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Procedure for separation of GM2 ganglioside species with different ceramide structures by a flash reversed-phase silica gel liquid chromatography

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Abstract

GM2 ganglioside, β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)-]β-Gal-(1-4)-β-Glc-(1-1)-Cer, is the main ganglioside in the brain of Tay-Sachs patients. In this work, GM2 ganglioside was extracted from a Variant B Tay-Sachs human brain, purified to homogeneity of the oligosaccharide moiety by silica gel chromatography. It was further fractionated for the first time into the molecular species differing in the ceramide structures by reverse-phase flash chromatography. The GM2 ganglioside species were characterized by gas-chromatography, nuclear magnetic resonance spectroscopy, and mass spectrometry. The major GM2 species contained the ceramides with *d*18:1–18:0 (40.5% of the total GM2 species), *d*20:1–18:0 (31%) and *d*18:1–20:0 (12%). We also found minor GM2 species with the ceramides with *d*18:1–24:1 (4%), *d*18:1–22:0 (2%) and *d*18:2–24:1 (1%), which have not been reported previously.

Keywords: GM2 ganglioside; Ceramide

1. Introduction

Gangliosides [1], sialic acid containing glycosphingolipids, are components of the plasma membrane of vertebrate cells and are particularly abundant in the central nervous system (CNS), where they are asymmetrically located in the outer lipid layer of neuronal plasma membrane [1]. In the CNS, the pattern of oligosaccharide moieties of gangliosides is complex and each hydrophilic oligosaccharide chain is linked to hydrophobic ceramides with different structures. Both the hydrophilic and the hydrophobic portions of gangliosides could modulate the membrane domain organization and the properties of cell membranes [2,3].

Gangliosides are commonly purified to homogeneity based on the oligosaccharide moiety by

Abbreviations: FA, fatty acids; DMSO, dimethylsulfoxide; Ceramide, *N*-acyl-sphingosine; Sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene; LCB, *d*18:0 or other structures with longer hydrocarbon chain; Long chain base, *d*18:1 or (2*S*,3*R*)-2-amino-1,3-dihydroxy-octadecane

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ion-exchange and silica gel-column chromatographies [4,5]. Both procedures are high resolution and allow the preparation of gangliosides, each with a uniform oligosaccharide moiety. However, these methods are not able to separate the molecular species that have the same oligosaccharide chain but differ in ceramide structures. The variation in ceramides can be in both the long chain base (LCB) and fatty acid (FA). The brain ganglioside species with homogeneous oligosaccharide chain have been fractionated by analytical or semi-preparative high performance reverse-phase chromatography (C8 or C18) due to the different hydrophobicity of the ceramide moieties [6,7]. However, a high resolution could be achieved only when a few mg of gangliosides were fractionated using a large and long column [8].

Tay-Sachs disease is caused by the impaired catabolism of ganglioside GM2 due to the deficiency of either β-hexosaminidase A [9–11] or GM2-activator protein [12–14]. The metabolic block caused by these deficiencies does not interfere with the biosynthesis of complex gangliosides. As a consequence, GM2 ganglioside, which is a minor ganglioside of normal brains or non-neuronal tissues, accumulates excessively about 100-fold in Tay-Sachs brains [15,16]. Thus, Tay-Sachs brains are the best source for GM2 ganglioside preparation.

In this paper, we present the preparative reverse-phase flash chromatography of GM2 ganglioside that was extracted from the brain sample of a Tay-Sachs patient and the characterization of the GM2 species differing in the ceramide structure by gas-chromatography, ^{1}H NMR spectroscopy, and mass spectrometry.

2. Experimental

2.1. Chemicals and reagents

Commercial chemicals were of analytical grade or the highest grade available. Common solvents and water were re-distilled using a glass apparatus. LiChroprep RP18 for column chromatography (particle size 40–63 μm) and high performance silica gel and reverse-phase pre-coated thin-layer plates (HPTLC Kieselgel 60, 10 cm × 10 cm; HPTLC RP18, 10 cm × 10 cm) were purchased from Merck GmbH.

2.2. Isolation of GM2 ganglioside

Total gangliosides were prepared according to the published method [17] from 50 g of Variant B Tay-Sachs brain sample. The crude ganglioside preparation was further fractionated using a Q-Sepharose column chromatography [18]. About 600 mg of a partially purified GM2 were obtained from 50 g of the brain sample.

