Acetonitrile-hydrochloric acid hydrolysis of gangliosides for high performance liquid chromatographic analysis of their long chain bases

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Abstract An improved method for the hydrolysis of long chain bases from gangliosides with aqueous acetonitrile-HCl is described. The long chain bases released from brain gangliosides were derivatized with biphenylcarbonylchloride and resolved by high performance liquid chromatography on a C_{18} reversed-phase column. Components of individual peaks were identified by gas–liquid chromatography–mass spectrometry as their trimethylsilyl derivatives. The acetonitrile-HCl hydrolysis procedure resulted in no formation of O-methyl ethers of long chain bases and a significant decrease in the level of secondary products.—**Kadowaki, H., E. G. Bremer, J. E. Evans, F. B. Jungalwala, and R. H. McCluer.** Acetonitrile-hydrochloric acid hydrolysis of gangliosides for high performance liquid chromatographic analysis of their long chain bases. *J. Lipid Res.* 1983. **24:** 1389–1397.

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Gangliosides are defined as glycosphingolipids that contain sialic acid. They were first identified by Klenk (1) as components of brain and have since been found to occur at lower concentrations in all extraneural tissues (2). Brain gangliosides are known to contain characteristically high concentrations of C_{20}-sphingosine (3). Studies with rats and humans indicate that, at birth, brain gangliosides have mainly C_{18}-sphingosine and the proportion of C_{20}-sphingosine increases during maturation eventually reaching 50% (4, 5). Studies on individual gangliosides from human brain have indicated similar changes (6, 7). The ceramide residues of individual bovine brain gangliosides are reported to have an increased content of C_{20}-sphingosine corresponding to the increased number of sialic acid residues in the sugar moieties (8).

The sphingosine content and composition of glycosphingolipids have been analyzed by a variety of methods (9). These analyses are complicated by the occurrence of allylic rearrangements during acid hydrolysis which apparently take place as a result of the formation of a carbonium ion. The carbonium ion can give rise to diastereomers at carbon atom 3, migration of the double bond and diastereomers at carbon 5, formation of analogous O-methyl ethers when the hydrolysis is performed in acidic methanol, and the formation of dehydration products (9, 10). A commonly used method for the liberation of long chain bases (LCBs) is aqueous acid methanolysis described by Gaver and Sweeley (11) which minimizes the production of O-methyl ethers. These conditions, suitable for most sphingolipids, do not completely hydrolyze gangliosides. Ando and Yu (12) have utilized stronger acid conditions to liberate ganglioside LCBs. Analysis of the ganglioside LCBs by HPLC of their biphenylcarbonyl derivatives, a procedure recently developed in this laboratory (13), has revealed that these stronger aqueous acid methanolysis conditions result in the formation of an excessive number of secondary LCB products, particularly the O-methyl ethers. In order to eliminate the production of O-methyl ethers during the liberation of ganglioside LCBs, we have devised a procedure for the hydrolysis of gangliosides in the aprotic solvent acetonitrile. This hydrolysis procedure coupled with the HPLC analysis has the advantages of low yield of secondary products, high sensitivity, and nondestructive analysis of the LCBs. We report here the conditions for the hydrolysis of gangliosides, HPLC separation of the liberated bases, their identification by mass spectrometry, and the use of this procedure for quantitative analysis of ganglioside LCBs.

Abbreviations: HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; LCB, long chain base; MS, mass spectrometry. The LCB abbreviations as suggested by Breimer et al. (21) are utilized throughout.

