Valdes-Gonzalez et al. J Glycom Lipidom 2012, S2
DOI: 10.4172/2153-0637.S2-004

Journal of Glycomics & Lipidomics

Research Article Open Access

Imaging Technology of Complex Lipid Molecular Species by a Combination of TLC-Blot and MALDI-TOF –Special Reference to Human Brain Ganglioside Molecular Species

Tania Valdes-Gonzalez[1]*, Naoko Goto-Inoue[2], Takahiro Hayasaka[2], Hironobu Ishiyama[1], Mitsutoshi Setou[2] and Takao Taki[3]*

[1]*Third Institute of New Drug Discovery, Institute of Biomedical Innovation, Otsuka Pharmaceutical Co. Ltd., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan*
[2]*Department of Molecular Anatomy, Molecular Imaging, Frontier Research Center; Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku Hamamatsu, Shizuoka 431-3192, Japan*
[3]*Institute of Biomedical Innovation, Otsuka Pharmaceutical Co. Ltd., 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan*

Summary

Glycosphingolipids and phospholipids from white matter of human hippocampus, were analyzed by a sequential procedure of two-dimensional TLC (2d-TLC), transference of separated lipids to a PVDF membrane by a TLC-Blot equipment and mass spectrometry (MS) analysis with an ion-trap type MALDI-TOF equipment. The method is simple and quick; very small amount of sample (0.1 mg of brain tissue) is enough to analyze all lipid components. The 2d-TLC provided excellent separation and the MS analyses allowed identifying the characteristic profile of molecular species for individual glycolipids and phospholipids. The results of MS analyses on gangliosides showed that di- and tri-sialogangliosides are richer in d20:1 sphingosine-containing ceramide than monosialogangliosides, suggesting the presence of sialylation selection after GM1 gangliosides. Then we analyzed ganglioside molecular species obtained from different brain regions by using MS imaging technology. The MS images of individual gangliosides provided clear visual profiles in terms of molecular species distribution. The imaging profiles were region dependent and also indicated that the sialyltransferase toward GM1 ganglioside prefers to select d20:1 sphingosine containing molecule. This technology provides visual characterization of individual phospholipid and glycosphingolipid molecular species and informs us about the metabolic characterization of target tissue, opening a new gate for colorful lipidomics research.

Keywords: TLC-Blot; Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS); Ion imaging; Glycosphingolipids; Gangliosides; Lipidomics; Brain (up to 6 keywords)

Abbreviations: MALDI-TOF MS: Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry; TLC: Thin Layer Chromatography; HPTLC Plate: High Performance Thin Layer Chromatography Plate; GSLs: Glycosphingolipids; PVDF: Polyvinylidene Difluoride; NeuAc: N-acetylneuraminic Acid

Introduction

The gangliosides were named according to the nomenclature of Svennerholm [1]. The glycosphingolipids (GSLs) are amphiphatic molecules consisting of one hydrophilic sugar chain and one hydrophobic ceramide moiety. Mostly they are located in the outer leaflet of the membrane, and anchored by the ceramide moieties with the sugar chains directed toward the cell exterior. GSLs are involved in a wide range of events such as cell-surface recognition, microbial pathogenesis [2,3], cell differentiation, and immunological recognition [4-6]. Also they have been identified as components of raft microdomain [7], where they play important roles in the early events of signal transduction by arranging the environment of receptor or ligand molecules [8]. In these events, not only their carbohydrate moieties but also ceramide moieties are very important to facilitate the formation of microdomain.

A widely used thin layer chromatography (TLC) allows us to separate the GSLs and phospholipids in mixtures according to their hydrophobic properties [9-11]. The TLC is rather old but still the best technology for lipid biochemistry even if HPLC system showed great progress, because of its rapid, simple and excellent separation potentials. On the other hand, mass spectrometry technology showed tremendous progress by introduction of various kinds of ionization technology together with progress of information processing system by computer. Especially, molecular ions and fragmentation profiles of individual lipid during the ionization are quite informative for identification of lipids. The combination of TLC separation properties and the spectra obtained from MS analysis has been used as complementary structural information [12-16]. In the present study, we also combined these two technologies, and for new lipidomics research, we introduced two more technologies, TLC-Blot which is a transfer of lipids separated on HPTLC-plate to PVDF membrane and MS imaging to the blotted lipid bands on the membrane. As shown in a previous paper [18], the lipids blotted on the PVDF membrane could be analyzed directly with high efficiency. Blotted GSLs have shown to be stable on the membrane and concentrated one side of the membrane [17]. These characteristics make possible not only binding experiments, but also the MS analysis of GSLs directly on the membrane (TLC-Blot-MS) [17-20]. It has been reported that the parameters such as sensitivity, mass resolution and background interference are better in TLC-Blot-MS than in TLC-MS [20]. We presented the usefulness of this TLC-Blot/MALDI-TOF MS system for detail structural analysis of complex GSLs such as gangliosides with high sensitivity [21].

