, with appreciable amounts of C_{18}-sphinganine and C_{20}-sphingenine. The peak cor-

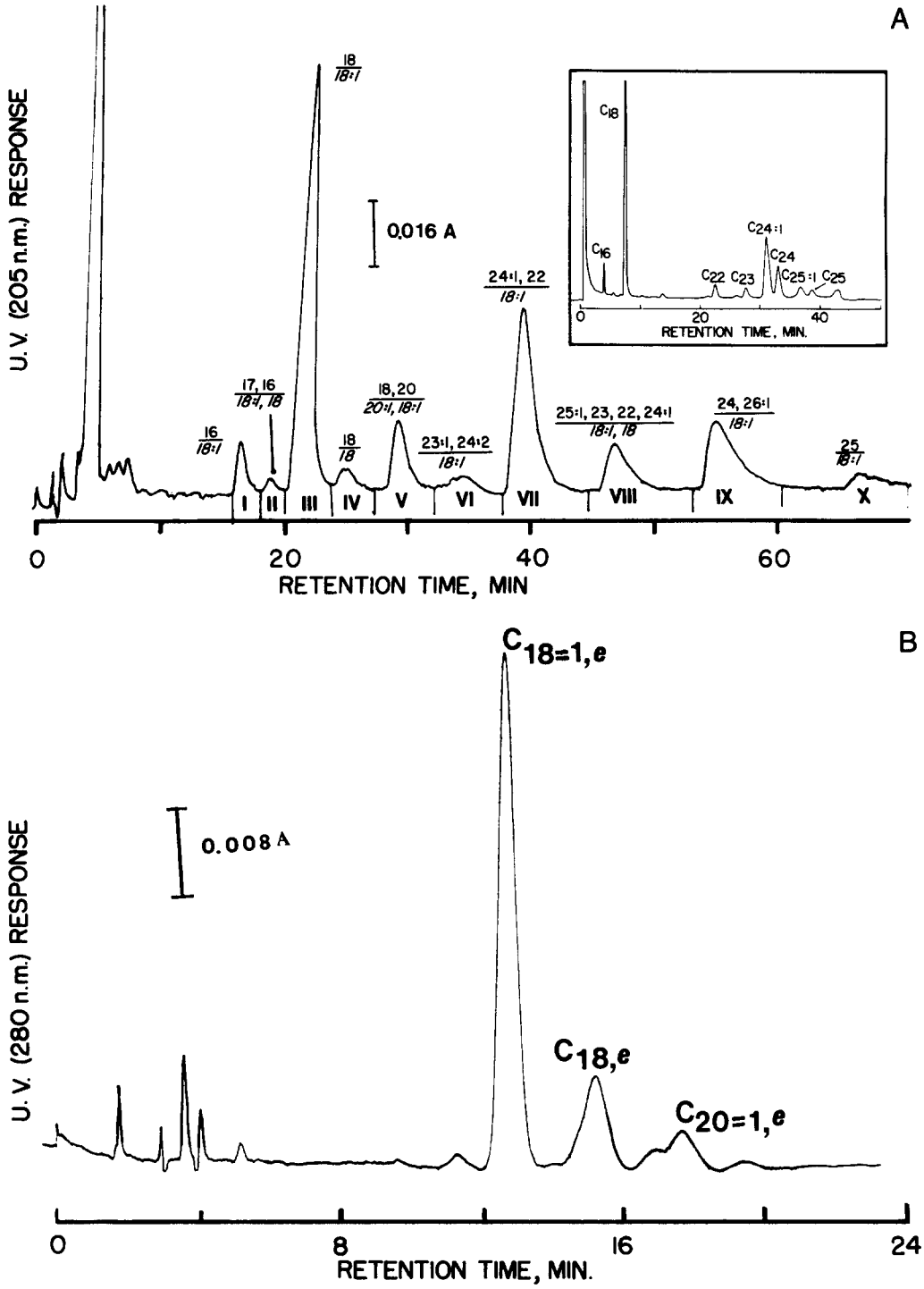

Fig. 1. *A*. HPLC analysis of bovine brain sphingomyelin on Nucleosil-5-C_{18} column. The solvent was methanol–5 mM phosphate buffer, pH 7.4, 97:3 (v/v) with a flow rate of 1 ml/min. Sphingomyelin, 350 μg dissolved in dichloromethane–methanol 1:1 (v/v), 35 μl, was injected. The sphingomyelin species eluted between marked peak areas were collected and analyzed (see Table 1). The major fatty acid (top lettering) and sphingoid (bottom lettering) composition of the sphingomyelin in an individual peak is given near the peak. Fatty acid and sphingoid in excess of 10% of the total in the peak area are indicated. *Inset*. GLC analysis of fatty acid methyl esters of bovine brain total sphingomyelins on OV-1 column. The chromatographic conditions are given in the text. The identification of the fatty acid is given above each peak. *B*. HPLC analysis of biphenylcarbonyl derivatives of sphingoid from bovine brain total sphingomyelin on a "Fatty Acid Analysis" column. The solvent was tetrahydrofuran–methanol–H_2O 25:40:40 (by vol) with a flow rate of 1 ml/min. $C_{18=1,e}$ is erythro-C_{18}-sphingenine; $C_{18,e}$ is erythro-C_{18}-sphinganine; $C_{20=1,e}$ is erythro-C_{20}-sphingenine.

TABLE 1. Percentage fatty acid and sphingoid composition of bovine brain sphingomyelin fractions obtained by HPLC[a]

Peak No.	Fatty Acid												Sphingoid		
	16:0	18:1	17:0	18:0	19:1	20:0	22:1	23:1	24:2	22:0	24:1	23:0	25:1	24:0	26:1	25:0	C_{18}-Sphingenine	C_{18}-Sphinganine	C_{20}-Sphingenine
I	94	6														100			
II	33		67													Traces[b]		Traces[b]	
III	2			98												93		6	1
IV				93	7											6		94	
V				75		20	5									24			76
VI								70	30							Traces[b]			
VII										15	85					99		1	
VIII										9	6	44	41			85		15	
IX														69	31	97		3	
X														3		97		3	

[a] The fatty acid methyl esters were analyzed by GLC on OV-1 and SP-2340 columns as described in the text.
[b] Quantitative analysis was not possible due to paucity of the materials associated with these peaks.