2.3. Fractionation of GM2 molecular species with different ceramide structures

GM2 (400 mg) was dissolved in 10 ml of methanol–water–triethylamine (15:2:1, v/v), and applied onto a 3 cm × 12 cm LiChroprep RP18 column, which had been washed in sequence with 1 l of water, 1 l of methanol–water (1:1, v/v), and 1 l of methanol–water–triethylamine (15:4:1, v/v). The column was eluted using the solvent system of methanol–water–triethylamine (15:2:1, v/v) at a flow rate of 35 ml/min under a nitrogen pressure of 1.2 bar.

2.4. Analytical procedures

The amount of ganglioside-bound sialic acid was determined by the resorcinol-HCl method [19,20], using Neu5Ac as the standard.

TLC of GM2 ganglioside was carried out on silica gel HPTLC plates using the solvent system chloroform–methanol–18 mM aqueous $CaCl_2$–50 mM aqueous KCl (50:50:4:8, v/v) and on RP18 HPTLC plates using the solvent system methanol–water–triethylamine (15:2:1, v/v). Gangliosides on the TLC plate were made visible by spraying the plate with a *p*-dimethylaminobenzaldehyde reagent [21] followed by heating at 120 °C for 20 min [21], or by spraying with 5% ammonium sulfate followed by heating at 160 °C. The amount of ganglioside on each spot was estimated by scanning the plate with a BioRad 700 imaging densitometer.

Gas-chromatographic analyses [22] of the ceramide moieties of GM2 were carried out as follows. GM2 ganglioside (0.5 mg) was solubilized in 0.5 ml of methanol–10 M HCl (9:1, v/v), and maintained at 75° for 16 h. After three extractions of the FA methyl esters with 1.5 ml of *n*-hexane, the methanolic phase was evaporated to dryness under a stream of

nitrogen. The residue was dissolved in 0.5 ml of 5 M NaOH, followed by addition of 1.5 ml of water. LCB were extracted three times with 3 ml of diethyl ether and the organic phase was evaporated. The residue was submitted to trimethylsilylation by addition of 10 μl of trimethylsilylimidazole and left for 15 min at 80 °C. FA methyl esters and trimethylsilyl–LCB were separated on a 25 m SE-54 capillary column using a Dani-3865 gas-chromatographic system equipped with a program temperature vaporization injector. Analyses were carried out using a total injection program. FA methyl esters and trimethylsily–LCB were analyzed at 5 °C/min, from 180 to 240, and at 230 °C, respectively.

^{1}H NMR of GM2 species was carried out at 500 MHz on a Bruker AM500 spectrometer as previously described [23,24].

ESI-MS of the GM2 species was carried out in negative mode on a ThermoQuest Finnigan LCQ_{deca} mass spectrometer equipped with an electrospray ion source and a Xcalibur^{TM} data system. Samples were dissolved in methanol at a concentration of 20–200 ng/μl and introduced into the electrospray needle by mechanical infusion through a microsyringe at a flow rate of 3 μl/min. Ionization was performed under the following conditions: spray voltage, 4 kV; sheath gas flow rate, 50 arbitrary units; capillary temperature, 260 °C; capillary voltage, −42 V. The scanning range was *m*/*z* 200–1600, and fragmentor voltage for collision induced dissociation was 25–90%.

3. Results and discussion

Ganglioside GM2 was extracted from a brain sample of human Tay-Sachs patient and partially purified by ion-exchange column chromatography. Fig. 1 shows the silica gel TLC of Tay-Sachs GM2 obtained after the DEAD-chromatography. Under the reported experimental conditions, the partially purified GM2 was fractionated into one major component and two minor components, of which one was faster and the other, slower in chromatographic mobility. The three spots were designated as, a, b and c, respectively, from the top (Fig. 1). Further attempts to improve the separation of a and b by changing the solvent systems for TLC were unsuccessful. A better separation, however, was achieved using C18 reverse-phase TLC plates. The pattern of the reverse-phase TLC of the Tay-Sachs GM2 is shown in Fig. 2. Three spots designated 1, 2 and 3 were observed (Fig. 2A) and their quantities were estimated by densitometry to be 43, 49 and 8% from the top. Fig. 2B shows the preparative reverse-phase silica gel-column chromatography of this Tay-Sachs GM2. The column chromatography showed the resolution similar to that of HPTLC, and the mixture was resolved in three fractions having the same mobility as spots 1, 2 and 3 on TLC analysis. The three fractions were also analyzed by normal-phase TLC as shown in Fig. 3 to compare with the fractions a, b, and c in Fig. 1. Fractions 1 and 2 had the same chromatographic mobility as spot b shown in Fig. 1, and fraction 3 had the same mobility as spot a. When Fraction 1 was heavily applied on the TLC plate, a small amount of spot c could also be seen. Most of spot c was eliminated when Fraction 1 was collected. The heavy loading of samples on TLC plate showed that each fraction still contained a small percentage of other fractions. Fraction 1 contained about 4% of

