Characterisation of galactosyl and lactosyl sulfatide species in human serum by MALDI-TOF mass spectrometry

Atsushi Hori [1,2], Makoto Yamaura [1,2], Sunao Morita [3], Takeshi Uehara [2], Takayuki Honda [2], Hiroya Hidaka [1]

[1] Department of Biomedical Laboratory Science, School of Health Sciences, Shinshu University School of Medicine, Japan

[2] Department of Laboratory Medicine, Shinshu University School of Medicine, Japan

[3] Department of Clinical Laboratory, Iida Municipal Hospital, Japan

[1-2] 3-1-1 Asahi, Matsumoto, 390-0821, Japan,

[3] 438 Hachiman, Iida, 395-8502, Japan

(Abstract word count: 199 words)
(Article word count: 2903 words)

Corresponding author:

Hiroya Hidaka, Ph.D.

Department of Biomedical Laboratory Science, School of Health Sciences, Shinshu University School of Medicine, Japan

E-mail : hiroyan@ shinshu-u.ac.jp

Abstract

Background

Sulfatides are found in a variety of tissues and serum lipoproteins. Sulfatide is a molecular species composed of various sphingoid bases, fatty acids and sugar chains; therefore rapid analysis of the qualitative structure is important in clinical assessment.

Methods

In this study, sulfatide-rich fractions were isolated from serum lipids and the sulfatide species analysed by negative ion mode using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Results

Sulfatide species identified in human serum included two different sugar chains, eight sphingoid molecules and various fatty acid side chains including hydroxy fatty acids. In total, 64 galactosyl sulfatides (SM4s) and 49 lactosyl sulfatides (SM3) were identified. Quantitatively, the amount of SM3 was less than 1% of the amount of SM4s. The fatty acids of SM4s of healthy serum (n = 8) were predominantly C16:0 and a hydroxylation C16:0 (C16:0h), followed by very long chain fatty acids (VLCFAs) predominant species, and SM3 was a major component of VLCFAs

Conclusion

This present study described a simple method of human serum sulfatide analysis using MALDI-TOF MS. This method is suitable for clinical laboratories and is likely to increase understanding of the roles of sulfatide species in both physiological and disease states.

Keywords： mass spectrometry, sphingolipid, sulfatide, hydroxy fatty acid, serum lipids.

Introduction

A sulfatide is a glycosphingolipid containing a sulfate ester on a sugar chain. Galactosyl sulfatide

(SM4s) and lactosyl sulfatide (SM3) have galactose and lactose as sugars, respectively.[1] Sulfatides

are found in a range of human tissues, plasma lipoproteins and peripheral blood cell membranes.

SM4s is mainly found in the brain, nervous system, kidney and gastrointestinal tract, and is

associated with renal function, Langerhans islet function, nervous system and immune system,

thrombosis, and virus infection.[2, 3] In humans, SM3 is restricted to the kidney and liver tissues,[4, 5]

and functions for cell adhesion.[6] Sulfatide in serum lipoprotein is mainly synthesised in the liver

and then secreted into the circulation.[7] Sulfatide levels in serum have been reported to correlate with

arteriosclerosis, terminal renal insufficiency and type-2 diabetes mellitus.[8-11]   Sulfatide levels in

serum may be a marker of abnormal organ metabolism and tissue disorders.

Sulfatide species vary in terms of their sphingoid base and fatty acid composition, and species

distribution in the body is tissue-specific. SM4s containing a C22: 0 fatty acid, SM4s (d18: 1 C22:

0), is abundant in the human kidney,[12] whereas SM4s (d18: 1 C24: 0) is mostly found in the human

brain.[13] Levels of SM4s (d18: 1 C22: 0 and C24: 0) are higher in human serous papillary ovarian

carcinoma tissues than normal ovarian stromal tissue.[14] In rats, SM4s (t18: 0 C24: 0h) containing

hydroxyl fatty acids was found in the stomach, while that containing phytosphingosine was found

in the colon.[15] Although sulfatide species differ in terms of fatty acid and sphingoid base, the

functional significance of these differences remains unclear.

A number of sulfatide analysis methods have been reported, including liquid chromatography mass spectrometry (LC-MS). Although LC-MS analysis obviates the need for sample pre-processing, it requires a customised analysis system and is a complicated procedure. In contrast, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has the advantage of a simple and rapid protocol for lipid analyses. MALDI-TOF MS can be applied to clinical assessments.

The present study was performed to develop a simple and sensitive method for identifying sulfatide species in human serum using MALDI-TOF MS. The procedure involved lipid hydrolase treatment, partial purification by hydrophobicity chromatography, and two stages of MALDI-TOF MS.

Materials and Methods

Materials

Healthy volunteers were recruited from the medical department of our university. The subjects gave informed consent for participation. The subjects (n = 8) were 20.4 ± 0.5 year old women. Body mass index (BMI) of subjects was 20.3 ± 1.7 (mean ± standard deviation; SD).