***Corresponding authors:** Tania Valdes-Gonzalez, Finlay Institute, Av 27 No: 19805 esq 198 LA Coronela, La Lisa 11600 Habana, Cuba, Tel: 208 6086; Fax 2086075; E-mail: tvaldes@finlay.edu.cu

Takao Taki, Institute of Biomedical Innovation, Otsuka Pharmaceutical Co. Ltd. 463-10 Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan, Tel: +81-88-665-2126; Fax: +81-88-665-6976; E-mail: t_taki@research.otsuka.co.jp

Received April 20, 2011; **Accepted** July 15, 2011; **Published** July 22, 2011

Citation: Valdes-Gonzalez T, Goto-Inoue N, Hayasaka T, Ishiyama H, Setou M, et al. (2012) Imaging Technology of Complex Lipid Molecular Species by a Combination of TLC-Blot and MALDI-TOF –Special Reference to Human Brain Ganglioside Molecular Species. J Glycomics Lipidomics S2:004. doi:10.4172/2153-0637.S2-004

In this study, we separated a total lipid mixture of normal human brain into each lipid component with 2d-TLC by using multi developing system and analyzed individual lipids using TLC-Blot/MALDI-TOF MS system. GSLs and phospholipids were separated and transferred clearly to the PVDF membrane. The direct analysis of the lipid component by MALDI-TOF MS showed very clear MS spectra.

In the course of this study, we found characteristic profiles of molecular species for individual lipids. Especially, two major fragment peaks derived from d18:1 sphingosine and its d20:1 derivative are quite characteristic for all gangliosides of the brain specimen. This fact attracted us to analyze these peaks directly on the blotted membrane by using imaging MS technology, because gangliosides in hippocampus have been reported to have important roles in learning and memory and the changes in their metabolic pathways are related to several disorders [22]. For this purpose, we developed a very good blotting solvent system and procedure for gangliosides. By using this TLC-Blot system, we challenged to make the profile of MS imaging in terms of molecular species for each major ganglioside individually in gray and white matter, obtained from different human brain regions. The ion images obtained for the ganglioside showed to provide clear visual information about the distribution of the different molecular species. The present system will provide a key to open a new lipidomics field.

Material and Methods

Materials

Primuline and all the solvents used for TLC-Blot were of HPLC analytical grade and were purchased from Sigma-Aldrich Japan (Tokyo, Japan). High performance thin layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany). The ganglioside standards were purchased from Sigma (USA) and the standards of sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), phosphatidylseine (PS), sphingomyelin (SM) and cardiolipin (Car) were from Doosan Serdary Research Laboratory (Toronto, Canada).

All the solvents and reagents for MS were of HPLC grade and were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Bradykinin, angiotensin-II, and ACTH were obtained from Sigma-Aldrich Japan (Tokyo, Japan) and used as calibration standards. 2,5-Dihydroxy benzoic acid (DHB) obtained from Bruker Daltonics (Leipzig, Germany) was used as the matrix.

Lipid extraction

The brain tissues were obtained from The Netherland Brain Bank (NBB) (The Netherlands). All samples were anonymous and were obtained under conditions of informed consent according to the ethical guidelines of the NBB. White mater and gray matter were carefully dissected from frozen brain (inferior frontal gyrus or hippocampus). Tissue (approximately 0.1g) was homogenized with twenty fold volume of chloroform/methanol (2/1, by vol.) and sonicated for 5 min. Then, the lipids were stored at 4°C. Aliquots of the supernatant were used for further analyses.

Thin layer chromatography (TLC)

For two-dimensional TLC the samples and the standards were applied on to a silica gel 60 HPTLC plates. The plate was developed to 3cm from the origin with a solvent mixture of chlorofrom/methanol/0.2%$CaCl_2$ (60/40/9, by vol.) (Solvent 1). After the plate was carefully air-dried, it was developed to 5cm to the same direction from the origin, with a solvent mixture of methyl acetate/propanol/chloroform/methanol/0.25% KCl (25/25/25/10/9, by vol.) (Solvent 2). Next, the plate was completely dried and developed to 5cm from the origin to the second direction using the Solvent 2 (Figure 1). For lipid visualization, the primuline reagent was prepared by dissolving 1mL of 0.1% primuline aqueous solution in 100ml of a mixture of acetone: water (80/20, by vol.). The plates were sprayed with the primuline reagent and then, air-dried thoroughly. Then, the bands were visualized under UV at 365 nm with the Fluorchem™ analyzer (Alpha Innotech Corporation, Tokyo, Japan). All the bands visualized were marked with a soft pencil.

For white and gray matter comparison, the plates were developed to 6 cm with Solvent 1, then, after carefully air-drying, to 8 cm with Solvent 2. The separated lipids were visualized with primuline reagent, as describe above.

TLC-Blot

The HPTLC plate for the two-dimensional TLC was dipped for 10s in a blotting solvent containing 2-propanol/0.2% aqueous $CaCl_2$/methanol, (40/20/7, by vol.). Immediately after, a PVDF membrane (ATTO, Tokyo, Japan), a Teflon membrane (ATTO, Tokyo, Japan), and then, a glass fiber filter sheet (ATTO, Tokyo, Japan) were placed over the HPTLC plate. This assemblage was pressed for 30s at 180°C with a TLC thermal blotter (ATTO, Tokyo, Japan). The PVDF membrane was removed from the HPTLC- plate and air-dried. The lipids were located on the reverse of the membrane side attached to the plate.