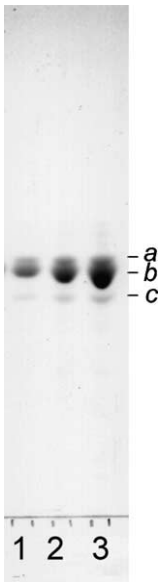

Fig. 1. Normal-phase silica gel TLC of the GM2 ganglioside purified from a human Tay-Sachs brain. Lanes 1, 2 and 3: 0.2, 0.6 and 1.2 μg of Tay-Sachs GM2, as lipid bound Neu5Ac. The solvent system used was chloroform–methanol–18 mM aqueous $CaCl_2$–50 mM aqueous KCl (50:50:4:8, v/v). Three spots designated as a, b and c were detected by *p*-dimethylaminobenzaldehyde spray reagent [21].

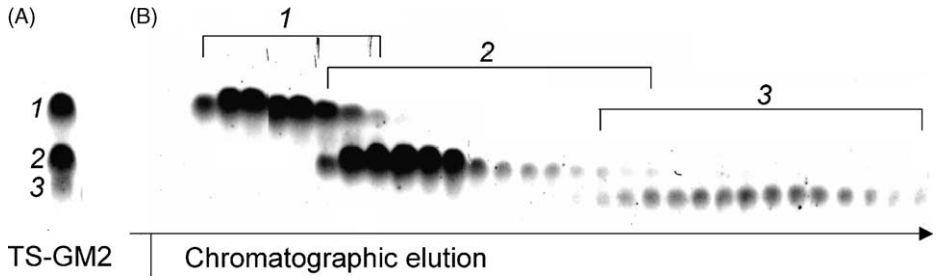

Fig. 2. Panel A: reverse-phase silica gel HPTLC of Tay-Sachs GM2. Spots 1, 2 and 3 were detected by staining with *p*-dimethylaminobenzaldehyde spray reagent [21]. Panel B: reverse-phase silica gel-column chromatography of Tay-Sachs GM2. Fractions were analysed by reverse-phase silica gel HPTLC as in panel A. The solvent system used was methanol–water–triethylamine (15:2:1, v/v). Experimental conditions as described in Section 2.

fraction 2, and fraction 2 contained about 2% of fraction 1 and 4% of fraction 3, whereas, fraction 3 contained about 5% of fraction 2.

The ^{1}H NMR spectra of fractions 1 and 2 were identical with that of the GM2 previously characterized [23,24]. The ^{1}H NMR spectrum of fraction 3, which was dissolved in DMSO-*d*6, is shown in Fig. 4. In this spectrum, an additional resonance at 5.3 ppm was observed. This resonance was also observed during the analysis of a sample dissolved in DMSO-*d*6/D_2O, indicating that this proton was resistant to deuterium exchange. This resonance was not connected to those assigned to protons on carbons 1–5 of sphingosine, but was connected to the methylene groups of the alkyl chains. The intensity of this signal was similar to that of a single proton. These results suggest that fraction 3 is a mixture of species and that one or more of them contains unsaturated FA.