responding to C_{18}-sphinganine (Fig. 1*B*, $C_{18:0,e}$) is slightly asymmetrical due to possible contamination by small amounts of 3-*O*-methyl derivative of C_{18}-sphingenine. Other minor peaks were not characterized. Upon HPLC analysis of the sphingomyelin, three separate peaks containing 18:0 fatty acid (peaks III–V, Table 1) were identified. These peaks had distinctly different sphingoid compositions. Peak III contained mainly C_{18}-sphingenine, peak IV had mainly C_{18}-sphinganine, while peak V had 76% C_{20}-sphingenine. Peak IV also contained 7% 19:1 fatty acid while peak V had 20% 20:0 and 5% 22:1 fatty acids (Table 1). These results showed that the sphingomyelin species were resolved not only on the basis of fatty acid composition but also through differences in the sphingoid moiety.

The major C_{18}-sphingenine base was associated with various fatty acid chains in bovine brain sphingomyelins (Table 1). C_{18}-Sphinganine was mostly associated with 18:0 (peak IV), 22:0, and 24:1 (peak VIII) fatty acids. However, C_{20}-sphingenine was apparently exclusively associated with the 18:0 fatty acid (peak V). Other major fatty acids such as 24:1 (24%) and 24:0 (13%), besides 18:0 (37%), showed no trace of C_{20}-sphingenine base. This is the first demonstration of specific association of C_{20}-sphingenine with 18:0 fatty acid. Essentially the same results were obtained when pig brain sphingomyelins were analyzed by HPLC (not shown).

Sheep erythrocyte sphingomyelin

The HPLC separation of sheep erythrocyte sphingomyelin is shown in **Fig. 2.** The GLC analysis of fatty acids of the sphingomyelin is shown in Fig. 2 inset. By HPLC, the sphingomyelin species were resolved into 11 peaks. The fatty acid and sphingoid compositions of the individual peaks are listed in **Table 2.** The major fatty acids of the sheep erythrocyte sphingomyelins were 16:0, 24:1, 24:2, and 24:0. Peak I, which showed several shoulders, appeared to be a mixture of 16:0, 18:1, and 18:0 fatty acids with C_{18}-sphingenine base. Both peaks I and II contained large percentages of 16:0 and C_{18}-sphingenine. The basis of this separation is not clear, although it may be that, since the minor peak I is not well resolved from major peak II, significant amounts of 16:0 in Peak I could be due to overlap from peak II. Peak III, though not very well resolved from Peak II 16:0, also contained 18:1, 17:0, and 18:0 fatty acids with 83% C_{18}-sphinganine. Peaks V–VII were minor peaks and the quantitative fatty acid and sphingoid analysis of these peaks by GLC was difficult. Nevertheless they are sphingomyelin species as they were also present in HPLC of benzoylated

