Characterization of glycosphingolipids by direct inlet chemical ionization mass spectrometry

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Abstract Permethylated derivatives of cerebrosides and ceramide di-, tri-, tetra-, and penta-hexosides were analyzed by the direct inlet ammonia chemical ionization (CI) mass spectrometry. In the CI mass spectra, the fragment ions produced by the loss of methanol from the protonated molecular ion were observed in all of glycosphingolipids. Other fragment ions due to the cleavage of glycosidic moiety were major ones under the CI conditions. These ions provided information on the molecular species of glycosphingolipids and the sugar sequence of their oligosaccharides. Glycosphingolipids with hydroxy fatty acids could also be differentiated from those with nonhydroxy fatty acids by comparing the intensities of characteristic fragment ions. The CI method should be particularly useful in structural studies of glycosphingolipids from natural sources.


Supplementary key words glucosyl ceramide · galactosyl ceramide · galactosyl glucosyl ceramide · galactosyl galactosyl glucosyl ceramide · globoside · Forssman glycolipid

Glycosphingolipids are located primarily on cell membranes and have various biological functions. They have specific immunological properties, and are a part of cell surface receptors functioning in cellular recognition, adhesion, and growth control (1, 2). Glycosphingolipids are minor components in most tissues, therefore the development of reliable micro methods for the qualitative and quantitative analyses of these lipids is often required.

During the last decade, mass spectrometry has proved to be a powerful technique for the structural analysis of lipids. The analysis of a number of complex glycosphingolipids has been performed by conventional electron impact ionization (EI) mass spectrometry (3–16). In the EI mass spectrometric analysis of these lipids, the molecular ions in the high mass range are generally extremely small or absent. Hence its usefulness is somewhat limited.

On the other hand, chemical ionization (CI) mass spectrometry has recently been employed successfully in the structural studies of glycosphingolipids (17–20). We have also applied this technique for the analysis of trimethylsilyl derivatives of cerebrosides and have shown that it can provide important information, particularly in the high mass range, on qualitative and quantitative analyses of cerebroside molecular species (20).

In this paper, we describe the analysis of some complex glycosphingolipids by direct ammonia CI mass spectrometry, and discuss the advantages of using permethylated derivatives for the structural elucidation of these lipids.

MATERIALS AND METHODS

Palmitoyl GL₃ₙ was synthesized from glucosyl sphingosine which was obtained by strong alkaline hydrolysis of Gaucher’s GL₃ₙ according to the method

Abbreviations: GLC, gas-liquid chromatography; CI, chemical ionization; MS, mass spectrometry; EI, electron impact ionization; GL₃ₙ, glucosyl ceramide; GL₄ₙ, galactosyl ceramide; GL₅ₙ, galactosyl glucosyl ceramide; GL₆ₙ, galactosyl galactosyl glucosyl ceramide; GL₇ₙ, globoside; GL₈ₙ, Forssman glycolipid; TLC, thin-layer chromatography; sph, sphingosine; h₂₄:₀ etc., hydroxy fatty acid 24:₀ etc.

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Methylation

Permethylation of glycosphingolipids (100–500 µg) was carried out with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide (24). The permethylated derivatives of glycosphingolipids were purified by silica gel TLC with a solvent system of chloroform–methanol 9:1 (by volume).

Chemical ionization mass spectrometry

A Shimadzu-LKB gas chromatography–mass spectrometer 9000A equipped with a CI source was employed. Ammonia was used as the reagent gas. The pressure in the ion source was adjusted to 0.7 torr. The temperature of the ion source was kept at 220°C. The mass spectra were obtained at an electron energy of 500 eV, an emission current of 500 pA, and an accelerating voltage of 1.75 kV. Samples of permethylated glycosphingolipids (50–100 µg) were introduced through a direct probe. The temperature of the direct probe inlet was programmed from 100°C to 400°C at the rate of about 50°C per minute. During the heating of the probe, total ion collector and mass spectra were monitored continuously. The mass marker was calibrated by measuring the high boiling fraction of perfluorokerasene (Koch-Light Laboratories, England) and tris(pentadecafluoroheptyl)-s-triazine (MW = 1185, E. Merck, F.R.G.).