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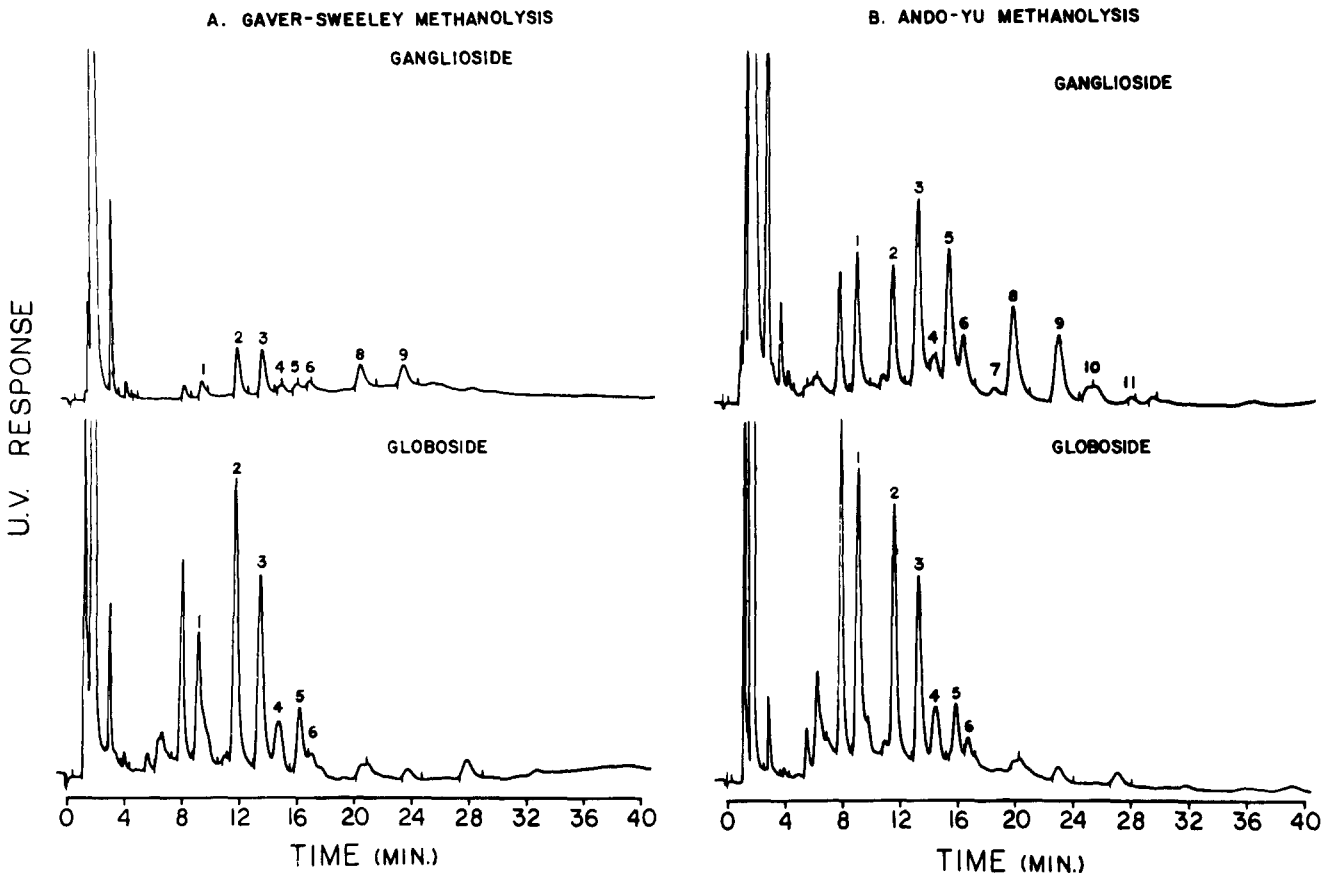

Fig. 1. HPLC of LCBs obtained from globoside and ganglioside by the aqueous methanolic-HCl procedures of Gaver and Sweeley (A) and Ando and Yu (B). After hydrolysis, the LCBs were extracted, derivatized with biphenylcarbonyl chloride, and separated by HPLC. The mobile phase was tetrahydrofuran–methanol–water 25:40:20 (by volume) with a flow rate of 2 ml/min and with a sensitivity of 0.16 absorbance unit full scale (AUFS). The ganglioside LCB peaks from the Ando and Yu hydrolysis were collected and identified by GLC–MS. The compositions of the peaks are indicated as: 1, d18:1(5-OH) with small amounts of d18:2; 2, *erythro*-d18:1; 3, *threo*-d18:1; 4, d18:1(5-O-methyl) (major component) plus unidentified LCB; 5, d18:0 plus d18:1(5-O-methyl); 6, d18:1(3-O-methyl) (major component) with d20:1(5-OH); 7, unidentified LCB; 8, d20:1 (major component) with unidentified LCB; 9, *threo*-d20:1 (major component) with unidentified LCB; 10, d20:1(5-O-methyl) with d20:0; 11, d20:1(3-O-methyl). The component which elutes just before peak 1 is from the reagent.

MATERIALS AND METHODS

Chemicals

The solvents used for HPLC were spectral grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI). Solvents used for extraction and TLC were reagent grade (Fisher Chemical Co., Fairlawn, NJ) and were used without prior distillation. 4-Biphenylcarbonyl chloride was obtained from Aldrich Chemical Co. (Milwaukee, WI) and bis(trimethylsilyl)trifluoroacetamide was from Supelco (Bellefonte, PA). Gangliosides were prepared from human and beef brains as previously described (14) or by the methods of Svennerholm (15). Globoside ($GbOse_4Cer$) was prepared from human erythrocytes as previously described (16).

