Analysis of sphingoid bases by reversed-phase high performance liquid chromatography

Firoze B. Jungalwala,[1] James E. Evans, Eric Bremer, and Robert H. McCluer

Department of Biochemistry, Eunice Kennedy Shriver Center for Mental Retardation, Waltham, MA 02254, and Department of Neurology, Harvard Medical School, Boston, MA 02115

Abstract A reversed-phase high performance liquid chromatography (HPLC) technique was developed for the analysis of sphingoid bases as their biphenylcarbonyl derivatives. The sphingoid bases (0.3–500 nmol) obtained from sphingolipids after hydrolysis were quantitatively derivatized with biphenylcarbonyl chloride at room temperature for 90 min. After removal of excess reagent by partitioning, the derivatized products were quantitatively analyzed on a reversed-phase HPLC column, with detection at 280 nm. A mixture of biphenylcarbonyl C_{18}-5-hydroxysphinganine, C_{18}-*erythro* and *threo* sphingenine, and sphinganine, C_{18}-5-O-methyl and 3-O-methyl sphingenine, C_{20}-sphingenine, sphingosyl phosphorylcholine, and psychosine and were well resolved from one another. The method was employed for the analysis of less than nanomole quantities of sphingoid bases from spingomyelin and cerebrosides.—**Jungalwala, F. B., J. E. Evans, E. Bremer, and R. H. McCluer.** Analysis of sphingoid bases by reversed-phase high performance liquid chromatography. *J. Lipid Res.* 1983. **24:** 1380–1388.

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All sphingolipids contain one molecule of sphingoid base per molecule of sphingolipid (1). The nature of the sphingoid base in the sphingolipids varies. Most commonly occurring mammalian sphingolipids have generally C_{18} or C_{20}-sphingenine or C_{18}-hydroxysphinganine (phytosphingosine) as the major long chain base, with small amounts of C_{18} or C_{20}-sphinganines. Several methods have been published on the analysis of total sphingoid base produced after acidic methanolysis of sphingolipids (2–7). However, only a few procedures for the analysis of individual molecular species of the bases have appeared. Although thin-layer chromatographic procedures for the separation of the sphingoids have been described, the separations are not quite adequate and the method is not quantitative (8, 9). The most commonly used procedures are based upon the gas–liquid chromatographic analysis of the aldehydes liberated after periodate oxidation (10) or trimethylsilyl ether and N-acetyl trimethylsilyl ether derivatives of the sphingoid bases (11, 12). Although good separation of the individual sphingoid bases was obtained by the GLC procedure, some of the isomers were not well resolved when a mixture of sphingoid bases was present. Again, several clearly resolved peaks by the GLC analysis of the sphingolipid hydrolysate (side products) were not identified. Thus the analysis of the sphingoid bases from a sphingolipid hydrolysate by such a procedure was not quite quantitative. The lower limit of detection of a sphingoid base by the GLC method previously reported was about 15 nmole (12).

We have developed several HPLC methods for the analysis of glycosphingolipids and phospholipids (reviewed in 13 and 14). Here we describe a simple HPLC method for the quantitative analysis of molecular species of sphingoid bases obtained from the acid hydrolysates of the sphingolipids. The method is based upon the quantitative formation of stable N-biphenylcarbonyl derivatives of the sphingoid bases and reversed-phase HPLC analysis of the derivatives with an isocratic solvent system. Most of the commonly occurring sphingoid bases are well resolved from one another and the small amounts of the side products that are usually formed during the acid hydrolysis of a sphingolipid have been identified. Gangliosides are an exception in that more vigorous hydrolysis conditions are required for quantitative liberation of the sphingoid bases and more side products are formed. The conditions for the hydrolysis of gangliosides are considered in a separate publication (15). The method also allows analysis of as little as 0.1 μg (0.33 nmol) of the long chain bases.

EXPERIMENTAL

Materials

C_{18}-Sphingenine, C_{18}-sphinganine (*erythro* and *threo* isomers), C_{18}-phytosphingosine, bovine brain cerebro-

Abbreviations: HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

[1] To whom correspondence should be directed at the Eunice Kennedy Shriver Center, 200 Trapelo Road, Waltham, MA 02254.

sides, and sphingomyelin were purchased from Supelco, Bellefonte, PA. C_{20}-Sphingenine, 3-O-methyl-C_{18} and C_{20}-sphingenine, and the corresponding 5-O-methyl derivatives were prepared after acid hydrolysis of brain gangliosides and purified according to Sambasivarao and McCluer (9). 4-Biphenylcarbonyl chloride was purchased from Aldrich Chemical Co, Milwaukee, WI. HPLC solvents and other chemicals were purchased from Fisher Scientific Co. (Medford, MA).

Hydrolysis of sphingomyelin and cerebroside

The hydrolysis of sphingomyelin was according to Gaver and Sweeley (11) with the following modification. Sphingomyelin (500 μg or less) was reacted with 1 ml of 1 N HCl in aqueous methanol (water–methanol 67:33, by volume, i.e., 37 M H_2O in methanol) at 70°C for 16 hr. After the reaction, 5 ml of chloroform was added followed by 1 ml of 0.05 N NaOH in methanol–0.9% saline–chloroform 48:47:3 (by volume). The two phases formed were vigorously shaken and centrifuged at 3000 *g* for 2–3 min. The upper phase was removed and the lower phase was rewashed three times with 1 ml of the same 0.05 N NaOH solution, followed by two washes with the same solution without NaOH. The lower phase was dried by gently blowing N_2 at 45–50°C.