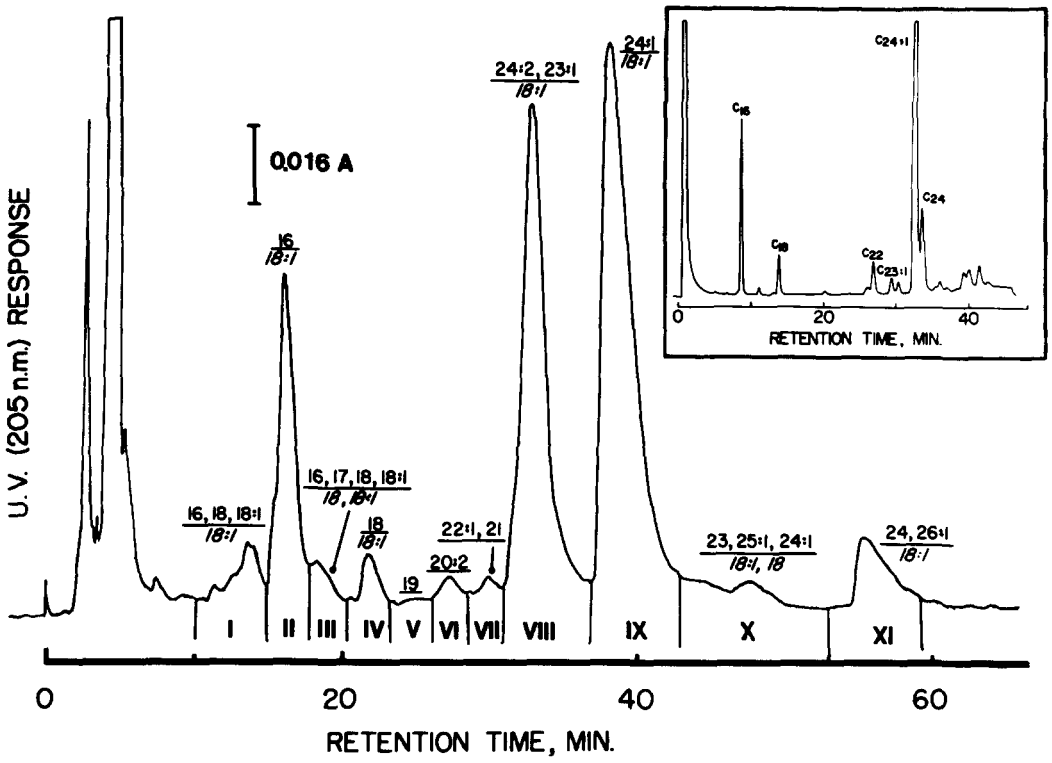

Fig. 2. HPLC analysis of sheep erythrocyte sphingomyelin on Nucleosil-5-C_{18} column. Sphingomyelin, 500 μg dissolved in dichloromethane–methanol 1:1; 50 μl was injected. The chromatographic conditions and other details are the same as in Fig. 1*A*. The fatty acid and sphingoid composition of each peak is given in Table 2. The sphingoid composition for peaks V, VI, and VII was not determined. Peak VII was a mixture of 20:0, 22:1, and 21:0 fatty acids with overlapping fatty acids from peak VIII. *Inset*. GLC analysis of fatty acid methyl esters of sheep erythrocyte total sphingomyelin on OV-1 column. The chromatographic conditions are given in the text.

sphingomyelin from sheep erythrocytes (data not shown). Peaks IX and X both contained 24:1 fatty acid. Sphingoid analysis showed that peak IX contained almost all C_{18}-sphingenine, while peak X contained 45% C_{18}-sphinganine. C_{20}-Sphingenine was not found in sheep erythrocyte sphingomyelin.