For ganglioside analysis, propanol/ ethyl acetate/ 0.2% aqueous $CaCl_2$/ methanol (40/5/13/18, by vol.) was used as blotting solvent. The plate was dipped in the solvent for 15s. Immediately after a PVDF membrane, a Teflon membrane, and then, an absorbent paper sheet were placed over the HPTLC plate. The assemblage was pressed for 50s at 170°C with the TLC thermal blotter. The PVDF membrane was removed from the HPTLC plate and air-dried for MS analysis.

For visualization of the gangliosides transferred to the membrane, 0.01% primuline (aqueous solution) was used. In this case, HPTLC plate was applied to the Blotter without visualizing the lipid bands with primuline, instead of that, after the lipid blotting, the membrane was dipped in the primuline solution for 2 min, then, washed out with water for 5 min, air-dried, and the bands visualized directly on the membrane with the Fluorchem™ analyzer.

MALDI-QIT-TOF-MS

MALDI-QIT(quadrupole ion trap)-TOF analyses were performed using AXIMA-QIT mass spectrometer (Shimadzu, Kyoto, Japan). Ionization was performed with a 337-nm pulsed N_2 laser. The ion trap chamber was supplied with two separates and independent gases, helium and argon. A continuous flow of helium gas was used for collisional cooling. The pulsed gas, argon, was used for collision-induced fragmentation [23]. Precursor and fragment ions obtained by collision-induced dissociation (CID) were ejected from the ion trap and analyzed by a reflectron TOF detector.

The MS spectra were calibrated externally using a standard peptide calibration mixture containing 10pmol/μL each of bradnikynin peptide fragment (amino acid residue 1-7) human ACTH peptide fragment (amino acid residue 18-39). 50mg/mL DHB in methanol/0.1%TFA solution (1/1, vol.) was used as the matrix. DHB solution (totaling 10μL) was applied onto each lipid band in the PDVF membrane.

The crystallization process was accelerated under a gentle stream of cold air. To achieve good mass spectra, we pressed the membrane with a paper tissue, in order to uniform the membrane surface and remove

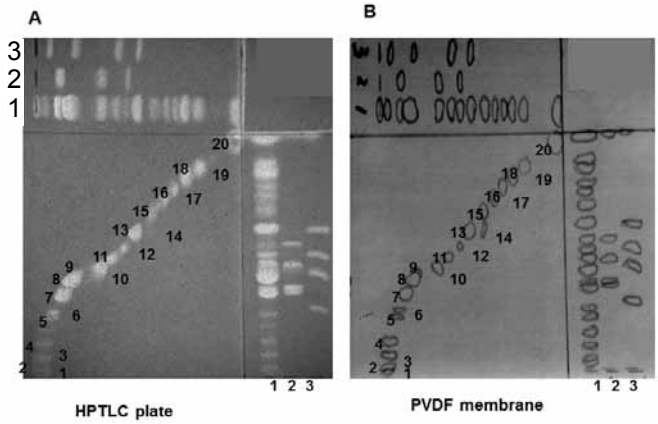

Figure 1: The two-dimensional TLC-Blot of lipids from human hippocampus white matter. A, TLC of lipid from human hippocampus white matter, visualized with primuline. Lane 1,human hippocampus white matter; Lane 2, standard phospholipids, Car, PS, and SM from upper to lower band; Lane 3, standard phospholipids, PE, PI, PC, and LPC from upper to lower band. B, The visualized bands were marked with soft pencil and transferred to the PVDF membrane.

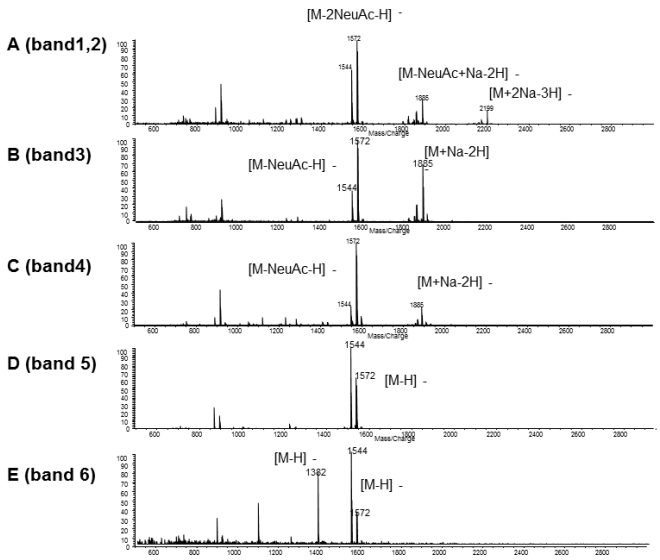

Figure 2: TLC-Blot/MALDI-QIT-TOF MS spectra of bands 1 to 6. The transferred lipids on the membrane were analyzed by MALDI-TOF MS in negative mode. A, bands 1and 2 (corresponding to GT1b); B, band 3 (GD1b); C, band 4(GD1a); D, band 5(GM1) and E, band 6(corresponding GM1and GM2).

the excess of matrix crystals. The PDVF membrane was attached to a MALDI sample plate with electrified double adhesive tape to reduce the charge-up of the plate.