The optimal hydrolysis conditions for cerebroside (40–500 μg) were with 0.5 ml of aqueous 3 N HCl in aqueous methanol (water–methanol 1:1, by volume, i.e., 28 M H_2O in methanol) at 60°C for 1.5 hr. The liberated sphingoids were isolated as previously described for sphingomyelin.

Derivatization of sphingoid bases

Standard sphingoid bases (0.3–500 nmol) and sphingoids liberated from the sphingolipids were reacted with 50 μl of 1% biphenylcarbonylchloride in tetrahydrofuran and 100 μl of a saturated solution of sodium acetate in water (16). The biphasic reaction mixture was vigorously agitated at room temperature for 1.5 hr. Five ml of "Folch" lower phase (prepared by mixing chloroform–methanol 2:1 with 0.2 vol of 0.9% saline solution and removing the separated upper phase) was added to the reaction mixture, followed by 1 ml of theoretical upper phase containing 0.05 N NaOH (prepared by mixing methanol–0.9% saline–chloroform–2.5 N NaOH 48:47:3:2, by volume). The mixture was shaken, centrifuged, and the upper phase was removed. The lower phase was washed twice with the same upper phase and three times with upper phase without 0.05 N NaOH. The lower phase was evaporated to dryness under N_2, dissolved in 100 μl of carbon tetrachloride or methanol, and an aliquot was used for HPLC analysis.

HPLC

The HPLC analysis was performed with a Water's Associates (Milford, MA) model 6000A solvent delivery system and model U-6K injector. A variable wavelength spectromonitor from Schoeffel Instrument Co. (Westwood, NJ) was used at 280 nm.

For determination of total sphingoid bases, the biphenylcarbonyl derivatives of sphingoid bases were injected (5–20 μl volume) on MicroPak SI-10 (50 cm × 2.1 mm, i.d.) column. The solvent was acetonitrile–dichloromethane 7.5:2.5 (v/v) pumped at a flow rate of 0.5 ml/min.

The individual molecular species of sphingoid bases were analyzed and quantitated on a reversed-phase column. For this purpose Water's Associates "Fatty Acid Analysis" column (30 cm × 4 mm) was used initially. In later experiments an Ultrasphere-ODS 5-μm column (25 cm × 4.6 mm) from Altex Scientific Co. was used. The derivatized sphingoid bases were usually injected in 5–20 μl of carbontetrachloride or methanol. The solvent, tetrahydrofuran–methanol–water 25:40:35 or 25:40:20 (by volume), was pumped at a flow rate of 1–1.5 ml/min. In later experiments better HPLC resolution was achieved with an Accupak-ODS 3-μm (10 cm × 4.6 mm) column (Rainin Instruments, Woburn, MA) with either tetrahydrofuran–methanol–water 25:40:20 (by volume) or with methanol–water 94:6 (v/v) as the solvent. With the former solvent the flow rate was 1–2 ml/min; with the latter it was 0.4–1 ml/min. The peaks were integrated by a Spectra Physics computing integrator or by the "cut and weigh" method of the paper charts reproduced with a Xerox copier. The peak identification was with known standards and mass spectrometric analysis, when unknown peaks were observed.

TLC

The purity of the sphingoid bases and completeness of the derivatization reaction was checked by TLC on Merck or Analtech silica gel G thin-layer plates developed in either chloroform–methanol–water–15 M ammonia 7.2:4.8:0.9:0.2 (by volume) or chloroform–methanol–2 N ammonia 10:2.5:0.25 as the solvent. The sphingoid bases were detected by completely drying the plates and spraying with 0.05% fluorescamine (4-phenylspiro (furan-2(3H), 1'-phthalan)-3,3-dione) in acetone and viewing the plates under long wave-length (366 nm) ultraviolet light (17). By this procedure, as little as 500 pmol of a compound containing a free primary amine could be visually detected (17). To check the yield of the reaction each sphingoid base (100 nmol) was reacted with biphenylcarbonyl chloride as described previously and the products of the reaction were washed with theoretical upper phase containing NaOH. The reaction

products were spotted on a silica gel G TLC plate along with the unreacted sphingoid bases (500 pmol). After developing the plates in appropriate solvents and spraying with fluorescamine reagent, the spots were visualized by ultraviolet light. The plates were then sprayed again with 0.1% aqueous 8-anilinonaphthalene-1-sulfonate solution and viewed again under ultraviolet light (18). Bright fluorescence of the derivatized products could be seen. The reaction products were also analyzed on silica gel GF TLC plates. The derivatized sphingoids appeared as intense dark brown spots on the fluorescent background of the TLC plate under short ultraviolet light.