Fractions 1, 2 and 3 were first submitted to methanolysis. Then, the methyl esters of FA and LCB were converted to trimethylsilyl derivatives and subjected to GC analysis. Within the GC peaks of LCB derivatives we identified, by mean of reference standards, four main structures corresponding to *d*18:0, *d*18:1, *d*20:0, *d*20:1. Other minor peaks were in spectrum but could not be identified. Within the FA methyl ester GC peaks, we could identify, by mean of reference standards, five main structures corresponding to 18:0, 20:0, 22:0, 22:1 and 24:1. The compositions of LCB and FA in GM2 species are listed in Table 1. Fraction 1 contained 93% of *d*18:1 LCB, 7% of *d*18:0 LCB and close to 100% of stearic acid. In fraction 2, we identified three LCBs with *d*18:1 (29%), *d*20:1 (63%), and *d*20:0 (8%), and two main FA with 18:0 (71%) and 20:0 (24%). Based on these FA and LCB contents, and on the fact that the species have similar

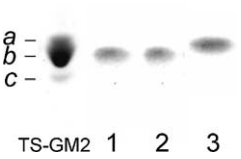

Fig. 3. Normal-phase HPTLC of Tay-Sachs GM2 molecular species purified by reverse-phase chromatography. Lane TS-GM2: the same material as that used in Fig. 1. Lanes 1, 2 and 3 are fractions 1, 2 and 3 of Fig. 2, respectively. The solvent system used was chloroform–methanol–18 mM aqueous $CaCl_2$–50 mM aqueous KCl (50:50:4:8, v/v).

Table 1
Relative distribution of fatty acids and long chain bases of GM2 species of the three fractions separated by the reverse-phase chromatography

	Fraction 1	Fraction 2	Fraction 3
LCB			
*d*18:0	7.2		
*d*18:1	92.8	29.1	100
*d*20:0		8.0	
*d*20:1		62.9	
FA			
18:0	99.4	70.6	
20:0		24.2	
22:0			32.3
22:1		1.8	
24:1		3.0	65.6
Others	0.6	0.4	2.1

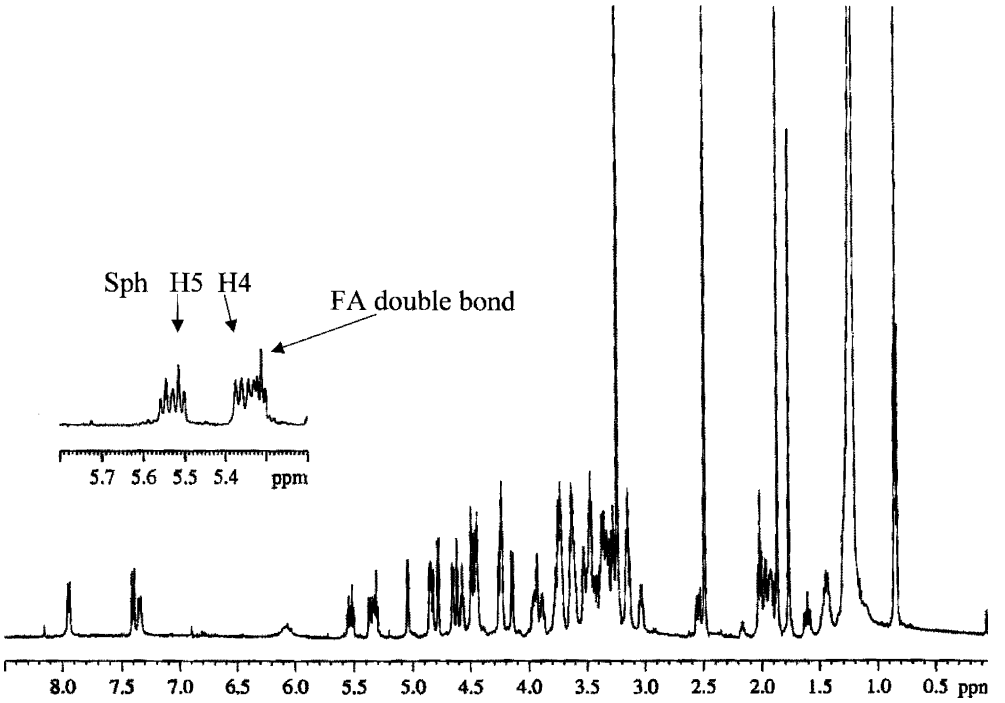

Fig. 4. ^{1}H NMR spectrum of fraction 3 in DMSO-*d*6 at 305 K.

