Lipids "methods"

Title

Mass spectrometric analysis of sphingomyelin with *N*-α-hydroxy fatty acyl residue in mouse tissues

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Running title:

Analysis of hydroxy sphingomyelin

Keyword: Sphingomyelin, Molecular species, liquid chromatography-tandem mass spectrometry, α-Hydroxy fatty acid, Skin

Abstract

Sphingomyelin (SM) with *N*-α-hydroxy fatty acyl residues (hSM) has been shown to occur in mammalian skin and digestive epithelia. However, the metabolism and physiological relevance of this characteristic SM species have not been fully elucidated yet. Here, we show methods for mass spectrometric characterization and quantification of hSM. The hSM in mouse skin was isolated by TLC. The hydroxy hexadecanoyl residue was confirmed by electron impact ionization-induced fragmentation in gas chromatography-mass spectrometry. Mass shift analysis of acetylated hSM by time of flight mass spectrometry revealed the number of hydroxyl groups in the molecule. After correcting the difference in detection efficacy, hSM in mouse skin and intestinal mucosa were quantified by liquid chromatography-tandem mass spectrometry, and found to be 16.5 ± 2.0 and 0.8 ± 0.4 nmol/μmol phospholipid, respectively. The methods described here are applicable to biological experiments on hSM in epithelia of the body surface and digestive tract.

Abbreviations used: CER, ceramide; CL, cardiolipin; C1P, ceramide 1-phosphate; ESI-TOF MS, electrospray ionization/time-of-flight mass spectrometry; FA, fatty acid; GC-MS, gas chromatography-mass spectrometry; hCER, CER with 2-hydroxyl fatty acyl residue; hC1P, C1P with 2-hydroxyl fatty acyl residue; hSM, SM with 2-hydroxyl fatty acyl residue; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SM, sphingomyelin.

Footnotes: The long-chain base of ceramide is designated as: sphingosine, *d*18:1. The *N*-acyl residues of ceramide are designated as: α-hydroxy palmitoyl, h16:0.

Introduction

Sphingolipids serve as constituents of functional domains in the plasma membrane. They also serve as precursors of intra- and inter-cellular signaling molecules and participated in proliferation, senescence and apoptosis of animal cells [1,2]. The *N*-acyl residues of sphingolipids are somewhat different from the acyl residues in glycerolipids. In general, saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids with chain lengths from C14 to C22 are the major fatty acids (FA) of mammalian glycerolipids. In contrast, very long saturated and monounsaturated FA, such as C24:0 and C24:1, are characteristically found as major *N*-acyl residues in the sphingolipids of many tissues [3,4]. α-Hydroxy FA (hFA) is also a typical FA found in sphingolipids [5]. The hFA is synthesized by hydroxylation at α-position of a FA by FA 2-hydroxylase. The CoA ester of hFA is then *N*-acylated by ceramide synthases to form dihydroceramide [6]. The resulting hFA-containing ceramide (hCER) is attached to sugars or phosphocholine to form glycosphinglipids or sphingomyeline (SM), respectively [5]. It is known that UDP-galactose: ceramide galactosyltransferase has preference for hCER [7]. This is one of the mechanisms for abundance in hFA-containing glycosphingolipids in brain [5]. The occurrence of SM with hFA residues is reported in several tissues, such as skin, testis and digestive mucosa [8-15]. However, the mechanisms of biosynthesis of hSM in these tissues are not fully elucidated yet.

Ceramide 1-phosphate (C1P) is a signaling molecule that mediates anti-apoptosis and cell migration [16]. Previously, we demonstrated the presence of C1P with 2-hydroxypalmitoyl residue (hC1P) in mouse skin. Interestingly, the molecular species composition of SM was almost the same as that of C1P in the skin, indicating a possibility that the hC1P is produced from the hSM as a precursor [15].