Blood was collected from healthy volunteers after overnight fast and allowed to clot at room temperature. Samples were then centrifuged at 1000 × g for 10 minute and serum was collected

and stored at -30 °C until analysis. The study protocol was approved by the Ethical Review Board of Shinshu University School of Medicine, and included volunteer anonymity.

Serum lipids and glucose concentrations were measured enzymatically using commercial reagent kits (Sekisui Med, Co.,Ltd., Tokyo, Japan and Mizuho Medy Co.,Ltd. Saga, Japan) by a biochemistry autoanalyzer (JCA-BM6070; JEOL Ltd, Tokyo, Japan). Levels of high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C) was measured using direct (or homogeneous) method. Triglyceride was measured using glycerol removal method. Glucose was measured using hexokinase method. Total cholesterol, HDL-C, LDL-C, triglyceride, and glucose (mean ± SD) were 181.5 ± 20.6, 70.8 ± 11.9, 89.0 ± 14.9, 55.0 ± 29.7 and 86 .4 ± 7.2 (mg / dL), respectively.

Chemical reagents

Chloroform and methanol (HPLC grade), phospholipase A_2 (PLA_2) (Type I from Crotalus adamanteus; 510 U/mg), calcium chloride ($CaCl_2$) and hydrochloric acid (HCl) were purchased **from Wako Pure Chemical Co. (Osaka, Ja**pan). Tris-base and 9-aminoacridine (9-AA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Phenyl-Sepharose gel was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The internal standard material (IS) [SM4s (d18: 1 C12: 0)] was purchased from Avanti (Alabaster, AL).

5

Phospholipase A$_2$ treatment of serum

To 500 μL of serum was added 250 μL of Tris-HCl buffer (45 mM, pH 8.0, containing 10 mM CaCl$_2$) and 10 μL PLA$_2$ (0.56 U/mL). The mixture was then sealed under N$_2$ gas and incubated for 20 hours at 37 °C.

Extraction of lipids

Following PLA$_2$ treatment, 5 mL of chloroform/methanol solution (C/M, 2:1 v/v) was added to the sample solution (760 μL), and the mixture was shaken vigorously **for 1 minute. Distilled wate**r (1.0 mL) was then added, the suspension was centrifuged at 855× g for 10 minute, and the lower phase was collected and placed in a glass tube. The lower phase was dried by centrifugation under vacuum, after which the lipid residue was dissolved in 950 μL of C/M (9:1 v/v) and sonicated for 1 minute.

Preparation of a sulfatide-rich fraction

Lipid samples were added to a Phenyl-Sepharose gel (bed capacity: ϕ 5 × 10 mm) equilibrated with C/M (9:1 v/v), and the unbound fraction was eluted using three column volumes of C/M (9:1 v/v). The sulfatide rich fraction was eluted in two stages, the first using C/M (85:15 v/v) and the

second using 100% methanol (five column volumes for each stage). The two eluted sulfatide-rich

fractions were combined and dried by centrifugation under vacuum. That lipid was then dissolved

in $10\,\mu\mathrm{L}$ of matrix solution (10 mg 9-AA in 1 mL of isopropanol/acetonitrile, 3:2 v/v).

Mass spectrometry

Mass spectrometric measurements were performed using a MALDI-TOF mass spectrometer

(ABSCIEX TOF/TOF TM5800 system) in reflector mode. The lipid sample ($0.2\,\mu\mathrm{L}$) was applied to

the metal sample plate, which was then inserted into the MALDI-TOF MS analyzer. The following

parameters were used for analysis: negative ion mode, 1600 shots / spectrum and 3800 – 4600

absorption laser intensity. In addition, measurements were made in the MS/MS mode at 5600

absorption laser intensity.

Sulfatide species in serum

The internal standard material (IS; $1\mu\mathrm{g/mL}$, $50\,\mu\mathrm{L}$) was added to serum obtained from healthy

volunteers (n = 8) after overnight fast, and the samples were pretreated with PLA_2 as described

above. After delipidation of each serum sample, sulfatide-rich fractions were partially purified by

Phenyl-Sepharose gel chromatography, and then sulfatide species were measured by MALDI-TOF

MS. The SM4s species was measured quantitatively with the internal standard material, and then

the peak ratios of SM4s and SM3 were calculated independently.

Results

Mass spectrum of partial purified sulfatide

To increase of the measurement sensitivity of mass spectrometry, sulfatide species in serum were partially purified by hydrophobic interaction chromatography, Phenyl-Sepharose gel chromatography. The sulfatide-rich fraction prepared from serum lipid (serum: 500 μL) by Phenyl-Sepharose gel chromatography was measured in the region of m/z 710 − 1100 by MALDI-TOF MS in negative ion mode. We detected major peaks at m/z 750.6 − 936.8, and very small peaks were detected at m/z 938.7 − 1098.8 (Fig. 1). The internal standard peak was observed at m/z 722.5. Subsequently, we divided the measurement region into m/z 750 − 937 and m/z 930 − 1100, and re-analyzed the peaks (Fig. 2A, 2B). The results indicated 64 ion peaks in the m/z 750 − 937 region, with another 49 peaks in the m/z 930 − 1100 region (Tables 1, 2).