RESULTS

Fig. 1 shows the TLC of isolated glycosphingolipids with the developing solvent system of chloroform–methanol–water 65:25:4 (by volume). GLb, GLa, and GL3 migrated as double bands, presumably due to the presence of hydroxy and nonhydroxy fatty acids in these lipids.

Mass spectra of monohexosyl ceramide

In ammonia CI mass spectra of the permethylated derivative of synthetic palmitoyl GL3, the protonated molecular [MH]+ ion at m/e 784 is extremely weak. This suggests that it is an unstable ion species. However the ions at m/e 752 and 720 derived by successive losses of methanol from [MH]+ permit the assignment of the molecular weight. The adduct ion at [M + 18; M + NH4]+ due to the addition of the reagent gas is also extremely weak (Fig. 2). The ion at m/e 720 shows less than 5% intensity of the base peak at m/e 752 [MH-32; MH-CH3OH]+. The characteristic ion at m/e 533 derived by the loss of glucosyl moiety [m/e 219] from [MH-32]+ provides information on the ceramide structure. The ions at m/e 219 and 187 [219-32; 219-CH3OH]+ are characteristic of the hexose moiety. A primary ion at m/e 253 [CH3(CH2)12-
Chemical ionization mass spectra of synthetic palmitoyl GL$_{18}$.

Fig. 2. Chemical ionization mass spectra of synthetic palmitoyl GL$_{18}$.

CH=CHCHOHCH$_3$ gives structural information about the long chain base, which is 1,3-dihydroxy-2-amino-4-octadecene (C18-sphingosine). Similar ions were characterized in the CI mass spectra of peracylated and trimethylsilylated derivatives of various sphingolipids (17, 18, 20). GL$_{1b}$ from bovine brain contains 2-hydroxy fatty acids as well as nonhydroxy fatty acids. Figs. 3A and 3B show the mass spectra of

Fig. 3. Chemical ionization mass spectra of GL$_{1b}$ from bovine brain white matter. [A], GL$_{1b}$ with nonhydroxy fatty acid; [B], GL$_{1b}$ with hydroxy fatty acid.

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GLb containing nonhydroxy fatty acids and hydroxy fatty acids, respectively. The CI mass spectrum in Fig. 3A gives two recognizable ion groups, ranging from m/e 752 to 906, and m/e 533 to 687, which provide information on the molecular species of GLb containing nonhydroxy fatty acids. The ions [MH-32]+ ranging from m/e 752 to 906 are formed by the sequential loss of methanol from the protonated molecular ions ranging from m/e 784 to 938. These ions represent the GLb with fatty acids ranging from C16:0 to C27:0. Since the protonated molecular ions are unstable, the molecular ions at m/e 896 and 894 (GLb with C24:0 and C24:1) are only barely detectable. In addition, in glycosphingolipids with monounsaturated fatty acids, all these ions are recorded at two mass units less than the corresponding saturated compounds. The ions ranging from m/e 533 to 687, formed by the elimination of the hexose moiety [m/e 219] from the ion groups at [MH-32]+, are useful for determining the molecular species of ceramides.

In the case of GLb with hydroxy fatty acids, as shown in Fig. 3B, the fatty acid containing ion groups should be shifted by 30 mass units from those observed in Fig. 3A. Namely, the recognizable ions ranging from m/e 810 to 936 are derived from the elimination of methanol from [MH]+ ranging from m/e 842 to 968. The ions correspond to GLb with hydroxy fatty acids from C18:0 to C27:0. These ion groups, and ion groups ranging from m/e 563 to 717 produced by the cleavage of glycosidic linkage, also provide information on the distinct molecular species of GLb with hydroxy fatty acids. In the fragment ions less than m/e 500, the fragment ions at m/e 219, 187, and 253 are detectable. The most prominent feature in both mass spectra is that GLb with C24:0 and C24:1 is present in large proportions (20).