Liberation of LCBs

The method of Gaver and Sweeley (11), which involves heating the ganglioside with aqueous methanolic HCl (1 N HCl and 10 M H_2O) at 70°C for 18 hr, and the method of Ando and Yu (12), which involves the same hydrolysis conditions but with aqueous methanol containing 2 N HCl, were used. The aqueous acetonitrile-HCl reagent contained 0.5 N HCl and 4 M H_2O in acetonitrile. Gangliosides (10–200 μg) were placed in 8-ml Teflon-lined screw-capped tubes and 0.3 ml of the aqueous acetonitrile-HCl reagent was added. The tubes were flushed with nitrogen, capped, and heated at 75°C for 2 hr with occasional shaking. After hydrolysis, the samples were taken to dryness with a stream of nitrogen and the LCBs were extracted and derivatized with biphenylcarbonyl chloride as described by Jungalwala et al. (13). Briefly, these procedures involved suspension of the dried sample in 1 ml of solvent A (methanol–0.9% saline–chloroform 48:47:3, by volume) containing 0.05 N NaOH and extraction with 5 ml of solvent B (chloroform–methanol–water 86:14:1, by volume). The organic lower phase was washed three

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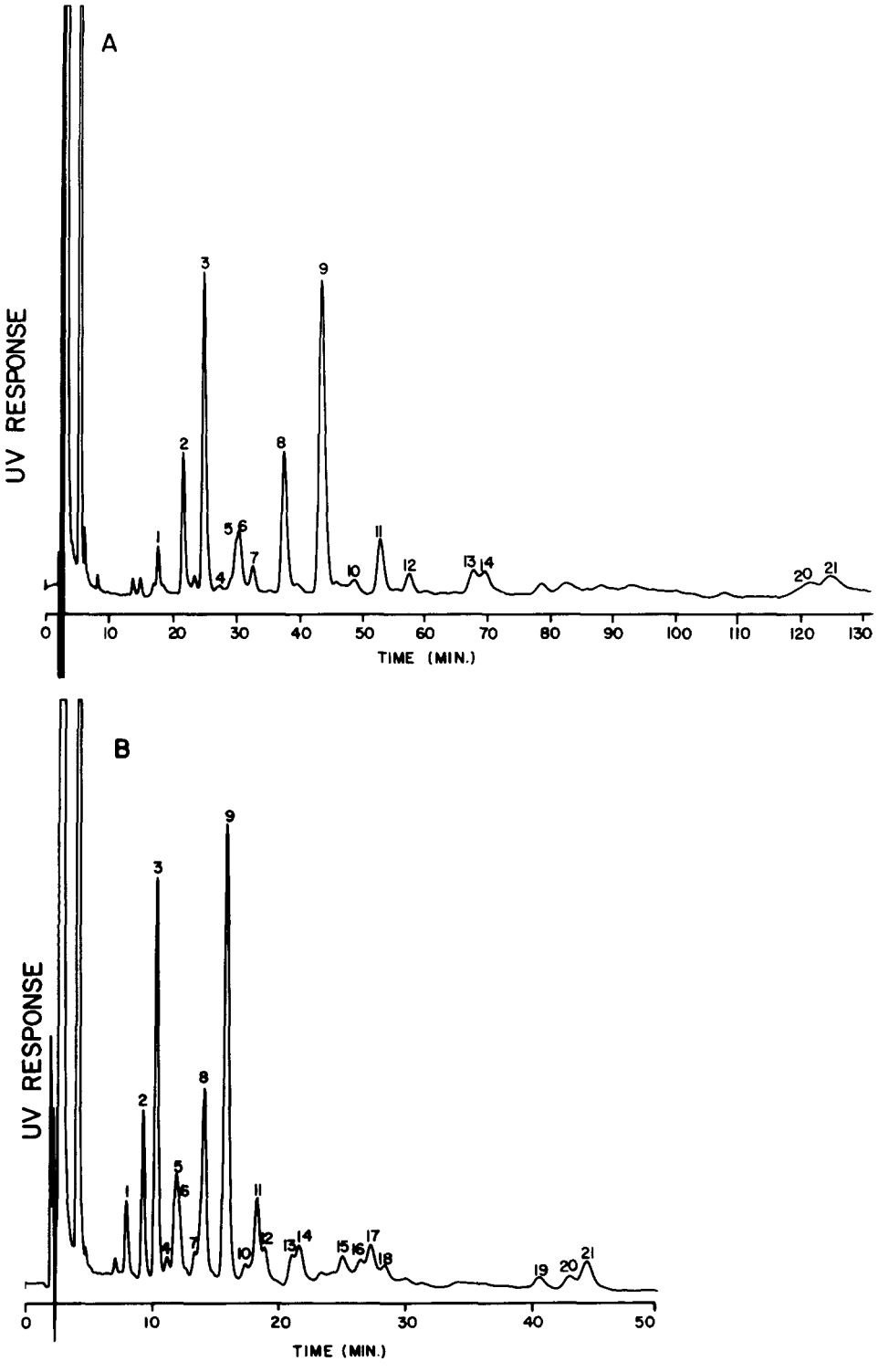

Fig. 2. HPLC of LCBs obtained from human brain gangliosides by aqueous acetonitrile-HCl hydrolysis. The LCBs after hydrolysis were extracted and derivatized with biphenylcarbonyl chloride as described in Materials and Methods. The mobile phases were (A) tetrahydrofuran–methanol–water 25:40:20 (by volume) with a flow rate of 1.0 ml/min, and (B) methanol–water 94:6 (by volume) with a flow rate of 1.0 ml/min. In the example shown, LCBs equivalent to 50 μg (A) and 30 μg (B) of human brain gangliosides were chromatographed with 0.1 AUFS. Peaks are numbered in sequence of elution and are identified in Table 2. Peaks in Fig. 2A which do not correspond to those in Fig. 2B were not numbered.