Product ion analysis of sulfatide

Product ion analysis was performed to obtain information regarding the types of sugar sulfate and sphingoid base (including phytosphingoid base), and fatty acids present in the various sulfatide

8

species by MALDI-TOF/TOF MS (MS/MS). The m/z 794.7 peak in Figure 2A showed peaks at m/z

97.0, 119.1, 241.1 and 259.1 on product ion analysis (Fig. 3). The peaks at m/z 97.0 were product

ions comprising sulfatides with the ions HSO_4^-. The peak at m/z 119.1 was $NaSO_4^-$ ion. The peaks

at m/z 241.1 and 259.1 were product ions consisting of the dehydrated galactose sulfate anion and

the hydrated galactose sulfate anion, respectively. Based on these findings, we assigned the ion

peak at m/z 794.7 as SM4s. Further analysis indicated the peaks in m/z 750 – 937 region and m/z

930 – 1100 region to represent different species of SM4s and SM3, respectively, according to

whether the sugar sulfate ion was a monosaccharide or disaccharide. In a similar way 113 peaks

were estimated to be sulfatide with detection of sulfate and sugar sulfate ions.

Product ion analysis of the m/z 794.7 peak revealed peaks at m/z 280.3 and 296.4 other than the ion

mentioned above (Fig. 3), and these corresponded to ion peaks of fatty acids (C16:0) and (C16:0 h),

respectively. In addition, other peaks were observed at m/z 522.3, 540.3 and 568.3 (Fig. 3). This set

of peaks corresponded to sulfatide species containing types of sphingosine, (d18:1). The peaks at

m/z 522.3, 540.3 and 568.3 were fragment ions of sphingosine (d18:1) containing hydroxyl fatty

acid and its degradation products. The molecular degradation process is shown in the insert of

Figure 3.

Assignment of sulfatide species

Table 1 shows the SM4s species assigned to the various m/z peaks and Table 2 shows the SM 3 species assigned to the various m/z peaks. The assignments were mainly made based on the m/z, sulfate, sphingosid base and fatty acid data. In the tables, bold characters represent sulfatide species assigned based on both m/z and product ions, sphingoid base and sulfate data. The circle symbol marks indicated the sulfatide species estimated based on m/z data and sulfate data. The ratio shows the average of composition ratios of SM4s and SM3 species, respectively.

Sulfatide species in human serum

Reproducibility in a well of the sample plate, and linearity of measurement of the main 15 molecular species of sulfatide in serum were acceptable.[16] The precision check was used pool serum from healthy volunteers. The between-day assay was examined for 4 days with dual measurements. The reproducibility of the eight major molecular species (ionic strength ratio of 3.5 to 17.7% and 5.5 to 12.7%, respectively) of SM4s and SM3 were CV 3.5 to 17.7% and CV 4.9 to 14.1%, respectively, We constructed sulfatide species in serum based on our assignments from healthy young volunteers (n = 8) (Fig. 4). The SM4s species were bound to C16 fatty acid most dominantly, and secondarily long chain fatty acid of C24 and then C22. The SM4s species with C16 fatty acids, (d18: 1 C16: 0h), (d18: 1 C16: 0), (d18: 1 C16: 1h), (t18: 0 C16: 0) and (t18: 0 C16: 0h), accounted for approximately 25.9%, 6.9%, 4.2%, 4.6% and 3.9% (average) of the total SM4s, respectively, and then (d18: 1 C24:

10

1h), (d18: 1 C24: 0h), (d18: 1 C24: 1), and (d18:1 C22: 0h) accounted for approximately 8.6%, 6.0%, 2.2%, and 4.6%, respectively.

In contrast, SM3 including the very long chain fatty acids (VLCFAs), C24 and C22, were more dominant than C16 fatty acids. The SM3 species at m/z 1050.8 and 1052.8 accounted for approximately 14.9% and 10.9% (average) of the total SM3, respectively, and were assigned as (d18:2 C24:0, d18: 1 C24: 1 and d18: 0 C24: 2) and (d18: 1 C24: 0, d18: 0 C24: 1, d20: 1 C22: 0), respectively. That of (d18: 1 C16: 0) at m/z 940.7 accounted for 10.0 %. The fatty acids of SM4s were predominantly C16: 0 and hydroxyl C16: 0h, and SM3 was the major component of VLCFAs.

Discussion

We have demonstrated the presence of a wide variety of sulfatide species of 64 SM4s and 49 SM3 in serum by negative ion mode analysis using MALDI-TOF MS. Galactosyl sulfatide (SM4s) was the major sulfatide, SM3 was present in trace amounts (approximately 1% of sulfatides in serum), and the fatty acid profile of both was different.

