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Structural characterization of neutral glycosphingolipids using high-performance liquid chromatography-electrospray ionization mass spectrometry with a repeated high-speed polarity and MS^n switching system

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Abstract Four types of neutral glycosphingolipids (LacCer, Gb_3Cer, Gb_4Cer, and $IV^3\alpha GalNAc\text{-}Gb_4Cer$; 10 pmol each) were analyzed using high-performance liquid chromatography (HPLC)-electrospray ionization quadrupole ion trap time-of-flight (ESI-QIT-TOF) mass spectrometry (MS) with a repeated high-speed polarity and MS^n switching system. This system can provide six types of mass spectra, including positive and negative ion MS, MS^2, and MS^3 spectra, within 1 s per cycle. Using HPLC with a normal-phase column, information on the molecular weights of major molecular species of four neutral glycosphingolipids was obtained by detecting $[M+Na]^+$ in the positive ion mode mass spectra and $[M-H]^-$ in the negative ion mode mass spectra. Sequences of glycosphingolipid oligosaccharide were obtained in the negative ion MS^2 spectra. In addition, information on the ceramide structures was clearly obtained in the negative ion MS^3 mass spectra. GlcCer molecular species were analyzed by HPLC-ESI-QIT-TOF MS with a reversed-phase column using 1 pmole of GlcCer. The structures of the seven molecular species of GlcCer, namely, d18:1-C16:0, d18:1-C18:0, d18:1-C20:0, d18:1-C22:0, d18:1-C23:0, d18:1-C24:1, and d18:1-C24:0, were characterized using positive ion MS and negative ion MS, MS^2, and MS^3. The established HPLC-ESI-QIT-TOF MS with MS^n switching and a normal phase column has been successfully applied to the structural characterization of LacCer and Gb_4Cer in a crude mixture prepared from human erythrocytes.

Keywords Glycosphingolipids · LC-MS · QIT-TOF · High-speed MS^n switching · Polarity switching

Abbreviations

GlcCer	Glucosylceramide
LacCer	Lactosylceramide
Gb_3Cer	Globotriaosylceramide
Gb_4Cer	Globotetraosylceramide
$IV^3\alpha GalNAc\text{-}Gb_4Cer$	Forssman antigen
GSLs	Glycosphingolipids
HPLC	High-performance liquid chromatography
ESI	Electrosprayionization
QIT-TOF	Quadrupole ion trap time-of-flight
MS	Mass spectrometry

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Introduction

Glycolipids are present ubiquitously in mammalian tissues and cell membranes. Cell membranes contain glycolipids in the form of glycosphingolipids (GSLs). GSLs, consisting of a hydrophobic ceramide and a hydrophilic carbohydrate chain, are typically located in the outer leaflet of the lipid bilayer of cell membranes, and their carbohydrate chains extend into the extracellular space. Recent studies have explored the biological functions of GSLs as a component of various types of microdomains or lipid rafts, which are formed together with cholesterol and sphingomyelin. Microdomains are thought to play a critical role in cellular functions such as cell–cell recognition, cell growth, and signal transduction [1–8]. To elucidate the physiological roles of GSLs, characterization of both carbohydrate structures and ceramide species is essential [9–12].

Mass spectrometry (MS) is an indispensable tool for the structural characterization of GSLs because it provides information on molecular weight, carbohydrate sequences, and ceramide structures of GSLs prepared from biological materials, even in small amounts [13–16]. Recently, electrospray ionization quadrupole ion trap time-of-flight (ESI-QIT-TOF) MS, which can perform collision-induced dissociation (CID)-MS^n analysis, was developed and applied to the fine structural characterization of organic compounds [17, 18].

We previously reported the structural characterization of gangliosides using QIT-TOF MS equipped with an atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI) source and repeated high-speed polarity and MS^n switching. This method can maintain analytes under atmospheric conditions and perform MS^n analysis with both positive and negative ion modes. Using this technique, the structures of gangliosides, including GM1, GM2, GD1a, GD1b, and GT1b, were characterized [19].

In this study, we applied ESI-QIT-TOF MS equipped with high-performance liquid chromatography (HPLC) and the high-speed polarity and MS^n switching system to structurally characterize a mixture of neutral GSLs. This HPLC-MS system provides positive- and negative-MS, MS^2, and MS^3 spectra within 1 s per cycle. In this report, a mixture of GSLs was analyzed using this HPLC-MS method with a normal-phase column, and molecular species of GlcCer were separated and analyzed with a reversed-phase column [20–21]. We have applied this established method with a normal phase column to the structural characterization of LacCer and Gb_4Cer in a crude mixture prepared from human erythrocytes.

Materials and methods

Materials

HPLC-grade isopropanol, *n*-hexane, water, acetic acid, chloroform, and methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). GlcCer (glucosylceramide, Glcβ1-1′Cer) purified from the spleen of a patient with Gaucher's disease was kindly supplied by Dr. M. Oshima (Tohoku University of Community Service and Science). LacCer (lactosylceramide, Galβ1-4Glcβ1-1′Cer), Gb_3Cer (globotriaosylceramide, Galα1-4Galβ1-4Glcβ1-1′Cer), and Gb_4Cer (globotetraosylceramide, GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer) were purified from human erythrocyte stroma [22], and IV^3αGalNAc-Gb_4Cer (Forssman antigen, GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1′Cer) was purified from sheep erythrocyte stroma [23] in our laboratory. We purified several fractions of LacCer with different ceramide structures and we used in this experiment, one of the late fractions obtained from a silica column. Then, the major molecular species is LacCer (d18:1–16:0).