Gas–liquid chromatography–mass spectrometry

The biphenylcarbonyl derivatives of sphingoid bases (5–100 μg) were trimethylsilylated by adding 5–100 μl of hexamethyl disilazane–trimethylchlorosilane–pyridine–N,O-bis (trimethylsilyl) trifluoroacetamide 5:2:3:1 (by volume) and heating at 60°C for 15 min. The silylated derivatives were introduced into the mass spectrometer either after GLC or by the direct probe method. The GLC was performed on 1% OV 1 (3 ft × 2 mm i.d.) column heated isothermally at 320°C with helium as the carrier gas flowing at 20 ml/min. In some cases the GLC was also performed on 1% Dexsil 300 on 100–120 mesh Supelcoport (4 ft × 2 mm i.d.) heated from 280–320°C linearly at 5°C per min. When the direct probe method was used, 1–5 μg of the silylated sample was introduced in a cup and the probe was heated from 100 to 270°C at rate of 50° per min. The mass spectra were obtained with a Finnigan model 4000 quadrupole mass spectrometer equipped with Teknivent, model 56K data system. It was operated with an ionizing current of 0.5 MA and an ionizing voltage of 70 ev. The ionizing temperature was 250°C. Repetitive scans of the mass range from 100 m/e to 650 m/e were acquired at 5-sec intervals.

Comparison of quantitative results with a previously published method

For this purpose bovine brain sphingomyelin and cerebrosides, 0.5 mg each, in duplicate samples were hydrolyzed as described above. From each sample the

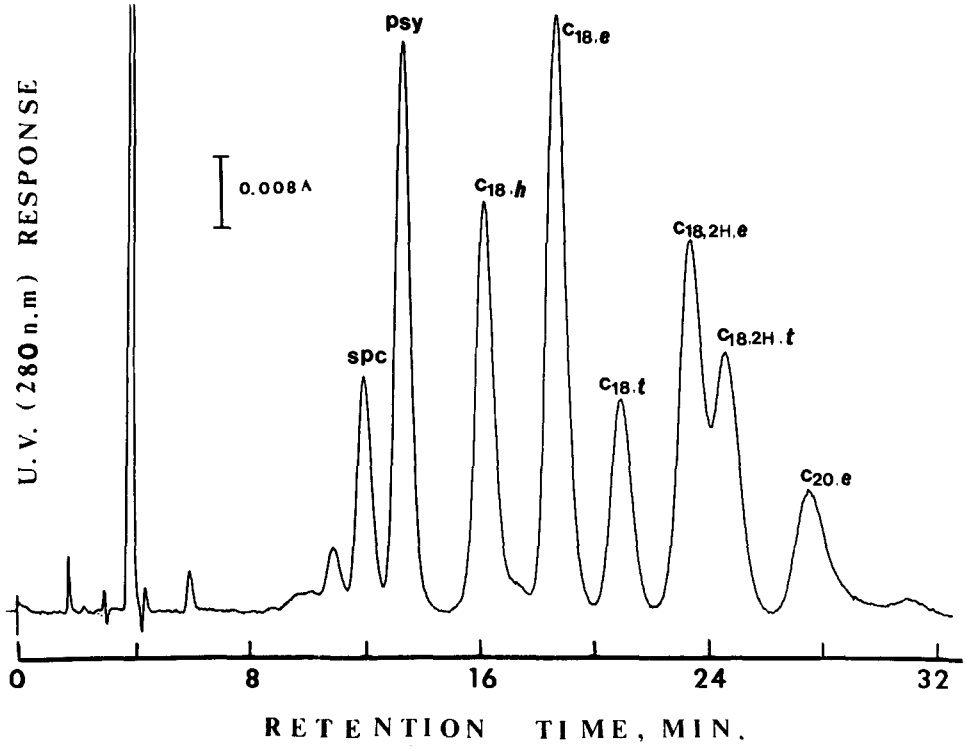

Fig. 1. HPLC analysis of biphenylcarbonyl derivatives of sphingosyl phosphorylcholine (SPC), 1.6 nmol; psychosine (PSY), 3.6 nmol; C_{18}-5-hydroxy sphinganine (C_{18},h), 3.3 nmol; *erythro*-C_{18}-sphingenine (C_{18}e); *threo*-C_{18}-sphingenine (C_{18},t) (total 7.6 nmol); *erythro*-C_{18}-sphinganine (C_{18}2H,e); *threo*-C_{18}-sphinganine (C_{18}2H,t) (total 7.2 nmol); and *erythro*-C_{20}-sphingenine (C_{20},e), 1.8 nmol on a fatty acid analysis column with tetrahydrofuran–methanol–water 25:40:35 (by volume) as the solvent pumped at 1 ml/min. C_{18}-sphengenine purchased from Supelco contained some *threo* isomer, whereas C_{18}-sphinganine contained almost 40% *threo* isomer. The mixture was dissolved in methanol and 20 μl was injected.

sphingoids obtained were equally divided into two portions. One half was reacted with biphenylcarbonyl chloride as described above and the other half of each sample was reacted to form the trimethylsilyl ether derivatives or N-acetyl trimethylsilyl ether derivatives and analyzed by GLC as described by Carter and Gaver (12).