**Mass spectra of complex glycosphingolipids**

Figs. 4 and 5 show the mass spectra of permethylated GL2a and GL3 from hog erythrocyte membranes. Both glycosphingolipids contain nonhydroxy and hydroxy fatty acids. The mass spectrum of GL2a shows three recognizable ion groups that

![Mass spectra of complex glycosphingolipids](image-url)
are of diagnostic value in the structural elucidation. Molecular weight information on GL_{2n} with hydroxy and nonhydroxy fatty acids comes from the ion groups ranging from m/e 1012 to 1096 [MH-32]^+ and from m/e 1042 to 1126 [hMH-32]^+, which are GL_{2n} containing fatty acids with chain lengths ranging from C20:0 to C26:0, respectively. The ion groups ranging from m/e 793 to 877 and m/e 825 to 907 afford unequivocal information on the GL_{2n} structure, originated from GL_{2n}. These ions are produced by the elimination of the terminal galactose moiety from the GL_{2n} molecules. The ion groups ranging from m/e 589 to 673 and from m/e 619 to 703 are also useful in determining the molecular species of ceramides. The mass spectra of GL_{2n} with nonhydroxy and hydroxy fatty acids indicate that GL_{2n} contains large proportions of fatty acid C24:1 and saturated fatty acids, such as C22:0, C23:0, C24:0, and C26:0. This finding correlates with fatty acid compositions of GL_{2n} from hog erythrocyte membranes which were reported by Taketomi and Kawamura (25). In the low mass range, the fragment ions at m/e 219, 187 and 423 were useful in determining the sugar moiety.

In permethylated GL_{3}, four recognizable ions are demonstrated. These ions include fatty acids whose chain lengths are distributed from C20:0 to C26:0. The ion groups ranging from m/e 1216 to 1300 [MH-32]^+ and from m/e 1246 to 1330 [hMH-32]^+ provide their molecular weight information. The mass spectra of GL_{3} which has a slower migratory rate than the major GL_{3} on silica gel TLC plate are often complicated. Presumably this slow moving GL_{3} is not homogeneous and contains considerable amounts of nonhydroxy fatty acids in addition to hydroxy fatty acids. By the characteristic fragment ions, slow migrated GL_{3} can be discriminated from GL_{3} with nonhydroxy fatty acids (Figs. 5A and 5B). The fragment ions at m/e 219 and 187 are also detected.

Figs. 6 and 7 show mass spectra of permethylated GL_{4} and GL_{5}, respectively. Both glycosphingolipids are homogeneous on silica gel TLC plate (Fig. 1). However, examination of their mass spectra indicates that both glycosphingolipids contain hydroxy and nonhydroxy fatty acids. Both mass spectra are characterized by the presence of five recognizable ion groups. In GL_{5}, the ions corresponding to the loss of two moles of galactosamine moiety are easily dis-
cernible. However the fragment ions corresponding to the loss of only one mole of galactosamine are almost always undetectable. GL₄ and GL₅ with C24:0 and hC24:0 give rise to the fragment ions at m/e 1517 [MH-32]+, 1547 [hMH-32]+, and m/e 1766 [MH-32]+, 1796 [hMH-32]+, respectively. The fragment ions at m/e 260 and 228 [260-32; 260-CH₃OH]+ are characteristic of the hexosamine moiety from GL₄ and GL₅. Other fragment ions at m/e 464 (galactosaminogalactose) and 432 [464-32]+ in GL₄ and m/e 505 (galactosamine-galactosamine) and 709 (galactosamine-galactosamine-galactose) are of diagnostic value in determining sugar moiety.