Pig erythrocyte sphingomyelin

The HPLC separation of pig erythrocyte sphingomyelin is shown in **Fig. 3.** The GLC analysis of fatty acids of the sphingomyelin is shown in Fig. 3 inset. By HPLC the sphingomyelin species were resolved into 12 different peaks. The fatty acid and sphingoid base compositions of the individually collected peaks are listed in **Table 3.** The major fatty acids were 16:0, 18:0, 24:1, and 24:0. Peak I was very small and contained a 16:0, 18:1, and 18:0 fatty acid mixture with mostly C_{18}-sphingenine as the base. Most of the sphingomyelin with 16:0 fatty acid and C_{18}-sphingenine was resolved as peak II. The basis of the separation of peaks I and II, as in the case of sheep erythrocyte sphingomyelin, is unclear. The sphingomyelin with 16:0 fatty acid and C_{18}-sphinganine was present in peak III. Peak V was due to sphingomyelin with 18:0 fatty acid and C_{18}-sphinganine along with some 19:0 and 20:0 fatty acids. Peak VII, which contained mostly sphingomyelin with 24:2 fatty acid, was well resolved from peak IX which contained sphingomyelin with 24:1 and 22:0 fatty acids. C_{20}-Sphingenine was not present in pig erythrocyte sphingomyelin.

General comments

The analysis of several different types of sphingomyelin by reversed-phase HPLC revealed that separations were obtained according to the number of carbon atoms as well as by the number and type of double bonds in the hydrophobic chains. The retention times of sphingomyelin species were progressively increased as the number of carbon atoms in the hydrophobic chains was increased in the homologous series. The presence of one or more double bonds in either the sphingoid or fatty acid chain reduced the retention time of the sphingomyelin species. Thus the retention time of sphingomyelin with C_{18}-sphingenine

TABLE 2. Percentage fatty acid and sphingoid composition of sheep erythrocyte sphingomyelin fractions obtained by HPLC[a]

	Fatty Acid																	Sphingoid	
Peak No.	16:0	18:1	17:0	18:0	19:0	20:2	20:0	22:1	21:0	23:1	24:2	22:0	24:1	23:0	25:1	24:0	26:1	C_{18}-Sphingenine	C_{18}-Sphinganine
I	63	10		27														98	2
II	96			4														97	3
III	30	20	26	24														17	83
IV	2			98														99	1
V					Traces[b]														
VI						Traces[b]													
VII							6	25	12	17	40								
VIII										21	79							98	2
IX												5	95					99	1
X													30	37	33			55	45
XI																78	22	98	2

[a] The fatty acid methyl esters were analyzed by GLC on OV-1 and SP-2340 columns, the sphingoids were analyzed by HPLC of biphenylcarbonyl derivatives as described in the text.

[b] Quantitative analysis was not possible due to paucity of the materials.

TABLE 3. Percentage fatty acid and sphingoid composition of pig erythrocyte sphingomyelin fractions obtained by HPLC[a]

	Fatty Acid																Sphingoid	
Peak No.	16:0	18:1	17:0	18:0	19:0	20:0	22:1	24:2	23:1	22:0	24:1	26:2	23:0	25:1	24:0	26:1	C_{18}-Sphingenine	C_{18}-Sphinganine
I	78	9		13													100	
II	92			8													100	
III	51		27	22													47	53
IV				94		6											99	1
V				58	18	24											50	50
VI						80	20										98	2
VII						11		89									99	1
VIII									58		42						98	2
IX										34	66						100	
X										10	30	60					67	33
XI													80	20			99	1
XII															97	3	100	

[a] The fatty acid methyl esters were analyzed by GLC on OV-1 and SP-2340 columns, the sphingoids by HPLC of biphenylcarbonyl derivatives as described in the text.