One previous report described the use of MALDI-TOF MS to analyze sulfatides in serum;[17] the authors analyzed the sulfatides in serum as lysosulfatides produced by alkali treatment with saponification, since the sulfatide peaks on negative ion mode were obscured by glycerophospholipid peaks and the matrix reagent, 2,5-dihydroxybenzoic acid (2,5-DHB). This

11

approach allowed identification of the sulfatide sphingoid bases but not the various fatty acid molecular species. We were able to measure sulfatide species by partial purification of serum followed by negative ion mode analysis with 9-AA matrix using MALDI-TOF MS. MALDI-TOF MS methods for molecular analysis are relatively easy, and the separation performance sensitivity of ion peaks is very high.

After the collection of serum, the procedure began with hydrolysis of glycerophospholipids with phospholipase A_2 in order to avoid overlap of glycerophospholipid peaks. Furthermore, to increase measurement sensitivity of mass spectrometry, sulfatides were partially purified by Phenyl-Sepharose gel chromatography. Using this procedure, we were able to identify sulfatide species that differed based on sphingoid bases (d18: 0, d18: 1, d18: 2, t18: 0, t18: 1, d20: 0, d20: 1 and t20: 0), sugars (galactose or lactose), and various fatty acids, differing in carbon chain length, hydroxyl base addition, and fatty acid saturation. In product ion analysis, the HSO_4^- ions, the $NaSO_4^-$ ions, **dehydrated galactose sulfate anion, and galactose sulfate anion detected** at m/z 97.0, 119.1, 241.1 and 259.1, respectively, were ionic species corresponding to universal ion peaks for sulfatide molecular species, and these have been identified as SM4s. The ion peaks detected at m/z 403.1 and 421.1 corresponded to the dehydrated lactose sulfate anion and the lactose sulfate anion, respectively, and these mass spectrum peaks were identified as SM3. In addition, we showed that the sphingoid base ions in the sulfatide species with a 2-hydroxy-fatty acid were detected at m/z

522.3, 540.3 and 568.3, but no fragment ions were detected in the sulfatide species without hydroxy fatty acid. The fragment ion at m/z 568.3 represented the 2-hydroxy fatty acid dissociated from the sulfatide, and subsequently the dissociation of the ketone base resulted in the m/z 540.3 fragment ion, and finally dehydration produced the m/z 522.3 fragment ion,[18] as shown in the insert of Figure 3. The main sulfatide sphingoid bases was sphingosine (d18: 1), and small quantities of sphingoid bases (d18: 2), (d18: 0), (d20: 1), and (d20: 0) were detected or assigned. In addition, phytosphingoid bases (t18: 1), (t18: 0) and (t20: 0) were detected using MS/MS analysis.

The major sulfatide in serum, SM4s, is present in the body as a result of biosynthesis in the liver and dietary absorption across the small intestine, and accounts for the major lipoprotein glycosphingolipid. Galactosyl sulfatide (SM4s) in serum is present at a concentration of 0.64 nmol/mL in serum, and serum levels are reported to be reduced in renal insufficiency patients [19] and elevated in arteriosclerosis.[20] In the present study, SM4s in serum was found to have a high proportion of saturated and monounsaturated fatty acids from C16 to C24, of which about half had a **hydroxyl fatty acid, but not including polyunsaturated fatty acids. Kyougashima et al. reporte**d that ratios of SM4s species differed in rat spinal **cord, lung, colon, a**nd stomach. [15] Similarly, Cheng et al. found different SM4s species ratios in mouse plasma, liver, muscle, kidney cortex, kidney medulla, cortex, spinal cord, and sciatic nerve, and detected SM4s (d18: 1 C22: 2h) in mouse plasma and kidney, but not in other samples.[21] In relapsing-remitting multiple sclerosis, levels of

SM4s C18: 0 and C24: 1 in plasma correlated with disease status.[22] The present study identified

SM4s (d18: 1 C16: 0h) and SM4s (d18: 1 C24: 1h) species, neither of which has been reported as

present in mouse plasma. Other groups reported that serum SM3 levels were approximately 2% of

SM4s based on immunochemical techniques,[11] but the composition and functional details remained

unknown. We showed that SM3 in serum was less than 1% of SM4s as calculated from ion peak

ratios, and the fatty acid composition ratio was not similar between the two species. The analysis of

the variation in sulfatide species in serum may contribute to different cell functions of various

diseases.

It has been reported that hydroxylation at the C2 position of the fatty acid in glycosphingolipid is

important for maintenance of the myelin sheath in the mammalian nervous system, and is also

highly developed in the digestive organs and kidney and skin cells.[23, 24] The variation of sulfatide

species in serum including hydroxy fatty acid may be useful to understand sulfatide metabolism in

diseases of the above organs. It has also been reported that phytosphingoid bases are derived from

yeasts, plants, and mammalian tissues in vivo, and a biosynthetic materials of the odd number fatty

acids through 2-hydroxy palmitic acid.[25] Further, the detection of differences in sphingoid bases of

serum may become a useful marker of cellular metabolism, because phytosphingoid bases species

were thought to influence the cell membrane thickness and molecular spatial structure and

hydrophilicity.[26] Recently, it was reported that sphingolipids with specific fatty acid compositions

and frameworks function as ligands to receptors.[27] Our screening of numerous species of sulfatides in serum may help to determine the sulfatide species present and their associations with sulfatide metabolic disorders.