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TABLE 1. Major ions identified in mass spectra of trimethylsilyl biphenylcarbonyl long chain bases

	d18:1 (5-OH)	d18:1	d18:0	m18:2	d20:1 (5-OH)	d20:1	d20:0	m20:2
Molecular weight	623	623	625	533	651	651	653	561
Ions								
M				533 (2.7)[a]			653 (0.7)	561 (2.5)
M-15	608 (1.0)	608 (1.4)	610 (2.6)	518 (4.3)		636 (1.2)	638 (19.3)	546 (4.2)
M-90	533 (1.3)	533 (0.7)		443 (3.3)		561 (0.6)	563 (2.6)	471 (2.2)
A = (M-103)	520 (14.4)	520 (0.8)	522 (2.8)	430 (95.4)	548 (3.9)	548 (0.6)	550 (16.9)	458 (81.6)
M-2X90	443 (1.2)	443 (0.9)	445 (0.7)			471 (1.0)	473 (1.4)	
M-103-90	430 (17.6)	430 (7.7)	432 (3.2)		458 (6.3)	458 (9.1)	460 (12.2)	
B = (M-196)	427 (1.4)	427 (2.5)	429 (2.9)	337 (0.8)		454 (4.0)		364 (1.0)
C = (M-312)	311 (1.8)	311 (36.1)	313 (21.0)	221 (12.6)		339 (15.2)	341 (43.5)	249 (1.7)
M-C	312 (2.7)	312 (100.0)	312 (18.4)	312 (1.0)		312 (42.7)	312 (20.6)	312 (0.8)
M-C-89	223 (1.8)	223 (2.7)	223 (40.5)	223 (3.6)	223 (1.4)	223 (3.0)	223 (51.4)	223 (2.1)
M-C-90	222 (2.9)	222 (3.7)	222 (8.4)	222 (5.3)	222 (2.1)	222 (4.2)	222 (9.3)	222 (4.0)
B-D	243 (8.2)	243 (5.1)		178 (9.3)		243 (3.0)		
Biphenylamide	197 (5.4)	197 (4.8)	197 (10.8)	197 (12.9)	197 (6.6)	197 (6.4)	197 (8.0)	197 (7.7)
Biphenylcarbonyl	181 (100.0)	181 (86.5)	181 (100.0)	181 (100.0)	181 (59.0)	181 (73.7)	181 (97.9)	181 (100.0)

[a] Relative intensities of major ions are presented in the parentheses.

times with 1 ml of the alkaline solvent A and three times with 1 ml of solvent A without NaOH and then taken to dryness with a stream of nitrogen. Fifty μl of 1% biphenylcarbonyl chloride in tetrahydrofuran and 100 μl of 50% aqueous sodium acetate were added and the biphasic mixture was agitated at room temperature for 1.5 hr. After the agitation, 5 ml of solvent B was added to the reaction mixture. The mixture was then shaken and centrifuged; the upper phase was removed and the lower phase was washed twice with 1 ml of methanol–0.9% saline–chloroform–15 M NH_4OH 96:92:2:3 (by volume) followed by three washes with solvent A. The lower phase was evaporated to dryness and dissolved in methanol for HPLC analysis.

HPLC

The HPLC analysis was performed with a Water's Associates (Milford, MA) model 6000A solvent delivery system and model U6K injector, or with a Varian model 5020 liquid chromatograph. A Schoeffel (Westwood, NJ) variable wavelength monitor set at 280 nm was utilized for detection. The biphenylcarbonyl derivatives of the LCBs were separated by reversed-phase chromatography as described (13). An Ultrasphere-ODS 5–μm column (25 cm × 4.6 mm) from Altex Scientific (Berkeley, CA) was utilized. Two solvent systems, tetrahydofuran–methanol–water (25:40:20, by volume) and methanol–water (94:6, by volume), were used as indicated.

GLC–MS

The biphenylcarbonyl derivatives of LCBs were collected from HPLC, trimethylsilylated and analyzed by direct probe MS or GLC–MS as described previously (13). Mass spectrometry was performed with a Finnigan 4000 mass spectrometer equipped with a Technivent 56K data system.

Hydrogenation

Twenty mg of palladium chloride coated on charcoal (17) was stirred with 4 ml of methanol under hydrogen gas (1 atm) at room temperature for 3 hr. The $Pd(H_2)$-charcoal was collected by filtration and mixed with 500 μg of ganglioside in 3 ml of methanol and stirred overnight under hydrogen gas. The hydrogenated ganglioside-charcoal was collected by filtration and the gangliosides were separated from charcoal by washing with 10 ml of chloroform–methanol (2:1, by volume).