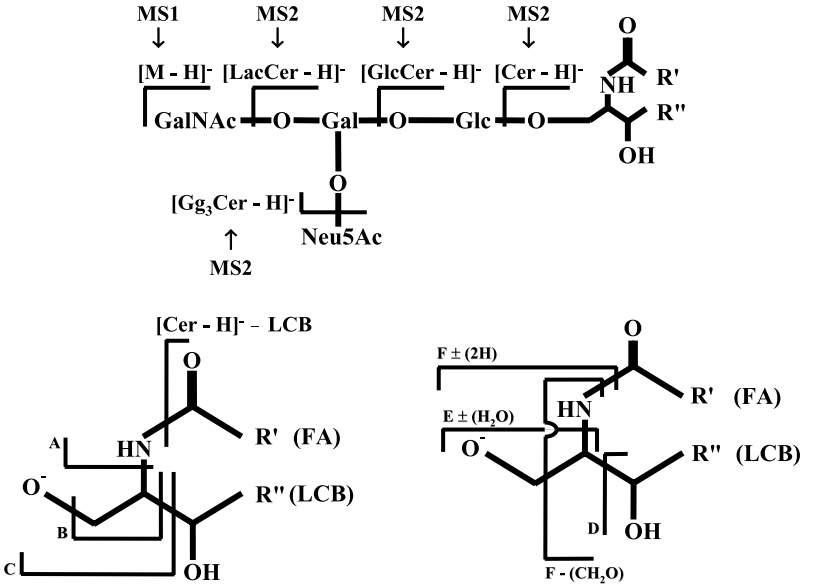

Fig. 5. Scheme of the MS ions for GM2 ganglioside (for more details see [30–34]). Fragments indicated with MS1 are obtained from the first MS, MS2 from a MS–MS, and MS3 from MS–MS–MS.

mobility in reverse-phase chromatography, fraction 2 should have the two main GM2 species containing the ceramides of *d*18:1–20:0 and *d*20:1–18:0 to provide the same number of carbons in the hydrophobic chains. In fraction 3, we identified *d*18:1 LCB (close to 100%) and the FAs of 22:0 (32%) and 24:1 (66%), suggesting the ceramide structure to be *d*18:1–22:0 and *d*18:1–24:1. According to these results, the hydrophobicity of the GM2 species in the three LC fractions should be increasing from fractions 1 to 3, which agrees with the reverse-phase chromatographic behavior shown in Fig. 2.

The structures of the Tay-Sachs GM2 species was confirmed by MS. Fig. 5 shows the ion fragmentation patterns previously suggested for gangliosides [25–29]. Fig. 6 shows the MS1 spectra (full scan mode) of the fractions 1, 2 and 3. Fractions 1 and 2 were characterized by a main pseudomolecular [$M - 1$] ion at *m*/*z* 1382 and 1410, respectively, and other minor pseudomolecular [$M - 1$] ions. The MS1 spectrum of fraction 3 was more complex. The MS2 spectrum (MS–MS) obtained from the ion at *m*/*z* 1382 of fraction 1 contained the ions corresponding to Gg_3Cer, LacCer, GlcCer and Cer derived from the sequential detachment of Neu5Ac, Neu5Ac and GalNAc, GalNAc-(Neu5Ac-)Gal, and GalNAc-(Neu5Ac-)Gal–Glc, respectively. The MS3 spectrum (MS–MS–MS) derived from the [Cer-H] ion at *m*/*z* 564 gave fragments supporting the single ceramide structure with *d*18:1–18:0. Fig. 7 shows MS spectra of fraction 2. The MS2 spectrum derived from the ion at *m*/*z* 1410 showed a series of ions corresponding to the sequential detachment of sugar moieties, suggesting a ceramide mass at *m*/*z* 592 (and