RESULTS

Various sphingoid bases after derivatization with biphenylcarbonyl chloride and appropriate partitioning between solvents as described in Methods gave a single lipid derivative, with each sphingoid base, as analyzed by thin-layer chromatography. Free sphingoid bases were not detected on the TLC plates after the derivatization and on spraying with sensitive fluorescamine reagent (17), whereas 0.5 nmol of underivatized sphingoids spotted alongside were clearly seen, indicating that, of the 100 nmol of each sphingoid base reacted, less than 0.5% may have remained underivatized.

An HPLC analysis of a mixture of standard sphingoid bases is shown in **Fig. 1.** The separation was not only on the basis of chain length but the stereoisomers were also resolved. *Erythro* and *threo* isomers of C_{18}-sphinganine were not completely resolved on the fatty acid analysis column, but base line separation was achieved on an Accupak 3-μm column with either solvent described in the method. The small peak prior to sphingosylphosphorylcholine at about 11 min had a mass spectrum similar to that of sphingosylphosphorylcholine and thus appears to be an isomer of this compound. Other minor peaks in the chromatogram were not identified but they were probably minor impurities in the standards. The peak at 4 min was due to solvent and a side product biphenylamide which is formed during the washing of the products with ammoniacal solvent used in early studies. Formation of biphenylamide is avoided if the products are washed with upper phase containing 0.05 M NaOH instead of ammonia. Relatively more polar sphingoid bases were eluted earlier than the less polar sphingoid bases. For example C_{18}-5-hydroxysphinganine (phytosphingosine) was eluted earlier than C_{18}-sphingenine.

The analysis of sphingoid bases from sphingomyelin and cerebroside was performed after aqueous methanolysis of the lipids. In each case 80–90% of the expected theoretical yield of the sphingoid base was recovered after the hydrolysis. An HPLC analysis of the derivatized sphingoid bases from sphingomyelin on an Ultrasphere-ODS 5-μm column is given in **Fig. 2.** The major peak was identified as biphenylcarbonyl derivative of *erythro*-C_{18}-sphingenine. This was also confirmed by GLC–mass spectrometry of the eluted material after

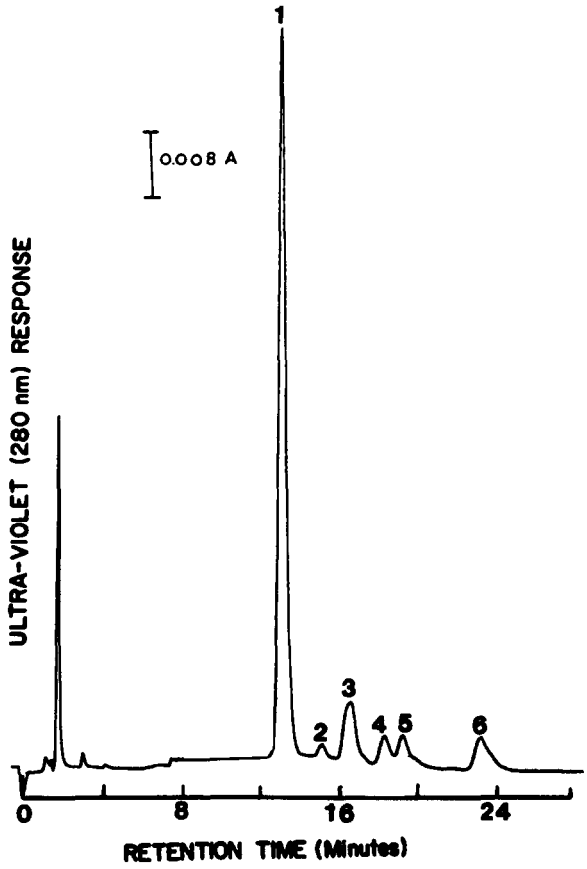

Fig. 2. HPLC analysis of sphingoid bases from bovine brain spingomyelin after derivatization with biphenylcarbonylchloride. Peak 1 is *erythro*-C_{18}-sphingenine; 2, *threo*-C_{18}-sphingenine; 3, 5-O-methyl-C_{18}-sphingenine; 4, *erythro*-C_{18}-sphinganine; 5, 3-O-methyl-C_{18}-sphingenine; and 6, *erythro*-C_{20}-sphingenine. The chromatography was on Altex Ultrasphere-ODS 5-μm column with tetrahydrofuran–methanol–water 25:40:20 (by volume) pumped at a flow rate of 1.5 ml/min. Sphingomyelin, 500 μg, was hydrolyzed as described in the methods section. The sphingoids obtained were dissolved in 500 μl of the chromatographic solvent and 5 μl was injected.

silylation. A minor peak 2 in the HPLC was identified as the biphenylcarbonyl derivative of *threo*-C_{18}-sphingenine. Peak 3 was identified as the biphenylcarbonyl derivative of 5-O-methyl-C_{18}-sphengenine from the GLC–MS analysis as shown in **Fig. 3A, Table 1.** The material eluted as peak 4 had retention time and GLC–MS identical to standard biphenylcarbonyl derivative of *erythro*-C_{18}-sphinganine. Peak 5 was identified to be a biphenylcarbonyl derivative of 3-O-methyl-C_{18}-sphingenine by GLC–MS analysis (Fig. 3b Table 1.). The sphingoid base eluted as peak 6 was the biphenylcarbonyl derivative of *erythro*-C_{20}-sphingenine. Under these chromatographic conditions, the *threo* isomer of C_{18}-sphinganine was not separated from 3-O-methyl C_{18}-sphingenine (peak 5). However, we saw little evidence of C_{18}-sphinganine in peak 5 by GLC–MS. As can be seen from the proportions of the *threo* isomer formed

