Journal of Chromatography B, 870 (2008) 170–176

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantitative evaluation of sphingomyelin and glucosylceramide using matrix-assisted laser desorption ionization time-of-flight mass spectrometry with sphingosylphosphorylcholine as an internal standard
Practical application to tissues from patients with Niemann-Pick disease types A and C, and Gaucher disease☆

Takehisa Fujiwaki*, Masaru Tasaka, Seiji Yamaguchi

Department of Pediatrics, Shimane University School of Medicine, 89-1 Enya-cho, Izumo 693-8501, Japan

ARTICLE INFO

Article history:
Received 26 November 2007
Accepted 6 May 2008
Available online 16 May 2008

Keywords:
Mass spectrometry
Niemann-Pick disease
Gaucher disease
Sphingolipid

ABSTRACT

Niemann-Pick disease types A and C, and Gaucher disease are glycolipid storage disorders characterized by the systemic deposition of glycosphingolipids, i.e., sphingomyelin in Niemann-Pick disease types A and C tissues and glucosylceramide in Gaucher disease ones, respectively. Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS), we analyzed the sphingolipids in liver and spleen specimens from patients with Niemann-Pick disease types A and C, and Gaucher disease. Crude lipids were extracted from tissue containing 5 mg protein with chloroform and methanol. After mild alkaline treatment of the crude lipids, a sphingolipid fraction was prepared and analyzed by MALDI-TOF/MS. The results were as follows: (a) ion peaks with *m/z* values corresponding to different sphingomyelin and ceramide monohexoside (CMH) species were clearly detected. (b) With sphingosylphosphorylcholine as the internal standard for quantification of sphingomyelin and CMH, the relative peak heights of sphingomyelin and CMH were calculated and plotted versus their contents. The relative peak heights of sphingomyelin and CMH showed linearity between 50 and 1500 ng sphingomyelin content, and between 5 and 150 ng CMH content, respectively. (c) Quantitative analysis revealed the accumulation of sphingomyelin in the liver and spleen specimens from the patients with Niemann-Pick disease types A and C. Striking accumulation of CMH was also detected in the liver and spleen specimens from the patients with Gaucher disease. This investigation indicated that accumulated sphingomyelin and CMH in small amounts of tissues from sphingolipidosis patients can be detected quantatively with the MALDI-TOF/MS method. This method will be useful not only for the diagnosis but also for biochemical pathophysiology evaluation of patients with various sphingolipidosis.

1. Introduction

The laser desorption time-of-flight mass spectrometer (TOF/MS) was developed to analyze non-volatile, thermally labile and high mass organic molecules. Matrix-assisted laser desorption ionization (MALDI) was reported to be a useful technique for the detection of high mass molecules first in 1987 by Tanaka et al. [1]. Delayed ion extraction was later introduced to MALDI-TOF/MS, which dramatically improved the resolution and accuracy of the mass spectra. MALDI-TOF/MS is sensitive and convenient for detecting glycosphingolipids including lysosphingolipids [2–4]. It was reported previously that MALDI-TOF/MS is a useful technique for quantitative analysis of biological materials with high sensitivity [5–11].

Niemann-Pick disease types A and C, and Gaucher diseases are all autosomal recessive inborn errors, sphingolipids storage diseases. Niemann-Pick disease type A is a lysosomal storage disorder that results from the deficient activity of acid sphingomyelinase and the accumulation of sphingomyelin [12]. Niemann-Pick disease type C comprises an error in cellular trafficking of exogenous cholesterol that is associated with lysosomal accumulation of unes-

Abbreviations: MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; 2,5-DHB, 2,5-dihydroxybenzoic acid; CMH, ceramide monohexoside; SPC, sphingosylphosphorylcholine.

☆ This paper was presented at the 32nd Meeting of the Japanese Society for Biomedical Mass Spectrometry, Kyoto, Japan, 27–28 November 2007.

* Corresponding author. Tel.: +81 853 20 2219; fax: +81 853 20 2215.
E-mail address: take-fujiwaki@k7.dion.ne.jp (T. Fujiwaki).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.05.013