Imaging MS

The raster scan of the PDVF membrane was performed automatically. The number of laser irradiations was 5 shots in each spot. The analyzed area was approximately 8mmx4mm; it was scanned in both the X and Y directions to produce and array of for gangliosides. The interval of data points was 200µm. A data converter (Axima2Analyze, Novartis, Basel, Switzerland) was developed to generate a BioMap readable data file from imaging data of AXIMA-QIT. Two dimensional ion density maps were created using the image reconstruction software (BioMap, Novartis).

Results

TLC-Blot-MS analysis of the lipids from white matter of human hippocampus

A sample extracted from the white matter of human hippocampus was separated by the 2d-TLC. A total of 20 bands were detected (Figure 1A). The lipid bands were marked with a soft pencil and transferred to a PVDF membrane (Figure 1B) and the MS analyses of these bands were conducted directly on the membrane (Figure 2-5).

The spectra were obtained in the negative mode for gangliosides and positive mode for other GSLs and phospholipids. The bands 1 and 2 showed almost same spectra, and were identified as GT1b from the mobility on HPTLC plate and MS analysis. The spectrum show the ion $[M+2Na-3H]^-$ *m/z* 2198 corresponding to the base eicosasphingenine (d20:1) and the ion $[M+2Na-3H]^-$ *m/z* 2170 corresponding to the base sphingenine (d18:1)–containing GT1b, respectively. Signals detected at *m/z* 1885 (d20:1/C18:0), 1857 (d18:1/C18:0), 1572 (d20:1/C18:0) and 1544 (d18:1/C18:0) are derived from the respective GT1b molecular species by the elimination of the sialic acids.

Band 3 was identified as GD1b from mobility on TLC and MS spectrum. GD1b molecular species $[M+Na-2H]^-$ with d20:1/C18:0 and d18:1/C18:0 appeared at *m/z* 1885 and 1857, respectively. The desialylated fragment for each GD1b specie appeared at *m/z* 1572 and 1544 respectively. Similarly, band 4 was identified as GD1a from TLC mobility and MS profile. The signals for GD1a molecular species $[M+Na-2H]^-$ with d20:1/C18:0 and d18:1/C18:0 at *m/z* 1885 and 1857, respectively, are weaker compared with those for GD1b. Signals appeared at *m/z* 1572 and 1544, are desialylated fragments, respectively.

The MS analysis for band 5 showed a $[M-H]^-$ ion, with *m/z* 1572 and *m/z* 1544, which are characteristic for GM1 molecular species with

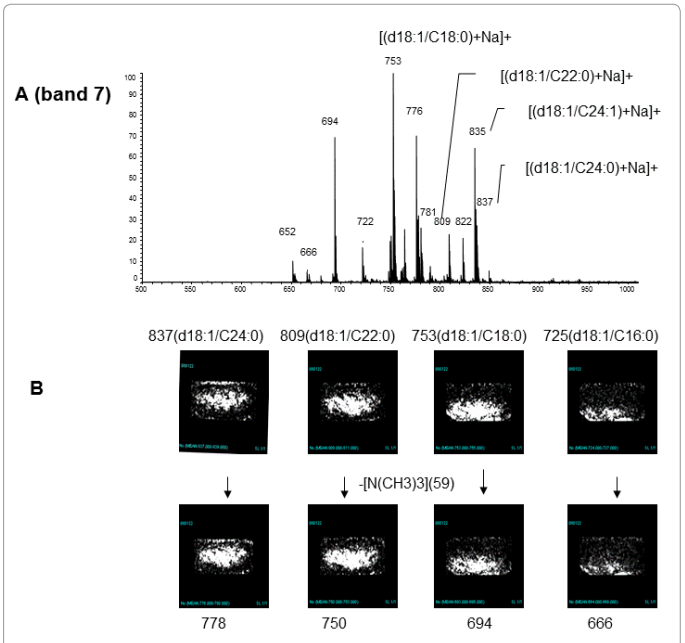

Figure 3: TLC-Blot/MALDI-QIT-TOF MS spectra bands 7. The spectrum was obtained by MALDI-TOF MS in positive mode. A, Spectrum of band 7 corresponding to SM. B, The ions of major SM molecular species and their fragment ions which are appeared by the reduction of the trimethylamine $[-N(CH_3)_3]$ from individual parental ions. These ions were scanned and their imaging profiles are shown.

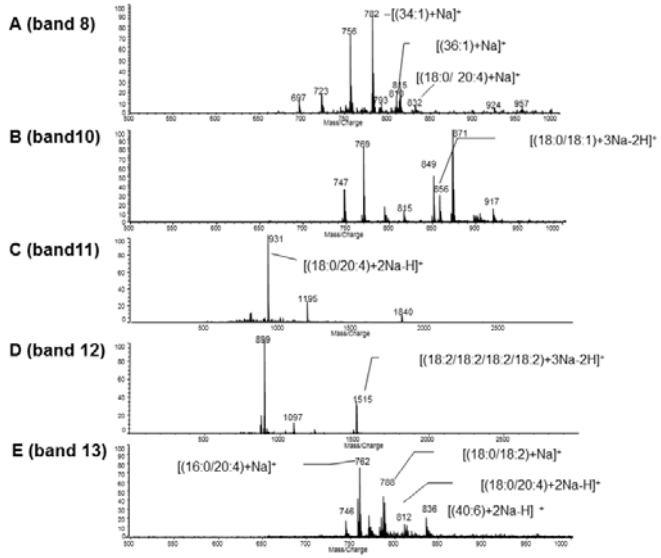

Figure 4: TLC-Blot/MALDI-QIT-TOF MS spectra of bands 8 to 13. A, band 8 (corresponding to PC); band 9 was also assigned as PC. B, band 10 (PS); C, band 11 (PI); D, band 12 (Car); E, band 13 (PE).