Hara and Taketomi have shown that concentrations of sulfatide in serum of WHHL rabbit (121nmol/ml), an animal model for human familial hypercholesterolemia, are much higher than in normal rabbit serum (3nmol/ml).[7] Sulfatides in human serum are present on all lipoprotein fractions. Mizoue has reported that when LDL is incubated with a high concentration of sulfatide, the sulfatide concentration of LDL increases.[28] Sulfatide in serum is suggested to be associated with cholesterol metabolism, but little has been reported on the relationship between cholesterol and sulfatide species. Thus the analysis of sulfatide species in serum will be helpful to elucidate cholesterol metabolism.

This present study identified various sulfatide species in healthy volunteer serum and revealed differences between SM4s and SM3. Use of this method permits enhanced understanding of the role of sulfatide species in both physiological and disease states.

References

1. Ishizuka I, Tadano-Aritomi K. Characterization of sulfoglycosphingolipids by direct probe electron impact mass spectrometry. Journal of biochemistry 1984; 96: 829-839.

2. Takahashi T, Suzuki **T. Role of sulfatide in normal and pathological cells and** tissues. Journal of Lipid Research 2012; 53: 1437-1450.

3. Martensson E. Sulfatides of human kidney. Isolation, identification, and fatty acid composition, Biochimica et biophysica acta 1966; 116: 521-531.

4. Kobayashi T, Honke K, Kuramitsu Y, Hosokawa M, **Miya**zaki T, Murata J, Saiki I, Ishizuka I, Makita A. Cell-surface sulfoglycolipids are involved in the attachment of renal-cancer cells to laminin. International Journal of Cancer 1994; 56: 281-285.

16

5. Sugita M, Dulaney JT, Moser HW. Structure and composition of sulfatides isolated from livers of patients with metachromatic leukodystrophy: galactosyl sulfatide and lactosyl sulfatide. Journal of lipid research 1974; 15: 227-233.

6. Wu XZ, Honke K, Zhang YL, Zha XL, Taniguchi N. Lactosylsulfatide expression in hepatocellular carcinoma cells enhances cell adhesion to vitronectin and intrahepatic metastasis in nude mice. International Journal of Cancer 2004; 110: 504-510.

7. Hara A, Taketomi T. Occurrence of sulfatide as a major glycosphingolipid in WHHL rabbit serum lipoproteins. Journal of Biochemistry 1987; 102: 83-92.

8. Hara A, Taketomi T. Characterization and changes of glycosphingolipids in the aorta of the Watanabe hereditable hyperlipidemic rabbit. Journal of Biochemistry 1991; 109: 904-908.

9. Li G. Hu R, Kamijo Y, Nakajima T, Aoyama T, Ehara T, Shigematsu H, Kannagi R, Kyogashima M, Hara A. Kidney dysfunction induced by protein overload nephropathy reduces serum sulfatide levels in mice. Nephrology 2009; 14: 658-662.

10. Merten A, Thiagarajan P. P-selectin in arterial thrombosis. Zeitschrift Fur Kardiologie 2004; 93: 8558-8563.

11. Buschard K, Fredman P, Bog-Hansen E, Blomqvist M, Hedner J, Rastam L, Lindblad U. Low serum concentration of sulfatide and presence of sulfated lactosylceramid are associated with Type 2 diabetes. The Skaraborg Project. Diabetic Medicine 2005; 22: 1190-1198.

12. Philippart M, Sarlieve L, Meurant C, Mechler L. Human urinary sulfatides in patients with sulfatidosis (metachromatic leukodystrophy). Journal of lipid research 1971; 12: 434-441.

13. O'Brien JS, Fillerup DL, Mead JF. BRAIN LIPIDS: I. QUANTIFICATION AND FATTY ACID COMPOSITION OF CEREBROSIDE SULFATE IN HUMAN CEREBRAL GRAY AND WHITE MATTER. Journal of lipid research 1964; 5: 109-116.

14. Liu Y, Chen Y, Momin A, Shaner R, Wang E, Bowen NJ, Matyunina LV, Walker LD,

McDonald JF, Sullards MC, Merrill Jr AH. Elevation of sulfatides in ovarian cancer: An integrated transcriptomic and lipidomic analysis including tissue-imaging mass spectrometry. Molecular Cancer 2010; 9: DOI: 10.1186/1476-4598-9-186.

15. Kyogashima M, Tamiya-Koizumi K, Ehara T, Li G, Hu R, Hara A, Aoyama T, **Kann**agi R. Rapid demonstration of diversity of sulfatide molecular species from biological materials by MALDI-TOF MS. Glycobiology 2006; 16: 719-728.

16. Hori A, Yamaura M, Hongo M, Honda T, Hidaka H. Analysis of serum sulfatide molecular profile using MALDI-TOF mass spectrometry. Japanese Journal clinical chemistry 2016; 45: 46-51.