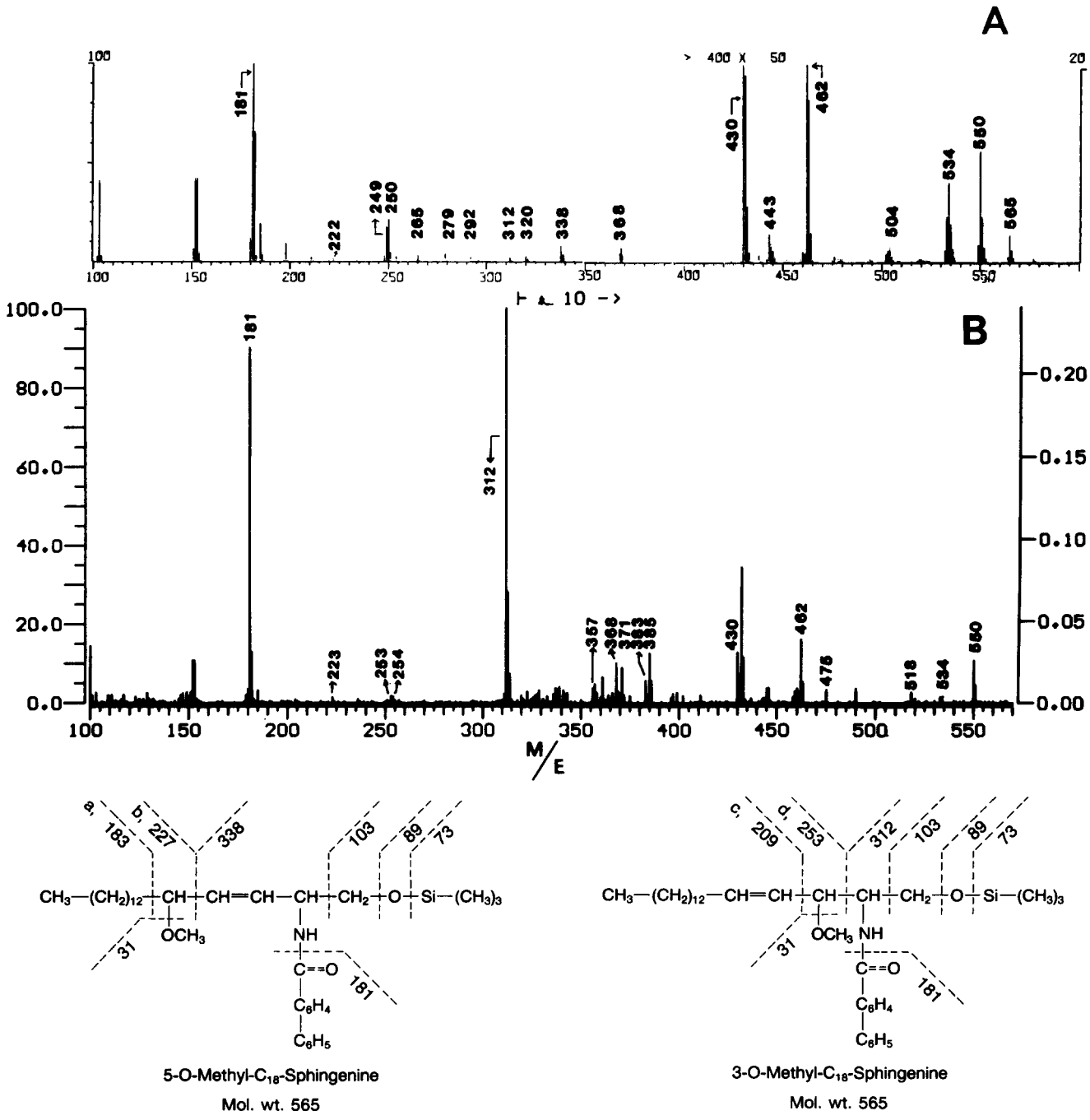

Fig. 3. Gas–liquid chromatography–mass spectrometry of the silylated biphenylcarbonyl derivative of 5-O-methyl-C_{18}-sphingenine, A; and 3-O-methyl-C_{18}-sphingenine, B. The GLC was performed on a 1% Dexsil 300 on 100–120 mesh Supelcoport as described in the methods section. The right ordinate represents percentage of the sum of total ion current.

from *erythro*-C_{18}-sphingenine (peak 1 and 2) it is likely that the formation of *threo*-C_{18}-sphinganine must have been negligible.

The HPLC analysis of sphingoid bases from bovine brain cerebroside is shown in **Fig. 4.** The sphingoid base pattern was similar to that described previously for sphingomyelin bases and the peaks were similarly characterized by GLC–MS analysis. Slightly more *threo*-C_{18}-sphingenine (peak 2) was formed from cerebroside than from sphingomyelin (Fig. 2). Peaks eluted between 8–12 min were not identified.