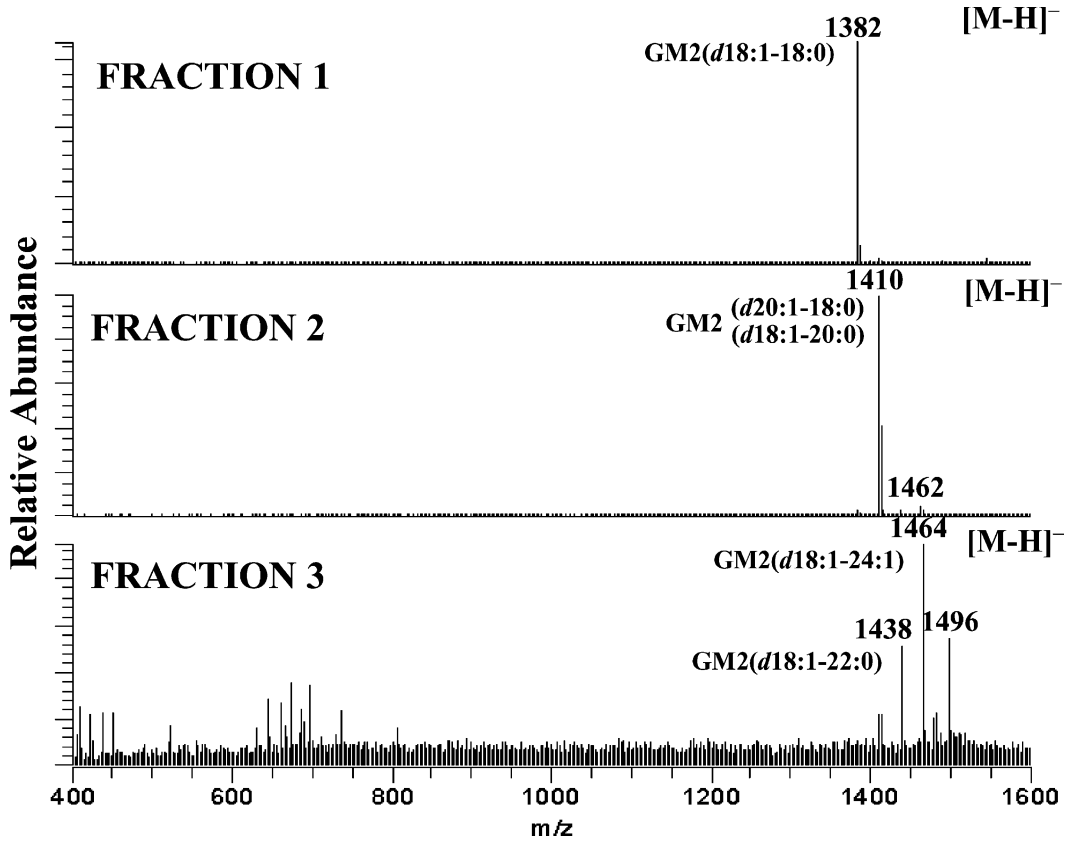

Fig. 6. MS1 spectra of fractions 1, 2 and 3 obtained by reverse-phase chromatography of Tay-Sachs GM2. The ion at *m*/*z* 1496 was not characterized.