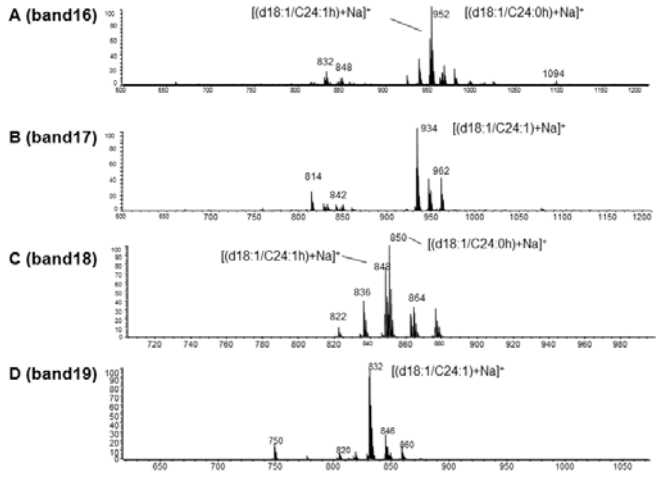

Figure 5: TLC-Blot/MALDI-QIT-TOF MS spectra of bands 16, 17, 18, and 19. A, band 16 (sulfatide with hydroxyl fatty acid containing ceramide); B, band 17 (sulfatide with normal fatty acid containing ceramide); C, band 18 (galactosylceramide with hydroxyl fatty acid containing ceramide); D, band 19 (galactosylceramide with normal fatty acid containing ceramide).

d20:1/C18:0 and d18:1/C18:0 in the ceramide residue. The signal for d18:1/C18:0-containing molecular specie in GM1 is much stronger than that of d20:1/C18:0 when compared with the higher ganglioside profiles.

In the MS spectra for band 6, signals appeared not only at *m/z* 1572 and 1544, but also at *m/z* 1382, corresponding to $[M-H]^-$ ion of GM2. Thus, band 6 is a mixture of GM1 and GM2. In the case of GM2 the signal for d18:1/C18:0-containing molecular specie is predominant.

From band 7 the MS measurement was change to positive mode. The band 7 was identified as SM from TLC mobility and MS analysis. The signals assigned are indicated in (Figure 3A). Major molecular species are as follows: d18:1/C24:0 at *m/z* 837, d18:1/C24:1 at *m/z* 835, d18:1/C22:0 at *m/z* 809 and d18:1/C18:0 at *m/z* 753 as identified from Lipid search (http://lipidsearch.jp/manual_search). All detected ions are $[M+Na]^+$ ions. The distribution of SM molecular species d18:1/C24:0, d18:1/C22:0, d18:1/C18:0 and d18:1/C16:0 were scanned on band 7 and illustrated in the (Figure 3B). In this small band area, the molecular species distribution was clearly characterized in terms of length of fatty acid, i.e., molecular species containing longer fatty acids appear at a higher position in the band, due to their higher hydrophobicity. Individual fragment ions derived by further characteristic elimination of trimethylamine $[-N(CH_3)_3]$ showed identical distribution in the band as their parental molecular ions.

For the identification of the phospholipids, mixtures of standards (Car, PS, SM (lane 2) and PE, PI, PC and LPC (lane 3 in Figure 1)) were applied to the TLC. The Figure 4 shows the spectra corresponding to the bands 8, 10, 11, 12 and 13.

The bands 8 and 9 were identified as PC from the mobility on HPTLC and MS fragmentation profile with different molecular species containing PC. The spectrum of the band 8 (A) shows the ions $[M+Na]^+$ *m/z* 832 (38:4), $[M+Na]^+$ *m/z* 810 (36:1) and $[M+Na]^+$ *m/z* 782 (34:1).

The band 10 was identified as PS from mobility on TLC and MS spectrum. PS molecular species [M+3Na-2H] with 18:0/18:1 appeared at *m/z* 856 (Figure 4B) [18]. The band 11 was identified as PI [24], with the predominant molecular specie $[M+2Na-H]^+$ at *m/z* 931 (18:0/20:4) (Figure 4C) and the band 12 was identified as Car, showing Car molecular species $[M+3Na-2H]^+$ at *m/z* 1515 (18:2/18:2/18:2/18:2) (Figure 4D) [25]. The Figure 4E shows the spectrum for band 13 which was identified as PE, with molecular species $[M+2Na-H]^+$ at *m/z* 836 (40:6) (from Lipid search) and $[M+2Na-H]^+$ at *m/z* 812 (18:0/20:4), $[M+2Na-H]^+$ at *m/z* 788 (18:0/18:2) or (18:1/20:4) as $[M+Na]^+$ and at *m/z* 762 (16:0/20:4) [26].