Sample preparation for the measurement of mass spectra of neutral glycosphingolipids

A mixture containing 5 pmol/μl of four neutral GSLs, including LacCer, Gb_3Cer, Gb_4Cer, and IV^3αGalNAc-Gb_4Cer, was prepared in chloroform–methanol (2:1, v/v). GlcCer was dissolved in chloroform–methanol (2:1, v/v) at 0.5 pmol/μl. The mixture solution (2 μl) of neutral GSLs and 2 μl of the GlcCer solution were injected into a HPLC and analyzed using HPLC-MS.

HPLC conditions

HPLC was performed with a model Prominence apparatus (Shimadzu Corp., Kyoto, Japan) and a normal-phase column (Develosil 100–7 micron silica gel, 2.0 mm i.d.×150 mm, Nomura Chemical Co., Ltd., Aichi, Japan) or a reversed-phase column (PEGASIL ODS, 2.0 mm i.d.×150 mm, Senshu Scientific Co., Ltd., Tokyo, Japan).

Normal phase HPLC separation of the neutral GSL mixture was performed with a programmed gradient elution with isopropanol–*n*-hexane–water–acetic acid from 10 % of 175:45:20:0.07 (by vol.) in 30:275:0.05:0.09 (by vol.) to 100 % within 10 min at a flow rate of 0.2 ml/min. GlcCer molecular species were analyzed using HPLC-MS with the reverse phase column and 5 mM acetic acid in methanol at a flow rate of 0.2 ml/min.

HPLC-ESI-IT-TOF MS with high-speed polarity and MS^n switching

All experiments were performed on an ESI-QIT-TOF mass spectrometer (Shimadzu Corp.) equipped with a high-performance liquid chromatograph and an electrospray ionization interface. The instrument consists of five main sections: an ion source, a lens system, an ion trap, a reflectron time-of-flight mass analyzer, and an ESI ion source. Ions generated in the ESI ion source passed through the lens system, accumulated in an ion trap, and were then discharged to time of flight. A pulsed gas, Ar, was used for ion cooling and CID. To maintain mass precision, the

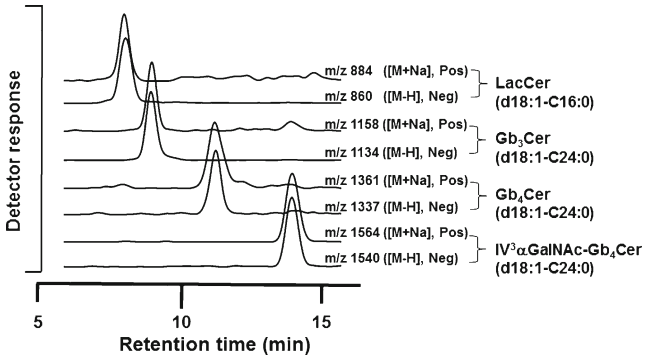

Fig. 1 Mass chromatograms of the major molecular species of LacCer, Gb_3Cer, Gb_4Cer, and $IV^3\alpha GalNAc\text{-}Gb_4Cer$ using $[M+Na]^+$ and $[M–H]^–$

temperature inside the mass spectrometer was controlled within 40±0.3 °C. ESI source voltage was set at 4.5 kV (positive ion mode) and −3.5 kV (negative ion mode), and the heated capillary temperature was 200 °C using nitrogen nebulizer gas. The polarity switching (positive/negative ion mode switching) speed was 0.1 s or less. The mass spectrum measurement cycle time for all positive and negative ion MS, MS^2, and MS^3 spectra (six types of mass spectra), including ion accumulation time, precursor ion selecting time, and CID time, was set to 0.9 s/cycle.

An application to the GSL analysis of human erythrocytes

Human erythrocytes were separated from 10 ml of commercially available human blood (AKC Co. Kyoto, Japan), and washed with 0.85 % NaCl solution three times by centrifugation at 1,600×g for 10 min at room temperature. Packed erythrocytes were suspended in 2 volumes of 0.85 % NaCl solution, 210 μl of the suspension equivalent to 70 μl of the packed erythrocytes were mixed well with 3 ml of methanol by a Vortex mixer, and 6 ml of chloroform was added and mixed well. After filtered with paper (No. 2, Whatman, Maidstone, England), the filtrate was mixed with 2 ml of water and the lower phase was separated from the upper phase. The lower phase lipids were dried under N_2 and treated with 1 ml of 0.1 N NaOH in methanol for 40 °C for 2 h. After neutralized with 1 ml of 0.1 N acetic acid, 2.7 ml of chloroform and 1.4 ml of methanol were added to make phase separation and to remove salts to the aqueous phase. The alkaline stable lipids of the lower phase were dried under N_2 and suspended with 100 μl of hexane-isopropanol (1 : 1). Aliquots were injected to a Develosil silica column (2.0×150 mm) and separated by the gradient elution as described. The selected ion mass chromatograms in negative ion mode for 1 μl injection monitored ions at m/z 860, 970, and 972 for LacCer, and 1335 and 1337 for Gb_4Cer. MS^2 and MS^3 spectra for each molecular species were obtained with 4 μl injection by a manual mode analysis monitoring m/z 860 and then 530 as precursor ions for MS^2 and MS^3, respectively. The same analysis was performed for m/z 970 and 646, and 972 and 648 for LacCer, and m/z 1335 and 646, and 1337 and 648 for Gb_4Cer.