Quantitation of total sphingoid bases

The total amount of biphenylcarbonyl derivative of sphingoid bases was analyzed by normal-phase HPLC (**Fig. 5**). All sphingoid bases were eluted as a single peak

TABLE 1. Major ions in mass spectra of 5-O-Methyl- and 3-O-Methyl-C_{18} and C_{20} sphingenines

	5-O-Methyl-C_{18}-Sphingenine	3-O-Methyl-C_{18}-Sphingenine	5-O-Methyl-C_{20}-Sphingenine	3-O-Methyl-C_{20}-Sphingenine
M	565		593 (0.6)	
M − 15	550	550	578 (2.1)	578 (2.0)
M − OCH_3	534	534	562 (1.2)	
M − OCH_3 − 16		518	546 (0.5)	546 (0.7)
M − OCH_3 − 30	504		532 (0.4)	
M − 89	476		504 (0.3)	504 (0.5)
M − 90	475	475	503 (0.3)	
M − 103	462	462	490 (8.5)	490 (1.4)
M − 90 − OCH_3 + 1	443		471 (1.4)	
M − 103 − OCH_3 + 1	430	430	458 (6.4)	458 (0.7)
*M + 73 − d		385		385 (0.5)
M − (181 + 1)		383		411 (0.5)
*M + 73 − d − 14		371		371 (1.5)
M − (196 + 1)	368	368	396 (2.3)	396 (2.7)
*M − b	338		338 (1.9)	
*M − d		312		312 (100)
M − b − 26	312		312 (5.0)	
*M − 183 − 90	292		292 (12.8)	
*M − c + 1		357		
*292 − CH	279		279 (6.0)	
*M − b − 18	320		320 (2.1)	
*M − b − 73	265			
M − d − 73 + 15		254		254 (3.1)
M − b − 26 − 73 + 15	254		254 (2.3)	
*M − 312		253		
*M − OCH_3 − 181 − 103	250		278 (15.1)	
*M − b − 89	249		249 (8.8)	
*M − d − 89		223		
*292 − OCH_3 − 39	222		222 (2.0)	
Biphenylcarbonyl	181	181	181 (100)	181 (91.1)

* Represents specific ions for either 3-O-methyl or 5-O-methyl sphingoid base. Numbers in parentheses represent percent relative intensity as compared to base ion which is 100%. a, Represents fragment m/z 183 or 211; b, 227 or 255; c, 209 or 237; and d, 253 or 281 for 5-O-methyl and 3-O-methyl C_{18} or C_{20}-sphingenine (see Fig. 3).

under these conditions and thus quantitated by comparison with known amounts of standard sphingoid bases. If one is interested in simply determining the total amount of sphingoid bases in a sample, this procedure is simple and rapid, rather than analysis of the bases by reversed-phase HPLC.

Quantitation of individual sphingoid bases

This was achieved after analysis of the biphenylcarbonyl derivatives on a reversed-phase HPLC column. Different amounts (0.33–495 nmol, i.e., 0.1–150 μg) of *threo*-C_{18}-sphinganine samples were reacted, in duplicate, with biphenylcarbonyl chloride as described above. A suitable portion, 3.3 nmol (1 μg) or, in the case of microassay, 0.165–0.33 nmol (0.05–0.1 μg), was injected in a 10-μl volume of carbon tetrachloride onto an Ultrasphere-ODS, 5-μm column, with tetrahydrofuran–methanol–water 25:40:20 (by volume) as the solvent at a flow rate of 1.5 ml/min. Each sample was injected twice. The detector response at 280 nm was measured with a sensitivity setting at 0.08 absorbance full scale. The peak area was determined by a "cut and weigh" method. The total peak area for the entire sample was calculated in terms of mg of paper weight. **Fig. 6** shows a linear relationship between the amount of the sphingoid base reacted and the total response obtained. The inset shows the lower end of the curve when plotted on a different scale. The response due to 167–495 nmol of *threo*-C_{18}-sphinganine was also linear (not shown). The coefficient of variation due to different samples (0.33–495 nmol) after the entire procedure, i.e., derivatization and HPLC analysis, was 7.8%. Thus it was possible to derivatize and quantitate as little as 0.33 nmol (1 μg) of sphingoid base by this method.