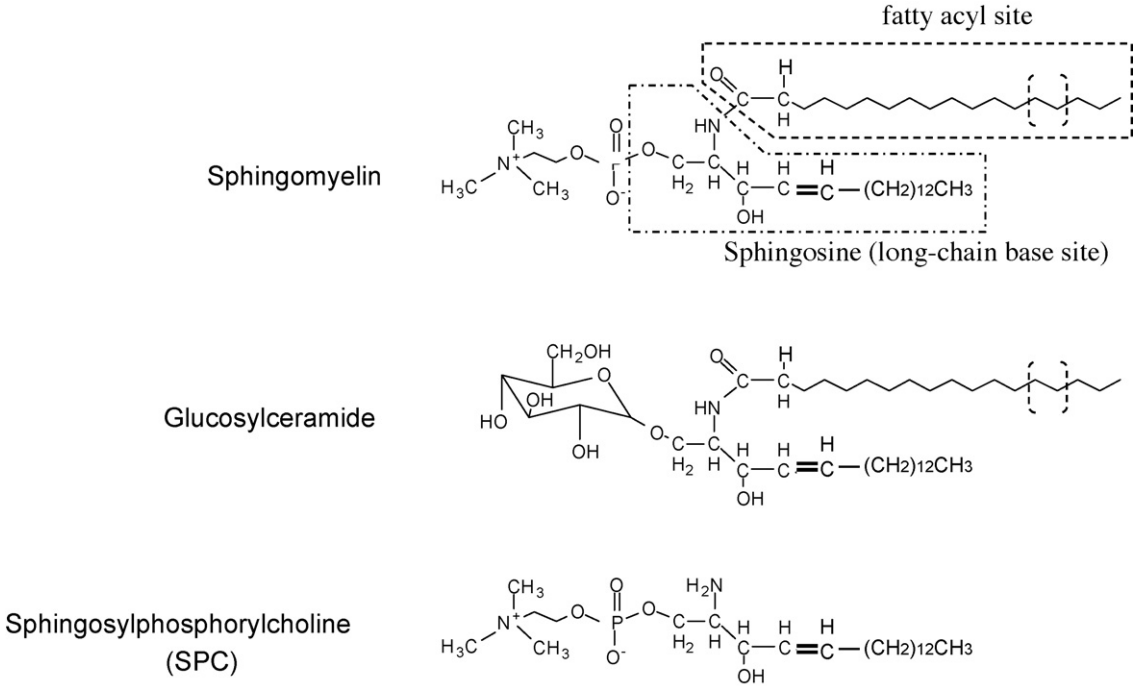

Fig. 1. General molecular structures of sphingomyelin, glucosylceramide (ceramide monohexoside), and sphingosylphosphorylcholine. The N-linked fatty-acyl region typically contains saturated and mono-unsaturated species with chain lengths of C_{14} to C_{26}.

terified cholesterol and sphingomyelin [13]. Sphingomyelin is a phospholipid composed of a long chain base, generally sphingosine, a long chain fatty acid of varying length, and a phosphocholine moiety. Gaucher disease is characterized by the accumulation of glucosylceramide, which is usually called ceramide monohexoside (CMH), as a result of insufficiency of acid β-glucosidase [14]. The structures of sphingomyelin and glucosylceramide are schematically shown in Fig. 1.

Previously, we reported that the qualitative analysis of sphingolipids by MALDI-TOF/MS was useful for the diagnosis of sphingolipidosis [15–18]. We also evaluated ceramide trihexoside quantatively in cardiac valves from a patient with Fabry disease using the MALDI-TOF/MS method [19]. Herein we report quantitative analysis of sphingomyelin and glucosylceramide in tissues from patients with Niemann-Pick disease types A and C, and Gaucher disease using MALDI-TOF/MS. In addition, we investigated the appropriate conditions for applying lipid samples and matrix solutions to TOF/MS sample plates for quantative analysis using MALDI-TOF/MS. The main aim of this study was to develop a method for the highly sensitive quantitative determination of a variety of sphingolipids in small amounts of lipid samples using MALDI-TOF/MS.

2. Materials and methods

2.1. Patients and specimens

The patients were nine pediatric patients with sphingolipidosis, i.e., one with Niemann-Pick disease type A (one female), four with Niemann-Pick disease type C (two males and two females), and four with Gaucher disease (four females). All patients had been diagnosed as having the respective diseases based on enzymatic diagnosis and/or diagnosis by DNA analysis. Autopsy samples from nearly age-matched six patients without metabolic diseases (two with sudden infant death syndrome, one with posthemorrhagic hydrocephalus, two with asphyxia, and one with biliary atresia) were used as controls. All patients and controls were Japanese, and age range was from 2- to 5-year old. Each sample was frozen and then stored at −80 °C until examination. All materials were collected with the informed consent of the patients and/or their parents.

2.2. Preparation of sphingolipids

The method used to prepare the sphingolipid fraction is schematically depicted in Fig. 2. Briefly, after determination of tissue protein concentration with Lowry method, about 5 mg/protein of each tissue sample was homogenized in 10 ml of chloroform–methanol, 2:1 (v/v), sonicated, left for 1 h, and then centrifuged at 1500 × *g* for 5 min, total crude lipids being obtained in the supernatant fluid. We added 2.5 ml of water to the supernatant fluid for Folch partitioning. Then, after centrifugation at 1500 × *g* for 5 min, the lower phase was evaporated to dryness under a nitrogen stream, and then treated with 1 ml of 0.1N NaOH in methanol at 55 °C for 1 h to decompose all esterified glycerolipids including glycerophospholipids and triacylglycerols. After acidification with 0.2 ml of 1N HCl in methanol, one drop of water and 1 ml of hexane were added, followed by centrifugation at 1500 × *g* for 5 min. After the upper phase had been removed, the lower phase was evaporated under a nitrogen stream. 0.2 ml of the theoretical lower phase, chloroform–methanol–water, 86:14:1 (v/v/v), and 0.8 ml of the theoretical upper phase, chloroform–methanol–water, 3:48:47 (v/v/v), were mixed for Folch partitioning. The resulting mixture was centrifuged at 1500 × *g* for 5 min. After the upper phase had been discarded to remove salts, the lower phase was evaporated under a nitrogen stream. The crude sphingolipids in the residue were dissolved in chloroform–methanol (2:1, v/v) to give a certain concentration for thin-layer chromatography and MALDI-TOF/MS.

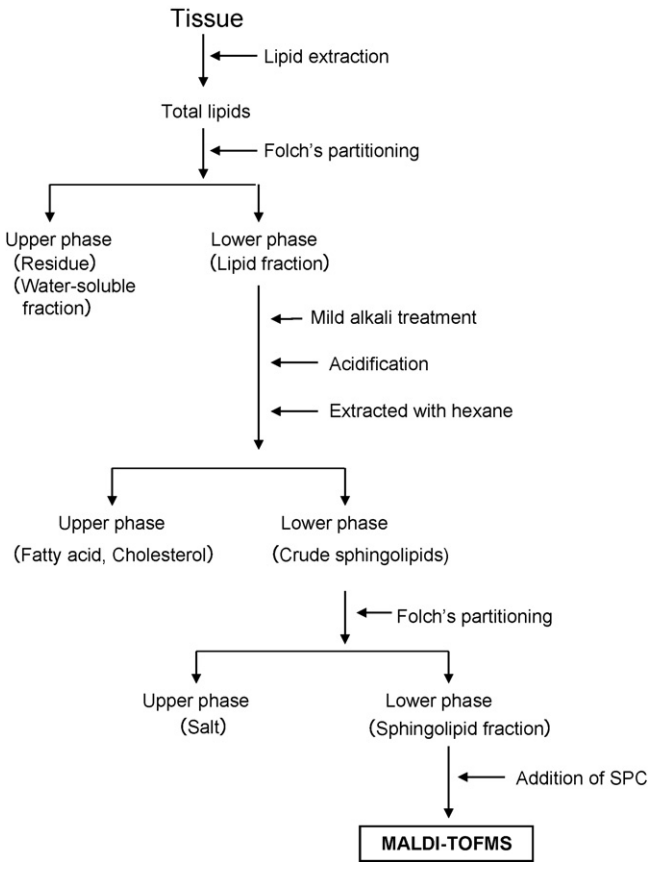

Fig. 2. Schema of preparation of sphingolipid fractions from liver and spleen.