Results

Mass chromatograms of a mixture of neutral GSLs, LacCer, Gb_3Cer, Gb_4Cer, and $IV^3\alpha GalNAc\text{-}Gb_4Cer$

Figure 1 shows mass chromatograms obtained by injection of the mixture containing 10 pmol each of LacCer, Gb_3Cer, Gb_4Cer, and $IV^3\alpha GalNAc\text{-}Gb_4Cer$. These mass chromatograms were recorded by selecting the major molecular related ions of $[M+Na]^+$ and $[M–H]^–$, with high-speed polarity switching. In the chromatograms, major molecular species of GSLs are clearly observed at *m/z* 884 as $[M+Na]^+$ and 860 as $[M–H]^–$ for LacCer (d18:1-C16:0), *m/z* 1158 as $[M+Na]^+$ and 1134 as $[M–H]^–$ for Gb_3Cer (d18:1-C24:0), *m/z* 1361 as $[M+Na]^+$ and 1337 as $[M–H]^–$ for Gb_4Cer (d18:1-C24:0), and *m/z* 1564 as $[M+Na]^+$ and 1540 as $[M–H]^–$ for $IV^3\alpha GalNAc\text{-}Gb_4Cer$ (d18:1-C24:0) [24–26]. These four GSLs were well separated within 15 min. Thus, the molecular weights of the major molecular species were 861 for LacCer (d18:1–16:0), 1135 for Gb_3Cer (d18:1-C24:0), 1138 for Gb_4Cer (d18:1-C24:0), and 1541 for $IV^3\alpha GalNAc\text{-}Gb_4Cer$ (d18:1-C24:0).

Negative ion MS^2 mass spectra of neutral GSLs, LacCer, Gb_3Cer, Gb_4Cer, and $IV^3\alpha GalNAc\text{-}Gb_4Cer$

Negative ion MS^2 mass spectra were obtained by selecting the major molecular ions at *m/z* 860 for LacCer, *m/z* 1134 for Gb_3Cer, *m/z* 1337 for Gb_4Cer, and *m/z* 1540 for $IV^3\alpha GalNAc$-

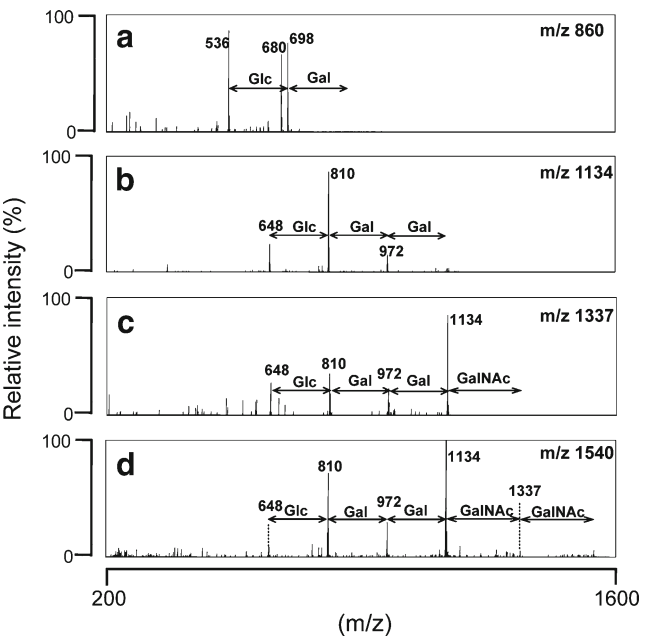

Fig. 2 Negative ion MS^2 mass spectra of the major molecular species of neutral glycosphingolipids. MS^2 was performed with selected precursor ions at *m/z* 860 for LacCer (**a**), *m/z* 1134 for Gb_3Cer (**b**), *m/z* 1337 for Gb_4Cer (**c**), and *m/z* 1540 for $IV^3\alpha GalNAc\text{-}Gb_4Cer$ (**d**)

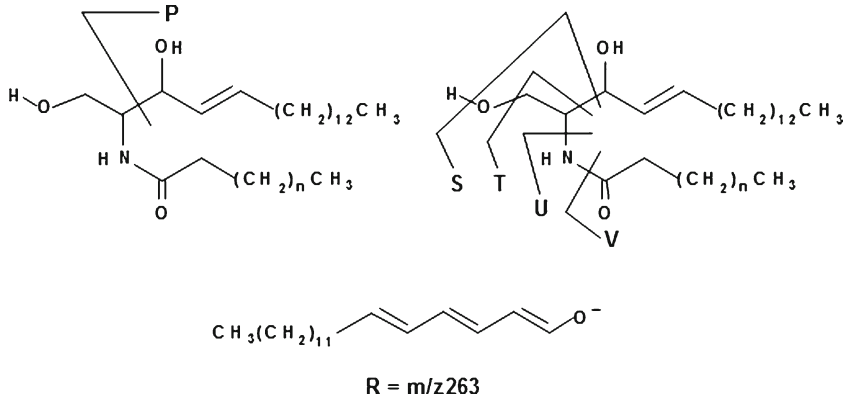

Fig. 3 Characteristic product ions in the negative ion mode for ceramide. The nomenclature used here was established by Ann and Adams [27, 28] and reported by Lee *et al.* [29]

Gb_4Cer as the precursor ions. As shown in Fig. 2a, sequential elimination of galactose (Gal) and glucose (Glc) from LacCer were observed at *m/z* 698 and 536, respectively. The ion at *m/z* 680 is the dehydrate ion of GlcCer. Sequential elimination of carbohydrate species from the molecules were also observed for Gb_3Cer at *m/z* 972, 810, and 648 (Fig. 2b), for Gb_4Cer at *m/z* 1134, 972, 810, and 648 (Fig. 2c), and for $IV^3\alpha GalNAc\text{-}Gb_4Cer$ at *m/z* 1337, 1134, 972, 810, and 648 (Fig. 2d). These were responsible for the elimination of Gal, Gal, and Glc from the oligosaccharide Gb_3Cer, *N*-acetylgalactosamine (GalNAc), Gal, Gal, and Glc from Gb_4Cer, and GalNAc, GalNAc, Gal, Gal, and Glc from $IV^3\alpha GalNAc\text{-}Gb_4Cer$, respectively.