17. Li G, Hu R, Kamijo Y, Nakajima T, Aoyama T, Inoue T, Node K, **Ka**nnagi R, Kyogashima M, Hara A. Establishment of a quantitative, qualitative, and high-throughput analysis of sulfatides from small amounts of sera by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Analytical Biochemistry 2007; 362: 1-7.

18. Hsu FF, Bohrer A, Turk J. Electrospray ionization tandem mass spectrometric analysis of sulfatide. Determination of fragmentation patterns and characterization of molecular species expressed in brain and in pancreatic islets. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism 1998; 1392: 202-216.

19. Wang L, Kamijo Y, Matsumoto A, Nakajima T, Higuchi M. Kannagi R, Kyogashima M, Aoyama T, Hara A. Kidney transplantation recovers the reduction level of serum sulfatide in ESRD patients via processes correlated to oxidative stress and platelet count. Glycoconjugate Journal 2011; 28: 125-135.

20. Li G, Hu R, Gu J, Wu HZ. Relationship between carotid artery atherosclerosis and sulfatide in hypertensive patients. Genetics and Molecular Research 2015; 14: 4840-4846.

21. Cheng H, Sun G, Yang K, Gross RW, Han X. Selective desorption/ionization of sulfatides by

MALDI-MS facilitated using 9-aminoacridine as matrix. Journal of Lipid Research 2010; 51: 1599-1609.

22. Moyano AL, Pituch K, Li G, van Breemen R, Mansson JE, Givogri MI. Levels of plasma sulfatides C18: 0 and C24: 1 correlate with disease status in relapsing-remitting multiple sclerosis. Journal of Neurochemistry 2013; 127: 600-604.

23. Hama H. Fatty acid 2-Hydroxylation in mammalian sphingolipid biology. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids 2010; 1801: 405-414.

24. Uchida Y, Hama H, Alderson NL, Douangpanya S, Wang Y, Crumrine DA, Elias PM, Holleran WM. Fatty acid 2-hydroxylase, encoded by FA2H, accounts for differentiation-associated increase in 2-OH Ceramides during keratinocyte differentiation. Journal of Biological Chemistry 2007; 282: 13211-13219.

25. Kondo N, Ohno Y, Yamagata M, Obara T, Seki N, Kitamura T, Naganuma T, Kihara A. Identification of the phytosphingosine metabolic pathway leading to odd-numbered fatty acids Nature Communications **2014**; 5: 5338 DOI: 10.1038/ncomms6338.

26. Westerlund B, Slotte JP. How the molecular features of glycosphingolipids affect domain formation in fluid membranes. Biochimica Et Biophysica Acta-Biomembranes 2009; 1788: 194-201.

27. Rhost S, Lofbom L, Rynmark BM, Pei B, Mansson JE, Teneberg S, Blomqvist M, Cardell SL. Identification of novel glycolipid ligands activating a sulfatide-reactive, CD1d-restricted, type II natural killer T lymphocyte. European Journal of Immunology 2012; 42: 2851-2860.

28. Mizoue T. Enhanced Macrophage Degradation of Low-Density Lipoprotein Modified with Sulfatide. Shinshu Medical Journal 1993; 41: 305 – 315.

Figure Legends

Table 1. Identification of galactosyl sulfatide species in human serum

The bold characters represent the assignment of galactosyl sulfatide (SM4s) species based on both

sulfatide ion m/z data and product ion data. The circle symbol marks indicated the sulfatide species

estimated based on m/z data, sulfate and/or galactose sulfate.

Table 2. Identification of lactosyl sulfatide species in human serum

The bold characters represent the assignment of lactosyl sulfatide (SM3) species based on both

sulfatide ion m/z data and product ion data. The circle symbol marks indicated the sulfatide species

estimated based on m/z data, sulfate and/or lactose sulfate.

Figure 1. Mass spectrum analysis of sulfatides in human serum.

Serum from healthy volunteers ($500\,\mu$L) was pretreated with phospholipase A_2, and the lipids were

extracted with chloroform/methanol (2:1 v/v). The lipids were subjected to Phenyl-Sepharose

chromatography (bed capacity: $\phi\,5\,\times\,10$ mm) to isolate the sulfatide-rich fraction. The

sulfatide-rich fraction with a 9-AA matrix was analyzed in the range from m/z 750 − 1100 by

MALDI-TOF MS in negative ion mode.

Figure 2. Mass spectrum at extended measurement range.

Panel A: Peaks observed in the range m/z 750 - 937.

Panel B: Peaks observed in the range m/z 930 - 1100.

Figure 3. Product ion analysis of sulfatides.

The peak at m/z 794.7 of the maximal ion strength peak of sulfatides in serum was chosen to undergo product ion analysis. The peaks at m/z 97.0 and 119.1 represented HSO_4^- and $NaSO_4^-$, and the peaks at m/z 241.1 and 259.1 corresponded to the dehydrated and hydrated galactose-sulfate moieties, respectively. The peaks at m/z 280.3 and 296.3 represented product ions of non-hydroxy fatty acid (C16: 0) and hydroxy fatty acids (C16: 0h), respectively. The peaks at m/z 522.3, 540.3, **and 568.3 were fragment ions of sphingosin**e (d18: 1) containing hydroxyl fatty acid and its degradation products. The molecular degradation process is shown in the insert. The peak at m/z 794.7 was identified as SM4s from the mass spectrum and product ion composition.