The Figure 5 shows the spectra for the bands 16, 17, 18 and 19 analyzed by the positive ion mode. The spectrum of band 16 (A) was identified as sulfatide from comparison with the standard, with signal at *m/z* 950 corresponding to (d18:1/C24:1h) $[M+Na]^+$ molecular specie. Band 17 (B) was identified as another sulfatide specie band with the ion $[M+Na]^+$ at *m/z* 934 (d18:1/C24:1). The bands 18 and was 19 (Figure 5C, D) were identified as GalCer molecular species with signals at $[M+Na]^+$ *m/z* 850 (d18:1/C24:0h), 848 (d18:1/C24:1h) and 832 (d18:1/C24:1) [15]. The bands 14, 15 and 20 could not be identified from the information of MS spectra.

MS molecular imaging of human brain gangliosides

Based on the analyses of white mater gangliosides, we found that signal of *m/z* 1572 is much higher than that of *m/z* 1544 in GT1b, GD1a and GD1b. On the contrary, in GM1, signal of *m/z* 1544 is higher than that of *m/z* 1572. As mentioned above signal derived from d18:1/C18:0 is predominant in GM2. These findings suggested that metabolic selection to synthesize the higher gangliosides seems to occur after GM1 ganglioside sialylation. Therefore, we next focused in the analysis of ganglioside molecular species using white and gray matter samples from human hippocampus and frontal gyrus. To perform this analysis we challenged to use a technology of MS imaging. The Figure 6 shows the total lipid profile on HPTLC-plate (Figure 6A) and its profile blotted on PVDF membrane (Figure 6B). For this analysis, we just concentrated in the transfer efficiency of ganglioside from HPTLC-plate to the PVDF membrane. As described in the Materials and Methods, we improved the blotting procedure for the ganglioside, trying different solvent systems, finally the solvent mixture propanol:ethyl acetate:$CaCl_2$:methanol (40:5:13:18, by vol.) was found to be the best.

Then, we scanned each ganglioside band to localize the major

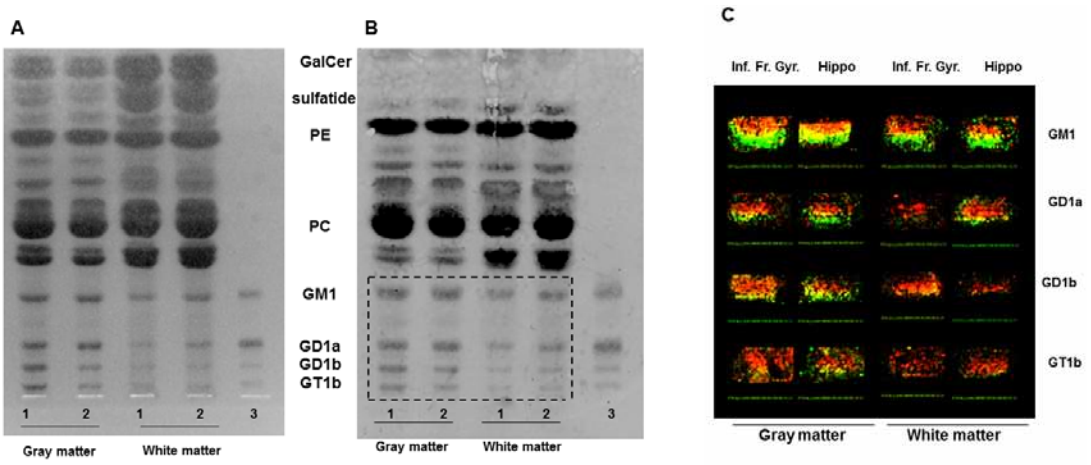

Figure 6: TLC-Blot of human brain lipids and ganglioside imaging of their molecular species. A, TLC of the lipids from gray matter and white matter extracted from normal human inferior frontal gyrus (1) and hippocampus (2). The bovine ganglioside standard was applied for reference (3). B, TLC-Blot of the lipids separated by TLC (the lipids transferred to the membrane were visualized with the primuline reagent). C, Ion imaging of the major molecular ions (*m/z* 1544 (green) and *m/z* 1572 (red)) of GM1, GD1a, GD1b and GT1b.

species derived from the ceramide species *m/z* 1544 (d18:1/C18:0) and *m/z* 1572 (d20:1/C18:0), and we profiled the ganglioside imaging for these two major peaks (Figure 6C). For every ganglioside band the ion corresponding to *m/z* 1572 was located in the upper band region compared with 1544 localization. As suggested in the analysis of Figure 2, we found that the ion *m/z* 1572 (shown as red dots) is denser in higher gangliosides such as GD1a, GD1b and GT1b. Especially in white matter this tendency is very significant. However, in the gray matter of hippocampus shows a balance in the appearance of the ion *m/z* 1544 (shown as green dots) and the ion *m/z* 1572, compared with those of other brain regions.

These results clearly indicate how easy become, using this kind of profiling, to understand the molecular species composition of an individual lipid and also the profile of metabolic difference between tissues or regions of tissue.

Discussion

Our principal findings in this study is that the combination of TLC-blot and MS provides complementary information about the complex lipid molecular species and allows visualizing in a very clear way, the differences in the composition and distribution of the individual molecular species when different samples are compared. While in TLC separation visualized with primuline reagent, each GSL and phospholipid appeared as a single band, the scanning with MS showed the distribution within the TLC band of the major molecular ions for every lipid species. This is especially useful in the analysis of complex sample total lipid mixtures.