RESULTS

HPLC analysis of ganglioside and globoside LCBs obtained by aqueous acid methanolysis procedures

Ganglioside G_{M1} and globoside were subjected to aqueous acid methanolysis according to Gaver and Sweeley (11) and to the stronger conditions recommended for gangliosides by Ando and Yu (12). The LCBs were isolated and analyzed by HPLC as their biphenylcarbonyl derivatives. These results are shown in **Fig. 1.** The yield of LCBs obtained by the Gaver and Sweeley method was over 95% for globoside, but was only about 20% of the expected quantity from ganglioside. The stronger acid conditions of the Ando and Yu method provided a high yield of products from both globoside and ganglioside, but the HPLC pattern for

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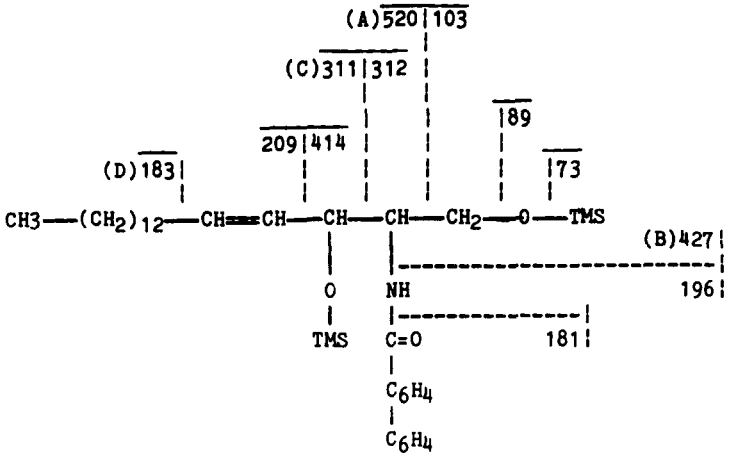
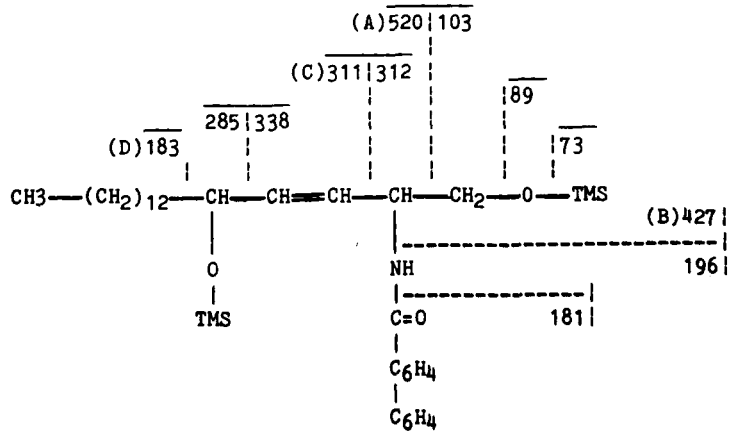


Trimethylsilylated biphenylcarbonyl C_{18}-erythro-sphingenine [d18:1]
Mol wt 623



Trimethylsilylated biphenylcarbonyl 1,5-dihydroxy-2-amino-3-octadecene [d18:1(5-OH)]
Mol wt 623

Fig. 3. MS fragmentation patterns of d18:1 LCBs. The differences in fragment ion intensities from 1,3- and 1,5-dihydroxy-LCBs are analogous to those reported for the O-methyl ethers of LCBs (13).

ganglioside LCBs was complex. The LCB derivatives from ganglioside were isolated by HPLC and analyzed by GLC-MS as their trimethylsilyl derivatives. These results indicated that many of the components separated by HPLC were heterogeneous, particularly the dihydrosphingosine (d18:0), C_{20}-*erythro*-sphingosine (*erythro*-d20:1), and C_{20}-*threo*-sphingosine (*threo*-d20:1). The peak heterogeneity resulted primarily from the presence of *erythro*- and *threo*-5-O-methylsphingosines, and *erythro*- and *threo*-3-O-methylsphingosines as well as the 5-OH rearrangement products of d18 and d20 LCBs. It was clear from the GLC–MS data, that the aqueous acid methanolysis was unsatisfactory for HPLC analysis of the LCBs from brain gangliosides.