The quantitative responses for equal nmole amounts of C_{18}-sphingenine, phytosphingosine, and psychosine were also determined by the same method. They were equivalent to that for C_{18}-sphinganine. This indicated that only one biphenylcarbonyl moiety was conjugated with the free amine of the spingoid bases and the response at 280 nm was almost entirely due to biphenylcarbonyl chromophore. In some cases, C_{18}-psychosine was used as an internal standard since it is eluted before the sphingoid derivatives with minimal interference and it contains galactose, which is easily calibrated by known methods (19). C_{18}-psychosine should be added to the

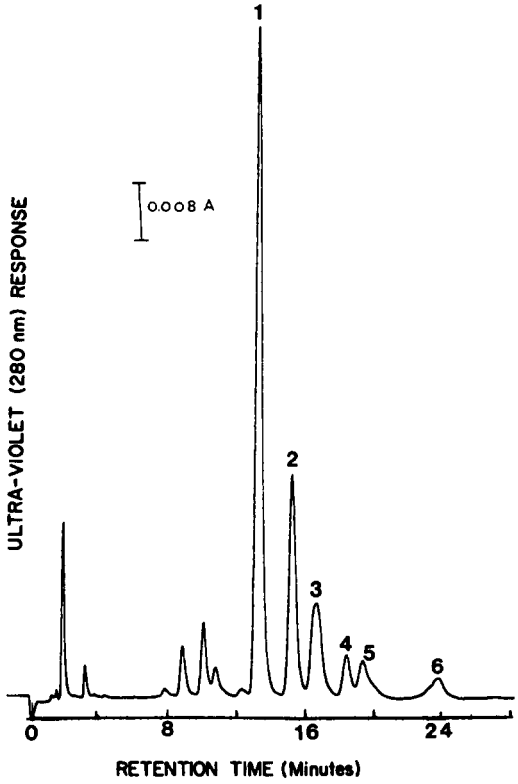

Fig. 4. HPLC analysis of sphingoid bases from bovine brain cerebrosides after derivatization with biphenylcarbonylchloride. Peak identification is as given in Fig. 2. The chromatographic conditions were the same as in Fig. 2. Cerebrosides, 500 μg, were hydrolyzed as described in the methods section. The sphingoids obtained were dissolved in 500 μl of the chromatographic solvent and 5 μl was injected.

sphingoid bases obtained after hydrolysis of spingolipid samples and prior to derivatization. The amount of C_{18}-psychosine added should be about equivalent to the expected amount of sphingoid bases from sphingolipids. If only relative percentage composition of sphingoids in a sphingolipid is desired, addition of psychosine is not necessary.

The percentage distribution of various sphingoid bases derived from bovine brain sphingomyelin and cerebroside is given in **Table 2.** The present method is compared with the results obtained by the GLC method previously published by Carter and Gaver (12). Since *threo* isomer of the sphingoid bases and 3-O and 5-O-methyl-C_{18}-sphingenine are formed from the corresponding *erythro* C_{18}-sphingenine, the relative percentages should be added up to represent total C_{18}-sphingenine. The results obtained by either of the methods were quite similar.

DISCUSSION

Although a number of procedures have been published for the analysis of sphingoid bases isolated from sphingolipids from natural sources, the most generally used procedure is the gas–liquid chromatographic analysis of the volatile trimethylsilyl derivatives of the bases (11, 12) or the aldehydes liberated by periodate oxidation (10). However, for the absolute assignment of the chemical structure of a variety of complex sphingoid bases present in a mixture, it is necessary to use combined gas–liquid chromatography–mass spectrometry procedure since gas–liquid chromatographic retention behavior by itself is less certain (20–24). Again, the quantitative analysis of spingoid bases isolated after acid methanolysis of the sphingolipids is further complicated by the variety of side products formed during the methanolysis. The major such side products were the 5-O-methyl and 3-O-methyl derivatives of the sphingoid bases, the *threo* isomers and sometimes a compound formed by migration of the 3-OH group of the sphingoid chain to form the 5-hydroxy isomer (25). We have described here an alternate procedure for the analysis of sphingoid bases by the use of HPLC. The resolution obtained with the reversed-phase HPLC columns was quite satisfactory since both *erythro* and *threo* isomers of the commonly occurring sphingoid bases were resolved. The quantitative analysis of sphingoids from bovine brain sphingomyelin and cerebrosides indicate that a larger amount of 5-O-methyl-C_{18}-sphingenine than 3-O-methyl-C_{18}-sphingenine was formed as a side product of methanolysis. The mass spectral analyses of both these compounds support their assigned structures. The base ion for the 3-O-methyl-C_{18}-sphingenine was at 312 which is obtained by the expected cleavage of carbon 2 and 3 of the sphingoid chain (Fig. 3, Table 1). This

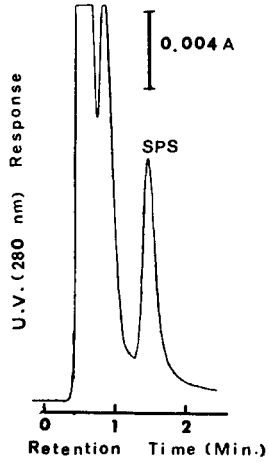

Fig. 5. HPLC analysis of biphenylcarbonyl derivative of total sphingoids (SPS) on a Micro Pak-SI-10 (50 cm × 2.1 mm) column with acetonitrile–dichloromethane 7.5:2.5 (by volume) pumped at 0.5 ml/min. Derivatized sphingoid bases, 0.33 nmol (0.1 μg), from sphingomyelin dissolved in 20 μl of carbon tetrachloride were injected.