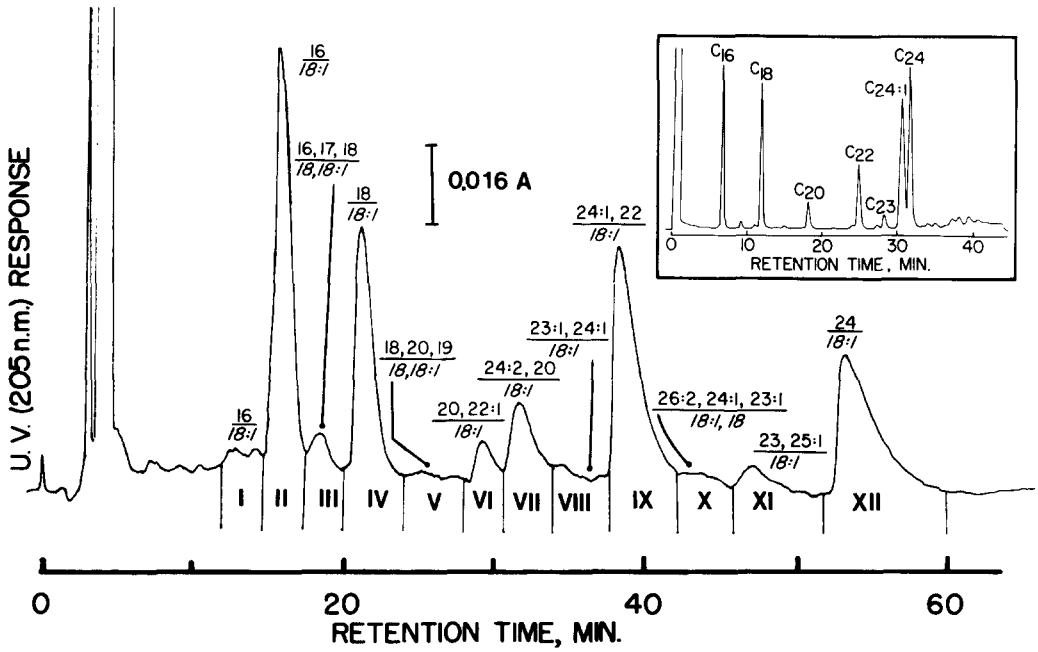

Fig. 3. HPLC analysis of pig erythrocyte sphingomyelin on Nucleosil-5-C_{18} column. Sphingomyelin, 500 μg in dichloromethane–methanol 1:1; 50 μl was injected. The chromatographic conditions and other details are the same as in Fig. 1*A*. The fatty acid and sphingoid composition of each peak is given in Table 3. *Inset*. GLC analysis of fatty acid methyl esters of pig erythrocyte total sphingomyelin on OV-1 column. The chromatographic conditions are given in the text.

and 24:0 fatty acid was about 53 min, while that of sphingomyelin species with C_{18}-sphingenine and 24:1 or 24:2 fatty acid were about 38 and 32 min, respectively. It was also noted that introduction of a second double bond in the fatty acid side chain did not reduce the retention time to the same extent as the first double bond in the saturated chain.

Introduction of a double bond in the sphingoid chain alone also reduced the retention time of sphingomyelin. Thus, the retention times of sphingomyelin with 18:0 and C_{18}-sphinganine or C_{18}-sphingenine were about 25 and 21 min, respectively. Similarly, the retention times of sphingomyelin with 24:1 fatty acid and C_{18}-sphinganine or C_{18}-sphingenine were about 44 and 38 min, respectively. It was noted that introduction of a double bond in the sphinganine side chain at the 4 position did not reduce the retention time of sphingomyelin to the same extent as introduction of the first double bond in the fatty acid side chain at the 9 position.

These differences in the hydrophobic interactions between the lipids and the ligand of the stationary phase could be due to the relative position and the nature of the double bond in two different types of side chains. However, it is more likely that the difference is due to the nature of the double bond, since it is known that the double bond in the sphingoid is *trans* while most of the fatty acyl side chains have *cis* double bonds. It was previously established that on the reversed-phase column the *trans* unsaturated fatty acids have retention times longer than the corresponding *cis* unsaturated fatty acids, as expected on the basis of their conformational differences (16, 17).

In order to understand the hydrophobic interaction between the ligand of the stationary phase and different molecular species of sphingomyelin, we calculated the "effective methylene number" (EMN) of each sphingomyelin species and related it to its retention time (18). EMN of lipids with one double bond was considered to be about the same as saturated lipid with two fewer methylene groups. Thus, EMN of sphingomyelin with C_{18}-sphinganine and 18:0 was considered to be 36, while that of C_{18}-sphingenine and 18:0 was considered to be 34. A plot of log retention time of sphingomyelin species vs. EMN is given in **Fig. 4.** It was noted that the log retention time of the sphingomyelin species with C_{18}- or C_{20}-sphingenine and saturated fatty acid or fatty acid having one double bond was linear with EMN (line B). However, sphingomyelin species with C_{18}-sphinganine (line C) had retention times shorter than expected (as in curve B). This could be due to differential reactivity of the neighboring OH group with the NH group in the sphingenine moiety as compared to the sphinganine moiety. Reversed-phase HPLC of the 3-benzoylated sphingomyelin