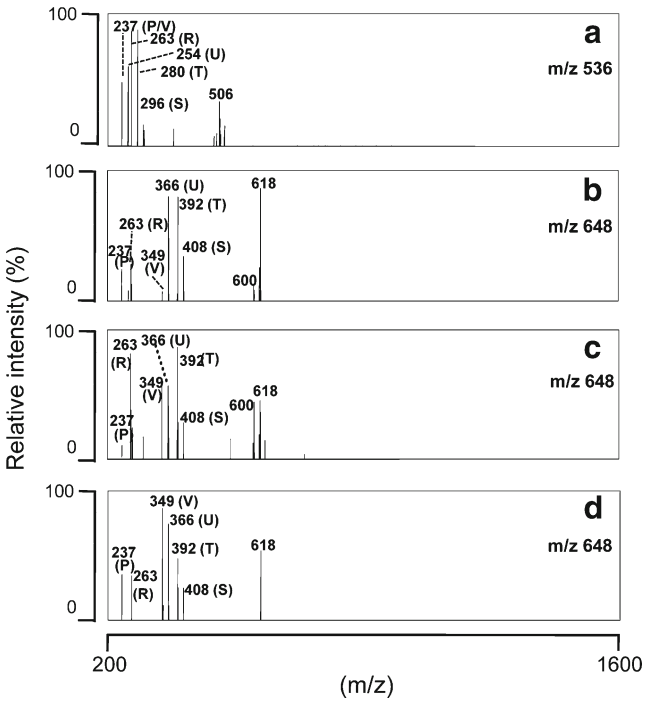

Fig. 4 Negative ion MS^3 mass spectra of the major molecular species of neutral glycosphingolipids. MS^3 was performed with select precursor ions at *m/z* 536 for LacCer (**a**), *m/z* 648 for Gb_3Cer (**b**), *m/z* 648 for Gb_4Cer (**c**), and *m/z* 648 for $IV^3\alpha GalNAc\text{-}Gb_4Cer$ (**d**)

Negative ion MS^3 (MS/MS/MS) mass spectra of neutral GSLs, LacCer, Gb_3Cer, Gb_4Cer, and $IV^3\alpha GalNAc\text{-}Gb_4Cer$

Characteristic product ions of ceramide generated in the negative ion mode [13], which were established by Ann and Adams [27, 28] and reported by Lee *et al.* [29], are shown in Fig. 3.

Figure 4 shows negative ion MS^3 mass spectra obtained by selecting the precursor ions for ceramides: *m/z* 536 for LacCer and *m/z* 648 for Gb_3Cer, Gb_4Cer, and $IV^3\alpha GalNAc\text{-}Gb_4Cer$. In the MS^3 spectrum of LacCer shown in Fig. 4a, fragment ions derived from fatty acid structures, S, T, U, and V (shown in Fig. 3), were clearly observed at *m/z* 296, 280, 254, and 237, indicating that the fatty acid structure of the ceramide of LacCer was C16:0 fatty acid. The ions responsible for the sphingosine structure of LacCer were detected at *m/z* 263 and 237 (R and P, shown in Fig. 3), indicating that the sphingosine structure of LacCer was identified as d18:1. These results indicated that the fine structure of LacCer was LacCer with d18:1 sphingosine and C16:0 fatty acid.

In the negative ion MS^3 spectrum of Gb_3Cer, as shown in Fig. 4b, the ions identified as S, T, U, V, R, and P were clearly observed at *m/z* 408, 392, 366, 349, 263, and 237, respectively,

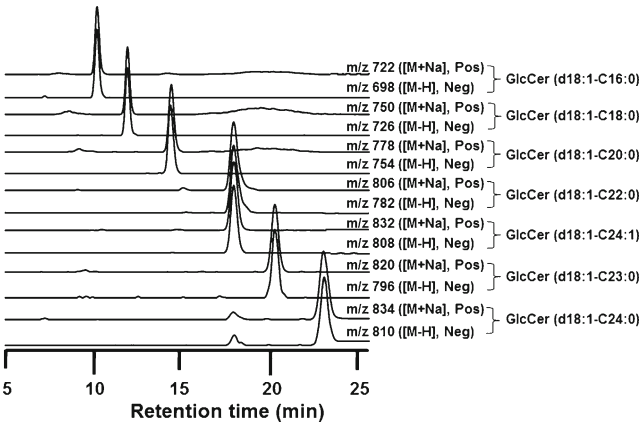

Fig. 5 Mass chromatograms of the major molecular species of GlcCer detected using $[M+Na]^+$ and $[M–H]^-$