2.3. Thin-layer chromatography

For comparison with MALDI-TOF/MS analysis, thin-layer chromatography was performed on silica gel 60 plates (Merck, Darmstadt) with a solvent system of chloroform–methanol–0.2% $CaCl_2$ in water, 60:30:6 (v/v/v). After development, spots were visualized with primuline reagent under ultraviolet light at a wavelength of 365 nm [20]. All reagents were of analytical grade.

2.4. MALDI-TOF/MS analysis

To 5 μl of the crude sphingolipid solution in a 1.5 ml Eppendorff tube, 5 μl of the matrix solution, i.e., 10 mg of 2,5-dihydroxybenzoic acid (2,5-DHB) in 1 ml of chloroform–methanol, 2: 1 (v/v) was added. The mixture was shaken vigorously on a vortex mixer, and then centrifuged in a microcentrifuge (Chibitan; Tomy Kogyo, Fukushima, Japan) at 2000 × *g* for 1 min. One microliter of the supernatant was loaded into a Voyager™ DE-RP (2.0 m flight length, reflector mode) Biospectrometry™ Workstation (Applied Biosystems, Framingham, MA, USA), and mass spectra of samples were obtained in the positive ion mode with an N_2 laser (337 nm; laser energy, 3.3 kv; radiation time per every laser pulse radiation, 3 μs) (delay, 100 ns; accelerating voltage, 25 kv; and scan average, 256). The resolution of the ion peak was more than 2000 and is represented as $M/\Delta M$ (peak centroid mass/right mass–left mass), the resolution calculator in the GRAMS/386 software (Galactic Industries Corp., Salem, NH) supplied with the instrument being used. Two-point external calibration was performed each time. In the positive ion mode with 2,5-DHB as the matrix, angiotensin I ($[M+H]^+$: 1296.6853) and des-Arg-bradykinin ($[M+H]^+$: 904.4681) were used for calibration of the instrument. Five-point Savitsky–Golay smoothing was applied to the mass spectra as described previously [2–4,21]. 2,5-DHB was purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade.

2.5. Quantitative analysis of sphingomyelin and glucosylceramide

Preceding quantitative analysis, we investigated appropriate conditions for crystallization on TOF/MS sample plates, the crystallization being from the crude sphingolipid solution and matrix (2,5-DHB) solution. When we dissolved 2,5-DHB in chloroform–methanol, 2:1 (v/v), to prepare the matrix solution, a mixture of an adequate amount of a lipid sample and the matrix solution (10 mg of 2,5-DHB in 1 ml of a chloroform–methanol, 2:1 (v/v)), which could be quickly dried with a dryer, showed an amorphous appearance on a TOF/MS sample plate (Fig. 3b). Under these conditions, a crystal showing the amorphous appearance was appropriate for detecting ion peaks in TOF/MS mass spectra, and for reducing variability of ion peak intensity from run to run and from spot to spot. When 2,5-DHB is dissolved in a 9:1 mixture of water–ethanol, the mixture of the lipid sample and the matrix solution naturally dried up in room air, or when the amount of the lipid sample is in excess as to the matrix solution in the mixture, the crystal on the sample plate did not show the amorphous appearance, and did not exhibit sufficient intensity or stable reproducibility (Fig. 3a and c).

Various amounts of authentic sphingomyelin with fatty acid C16:0, dihydroglucosylceramide with fatty acid C18:0, and 50 μg of SPC, as an internal standard, were added and analyzed with MALDI-TOF/MS to obtain a standard curve. A standard curve was obtained each time. MALDI-TOF/MS analysis was performed by the methods mentioned above. The relative peak heights of sphingomyelin and CMH as to that of SPC were calculated. Using the standard curve obtained, from the ion peak intensity ratio for each sphingomyelin, CMH species and SPC, the amounts of sphingomyelin and CMH in each sample tissue were determined. SPC was purchased from Calbiochem-Novabiochem (San Diego, CA, USA), and authentic sphingomyelin and dihydroglucosylceramide were purchased from Sigma Chemicals (St. Louis, MO, USA).

3. Results

The mass spectra of various amounts (50, 150, 500, 1500, and 2500 ng) of authentic sphingomyelin with fatty acid C16:0 and 50 μg of SPC, as an internal standard, obtained on MALDI-TOF/MS for making a standard curve are presented in Fig. 4. When we loaded 5 ng of sphingomyelin on the sample plate, the ion peak corresponding to sphingomyelin was not detected (data not shown). With increasing amounts of added authentic sphingomyelin, each ion peak with *m/z* values corresponding to sphingomyelin, $[M+H]^+$ and $[M+Na]^+$, shows higher intensity. The relative peak height of sphingomyelin, $[M+H]^+$ and $[M+Na]^+$, as to that of SPC was calculated to obtain a standard curve. The standard curve for sphingomyelin and glucosylceramide quantification is shown in Fig. 5. Each point is the mean value of six measurements of the relative peak height. The relative peak height value was plotted against the sphingomyelin and CMH contents. A good linear relationship was obtained between 50 and1500 ng of sphingomyelin and 5 and 150 ng of CMH, as illustrated in Fig. 5. The square of the regression coefficients of standard curve maintained over 0.89 in several times measurements both for sphingomyelin and CMH.