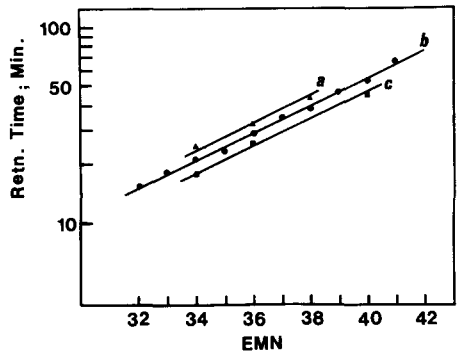

Fig. 4. Semilogarithmic plot of effective methylene number (EMN) vs. retention time of sphingomyelin species on Nucleosil-5-C_{18} reversed-phase column. The points in curve A are for sphingomyelin having C_{18}-sphingenine and two double bonds in the fatty acid side chain; curve B, sphingomyelin having C_{18}- or C_{20}-sphingenine and saturated fatty acids or fatty acids with one double bond; curve C, sphingomyelin with C_{18}-sphinganine and saturated fatty acid or fatty acids with one double bond.

showed that the relative retention times were similar to those described for the native sphingomyelin.[3] This suggested that the supposed differential reactivity of the OH group was not the cause for differential hydrophobic interaction. The alternate possibilities are that the 4 position of the double bond, being in the "inner core" of the sphingoid moiety, could have different hydrophobic interaction than the double bond in the fatty acid side chains which is mostly in the 9 position or that the *trans* double bond in the sphingoid could produce this effect as discussed previously.

Sphingomyelins having fatty acid containing two double bonds also behaved slightly differently with respect to retention time on the column (curve A), even though they had the same calculated EMN. The sphingomyelin having the same EMN but with a fatty acid having two double bonds eluted later than those having a saturated fatty acid or a fatty acid with one double bond. This indicated that the position and presence of a second double bond in the fatty acyl chain rendered the molecule more hydrophobic than expected, based solely upon the EMN. However, it has to be realized that introduction of one *cis* double bond in the fatty acid side chain of sphingomyelin reduces the contact surface area and thus the hydrophobic interaction. But introduction of the second *cis* double bond in the same chain, due to favorable physical conformation (16, 17), should provide more nonpolar contact area and thus better interaction of the lipid with the ligand of the stationary phase. Thus, the retention times are expected to be longer for such molecules, even though the effective methylene number is the same. Based upon these observations, it has become possible to predict the possible structure of unknown sphingomyelin species eluted from the HPLC reversed-phase column under defined conditions.

These observations suggest that the elution of the sphingomyelin from the reversed-phase column is dependent upon the hydrophobic interaction between the solute and the ligand of the stationary phase. The magnitude of this interaction is dependent upon the contact area between the ligand and the solute which in turn is determined by the molecular structure and conformation of the ligand and the solute. Since the ligand in the stationary phase is held constant, the HPLC method offers a simple way to determine the relative hydrophobic nature of the solutes in the mobile phase such as various membrane lipids. The ligand also could be varied by making reversed-phase HPLC columns with varying chain lengths (C_2, C_8, and C_{18} are commercially available) or other lipids such as cholesterol. Again, effects of other parameters such as various solvents, ions, ionic strength, temperature, or pH on the nature of hydrophobic interaction could be easily studied by this method.

The HPLC method described here for the separation of molecular species of intact sphingomyelin is more effective than any other procedure previously described (19–21). Though all the "critical pairs" were not resolved on the reversed-phase column, it is possible to separate these pairs either by catalytic hydrogenation and reinjection or by argentation thin-layer chromatography. We have also used the method for the large-scale preparation of molecular species of sphingomyelin either by repeated injection or by using Waters Associates preparative HPLC system-500. It is possible to load up to 3 mg of sample on the 20 cm × 4 mm C_{18}-column with good resolution. With a Waters preparative HPLC system-500, 2 g of sphingomyelin were loaded and resolved into the molecular species. The method also demonstrates great potential for the separation of individual molecular species of other complex phospholipids. We have recently shown that pig liver phosphatidylcholine could be resolved into 15 separate peaks by this method.[3]

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