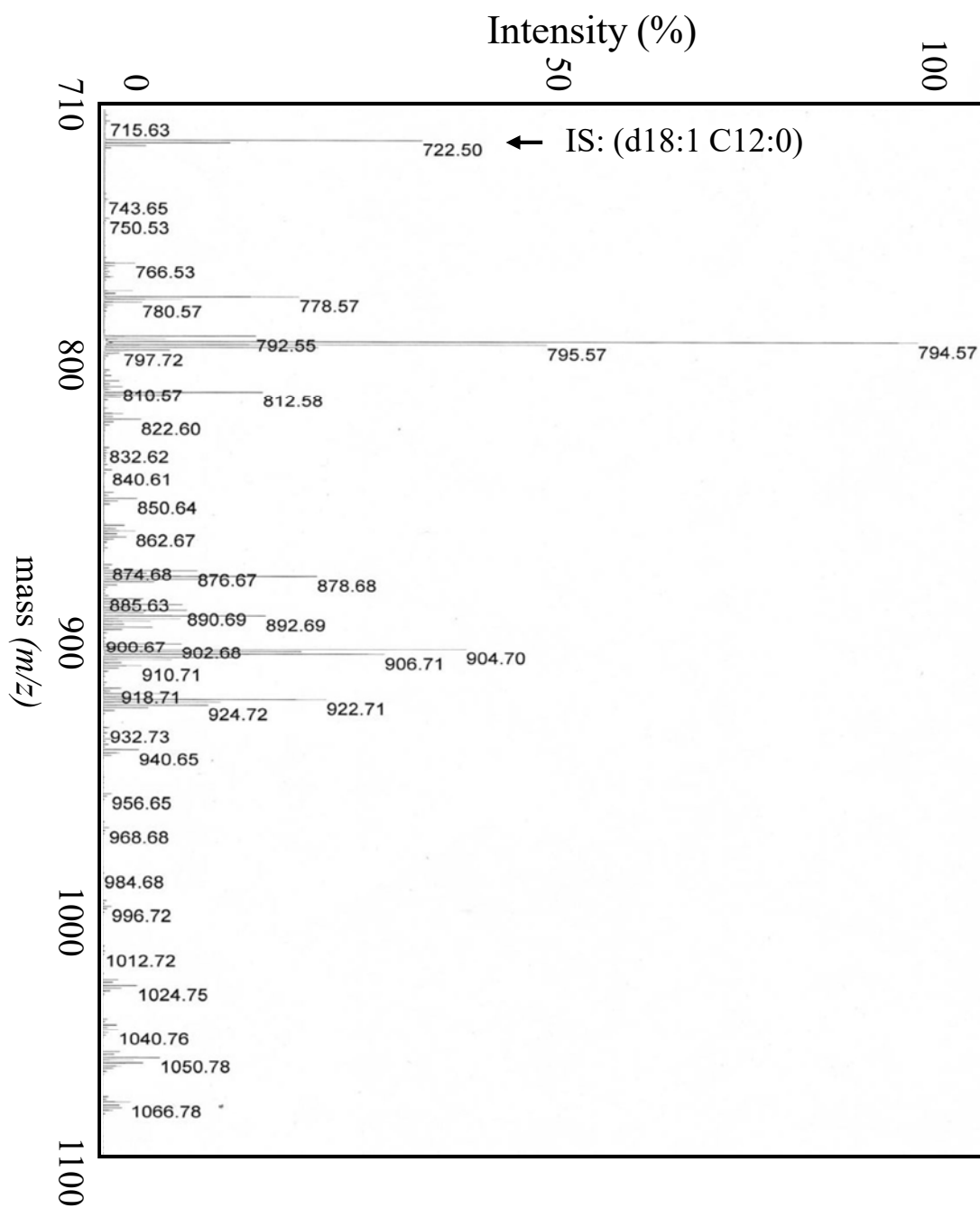Figure 4.   Galactosyl sulfatide and lactosyl sulfatide species of healthy human serum.