Acetonitrile hydrolysis

Because the O-methylsphingosines were responsible to a large extent for the heterogeneity of the components separated by HPLC, hydrolysis of ganglioside in the aprotic solvent acetonitrile was examined. Preliminary studies (18) revealed that mixtures of concentrated HCl and acetonitrile resulted in the liberation of LCBs without the formation of peaks previously identified as O-methyl derivatives. However, degradation products were produced under strong acid conditions and prolonged heating. The formation of these side products was found to be suppressed by increasing the water content of the hydrolysis reagent. Optimal yields of LCBs

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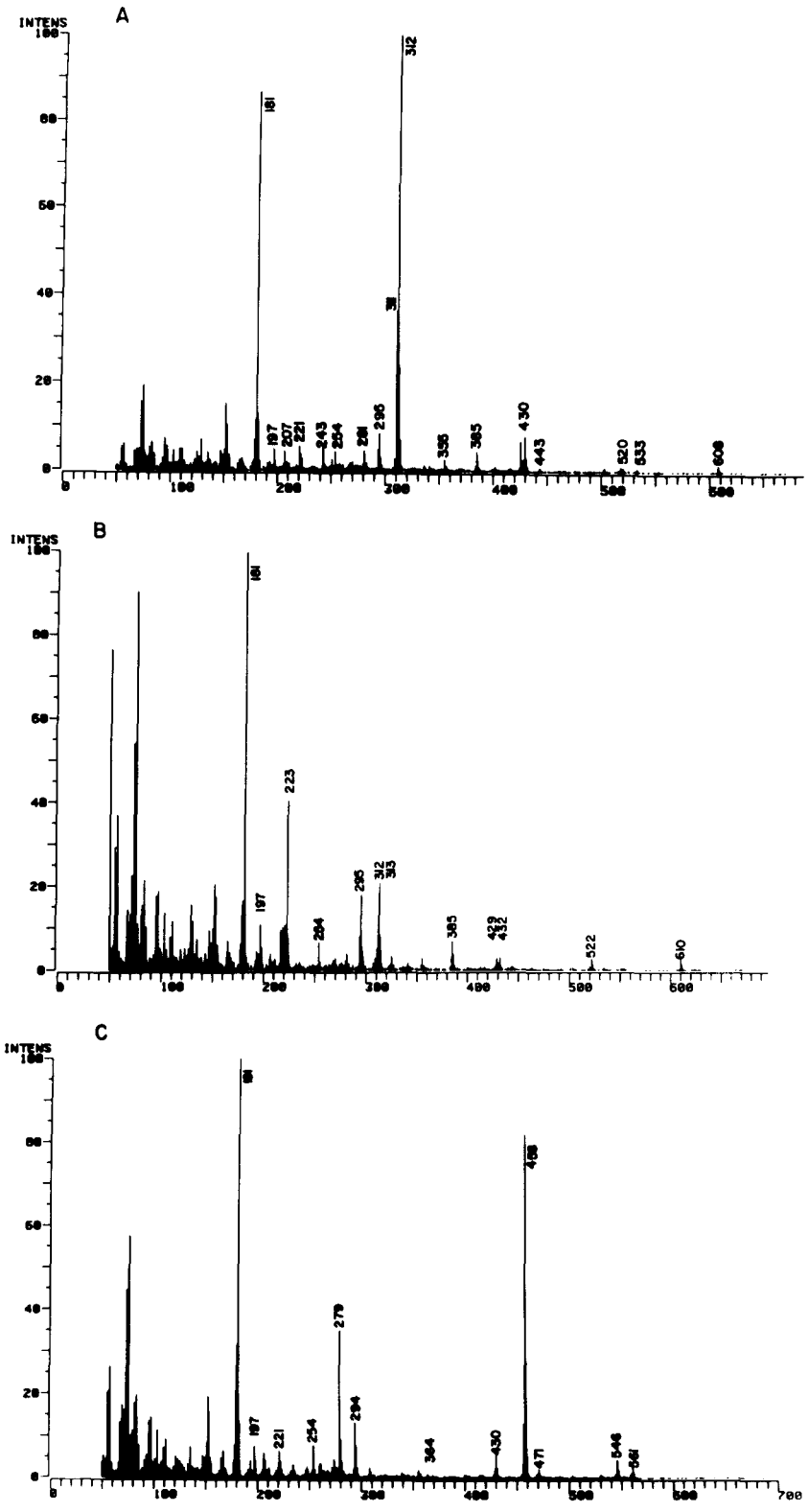

Fig. 4. Mass spectra of trimethylsilylated biphenylcarbonyl LCBs: (A), *erythro*-d18:1; (B), *erythro*-d18:0, (C), m20:2. The conditions for ammonia CI–MS were: electron energy, 70 eV; ion source pressure, 0.3 torr; ion source temperature, 250°C; conversion dynode 3 kV, preamplifier 10-7 Amp/V. Ordinate, relative intensity (percent); abscissa, m/z.

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with the least formation of degradation products were obtained by hydrolysis with 0.5 N HCl in aqueous (4 M) acetonitrile reagent at 75°C for 2 hr. HPLC of the ganglioside LCBs obtained under these conditions using two different mobile phases is shown in **Fig. 2:** tetrahydrofuran–methanol–water (solvent 1) and methanol–water (solvent 2). Solvent 1 was preferable for determination of LCB composition and solvent 2 was preferable for recovery studies and analysis of rearrangement products. Some differences in peak resolution between these two solvent systems were observed, but the calculated relative amounts of major components were essentially identical.