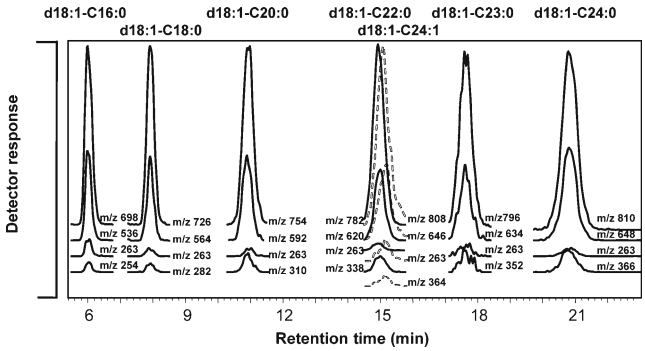

Fig. 6 Mass chromatograms of the molecular species of GlcCer using negative ion MS, MS^2, and MS^3. Molecular species containing d18:1 - 24:1 are shown on the dotted line

indicating that the structure of Gb_3Cer was Gb_3Cer containing d18:1-C24:0. As shown in Fig. 4c for Gb_4Cer, S, T, U, V, R, and P ions are clearly observed at *m/z* 408, 392, 366, 349, 263, and 237, respectively, indicating that the structure of Gb_4Cer was Gb_4Cer containing d18:1-C24:0. As shown in Fig. 4d for $IV^3\alpha GalNAc\text{-}Gb_4Cer$, S, T, U, V, R, and P ions were clearly observed at *m/z* 408, 392, 366, 349, 263 and 237, respectively, indicating that the ceramide structure of $IV^3\alpha GalNAc\text{-}Gb_4Cer$ was d18:1–24:0.

Mass chromatograms detecting ions of $[M+Na]^+$ and $[M–H]^–$ of glucosylceramide molecular species obtained using high-speed polarity switching MS

Figure 5 shows mass chromatograms of GlcCer molecular species obtained by injecting 1 pmol of GlcCer. Molecular ions of seven molecular species were clearly detected at *m/z* 722 ($[M+Na]^+$) and 698 ($[M–H]^–$) for GlcCer (d18:1-C16:0), 750 ($[M+Na]^+$) and 726 ($[M–H]^–$) for GlcCer (d18:1-C18:0), 778 ($[M+Na]^+$) and 754 ($[M–H]^–$) for GlcCer (d18:1-C20:0), 806 ($[M+Na]^+$) and 782 ($[M–H]^–$) for GlcCer (d18:1-C22:0), 832 ($[M+Na]^+$) and 808 ($[M–H]^–$) for GlcCer (d18:1-C24:1), 820 ($[M+Na]^+$) and 796 ($[M–H]^–$) for GlcCer (d18:1-C23:0), and 834 ($[M+Na]^+$) and 810 ($[M–H]^–$) for GlcCer (d18:1-C24:0). Thus, the molecular weights of the molecular species of GlcCer were determined to be 699 [GlcCer (d18:1-C16:0)], 727 [GlcCer (d18:1-C18:0)], 755 [GlcCer (d18:1-C20:0)], 783 [GlcCer (d18:1-C22:0)], 809 [GlcCer (d18:1-C24:1)], 797 [GlcCer (d18:1-C23:0)], and 811 [GlcCer (d18:1-C24:0)]. These seven molecular species of GlcCer were separated within 25 min.

Mass chromatographic fine structural characterization of molecular species of GlcCer obtained by high-speed polarity and negative ion MS, MS^2, and MS^3 switching

Figure 6 shows mass chromatograms of seven molecular species of GlcCer obtained by negative ion MS, MS^2, and MS^3. MS^2 analyses of seven molecular species of GlcCer were performed by selecting $[M\text{-}H]^-$ ions as the precursor ions at *m/z* 698 for d18:1-C16:0, *m/z* 726 for d18:1-C18:0, *m/z* 754 for d18:1-C20:0, *m/z* 782 for d18:1-C22:0, *m/z* 808 for d18:1-C24:1, *m/z* 796 for d18:1-C23:0, and *m/z* 810 for d18:1-C24:0, which were clearly detected in the mass chromatograms shown in Fig. 6. Ceramide ions were detected at *m/z* 536 for d18:1-C16:0, *m/z* 564 for d18:1-C18:0, *m/z* 592 for d18:1-C20:0, *m/z* 620 for d18:1-C22:0, *m/z* 646 for d18:1-C24:1, *m/z* 634 for d18:1-C23:0, and *m/z* 648 for d18:1-C24:0, as shown in the chromatograms. Structures of the ceramide of seven GlcCers were analyzed using negative ion MS^3 MS selecting the ceramide ions as the precursors: *m/z* 536, 564, 592, 620, 646, 634, and 648. As a result, ions due to the sphingosine base (R) and fatty acid (U) of GlcCer were clearly detected at *m/z* 263 and 254 for d18:1-C16:0, *m/z* 263 and 282 for d18:1-C18:0, *m/z* 263 and 310 for d18:1-C20:0, *m/z* 263 and 338 for d18:1-C22:0, *m/z* 263 and 364 for d18:1-C24:1, *m/z* 263 and 352 for d18:1-C23:0, and *m/z* 263 and 366 for d18:1-C24:0. These results indicated that the fine structures of the seven molecular species of GlcCer were GlcCer containing d18:1-C16:0, d18:1-C18:0, d18:1-C20:0, d18:1-C22:0, d18:1-C24:1, Cd18:1–23:0, and d18:1-C24:0.