 Panel A: Galactosyl sulfatide (SM4s). Panel B: lactosyl sulfatide (SM3)

No.	m/z	d18:2	d18:1	d18:0	t18:1	t18:0	d20:1	d20:0	t20:0
1	750.6		+	+					
2	764.6	+	+	+	+				
3	766.6		C14:0h	+	C14:0	+			
4	768.7			+		+			
5	776.7	C16:0	+				+		
6	778.7	+	C16:0	+	+		+	+	
7	780.7		+	C16:0	+	+		+	
8	782.7			+	+	+			
9	790.7	C16:1h	+				+		
10	792.7	C16:0h	C16:1h	+	C16:1		+	+	
11	794.7		C16:0h	+	C16:0	+	+	+	+
12	796.7			+	+	C16:0		+	+
13	802.7	+							
14	804.7	+	+				+		
15	806.7	+	C17:1h	+	+		+	+	
16	808.7		C17:0h	+	+	+	+	+	+
17	810.7			+	+	C16:1h		+	+
18	812.7					C16:0h			+
19	816.7	+							
20	818.7	+	+				+		
21	820.7	+	C18:1h	+	+		+	+	
22	822.7		C18:0h	+	+	+	+	+	+
23	824.7			+		+		+	C16:0
24	832.7	+	+				+		
25	834.7	+	+	+	+		+	+	
26	836.7		+	+	+	+	+	+	+
27	838.7			+	+	+		+	+
28	840.7					+			+
29	846.7	+	+				+		
30	848.7	C20:0h	C20:1h	+	+		+	+	
31	850.7		C20:0h	+	+	+	+	+	+
32	852.7			+	+	+		+	+
33	860.8	+	+				+		
34	862.8	+	C22:0	+	+		+	+	
35	864.8		+	+	+	+	+	+	+
36	866.8			+	+	C20:1h		+	+
37	868.8					C20:0h			+
38	874.8	C22:1h	+				+		
39	876.8	C22:0h	C22:1h	C23:1	+		+	+	
40	878.8		C22:0h	+	+	+	+	+	+
41	880.8			C22:0h	+	C22:0		+	+
42	884.8	+							
43	886.8	C24:1	+						
44	888.8	C23:1h	C24:1	+			+		
45	890.8	C23:0h	C23:1h	C24:1	+		+	+	
46	892.8		C23:0h	+	+	+	+	+	+
47	894.8			+	+	+		+	+
48	896.8					C22:0h			+
49	900.8	+							
50	902.8	C24:1h	C24:2h		+		+		
51	904.8	C24:0h	C24:1h	+	+	C24:2	+	+	
52	906.8		C24:0h	C25:0	+	+	+	+	+
53	908.8			C24:0h	+	+		+	+
54	910.8					+			+
55	914.8	+					+		
56	916.8	+	+				+	+	
57	918.8	+	**C25:1h**	+	+		+	+	
58	920.8		C25:0h	+	C24:1h	+	+	+	+
59	922.8			+	C24:0h	C24:1h		+	+
60	924.8					C24:0h			+
61	930.8	+	+				+		
62	932.8	+	+	+			+	+	+
63	934.8		+	+	+	+	+	+	
64	936.8			+	+	+		+	+

Bold characters: identified species, +: not identified, but deduced species.

No.	*m/z*	d18:2	d18:1	d18:0	t18:0	d20:1	d20:0	t20:0
1	938.7	C16:0	+		+			
2	940.7	+	C16:0	+	+	+		
3	942.7		+	+	+	+		
4	952.7	+	+		+			
5	954.7	+	+	+	+	+		
6	956.7		+	+	+	+	+	
7	958.7		+	+		+	+	
8	964.7	+						
9	966.7	+	+		+			
10	968.7	+	C18:0	+	+	+		
11	970.7		+	C18:0	+	+	+	
12	978.7	+						
13	982.7	+	+	+	+	+		
14	984.7		+	+	+	+	+	
15	986.7		+	+		+	+	
16	992.7	+						
17	994.7	C20:0	+		+			
18	996.7	+	C20:0	+	+	+		
19	998.7		+	+	+	+	+	
20	1008.7	+	+		+			
21	1010.7	+	+	+	+	+		
22	1012.7		+	+	+	+	+	
23	1014.7		+	+		+		
24	1020.7	C22:1						
25	1022.8	C22:0	+		+			
26	1024.8	+	C22:0	C22:1	+	+		
27	1026.8	+	C22:0	+	+	+	+	
28	1034.8	+						
29	1036.8	C23:0	C23:1		+			
30	1038.8	C22:0h	C23:0	+	+	+		
31	1040.8		C22:0h	C23:0	+	+	+	+
32	1042.8			+	+		+	+
33	1046.8							
34	1048.8	+						
35	1050.8	C24:1	+	C24:2	+			
36	1052.8	C24:0	C24:1	C24:1		C22:0	+	
37	1054.8		C24:0	C24:0	+	+	C22:0	+
38	1056.8		C23:0h	+	+		+	+
39	1062.8							
40	1064.8	+			C23:1			
41	1066.8	C25:0	C24:2h	+	+	C23:0	+	
42	1068.8	C24:0h	C24:1h	C25:0	+	C24:2	+	+
43	1070.8		C24:0h	+	+	C24:2	+	
44	1080.8		+		+	+		
45	1082.8	+	+	+	+	+	+	+
46	1084.8		+	+	+		+	+
47	1094.8		+		+	+	+	+
48	1096.8	+	+	+	+	+	+	+
49	1098.8		+	+	+		+	+

Bold characters: identified species, +: not identified、but deduced species

Hori, Fig.1

Hori, Fig.2

Hori, Fig.3

Hori, Fig.4