In this study, all lipids extracted from human brain, including, gangliosides, glycolipids and phospholipids were separated at one time by 2d-TLC, transferred on a PVDF membrane by TLC-Blot and analyzed by an ion-trap type MALDI-TOF MS directly on the membrane.

The method is simple and quick and very small amount of sample such as 0.1 mg of brain tissue is enough to analyze the all lipid components. The 2d-TLC provided a high separation efficiency of the different lipid molecular species. In the previous paper [21], we demonstrated that the MS/MS and MS/MS/MS analyses of the molecular and fragment ions on the blotted lipid on the PVDF membrane made it possible to identify the lipid structures and sugar sequences.

From the analytical data of hippocampus white matter phospholipids, it was obvious that each phospholipid class has very characteristic molecular species (Figure 3 and 4). Especially PI and Car showed quite simple and specific molecular species. In the case of PE and PS showed rather more complex profile of their fatty acid species. PS is known to be synthesized by base-exchange reaction between PE and serine and some part of PE can be synthesized from PS by decarboxylase reaction [27,28]. This indicates that there should be a very close relationship between PE and PS in terms of precursor and product. The data of our molecular species profile strongly support this kind of relationship. And furthermore, our data suggest that highly unsaturated fatty acid containing molecular species of these two lipids are involved in this precursor product relationship.

The spectra of gangliosides obtained in the 2d-TLC study showed mainly the peaks 1544 (d18:1/C18:0) and 1572 (d20:1/C18:0) corresponding to the GM1 molecule even in the disialyl and trisialyl gangliosides. The signal for the ion at *m/z* 1885 for GD1b was stronger than the signal for GD1a, due to the GD1a structure allows more easily sialic acid elimination. When we focused the analysis in the major desialylated fragments *m/z*, 1544 (d18:1/C18:0) and 1572 (d20:1/C18:0), the ganglioside spectra in Figure 2 were quite suggestive to us. Higher gangliosides, disialo and trisialogangliosides showed stronger signal at 1572 than at 1544. However, in the case of monosialogangliosides GM1 (Figure 2D and E), signal of *m/z*, 1544 was much higher than that of 1572, suggesting that the glycosylation after monosialoganglioside biosynthesis have a kind of tendency to select ceramide molecular species. To confirm this possibility, we focused our analysis in the ganglioside molecular species distribution using samples of gray matter and white matter from two different brain regions, the hippocampus and inferior frontal gyrus. First, we improved the transfer efficiency of gangliosides from HPTLC to the PVDF membrane. The result is shown in Figure 6B; the transfer efficiency was found to be the best by using a solvent mixture of propanol/ethyl acetate/0.2% aqueous $CaCl_2$/methanol (40/5/13/18, by vol.). Then MS imaging was performed using the target ions 1544 and 1572 as the representative fragments of individual gangliosides within a TLC band. We found that molecular species containing eicosasphingenine (d20:1) appeared

in the upper part of the band and in the lower part, the ganglioside species with sphingenine (d18:1)-containing ceramide (Figure 6C). This distribution was clearly observed even within such a small area of ganglioside band. As the imaging profiles (shown by red and green color) clearly indicate, ganglioside molecular species distribution is brain region dependent and also ganglioside class dependent. The data suggested in Figure 2 were confirmed by this MS image, furthermore, the MS image provided lots suggestive information.

This kind of characteristic distribution could give us the bases to understand the relationship between functional distributions of ganglioside molecular species. Namely, we could obtain important information about the combination of sugar chain variety and hydrophobic species by using the present MS imaging technology. Furthermore, it seems to be important a balance of appearance of gangliosides in terms of d18:1 and d20:1 sphingosin-containing in hippocampus for functional expression. Therefore, it would be interesting to investigate whether the molecular species distribution in individual gangliosides of hippocampus, could be related with the memory and learning [29], respectively. Finally, this ion imaging technology of GSL molecular species could provide valuable information for metabolic changes of lipids in diseases and aging and furthermore, opens a gate for a new lipidomics with molecular imaging.