An application to the GSL analysis of human erythrocytes

Figure 7a shows selected ion mass chromatograms detecting m/z 860, 970, and 972 for LacCer and 1315 and 1337 for Gb_4Cer. Figure 7b and c exhibit MS^2 and MS^3 spectra detecting fragment ions from m/z 970 and 646 as the precursor ions, respectively, providing sufficient information for the identification of oligosaccharide sequence and ceramide structure to be LacCer(d18:1–24:1). Figure 7d and e exhibit MS^2 and MS^3 spectra detecting fragment ions derived from m/z 860 and 536 as the precursor ions, respectively, identifying the structure to be LacCer(d18:1–16:0). LacCer(d18:1–24:0) was also identified by MS^2 and MS^3 spectra in a similar way (data not shown). Figure 7f and g show MS^2 and MS^3 spectra detecting fragment ions derived from m/z 1337 and 648 as the precursor ions, respectively, providing sufficient information for the identification of oligosaccharide sequence and ceramide structure to be Gb_4Cer(d18:1–24:0). Figure 7h and i show MS^2 and MS^3 spectra detecting fragment ions derived from m/z 1335 and 646 as the precursor ions, respectively, identifying the structure to be Gb_4Cer(d18:1–24:1). The fragment ion at m/z 237 (P) derived from ceramide in MS^3 was small or undetectable in the case of long chain fatty acid containing ceramides, as shown in Fig. 7i. However, the ion at m/z 263 (R) was detected, indicating that sphingosine is d18:1. It is clearly shown that identification of LacCer and Gb_4Cer by the present method is possible using alkaline stable lipids prepared from a small amount of erythrocytes equivalent to 70 μl of packed erythrocytes.

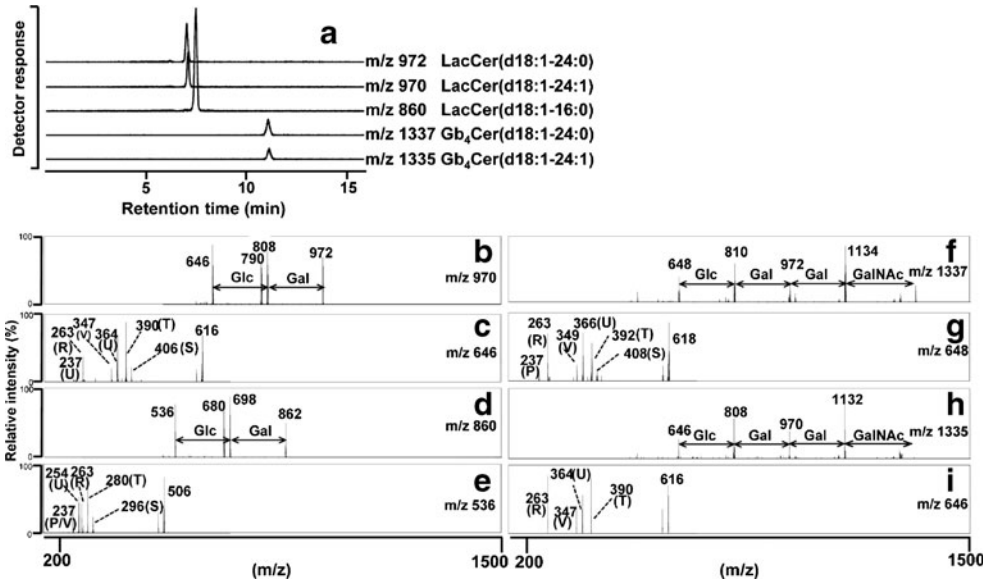

Fig. 7 An application to the GSL analysis of human erythrocytes. The selected ion-mass chromatogram **a** monitored signals at m/z 860, 970, 972, 1335, and 1337. MS^2 and MS^3 spectra, **b** for 970 and **c** for 646 as the precursors, respectively, clearly detected carbohydrate chain- and ceramide-derived fragments, characterizing LacCer(d18:1 - 24:1). MS^2 and MS^3 spectra, **d** for 860 and **e** for 536 as the precursors, respectively, detected fragments characterizing LacCer(d18:1 - 18:0). MS^2 and MS^3 spectra, **f** for 1337 and **g** for 648 as the precursors, respectively, detected fragments for characterizing Gb_4Cer(d18:1 - 24:0). MS^2 and MS^3 spectra, **h** for 1335 and **i** for 646 as the precursors, respectively, detect fragments for characterizing Gb_4Cer(d18:1 - 24:1)

Discussion

GSLs consist of a large number of molecules with different carbohydrate structures, as well as a variety of ceramide structures. Therefore, obtaining detailed structural information on ceramides as well as carbohydrates is important.

Kushi *et al*. [30] analyzed the molecular species of neutral GSLs using HPLC-MS with a reversed-phase column. We also reported the structural characterization and separation of a mixture of GSLs and gangliosides, focusing on differences in their carbohydrate structures using HPLC-MS with a normal-phase column [24]. Based on these HPLC-MS systems in the early stages of technical development, at least more than 100 pmol of GSLs or gangliosides were required to obtain information on the molecular weights and carbohydrate sequences. Furthermore, information on the ceramide structures of the GSLs or gangliosides were not obtained using these systems. Therefore, more sensitive and sophisticated HPLC-MS systems are required to provide information on molecular weights, carbohydrate sequences, and ceramide structures.

Previously, we reported that QIT-TOF MS coupled to an AP-MALDI source with positive and negative ion-MS, MS^2, and MS^3 switching has been successfully applied to the structural characterization of mono-, di-, and trisialogangliosides [19]. The system has high sensitivity and can be used to obtain information on the molecular weights, carbohydrate sequences, and ceramide structures of gangliosides. However, structural characterization can be applied for only purified gangliosides, indicating that the development of efficient and microscale separation methods are required to analyze mixtures of various types of gangliosides.

In this report, neutral GSLs were successfully separated using HPLC with a normal phase-column and analyzed by ESI-QIT-TOF MS with a repeated high-speed polarity and MS^n switching system. This method can provide information on the molecular weights, carbohydrate sequences, and ceramide structures of GSLs by injecting 10 pmol of each GSL as a mixture (Figs. 1, 2, and 4). Structural characterization of the GlcCer molecular species was also achieved with 1 pmol injection into the HPLC with a reversed-phase column (Figs. 5 and 6). These results indicated that structural characterization of GSLs is possible using a normal-phase or reversed-phase column.