To understand the metabolism and physiological relevance of hSM in these tissues, sensitive methods to analyze hSM are necessary. In this regard, standard hSM is indispensable for

establishment of structural and quantitative analysis. However, a synthetic standard of hSM is not commercially available at present. In this study, we isolated hSM from mouse skin, and confirmed it by electrospray ionization/time-of-flight mass spectrometry (ESI-TOF MS) and gas chromatography-mass spectrometry (GC-MS). Using the isolated hSM as a standard, we quantified hSM in several mouse tissues by several methods including liquid chromatography-tandem mass spectrometry (LC-MS/MS) after correcting its detection efficiency.

Materials and Methods

Materials

Synthetic 12:0 SM (*d*18:1/12:0), 16:0 SM (*d*18:1/16:0), 24:1 SM (*d*18:1/24:1) and SM from bovine brain were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Egg yolk phosphatidylcholine, soybean phosphatidylinositol, bovine brain phosphatidylethanolamine and porcine brain phosphatidylserine, and bovine heart cardiolipin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methyl 2-hydroxyhexadecanoate was purchased from Larodan Fine Chemicals (Malmo, Sweden). TLC plates coated with silica gel (Merck Art 5721, Darmstadt, Germany) were used. All reagents were of analytical grade and used without further purification.

Isolation of phospholipids from mouse tissue

All experimental procedures described below were approved by the Tokushima University Animal Care and Use Committee (No. 14036). Five-week-old male ICR mice (25g body weight) were obtained from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Brain,

small intestine, liver and back skin were isolated from euthanized mice. The intestinal mucosa was obtained by scraping the luminal surface of the small intestine with the edge of a glass slide. After weighing, the tissues were cut into small pieces, mixed with saline, and homogenized in an ultradisperser (LK-21, Yamato Scientific, Tokyo Japan) for 5 min in ice-cold vessels. Lipids were extracted from the homogenates by the method of Bligh and Dyer [17]. The extracted lipids were heated in 0.1 M KOH in 95 % methanol at 60 °C for 10 min for hydrolysis of glycerolipids. The alkaline lysates were acidified using a small amount of 5 M HCl, and extracted by the Bligh and Dyer method [17]. The extracted lipids were subjected to TLC developed with chloroform/methanol/28 % ammonia (60:35:8, by vol). The silica gel bands corresponding to SM and hSM were then scraped off the plate. These SMs were recovered from the silica gel by the Bligh and Dyer methods and subjected to electrospray ionization-time of flight MS (ESI-TOF MS) or GC as described below. For analysis of the phospholipid composition, aliquots of skin lipid extracts were subjected to two-dimensional (2d) TLC. The solvent systems of the first and second chromatography were chloroform/acetone/methanol/acetic acid/water (50:20:10:13:5, by vol) and chloroform/methanol/28 % ammonia (60:35:8, by vol), respectively. Lipid spots were visualized by spraying with primuline, followed by detection under UV light. Each lipid was recovered from the silica gel by the Bligh and Dyer methods. Isolated phospholipids were identified by co-migration on the TLC with standard phospholipids, and determined using the phosphorus colorimetric method based on phosphomolybdenum-malachite green formation [18].

Acetylation of SM

Acetylation of SMs was conducted as described previously [19]. In brief, SM or hSM was

dissolved in a small amount of acetic anhydride/pyridine (2:1, by vol) and incubated at 37 °C for 3 h. The acetylated SMs were extracted and subjected to ESI-TOF MS.

ESI-TOF MS

Structural conformation of hSM was conducted using ESI-TOF MS (LCT Premier, Waters, Milford, MA, USA) in positive ion detection mode. The isolated skin SM and hSM with or without acetylation were injected using a syringe pump. Voltages of the capillary and sample cone were 3,000V and 75V, respectively. Temperatures of desolvation and ion source were set at 150ºC and 100ºC, respectively.