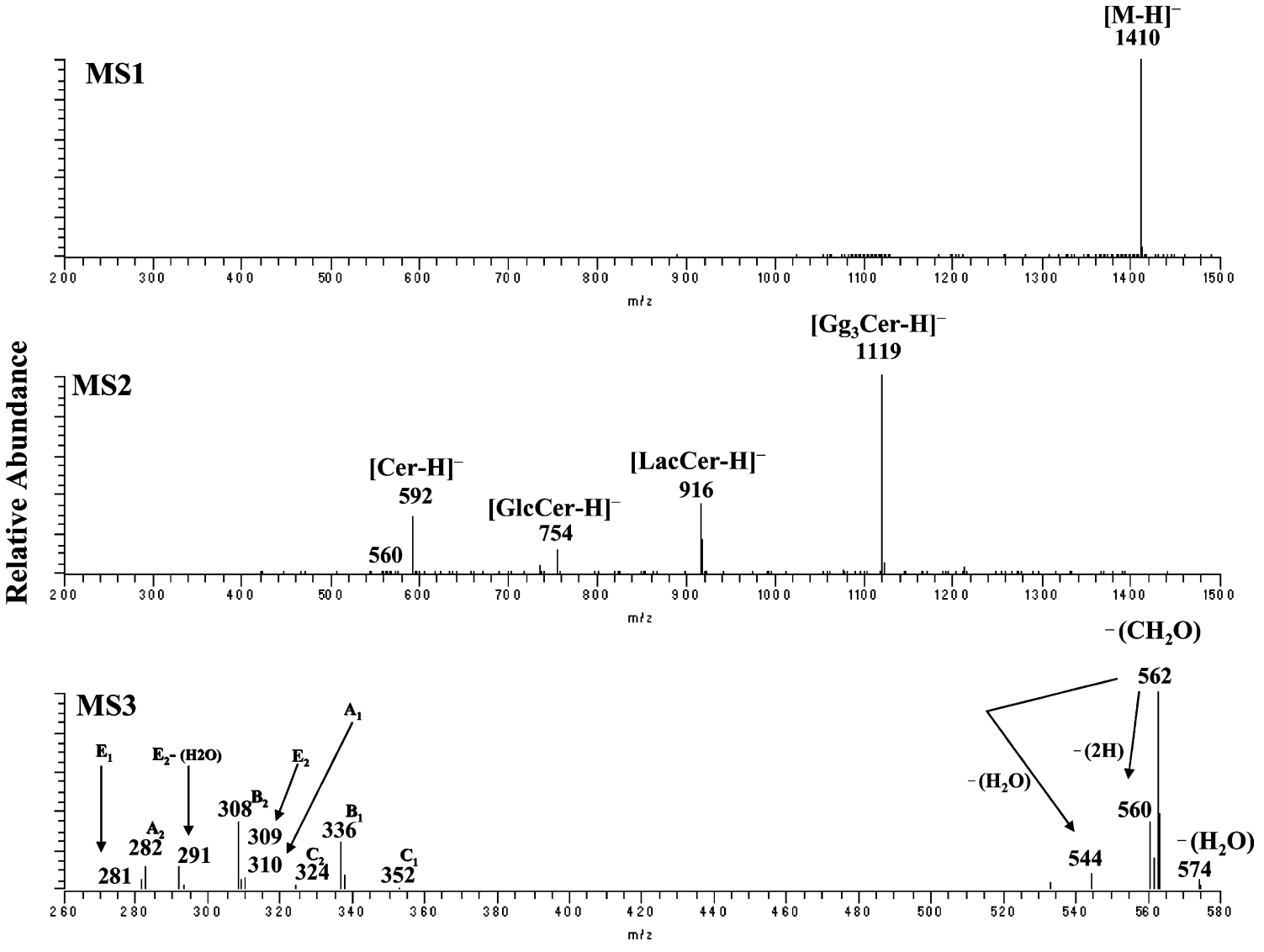

Fig. 7. MS1, MS2 and MS3 spectra of fraction 2 obtained by reversed-phase chromatography of Tay-Sachs GM2. MS2 is derived from *m/z* at 1410; MS3 from *m/z* at 592. Fragment nomenclature follows that used in Fig. 5.

Table 2
Molecular species of GM2 ganglioside extracted from human Tay-Sachs brain and their relative distribution

Percent on GM2 species	Species in fraction 1	Species in fraction 2	Species in fraction 3
3	*d*18:0–18:0		
40.5	*d*18:1–18:0		
12		*d*18:1–20:0	
2			*d*18:1–22:0
1		*d*18:1–22:1	
4			*d*18:1–24:1
1.5			*x*
1		*d*18:2–24:1	
4		*d*20:0–18:0	
31		*d*20:1–18:0	

Species *x* in fraction 3, with [$M-1$] ion at *m*/*z* 1496 (see Fig. 6), was not characterized.

derived 560). Furthermore, the MS3 spectrum derived from *m*/*z* 592 contained a set of ions A1, B1, C1, and E1 (see Fig. 5 for fragmentations) at *m*/*z* 310, 336, 352 and 281, together with a set of ions A2, B2, C2 and E2 at *m*/*z* 282, 308, 324 and 309, supporting the co-presence of ceramides of *d*18:1–20:0 and *d*20:1–18:0, respectively, i.e. with the same molecular mass. The ion related to fraction 2 at *m*/*z* 1462 was identified by derived MS2 and MS3 spectra as the specie containing the ceramide of *d*18:2–24:1. The two ions related to fraction 3 at *m*/*z* 1438 and 1464 were identified by derived MS2 and MS3 spectra as the species containing the ceramides of *d*18:1–22:0 and *d*18:1–24:1, respectively. The species represented by the ion at *m*/*z* 1496 has not been characterized.

Taken together the GC data for the components of FA and LCB (Table 1) and the MS data (Fig. 7), we calculated the distribution of the molecular species for Tay-Sachs GM2 differing in the ceramide structures and summarized the data in Table 2.