The HPLC-ESI-QIT-TOF MS with polarity and MS^n switching system provides six types of mass spectra, namely, MS, MS^2, and MS^3 spectra in the positive and the negative ion modes within 1 s per cycle. The MS^2 spectra of $[M+Na]^+$ for the GSLs provide fragment ions due to sequential monosaccharide elimination, giving information on glycan structures, but MS^3 spectra of ceramides as the precursor ions do not detect sets of fragments like the negative ion mode analysis, giving insufficient information on ceramide structures as reported previously [19]. Therefore, we selected negative ion mode for the present analysis. Selecting negative ion mode MS^n switching increases acquisition time for data collection and can improve spectra quality.

The developed method has been successfully applied to the structural analysis of LacCer and Gb_4Cer in a lipid mixture

prepared from a small amount of human erythrocytes. In this analysis, we have detected Cl-adduct ions which consist about 70 % of LacCer and Gb_4Cer molecular related ions. To obtain structural information of ceramides from the Cl-adduct molecular ions, we need to do MS^4 analysis. MS^2 and MS^3 spectra shown in Fig. 7b to i were obtained using $[M-H]^-$ ions as the precursor ions. Information on ceramide structures obtained by MS^3 of $[M-H]^-$ was much better than that obtained by MS^4 using Cl-adduct molecular ions as the precursor ions. When we analyzed the purified GSLs to establish the analytical method, Cl-adduct molecular ions were much smaller than in the case of crude GSLs, about 40 %, and we took $[M-H]^-$ ions for the further analysis. Reasons how Cl-adduct ions are formed are not clear at present but there are several possibilities; the formation of the Cl-adducts depends on GSL concentration in the electron spray droplets, and Cl may derive from silica column packing material, chloroform used for the extraction or sample solvent for the injection, or 0.85 % NaCl for erythrocyte washing. We have experienced that neutral GSLs rather likely form molecular-related adduct ions, compared to gangliosides, and we need to solve this problem to screen for better matrices.

Another issue is the quantification. Figure 7a clearly shows that the peaks of selected ion mass chromatograms are much higher in LacCer than Gb_4Cer, but it is well described that human erythrocytes contain Gb_4Cer in a much higher content than LacCer [31]. This is due to the difference in the ionization and detection efficiency in LC-MS analysis. Therefore, the quantitation requires stable isotope-labeled internal standards.

Thus, the developed HPLC-ESI-TOF MS could be applied to the structural characterization of neutral GSLs prepared from small amounts of biological materials including tissues, isolated cells, and membrane fractions. We are now applying this method for neutral GSL analysis of membrane fractions, sera, and body fluids. Enrichment of GSLs is required because GSLs are minor components, excluding GalCer and sulfatide in myelin, globo-series GSLs in the kidney, and gangliosides in the brain. GSLs are very well described as one of critical lipid components of microdomains [32], but biochemical methods for the isolation of different types of microdomains from membrane fractions are not available at present, indicating that structural analysis of GSLs as the member of microdomains is not possible. We consider that not the detergent insoluble fraction called the raft but membrane proteins keeping biological functions and purified from detergent soluble membrane fractions are the target for microdomain component analysis for tightly bound membrane lipids including GSLs. An excellent example can be seen for essential phospholipids to support enzymatic activity of cytochrome c oxidase [33]. We consider the same type of specific interaction is possible between GSLs and membrane functional proteins. Then, LC-MS is a powerful method to demonstrate the presence of GSLs in the purified membrane proteins because of its high sensitivity and applicability to GSLs in crude lipid mixtures.