On thin-layer chromatography analysis, the lipid material in the liver and spleen specimens from the patients with Niemann-Pick disease types A and C gave a distinct spot, which exhibited a similar

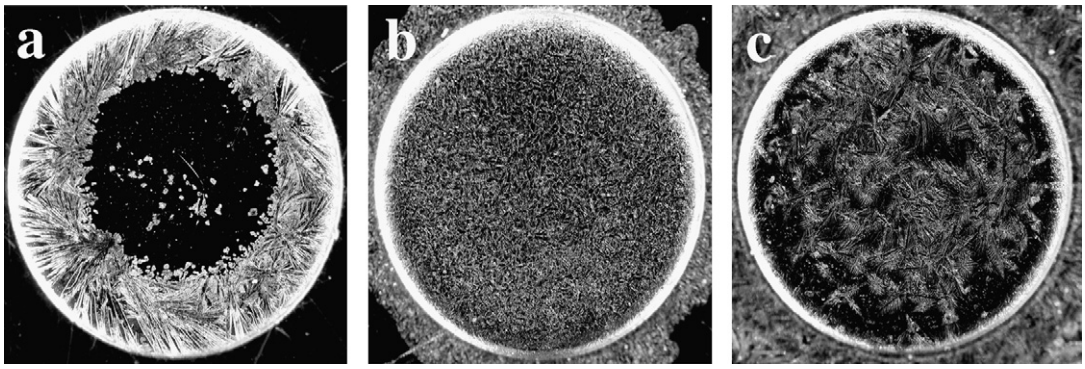

Fig. 3. Enlarged photos of TOF/MS sample plates. The mixture of the crude sphingolipid solution and the matrix (2,5-DHB) solution was loaded onto TOF/MS sample plates. (a) The matrix solution comprised 2,5-DHB in a 9:1 mixture of water–ethanol. The mixture of the lipid sample and the matrix solution, which naturally dried up in room air, crystallized in a ring shape. (b) The matrix solution comprised 2,5-DHB in chloroform–methanol, 2: 1 (v/v). The mixture of an adequate amount of the lipid sample and the matrix solution, which quickly dried up with a dryer, showed an amorphous appearance. (c) The matrix solution comprised 2,5-DHB in chloroform–methanol, 2: 1 (v/v). The mixture of an excess amount of the lipid sample and the matrix solution did not show an amorphous appearance. Crystal (b) was appropriate for detecting ion peaks and for quantification in TOF/MS mass spectra, but crystals (a) and (c) were not appropriate.

Rf value to that of the standard sphingomyelin and is present as a mere trace in the control lane. The lipid material in the liver and spleen specimens from the patients with Gaucher disease gave a distinct spot, that exhibited a similar Rf value to that of the standard CMH and is not present in the control lane.

The mass spectra of sphingolipids in the spleen specimens from the patients with Niemann-Pick disease types A and C, and Gaucher disease, and the control obtained on MALDI-TOF/MS are presented in Fig. 6. Table 1 shows the *m/z* values of the different $[M+Na]^+$ and $[M+H]^+$ ions in the mass spectra in Fig. 6, and the proposed sphingolipid species corresponding to these ions [22]. The *m/z* values of the 487, $[M+Na]^+$ and 465, $[M+H]^+$ ions in the mass spectrum corresponded to SPC in the lipid material in a control spleen specimen (Fig. 6D). The mass spectrum of control spleen showed scanty ion peaks corresponding to different sphingomyelin and CMH species. The mass spectra of the sphingolipids from the spleen specimens from the patients with Niemann-Pick disease types A and C, and Gaucher disease showed SPC ions similar to those in the control one and, in addition, prominent ions corresponding to different sphingomyelin and CMH species (Fig. 6A, B, and C). The contents of sphingomyelin and CMH in the patient's tissues determined by MALDI-TOF/MS are presented in Figs. 7 and 8, respectively.

Hence, the thin-layer chromatography and MALDI-TOF/MS results were compatible, indicating that sphingomyelin accumulated in the Niemann-Pick disease types A and C patients, and CMH accumulated in the Gaucher disease patients.

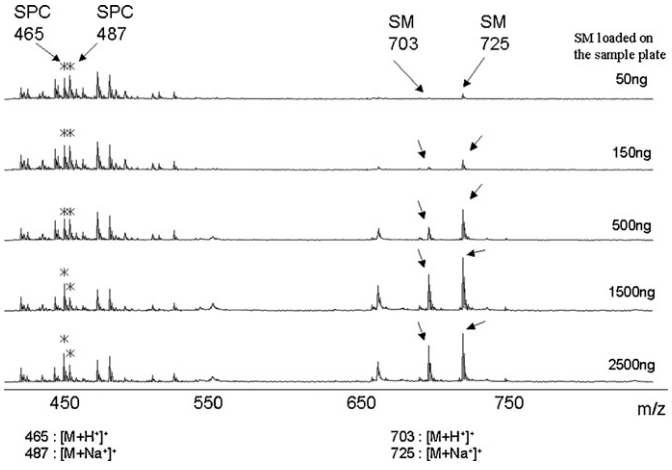

Fig. 4. TOF/MS mass spectra of various amounts of authentic sphingomyelin with fatty acid C16:0 and 50 μg of SPC, as an internal standard. The relative peak height of sphingomyelin as to that of SPC increased with the amount of sphingomyelin loaded on the sample plates. Subsequently, the ion peak intensity ratio of sphingomyelin/SPC was calculated to obtain a standard curve. When we loaded 5 ng of sphingomyelin on the sample plate, the ion peak corresponding to sphingomyelin was not detected (data not shown). Abbreviations: SPC, sphingosylphosphorylcholine; SM, sphingomyelin.