ion is not so prominent in the 5-O-methyl-C_{18}-sphingenine. The characteristic ions at 338, 320, 292, 279, 265, 269, 250, and 222 strongly support the presence of OCH_3 at carbon atom 5 and a double bond between carbon atoms 3 and 4 of the spingoid chain. These ions are not present to any significant extent in the mass spectrum of the 3-O-methyl-C_{18}-sphingenine. The characteristic ions at 312, 385, 371, 253, and 223 in the mass spectrum of the 3-O-methyl-C_{18}-sphingenine support the position of OCH_3 at 3 position and a double bond between carbon atom 4 and 5. The rest of the ions were common to both the compounds as expected. We have also analyzed the mass spectrum of 5-O-methyl and 3-O-methyl-C_{20} sphingenine (Table 1) obtained after the acid hydrolysis of mixed gangliosides from brain. The mass spectra were in complete agreement with the expected structures of these compounds. The reversed-phase chromatography elution pattern of these compounds also supported the proposed structures of the 3-O and 5-O-methyl-C_{18}-sphingenines. The biphenylcarbonyl derivative of 5-O-methyl-C_{18}-sphingenine eluted before the same derivative of the 3-O-methyl compound. The retention on the column is dependent upon the hydrophobic interaction between octadecyl residues of the adsorbent and mainly the alkyl chains of the sphingoid bases (26, 27). In the case of 5-O-methyl derivative the surface area of contact is reduced by two carbon atoms due to the position of O-methyl group at carbon 5, compared to that of the 3-O-methyl derivative.

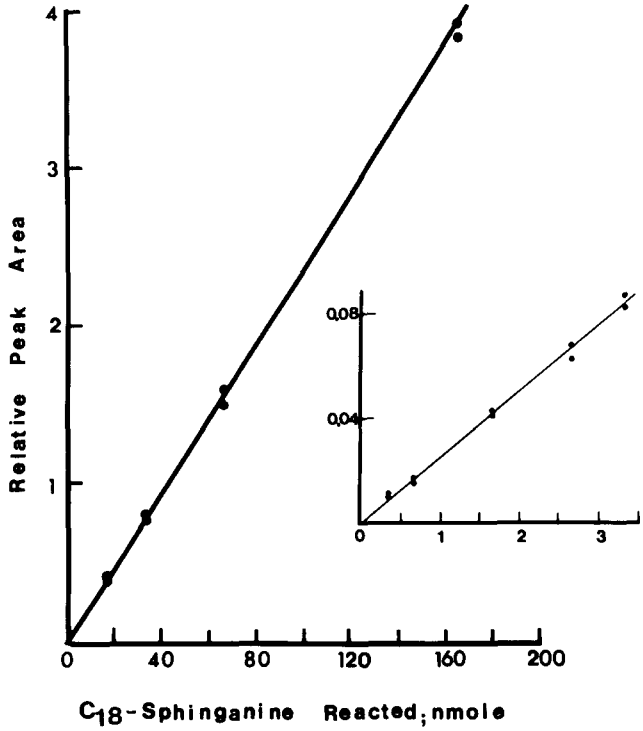

Fig. 6. Quantitative analysis of sphinganine by HPLC. The amounts indicated of C_{18}-sphinganine were reacted with biphenylcarbonyl-chloride in duplicate tubes, and a suitable portion of the sample was injected twice onto the Ultrasphere-ODS 5-μm column. The response was measured in terms of peak weight. The inset shows quantitative analysis between the range of 0.33–3.3 nmol of C_{18}-sphinganine. Other details are in the text.

TABLE 2. Percentage distribution of sphingoid bases in sphingomyelin and cerebroside of bovine brain

Base	Sphingomyelin		Cerebroside	
	HPLC	GLC	HPLC	GLC
	%		%	
C_{18}-Sphingenine				
-erythro	81.5		72.9	
-threo	0.5	79.0	6.6	80.1
-5-OCH_3	1.0		8.0	
-3-OHC_3	0.5	2.4	1.0	6.5
C_{18}-Sphinganine				
-erythro	7.0	9.1	8.5	11.2
C_{20}-Sphingenine				
-erythro	9.5	9.5	3.0	2.2

Sphingomyelin and cerebroside (0.5 mg each) were hydrolyzed in duplicate samples. One half of each sample was analyzed by HPLC as described in the methods section. The other half was analyzed by GLC procedure (12). The GLC procedure does not separate *erythro* and *threo* isomers as well as 5-O and 3-O-methyl species, therefore they are given together. The results are the average of two determinations.

The HPLC method described here for the analysis of sphingoid bases is relatively simple, specific, and sensitive. It is more specific than the GLC method since only compounds having free amines will react with the derivatizing reagent, biphenylcarbonylchloride, and be specifically analyzed by the HPLC method at 280 nm. With the GLC method, compounds having a number of reactive groups (such as OH, NH_2, or SH) are derivatized with the silylating reagents and analyzed by the nonspecific GLC detectors. The GLC method also does not resolve the stereoisomers satisfactorily (12). Again the range of the method is large so that one can analyze as little as 0.1 μg of the sphingoid base or scale up the procedure to prepare mg amounts of the bases for separation, collection, and determination of the structure of unknown sphingoid bases. The HPLC method has been applied to the analysis of sphingoids from various neutral glycosphingolipids and gangliosides from brain, sciatic nerves, kidney, and other tissues in our laboratory.

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