Fig. 8 shows a generalized scheme for the frag-
DISCUSSION

The analysis of a number of complex glycosphingo-
lipids has been performed by conventional EI mass spectrometry (3–16). Permethylated derivatives are preferred for mass spectrometric analysis because of the relatively small mass increase compared to other derivatives (5–12). In the EI mass spectrometric analysis of glycosphingolipids, the molecular ions and fragment ions in the high mass range are generally extremely small or absent. CI mass spectrometry generally enhances the intensities of these ions in the high mass range and has recently been employed with great success in the structural elucidation of glycosphingolipids (17–20). Markey and Wenger (17) first reported the direct methane CI mass spectra of peracetylated glycosphingolipids. However, the molecular ions and fragment ions in the high mass range were of insufficient intensity for the determination of molecular species of glycosphingolipids. They concluded that it might be advantageous to employ the combination of EI and CI mass spectrometry for the complete structural analysis of glycosphingolipids. Oshima, Ariga, and Murata (18) reported gas–liquid chromatography (GLC)-CI mass spectra of trimethylsilyl derivatives of psychosine, ceramide, and cerebroside from the spleen of a patient with Gaucher's disease. Isobutane CI mass spectrometry gave prominent molecular ions and characteristic fragment ions corresponding to the cleavage of long chain base, fatty acid, and the sugar moiety. These ions provided sufficient information for the complete determination of glycosphingolipid structure. Ando et al. (19) reported isobutane and ammonia CI mass spectra of oligosaccharides liberated from glycosphingolipids. In this case, the oligosaccharides were first reduced with sodium borohydride, and were analyzed as their permethylated, peracetylated, and trimethylsilylated derivatives. The isobutane and ammonia CI mass spectra gave prominent molecular ions and fragment ions due to the cleavage of glycosidic bonds, and they concluded that CI mass spectra could be used for elucidating the sugar sequence in oligosaccharides. Murata et al. (20) investigated the direct CI mass spectra of trimethylsilyl derivatives of cerebrosides. The direct CI mass spectrometry provided important information on the qualitative and quantitative analyses of cerebroside molecular species, including cerebrosides with hydroxy and nonhydroxy fatty acids. They concluded that this method should be more informative than GLC analyses of cerebrosides isolated from natural sources.

In this paper, we have analyzed permethylated derivatives of glycosphingolipids with one to five sugars by direct ammonia CI mass spectrometry. As described in results, in the CI mass spectra, the fragment ions produced by the loss of methanol from the protonated molecular ion were observed in all of glycosphingolipids and sometimes showed as the base peak (Figs. 2, 3A, and 3B). Other fragment ions produced by the successive loss of sugar moieties from the non-reducing end were prominent. In the CI mass spectrometric studies of oligosaccharides, the fragmentation due to ring opening, which is often observed in the EI mass spectrometry, is a rare occurrence whereas that due to the cleavage of glycosidic moiety is predominant (19, 26, Fig. 8). These ions provided information on molecular species of glycosphingolipids and sugar sequences of their oligosaccharides. Permethylated glycosphingolipids with hydroxy fatty acids are recorded at barely 30 mass units higher than those with nonhydroxy fatty acids. In addition, the combination of different fatty acids and different types of long-chain bases may complicate the interpretation of the fragmentation pattern. This problem can be alleviated by the use of

**Fig. 8.** Principal fragmentations of GL₃ with C24:0.
trimethylsilyl derivatives of cerebrosides, because cerebrosides with hydroxy fatty acids were recorded at 88 mass units higher than those with nonhydroxy fatty acids (20). Additionally, the separation of glycosphingolipids with hydroxy and nonhydroxy fatty acids by TLC prior to analysis may also be preferable (Fig. 1). In this study, however, we have shown that glycosphingolipids with hydroxy fatty acids can be differentiated from those with nonhydroxy fatty acids by comparing the intensities of characteristic fragment ions (Figs. 3A, 3B, 4A, and 4B). Karlsson, Samuelsson, and Steen (15) also reported the mass spectra of molecular species of cerebrosides isolated from bovine kidney and the advantage of using borate-impregnated silica gel column chromatography to separate six types of cerebrosides before mass spectrometric analysis.

In conclusion, the CI method is particularly valuable in quantitative studies of glycosphingolipids, because the mode of fragmentation is quite simple and informative, and the presence of glycosphingolipids is demonstrated. Quantitative analysis of the CI mass spectrometry has already been demonstrated for triglycerides (27) and cerebrosides (20). The authors are indebted to Miss Hisako Kojima, Department of Biochemistry, Kitasato University, for preparing palmitoyl glucosyl ceramide. The authors also acknowledge thanks to Dr. Robert K. Yu, Department of Neurology, School of Medicine, Yale University, for valuable discussions in preparing this manuscript.

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