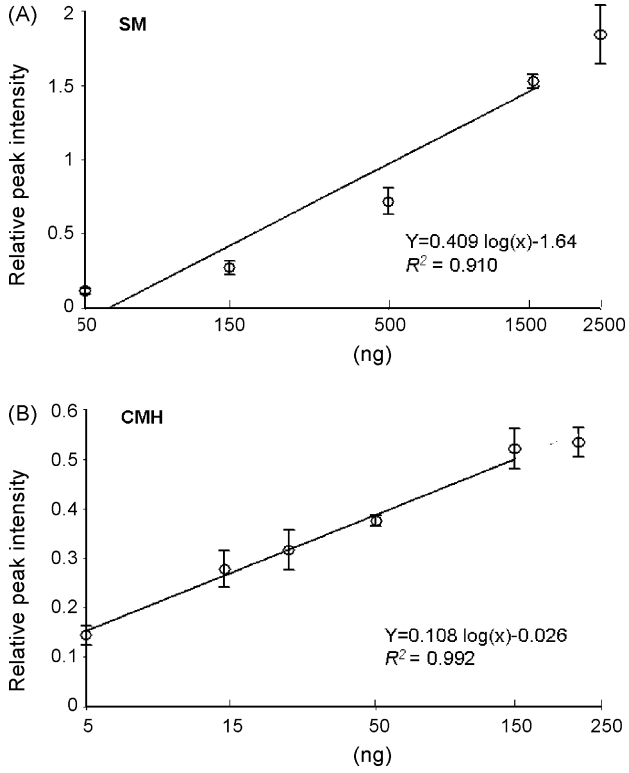

Fig. 5. Representative standard curves for sphingomyelin (A) and CMH (B). The transverse axis indicates the amount of sphingomyelin with fatty acid C16:0 (A) and dihydroglucosylceramide with fatty acid C18:0 (B) loaded on the sample plate. To various amounts of authentic sphingomyelin and CMH, 50 μg of SPC, as an internal standard, was added. Subsequently, MALDI-TOF/MS analysis was performed, and the relative peak heights of sphingomyelin and CMH ions as to that of SPC ions were calculated. The values are the means of six measurements, and the error bars show S.D. "*R*" means regression coefficient. The measure points observed in 2500 ng of sphingomyelin, and 250 ng of CMH, were over a saturation limit. The relative peak intensities of sphingomyelin and CMH showed linearity between 50 and 1500 ng sphingomyelin, and between 5 and 150 ng CMH, respectively.

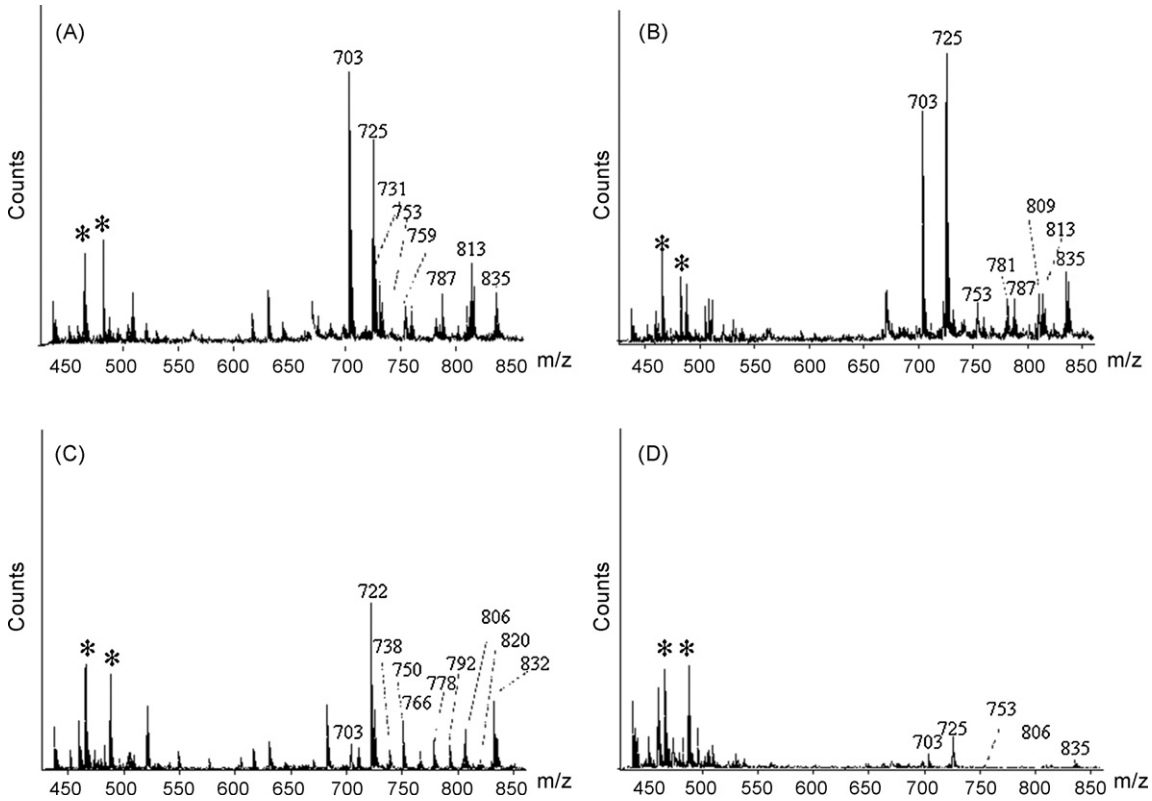

Fig. 6. Representative TOF/MS mass spectra of lipid samples from spleen specimens for Niemann-Pick disease type A (A), Niemann-Pick disease type C (B), Gaucher disease (C), and a control (D) in the positive ion mode magnified. The mass spectrum of the sphingolipids showed ions corresponding to sphingomyelin and CMH species. The mass numbers (*m/z*) and proposed molecular species are shown in Table 1. The matrix used was 2,5-DHB. *: Ion peaks derived from SPC, which was added as an internal standard. Ion peaks with *m/z* values corresponding to sphingomyelin species were dominantly detected in Niemann-Pick disease types A and C, and ones corresponding to CMH species were dominantly detected in Gaucher disease.