References

1. Svennerholm L (1963) Chromatographic separation of human brain gangliosides. J. Neurochem 10: 613-623.
2. Hakomori S, Igarashi Y (1993) Gangliosides and glycosphingolipids as modulators of cell growth, adhesion, and transmembrane signaling. Adv Lipid Res 25: 147-162.
3. Karlsson KA (1989) Animal Glycosphingolipids as Membrane Attachment Sites for Bacteria. Annu Rev Biochem 58: 309-350.
4. Hakomori S (2000) Traveling for the glycosphingolipid path. J Glycoconj 17: 627-647.
5. Karlsson KA (1995) Microbial recognition of target-cell glycoconjugates. Curr Opin Struct. Biol 5: 622-635.
6. Feizi T (2000) Carbohydrate-mediated recognition systems in innate immunity. Immunolgical Rev 173: 79-88.
7. Kasahara K, Sanai Y (1999) Possible roles of glycosphingolipids in lipid rafts. Biophys. Chem. 82: 121-127.
8. Psanasiewics M, Domek H, Hoser G, Kawalec M, Pacuszca T (2003) Structure of the ceramide moiety of GM1 ganglioside determines its occurrence in different detergent-resistant membrane momains in HL-60 cell. Biochem 42: 6608-6619.
9. Muting J (1996) High-resolution thin-layer chromatography of gangliosides. J Chromatogr A 720: 3-25.
10. Van Echten-Deckert G (2000) Sphingolipid Extraction and Analysis by Thin-Layer Chromatography Introduction. Methods Enzymol 312: 64-79.
11. Yu RK, Ariga T (2000) Ganglioside analysis by high-performance thin-layer chromatography. Methods enzymol 312: 115-134.
12. Distler U, Hulsewig M, Souady J, Dreisewerd K, Haier J (2008) Matching IR-MALDI-o-TOF Mass Spectrometry with the TLC Overlay Binding Assay and Its Clinical Application for Tracing Tumor-Associated Glycosphingolipids In Hepatocellular and Pancreatic Cancer. Anal Chem 80: 1835-1846.
13. Chai W, Leteux C, Lawson A M, Stoll MS (2003) On-line Overpressure Thin Layer Chormatographic Separation and Electrospray Mass Spectrometric Detection of Glycolipids. Anal Chem 75: 118-125.
14. O'Connor PB, Budnik BA, Ivleva VB, Kaur P, Moyer SC (2004) A High Pressure Matrix-Assisted Laser Desorption Ion Source for Fourier Transform Mass Spectrometry Designed to Accommodate Large Targets with Diverse Surfaces. J Am Soc Mass Spectrom 15: 128-132.
15. Ivleva VB, Elkin YN, Budnik BA, Moyer SC, O'Connor PB (2004) Coupling Thin-Layer Chromatography with Vibrational Cooling Matrix-Assisted Laser Desorption/Ionization Fourier Transform Mass Spectrometry for the Analysis of Ganglioside Mixtures. Anal Chem 76: 6484-6491.
16. Nakamura K, Suzuki Y, Goto-Inoue N, Yoshida-Noro C, Suzuki A (2006) Structural Characterization of Neutral Glycosphingolipids by Thin-Layer Chromatography Coupled to Matrix Assisted Laser Desorption/ Ionization Quadrupole Ion Trap Time-of-Flight MS/MS. Anal Chem 78: 5736-5743.
17. Taki T, Ishikawa D (1997) TLC Blotting: Application to Microscale Analysis of Lipids and as a New Approach to Lipid-Peptide Interaction. Anal Biochem 251: 135-143.
18. Taki T, Ishikawa D, Handa S, Kasama T (1995) Direct Mass Spectrometric Analysis on Glycosphingolipid Transferred to a Polyvinylidene Difluoride Membrane by Thin-Layer Chromatography Blotting. Anal Biochem 225: 24-27.
19. Furutani M, Itoh T, Ijuin T, Tsujita K, Takenawa T (2006) Thin Layer Chromatography-Blotting, a novel Method for the Detection of Phospholipids. J Biochem 139: 663-670.
20. Guittard J, Hronowski XL, Costello CE (1999) Direct matrix-assisted laser desorption/ionization mass spectrometric analysis of glycosphingolipids on thin layer chromatographic plates and transfer membranes. Rapid Commun Mass Spectrom 13: 1838-1849.
21. Goto-Inoue N, Hayasaka T, Sugiura Y, Taki T, Li YT, et al. (2008) High-sensitivity analysis of glycosphingolipids by matrix assisted laser desorption/ ionization quadrupole ion trap time of flight imaging mass spectrometry on transfer membranes. J Chromat B 870: 74-83.
22. Nagai Y (1995) Functional roles of gangliosides in bio-signaling. Behav Brain Res 6:99-104.
23. Li YT, Mascos K, Chou CW, Cole RB, Li SC (2003) Presence of an Unusual GM2 Derivative, Taurine-conjugated GM2 in Tay-Sachs Brain. J Biol Chem 278:35286- 35292.
24. Brugger B, Erben G, Sandhoff R, Wieland FT, Lehman WD (1997) Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry¨. PNAS 94:2339-2344.
25. Wang H-Y J, Jackson SN, Woods AS (2007) Direct MALDI-MS Analysis of Cardiolipin from Rat Organs Sections. J Am Soc Mass Spectrom 18: 567–577.
26. Petkovic M, Schiller J, Muller M, Benard S, Reichl S (2001) Detection of Individual Phospholipids in Lipid Mixtures by Matrix-Assisted Laser Desorption /Ionization Time-of-Flight Mass Spectrometry: Phosphatidylcholine Prevents the Detection of Further Species. Analyt Biochem 289: 202-216.
27. Taki T, Nishimura K, Matsumoto M (1973) Incorporation of ethanolamine into phosphatidylethanolamine in mouse liver by exchange reaction. Japan J Exp Med 43: 87-105.
28. Taki T, Matsumoto M (1973) Study of Exchange reaction between phospholipid-base and free base: incorporation of L-serine into phospholipid and decarboxylation of phosphatidylserine .Japan J Exp Med 43: 219-224.
29. Siegel GJ, Agranoff BW, Wayne Alberns R, Fisher SK, Uhler MD (Eds) (1999) Basic Neurochemistry. Molecular, cellular and medical aspects, Lippincott-Raven, Philadelphia.