The peaks, as shown in Fig. 2B, were collected and examined by GLC-MS as their trimethylsilyl ethers. Major ions identified by GLC–MS are presented in **Table 1** and MS fragmentation patterns of the derivatized d18:1 and d18:1(5-OH) LCBs, as examples, are shown in **Fig. 3.** Representative mass spectra of the naturally occurring LCBs, *erythro*-d18:1 (peak 2), *erythro*-d18:0 (peak 5), and of the side product of acid hydrolysis, m20:2 (peak 21), are shown in **Fig. 4.** The major secondary products of LCBs from gangliosides by aqueous acetonitrile-HCl hydrolysis appeared to be varieties of monohydroxydienes. To provide confirmation of monohydroxydiene structures, peaks 15, 16, 17, and peaks 19, 20, 21 (Fig. 2B) were collected together and reduced with hydrogen in the presence of Pd on charcoal. HPLC–MS analysis indicated that upon reduction the cluster of peaks 15, 16, 17 formed m18:0 and the cluster of peaks 19, 20, 21 formed m20:0. A summary of peak identifications is presented in **Table 2.** Although some dehydration products were formed by aqueous acetonitrile-HCl hydrolysis, 81.8 ± 2.0% (mean ± SD for seven observations) of LCBs were identified as naturally occurring bases or their isomerization products.

To verify that the isomers and dehydration derivatives of C_{18} and C_{20} sphingosine were all side products formed during acid hydrolysis as a consequence of the allylic double bond, the brain gangliosides were directly reduced with hydrogen over Pd on charcoal prior to aqueous acetonitrile-HCl hydrolysis. The products were examined by HPLC and GLC–MS. Only the *erythro*-isomers of d18:0 and d20:0 were detected along with products identified as monohydroxy LCBs (m18:0 and m20:0). The latter products presumably resulted from elimination of H_2O from the 3-OH group of LCBs during the hydrogenation procedure in a reaction analogous to that reported by Carter (19) for acetylsphingosine.

Hydrolysis conditions, yield, quantitation, and sensitivity

The optimum hydrolysis time was determined by measurement of the total LCB peak area obtained after periods up to 2 hr. Maximum yields were obtained after 100 min and remained constant up to 2 hr, and the ratios of the different LCBs remained constant at these hydrolysis periods. However, prolonged hydrolysis resulted in increased amounts of the side products. The yield of total LCBs from brain gangliosides during the optimum hydrolysis period was 81.3 ± 4.8% (mean ± SD for seven observations) of theoretical amounts on a weight basis using internal standard, phytosphingosine. The relationship between total LCB peak area and the amount of ganglioside hydrolyzed was linear between 10 and 200 μg of gangliosides.

TABLE 2. GLC–MS identification of long chain base peaks isolated by HPLC

Peak Number	Composition
1	1,5-Dihydroxy-2-amino-3-octadecene [d18:1(5-OH), 80%; unidentified LCB, 20%
2	C_{18}-*erythro*-sphingenine [d18:1]
3	C_{18}-*threo*-sphingenine [d18:1]
4	Unidentified C_{18}-LCB
5	C_{18}-*erythro*-sphinganine [d18:0]
6	1,5-Dihydroxy-2-amino-3-eicosene [d20:1(5-OH)]
7	Unidentified C_{18}-LCB
8	C_{20}-*erythro*-sphingenine [d20:1]
9	C_{20}-*threo*-sphingenine [d20:1]
10	Unidentified C_{20}-LCB (homolog of peak 4)
11	C_{20}-*erythro*-sphinganine [d20:0]
12	Unidentified LCB
13	Unidentified C_{20}-LCB (homolog of peak 7)
14	1-Hydroxy-2-amino-eicosadiene [m20:2]
15	Unidentified C_{18}-LCB
16	m18:2
17	m18:2
18	Unidentified LCB
19	Unidentified C_{20}-LCB (homolog of peak 15)
20	m20:2 (homolog of peak 16)
21	m20:2 (homolog of peak 17)

DISCUSSION

In this study, we have described an improved hydrolysis method for LCBs from brain gangliosides. A major advantage of this procedure is that no O-methyl ethers of LCBs are formed—these are the major secondary products formed by traditional acid methanolysis and they contribute significant complexity to ganglioside LCB HPLC chromatograms. In addition, the current method gives recoveries of LCBs from gangliosides comparable to that of aqueous acid methanolysis and decreases the secondary products to less than 20% of total yields.