4. Discussion

In patients with sphingolipidosis, sphingolipids corresponding to the defective enzymes are accumulated in tissues. For the diagnosis of such disorders, demonstration of accumulating sphingolipids in tissues and/or body fluids of presumptive patients is an important approach. We evaluated the usefulness of the MALDI-TOF/MS method for quantitative analysis of sphingomyelin and CMH in patients with Niemann-Pick disease types A and C, and Gaucher disease in this study.

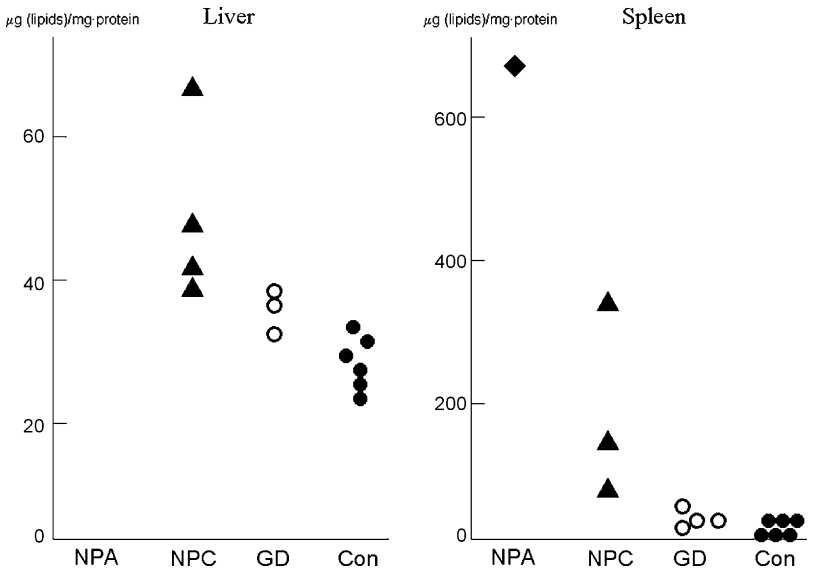

Fig. 7. Sphingomyelin contents in liver and spleen of patients with sphingolipidosis. Sphingomyelin was accumulated in the spleen in Niemann-Pick disease type A, and in both the liver and spleen in Niemann-Pick disease type C. Abbreviations: NPA, Niemann-Pick disease type A; NPC, Niemann-Pick disease type C; GD, Gaucher disease; Con, control. We could not obtain the liver sample from patient with Niemann-Pick disease type A, there is no data of liver sample with NPA.

Table 1
Measured mass-to-charge ratios (*m/z*) and proposed molecular species associated with sphingolipids

Sphingomyelin (*m/z*)	Ceramide monohexoside[a] (*m/z*)
703 (d18:1C16) $[M+H]^+$	722 (d18:1C16) $[M+Na]^+$
725 (d18:1C16) $[M+Na]^+$	738 (d18:1C16:h) $[M+Na]^+$
731 (d18:1C18)$[M+H]^+$	750 (d18:1C18) $[M+Na]^+$
753 (d18:1C18) $[M+Na]^+$	766 (d18:1C18:h) $[M+Na]^+$
759 (d18:1C20) $[M+H]^+$	778 (d18:1C20) $[M+Na]^+$
781 (d18:1C20)$[M+Na]^+$	792 (d18:1C20:1 h) $[M+Na]^+$
787 (d18:1C22) $[M+H]^+$	806 (d18:1C22) $[M+Na]^+$
809 (d18:1C22) $[M+Na]^+$	820 (d18:1C23) $[M+Na]^+$
813 (d18:1C24:1) $[M+H]^+$	832 (d18:1C24:1) $[M+Na]^+$
835 (d18:1C24:1) $[M+Na]^+$	
	SPC (*m/z*)[b]
	465 (d18:1) $[M+H]^+$
	487 (d18:1) $[M+Na]^+$

"d" indicates dihydroxy-sphingosine. "h" indicates hydroxy-fatty acid.
[a] Ceramide monohexoside (CMH) includes glucosylceramide.
[b] SPC: sphingosylphosphorylcholine.

Sugiyama et al. first reported the quantitative analysis of glycosphingolipids, including serum sulfatide, using MALDI-TOF/MS [23]. In that study, hydrogenated *N*-acetyl lysosulfatide was used as an internal standard, and the results corresponded well to the reported data obtained on gas-liquid chromatography. In our previous study, we evaluated the increase in sphingomyelin or CMH using MALDI-TOF/MS as the ion peak intensity ratio of CMH/sphingomyelin. Tissues [15], cultured cells [16], and body fluids [17] from Niemann-Pick disease patients showed a low ion intensity ratio of CMH/sphingomyelin, indicating the accumulation of sphingomyelin, and ones from Gaucher disease patients showed a high ion intensity ratio of CMH/sphingomyelin, indicating the accumulation of CMH. In this study, we confirmed more directly the accumulation of sphingomyelin in Niemann-Pick disease types A and C, and CMH in Gaucher disease, respectively.

We examined appropriate conditions for crystallization on TOF/MS sample plates. In general, when a sample is analyzed by MALDI-TOF/MS, ionization is a very important step. To reduce the variability from run to run and from spot to spot, it is necessary to obtain homogenous crystals on a sample plate. In our study, the best result was obtained when the matrix, 2,5-DHB, is dissolved in chloroform–methanol, 2:1 (v/v), the mixture of the lipid sample and the matrix solution subsequently quickly drying up with a dryer.

In this study, we used SPC as an internal standard. SPC is physiologically nonexistent in normal human tissues or body fluids except as an artifact. Sphingomyelin and glucosylceramide with fatty acid C17:0, which are candidate internal standards for quantitative analysis of sphingomyelin and glucosylceramide, might possibly exist in small amounts in organisms. Although sphingomyelin and glucosylceramide with fatty acid C17:0 need to be prepared for use, SPC is commercially available and convenient to use.