4. Conclusions

Ganglioside mixtures from different sources have been fractionated previously by ion-exchange and normal-phase silica gel-column chromatographies under varieties of conditions [4,5,18]. These procedures have been used for large-scale purification of gangliosides with different sugar components and complexity. The gangliosides obtained by these procedures usually have high homogeneity regarding the oligosaccharide chain. However, the hydrophobic moiety of gangliosides, ceramide (for the structure see Fig. 5), can be quite heterogeneous in both the LCB and FA due to the differences in chain length and the number of double bonds. The ion-exchange and normal-phase silica gel chromatographies are not capable to separate the ganglioside species differing in only the hydrophobicity of the ceramide moiety. Thus, the purified gangliosides can be homogeneous in the oligosaccharide chain, but still heterogeneous in the ceramide moiety. High performance reverse-phase chromatography procedures have been developed for purifying the ganglioside species homogeneous in both the oligosaccharide chain and the ceramide moiety [6–8]. However, only small amounts of gangliosides can be fractionated by these procedures. From our experience [8], not more than 5 mg of ganglioside homogeneous in the oligosaccharide chain could be applied to a column of 4.5 cm diameter using reverse-phase chromatography to achieve the separation of the ganglioside species having different ceramide structure.

In this paper, we report the use of a flash reverse-phase silica gel liquid chromatography for the preparation of large amounts of ganglioside molecular species. This was demonstrated by using a complex mixture of GM2 species from a Tay-Sachs disease brain, which contained ceramide structures varying in the number of carbons and/or the double bonds. In spite of the complexity of the mixture of the GM2 species, 400 mg of GM2 were very well fractionated into three fractions using a small 3 cm × 12 cm LiChroprep RP18 column. Each fraction contained GM2 species with very similar hydrophobicity and just different in ceramide structure. The fractionation was rapid, reproducible, and required only a few liters of solvents. Due to the fact that no specific and expensive instrument is needed for this procedure, it should be highly feasible to combine this procedure with the ion-exchange and normal-phase chromatography for the effective preparation of large amounts of gangliosides homogeneous in both the oligosaccharide and ceramide moiety. This will be important for the understanding of glycosphingolipid metabolism. In fact, it is well known that both glycosyltransferases [35,36] and glycosidases [37] show different activity toward

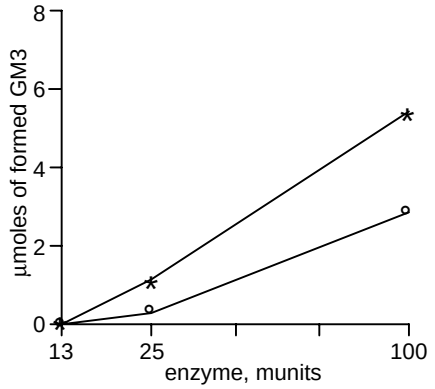

Fig. 8. Hydrolysis of GM2 fractions prepared by flash reverse-phase chromatography using human liver β-hexosaminidase A and GM2-activator protein. The incubation was performed in 50 μl incubation mixture that contained 10 μg of GM2, 10 mM sodium acetate buffer, pH 4.6, and 1 μg of GM2-activator protein at 37 °C for 135 min. In the absence of GM2-activator protein, there was little or no hydrolysis of GM2. Open circles, fraction 1 that mainly contains GM2 species with ceramide of *d*18:1–18:0; stars, fraction 3 that mainly contains GM2 species with ceramide of *d*18:1–22:0 and *d*18:1–24:1. For details on the protein preparations and enzymatic assay see [38].

various synthetic glycosphingolipids with different ceramide structures. We confirmed this in an in vitro assay of the hydrolysis of GM2 fractions prepared by flash reverse-phase chromatography using human liver β-hexosaminidase A and GM2-activator protein [38]. As shown in Fig. 8, the rate of hydrolysis of fraction 3 that contains GM2 species with ceramides of *d*18:1–22:0 and *d*18:1–24:1 was double of that of fraction 1, which mainly contains the GM2 species with ceramide of *d*18:1–18:0. Thus, our procedure for the preparation of ganglioside species can be considered a good alternative to the synthetic procedures [39,40] that usually have very low yield after multiple steps of reactions and purification.

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