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References

1. Wang, X.Q., Paller, A.S.: Lipid rafts: membrane triage centers. J. Inv. Dermatol. **126**, 951–953 (2006)
2. Hakomori, S.: Structure, organization, and function of glycosphingolipids in membrane. Cur. Opin. Hematol. **10**, 16–24 (2003)
3. Hakomori, S., Igarashi, Y.: Functional role of glycosphingolipids in cell recognition and signaling. J. Biochem. **118**, 1091–1103 (1995)
4. Iwabuchi, K., Nagaoka, I.: Lactosylceramide-enriched glycosphingolipid signaling domain mediates superoxide generation from human neutrophils. Blood **100**, 1454–1464 (2002)
5. Wang, T.Y., Silvius, J.R.: Sphingolipid partitioning into ordered domains in cholesterol-free and cholesterol-containing lipid bilayers. Biophys. J. **84**, 367–378 (2003)
6. Hoekstra, D., Maier, O., van der Wouden, J.M., Slimane, T.A., van IJzendoorn, S.C.: Membrane dynamics and cell polarity: the role of sphingolipids. J. Lipid Res. **44**, 869–877 (2003)
7. Gomez-Munoz, A.: Modulation of cell signalling by ceramides. Biochim. Biophys. Acta **1391**, 92–109 (1998)
8. Cremesti, A.E., Fischl, A.S.: Current methods for the identification and quantitation of ceramides: an overview. Lipids **35**, 937–945 (2000)
9. Rosenthal, M.D.: Fatty acid metabolism of isolated mammalian cells. Prog. Lipid Res. **26**, 87–124 (1987)
10. Sandhoff, K., Kolter, T.: Biosynthesis and degradation of mammalian glycosphingolipids. Philos. Trans. Roy. Soc. Lond. B Biol. Sci. **358**, 847–861 (2003)
11. Colsch, B., Afonso, C., Popa, I., Portoukalian, J., Fournier, F., Tabet, J.C., Baumann, N.: Characterization of the ceramide moieties of sphingoglycolipids from mouse brain by ESI-MS/MS: identification of ceramides containing sphingadienine. J. Lipid Res. **45**, 281–286 (2004)
12. Kaga, N., Kazuno, S., Taka, H., Iwabuchi, K., Murayama, K.: Isolation and mass spectrometry characterization of molecular species of lactosylceramides using liquid chromatography-electrospray ion trap mass spectrometry. Anal. Biochem. **337**, 316–324 (2005)
13. Hsu, F.F., Turk, J.: Characterization of ceramides by low energy collisional-activated dissociation tandem mass spectrometry with negative-ion electrospray ionization. J. Am. Soc. Mass Spectrom. **13**, 558–570 (2002)
14. Hsu, F.F., Turk, J., Stewart, M.E., Downing, D.T.: Structural studies on ceramides as lithiated adducts by low energy collisional-activated dissociation tandem mass spectrometry with electrospray ionization. J. Am. Soc. Mass Spectrom. **13**, 680–695 (2002)
15. Suzuki, Y., Suzuki, M., Ito, E., Goto-Inoue, N., Miseki, K., Iida, J., Yamazaki, Y., Yamada, M., Suzuki, A.: Convenient structural analysis of glycosphingolipids using MALDI-QIT-TOF mass spectrometry with increased laser power and cooling gas flow. J. Biochem. **139**, 771–777 (2006)
16. Hsu, F.F., Turk, J.: Structural determination of glycosphingolipids as lithiated adducts by electrospray ionization mass spectrometry using low-energy collisional-activated dissociation on a triple stage quadrupole instrument. J. Am. Soc. Mass Spectrom. **12**, 61–79 (2001)
17. Han, X., Cheng, H.: Characterization and direct quantitation of cerebroside molecular species from lipid extracts by shotgun lipidomics. J. Lipid Res. **46**, 163–175 (2005)
18. Han, X.: Characterization and direct quantitation of ceramide molecular species from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. Anal. Biochem. **302**, 199–212 (2002)
19. Ito, E., Tominaga, A., Waki, H., Miseki, K., Tomioka, A., Nakajima, K., Kakehi, K., Suzuki, M., Taniguchi, N., Suzuki, A.: Structural characterization of monosialo-, disialo- and trisialo-gangliosides by

negative ion AP-MALDI-QIT-TOF mass spectrometry with MS^n switching. Neurochem. Res. **37**, 1315–1324 (2012)
20. Sommer, U., Herscovitz, H., Welty, F.K., Costello, C.E.: LC-MS-based method for the qualitative and quantitative analysis of complex lipid mixtures. J. Lipid Res. **47**, 804–814 (2006)
21. Merrill Jr., A.H., Sullards, M.C., Allegood, J.C., Kelly, S., Wang, E.: Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. Methods **36**, 207–224 (2005)
22. Ando, S., Yamakawa, T.: Separation of polar glycolipids from human red blood cells with special reference to blood group-A activity. J. Biochem. **73**, 387–396 (1973)
23. Saito, T., Hakomori, S.-I.: Quantitative isolation of total glycosphingolipids from animal cells. J. Lipid Res. **12**, 257–259 (1971)
24. Suzuki, M., Yamakawa, T., Suzuki, A.: High-performance liquid chromatography-mass spectrometry of glycosphingolipids: II. Application to neutral glycolipids and monosialogangliosides. J. Biochem. **108**, 92–98 (1990)
25. Suzuki, M., Yamakawa, T., Suzuki, A.: A micro method involving micro high-performance liquid chromatography-mass spectrometry for the structural characterization of neutral glycosphingolipids and monosialogangliosides. J. Biochem. **109**, 503–506 (1991)
26. Suzuki, M., Sekine, M., Yamakawa, T., Suzuki, A.: High-performance liquid chromatography-mass spectrometry of glycosphingolipids: I. Structural characterization of molecular species of GlcCer and IV^3bGal-Gb_4Cer. J. Biochem. **105**, 829–833 (1989)
27. Ann, Q., Adams, J.: Structure determination of ceramides and neutral glycosphingolipids by collisional activation of $[M+Li]^+$ ions. J. Am. Soc. Mass Spectrom. **3**, 260–263 (1992)
28. Ann, Q., Adams, J.: Structure-specific collision-induced fragmentations of ceramides cationized with alkali-metal ions. Anal. Chem. **65**, 7–13 (1993)
29. Lee, M.H., Lee, G.H., Yoo, J.S.: Analysis of ceramides in cosmetics by reversed-phase liquid chromatography/electrospray ionization mass spectrometry with collision-induced dissociation. Rapid Commun. Mass Spectrom. **17**, 64–75 (2003)
30. Kushi, Y., Rokukawa, C., Numajir, Y., Kato, Y., Handa, S.: Analysis of underivatized glycosphingolipids by high-performance liquid chromatography/atmospheric pressure ionization mass spectrometry. Anal. Biochem. **182**, 405–410 (1989)
31. Yamakawa, T., Nagai, Y.: Glycolipids at the cell surface and their biological functions. Trends Biochem. Sci. **3**, 128–131 (1978)
32. Sonnino, S., Prinetti, A.: Membrane domains and the "lipid raft" concept. Curr. Med. Chem. **20**, 4–21 (2013)
33. Shinzawa-Itoh, K., Aoyama, H., Muramoto, K., Terada, H., Kurauchu, T., Tadehara, Y., Yamasaki, A., Sugimura, T., Kurono, S., Tsujimoto, K., Mizushima, T., Yamashita, E., Tsukihara, T., Yoshikawa, S.: Structures and physiological roles of 13 integral lipids of bovine heart cytochrome c oxidase. EMBO J. **26**, 1713–1725 (2007)