Sphingomyelin and glucosylceramide are heterogeneous glycosphingolipids that consist of numerous isoforms with various fatty acid moieties. In this study, a standard curve was obtained using sphingomyelin with fatty acid C16:0 and dihydroglucosylceramide with fatty acid C18:0, both of which were purchased commercially, from the calculated ion peak intensity ratio of each sphingomyelin, CMH species and SPC. Consequently, the amounts of sphingomyelin and CMH in the sample tissues were determined as sphingomyelin with fatty acid C16:0 or dihydroglucosylceramide with fatty acid C18:0. Each isoform of sphingomyelin and CMH with a different fatty acid moiety might exhibit a different ionization tendency. While our new approach with MALDI-TOF/MS has an advantage of the direct determination of ions of sphingolipids without requirement of many steps to purify the samples and/or conversion of ones to its lyso form, is disadvantageously under the influence of complicated fatty acid composition of sphingolipids. In future, ionization tendency of different molecular species involving different fatty acids in sphingomyelin and CMH should be investigated. Based on that study, more precise quantitative method will be established for the compounds of several molecular species such as clinical samples for example presented in this paper. However the method with MALDI-TOF/MS presented here was sufficiently

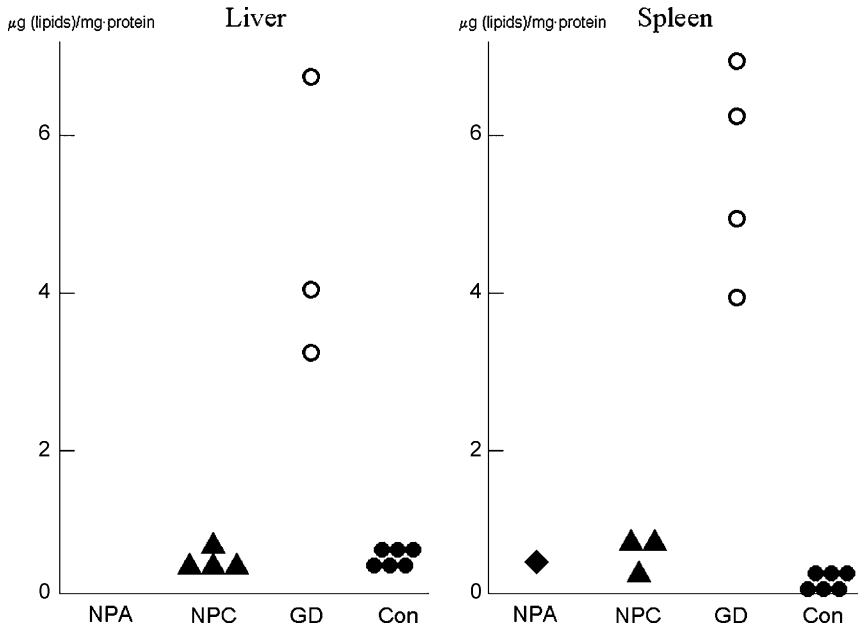

Fig. 8. Ceramide monohexoside (CMH) contents in liver and spleen of patients with sphingolipidoses. CMH includes glucosylceramide. CMH was accumulated in both the liver and spleen in Gaucher disease. Abbreviations: NPA, Niemann-Pick disease type A; NPC, Niemann-Pick disease type C; GD, Gaucher disease; Con, control. We could not obtain the liver sample from patient with Niemann-Pick disease type A, there is no data of liver sample with NPA.

valuable one for clinical diagnosis by quantitative lipid analysis of Niemann-Pick disease types A and C, and Gaucher disease.

In Fig. 4, the mass spectra for sphingomyelin standard, besides the two major ion peaks (*m/z*, 703 and 725), there is also another smaller peak (around 670) whose intensity also increased with the increased concentration of authentic sphingomyelin. It could be a fragment of sphingomyelin molecule broken during the ionization process. This smaller peak (around 670) was not detected in the mass spectra for CMH standard (data not shown). The origin of this smaller peak and impact it would have on the construction of the standard curve should be investigated hereafter with examination using by another analytical method, for example LC/MS–MS, or with alteration of laser intensity on MALDI-TOF/MS.

In previous reports concerning quantitative analysis of accumulating sphingolipids in sphingolipidosis patients' tissues, it was documented that the sphingomyelin levels may be elevated up to fifty-fold in tissues of Niemann-Pick disease type A patients [12], there are two- to five-fold increases in sphingomyelin accumulated in liver and spleen of Niemann-Pick disease type C patients [13], and there are twenty- to one hundred-fold increase in glucosylceramide in liver and spleen of Gaucher disease patients [14]. Our quantitative results presented here are almost consistent with results documented previously. Gas–liquid chromatography has been the main quantitative method for the detection of accumulating sphingolipids in sphingolipidosis patients. In comparison with the quantitative method involving gas-liquid chromatography, the MALDI-TOF/MS method we described in this paper has the following advantages: (a) Crude lipids extracted from about 5 mg/protein of each tissue sample could be subjected to quantitative analysis in this study. The MALDI-TOF/MS method is thus suitable for the analysis of small amounts of samples. (b) With the MALDI-TOF/MS method it is possible to obtain the isoform profiles of sphingomyelin and CMH concurrently with quantification. Additionally, mass spectra of Niemann-Pick disease types A and C, and Gaucher disease show some unknown ion peaks with between *m/z* values 600 and 700, which are not detected in control one (Fig. 6). These unknown ion peaks possibly might be derived from metabolites derived from accumulating sphingolipids. The origin of these unknown ion peaks detected by MALDI-TOF/MS is important and interesting subject, should be studied enthusiastically.