Considerable amounts of *erythro*-d18:1 and d20:1 are isomerized to the *threo* forms by the acid hydrolysis pro-

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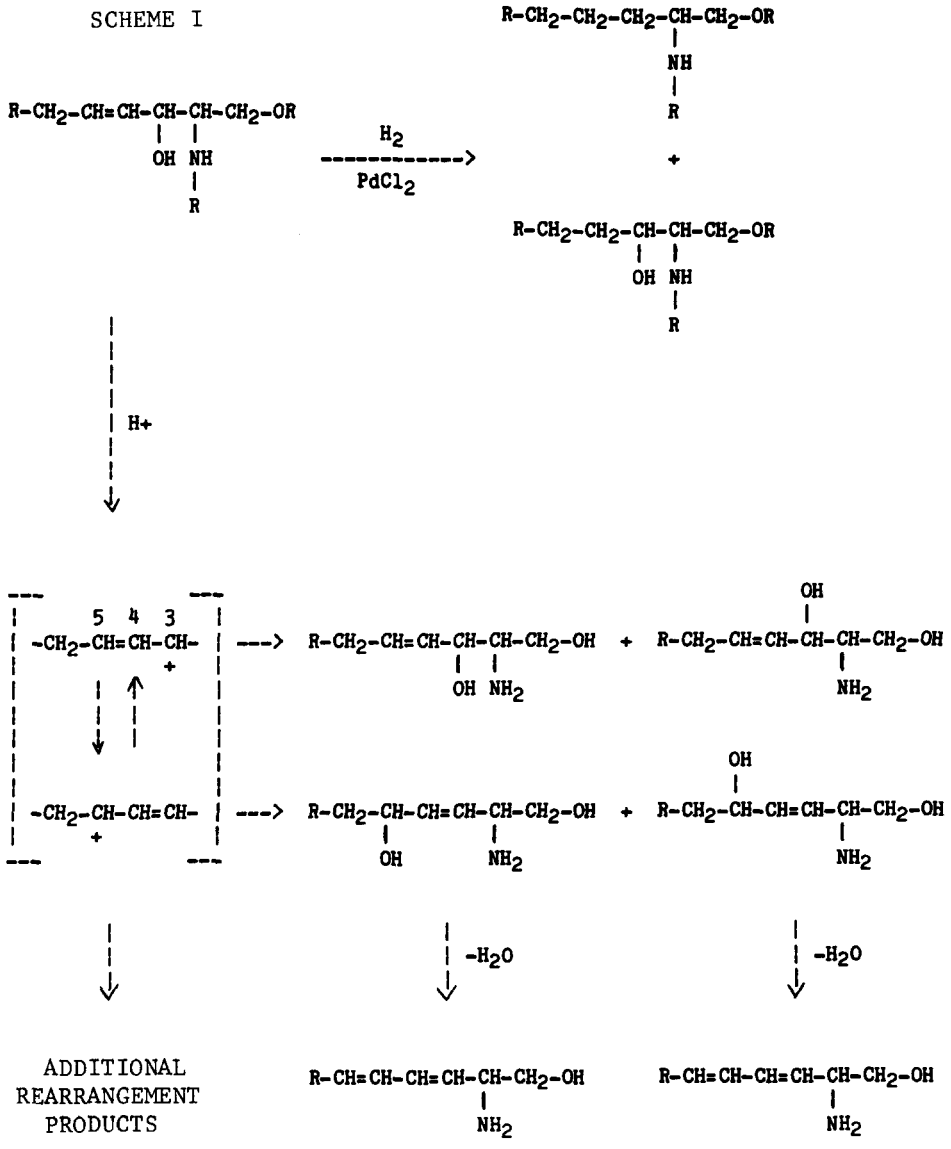
cedures. The results of direct hydrogenation of gangliosides over Pd on charcoal prior to the acid hydrolysis indicate that the isomerization and dehydration products derive from the natural *erythro*-sphingosine in gangliosides.

The major secondary products formed during aqueous acetonitrile-HCl hydrolysis were monohydroxydienes of LCBs. The several peaks, which were identified as monohydroxydienes by GLC–MS, probably differ in the position and/or configuration of the double bonds. The probable origins of LCBs isolated by HPLC as their biphenylcarbonyl derivatives and identified by GLC–MS are illustrated in Scheme I.

SCHEME I



The use of tetrahydrofuran–methanol–water as a mobile phase for HPLC analysis has some advantages over methanol–water, such as the clear resolution of d20:0 from the surrounding minor peaks of side products and, in general, better resolution of the earlier components. On the other hand, methanol–water results in better resolution of later peaks and shorter retention times with a comparable resolution of the major peaks.

The derivatization of the liberated LCBs with biphenylcarbonyl chloride and HPLC analysis provides highly sensitive detection. Theoretically, samples as small as 1 μg of brain gangliosides can be quantitatively analyzed if an internal standard is used to correct for sample loss during extraction and derivatization. Fatty acids released by aqueous acetonitrile-HCL hydrolysis

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can be extracted in suitable organic solvents for separate analysis as reported by Aveldaño and Horrocks (20).

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