Liquid chromatography–tandem mass spectrometry (LC/MS–MS) has also been applied to the analysis of sphingolipids in samples from patients with sphingolipidosis. Generally speaking, MALDI has advantages over the electrospray ionization (ESI) method, which is used for LC/MS–MS, for investigation of non-volatile and/or high mass molecules, and thus the two methods, MALDI-TOF/MS and LC/MS–MS, should be compared and investigated regarding sensitivity, reproducibility, convenience, and time of analysis for the quantification of glycosphingolipids. ESI method has been considerably established for quantification of volatile and low to middle mass molecules, involving glycosphingolipids. On the other side, MALDI-TOF/MS has some disadvantage against ESI, i.e. poor stability and reproduction of ion intensity result from difficulty of making a homogeneous crystallization of the solute in the matrix. Therefore MALDI-TOF/MS is still challenging stage for quantitative analysis. At present, Gas–liquid chromatography and LC/MS–MS have the advantage of accuracy and reproducibility; MALDI-TOF/MS has one of high degree efficiency of ionization and limit of detection for high mass molecules. In this paper, we indicate the clue for quantitative analysis of MALDI-TOF/MS method.

Quantitative analysis involving MALDI-TOF/MS with SPC as an internal standard is expected to be applicable to the quantification of various glycosphingolipids other than sphingomyelin and CMH in small amounts of tissues or body fluids, and to clinical examination.

5. Conclusion

We showed the accumulation of sphingomyelin in Niemann-Pick disease type A and C tissues, and CMH in Gaucher disease ones quantitatively, using MALDI-TOF/MS as well as thin-layer chromatography. The former method was simple, convenient, and practical for the diagnosis of this disease. MALDI-TOF/MS analysis with SPC as an internal standard may allow the quantification of even low levels of sphingolipids in tissues, and identification of the molecular species of accumulating sphingolipids.

Acknowledgements

We are indebted to Tamotsu Taketomi (Department of Biochemistry, Research Center on Aging and Adaptation, Shinshu University School of Medicine) for the helpful discussions. We also thank Drs. Haruo Takeshita (Department of Legal Medicine, Shimane University School of Medicine), Kazuko Sukegawa (Department of Pediatrics, Gifu University School of Medicine), and Hitoshi Kawahara (Department of Pediatrics, National Matsue Hospital) for the preparation of this paper. This study was partly supported by a grant for the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] K. Tanaka, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, Proc. Second Japan–China Joint Symposium on Mass Spectrom, 1987, p. 185.
[2] T. Taketomi, A. Hara, K. Uemura, H. Kurahashi, E. Sugiyama, Biochem. Biophys. Res. Commun. 224 (1996) 462.
[3] T. Taketomi, A. Hara, K. Uemura, E. Sugiyama, J. Biochem. (Tokyo) 120 (1996) 573.
[4] E. Sugiyama, A. Hara, K. Uemura, T. Taketomi, Glycobiology 7 (1997) 719.
[5] M.W. Duncan, G. Matanovic, A. Cerpa-Poljak, Rapid Commun. Mass Spectrom. 7 (1993) 1090.
[6] D.C. Muddiman, A.I. Gusev, A. Proctor, D.M. Hercules, R. Venkataramanan, W. Diven, Anal. Chem. 66 (1994) 2362.
[7] R. Wang, D. Sweeney, S.E. Gandy, S.S. Sisodia, J. Biol. Chem. 271 (1996) 31894.
[8] J.J. Pitt, J.J. Gorman, Anal. Biochem. 248 (1997) 63.
[9] M. Bizzarri, C. Cavaliere, P. Foglia, C. Guarino, R. Samperi, A. Laganà, Anal. Bioanal. Chem., in press.
[10] K.L. Howard, G.L. Boyer, Anal. Chem. 79 (2007) 5980.
[11] G. Li, R. Hu, Y. Kamijo, T. Nakajima, T. Aoyama, T. Inoue, K. Node, R. Kannangi, M. Kyogashima, A. Hara, Anal. Biochem. 362 (2007) 1.
[12] E.H. Schuchman, R.J. Desnick, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Vall (Eds.), The Metabolic and Molecular Basis of Inherited Diseases, 8th ed., McGraw-Hill, New York, 2001, p. 3589 (Chapter 144).
[13] M.C. Patterson, M.T. Vanier, K. Suzuki, et al., in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Vall (Eds.), The Metabolic and Molecular Basis of Inherited Diseases, 8th ed., McGraw-Hill, New York, 2001, p. 3611 (Chapter 145).
[14] E. Beutler, G.A. Grabowski, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Vall (Eds.), The Metabolic and Molecular Basis of Inherited Diseases, 8th ed., McGraw-Hill, New York, 2001, p. 3635 (Chapter 146).
[15] T. Fujiwaki, S. Yamaguchi, K. Sukegawa, T. Taketomi, J. Chromatogr. B Biomed. Sci. Appl. 731 (1999) 45.
[16] T. Fujiwaki, S. Yamaguchi, K. Sukegawa, T. Taketomi, Brain Dev. 24 (2002) 170.
[17] T. Fujiwaki, S. Yamaguchi, M. Tasaka, N. Sakura, T. Taketomi, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 776 (2002) 115.
[18] T. Fujiwaki, S. Yamaguchi, M. Tasaka, M. Takayanagi, M. Isobe, T. Taketomi, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 806 (2004) 47.
[19] T. Fujiwaki, M. Tasaka, N. Takahashi, H. Kobayashi, Y. Murakami, T. Shimada, S. Yamaguchi, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 832 (2006) 97.
[20] V.P. Skipski, Methods Enzymol. 35 (1975) 396.
[21] T. Taketomi, A. Hara, K. Uemura, H. Kurahashi, E. Sugiyama, J. Biochem. (Tokyo) 121 (1997) 264.
[22] T. Taketomi, E. Sugiyama, Methods Enzymol. 312 (2000) 80.
[23] E. Sugiyama, A. Hara, K. Uemura, Anal. Biochem. 274 (1999) 90.