GC-MS and GC

The FA methylesters derived from hSM in mouse skin were analyzed by GC-MS as described previously [16]. In brief, FA methylesters prepared from isolated hSM were analyzed by GC-MS (JEOL Automass mass spectrometer, Tokyo, Japan) equipped with a Hewlett Packard (Wilmington, DE, USA) GC5890 gas chromatograph. The capillary column used was a non-polar DB-1 column (30 m length × 0.25 mm internal diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). The column temperature was kept at 100°C for 1 min, increased at 20°C/min to 300°C, and maintained at this temperature. Helium was used as the carrier gas. The ionization energy was 70 eV. The FA methylesters from SM of skin were also analyzed by gas chromatography (GC) (Shimadzu GC-15A; Shimadzu, Kyoto, Japan) equipped with a capillary column (DB-225, 0.25 μm film thickness, 30 m length, 0.25 mm ID; Agilent Technologies, Santa Clara, CA, USA) [20]. In this analysis, isolated SM and hSM were combined, and synthetic 17:0/17:0 phosphatidylcholine was added to the sample as an internal

standard before methylesterification. The oven temperature was kept at 100°C for 0.5 min and increased to 195°C at a rate of 25°C/min. Then, it was increased to 205°C at 3°C/min followed by 240°C at 8°C/min, and kept 240°C for 10 min. The oven temperature was then decreased to 100°C prior to injection of the next sample.

LC-MS/MS

Lipids of the skin, brain, liver and intestinal mucosa of mouse were extracted by the Bligh and Dyer method as described above. A known amount of synthetic 12:0 SM was added to the homogenates as an internal standard. The lipids were dissolved in methanol and filtered through a non-polar filter (Chromatodisc 4 N, 0.2 μm, Kurabo, Osaka, Japan). The filtrate was dissolved in 0.1 ml of methanol/formic acid (99:1, by vol) containing 5 mM ammonium formate for LC-MS/MS. SMs were analyzed performed using a quadrupole-linear ion trap hybrid mass spectrometer 4000 Q TRAP (Applied Biosystems/MDS Sciex, Concord, ON, Canada) operating in a multiple reaction-monitoring positive ionization mode with an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington DE, USA) and HTSPAL autosampler (CTC Analytics AC, Zwingen, Switzerland). The multiple reaction monitoring transitions measured were m/z $[M+H]^+$ to m/z 184 $[phosphocholine]^+$. The lipid extracts from mouse skin were separated by LC with a Cadenza CD-C18 column (50×2 mm, 3μm, Imtakt Corp., Kyoto, Japan) at 42°C. The mobile phase used was methanol/formic acid (99:1, by vol) containing 5 mM ammonium formate at an isocratic flow of 300 μL/min.

Results

Isolation and structural confirmation of hSM

The lipid extract from mouse skin was separated by 2d TLC. As shown in Fig. 1a, SM with an α-hydroxy acyl residue (hSM) was successfully separated. After recovery of these phospholipids from the plate, isolated phospholipids were quantified by a conventional phosphorus colorimetric method. We found that hSM accounted for 1.7 ± 0.4 mol% of total skin phospholipid. This corresponds to 17 ± 4 nmol/μmol skin phospholipid. Based on the amount of extracted phospholipid from skin (5.9 ± 0.5 μmol/g wet weight), Amount of hSM was calculated to be 0.1 ± 0.02 μmol/g wet weight. The ratio of hSM in total SM was 11.5 mol% (Table 1). The ratio of hSM to total SM observed in this experiment is lower than that reported by Uchida who reported the ratio to be 45% in the skin of hairless mice [13]. The large difference in the hSM contents between our data and that of Uchida et al. may be caused by differences in the analysis method, mice strain and skin tissue preparation. Among these, tissue preparation was critically different. Epidermis and dermis were not separated in our preparation of skin, whereas, only epidermis was used in experiments of Uchida et al [13]. Relatively large amount of hSM was isolated from alkaline-treated skin lipids by preparative TLC (Fig. 1b).

The SMs isolated from the mouse skin were analyzed by ESI TOF-MS (Fig. 2a). The highest peak observed in the analysis of the SM fraction (Fig. 2a top) was *m/z* 703.6 followed by *m/z* 731.6 and *m/z* 813.7. They were assignable to *d*18:1/16:0, *d*18:1/18:0 and *d*18:1/24:1, respectively. In the analysis of the hSM fraction, *m/z* 719.6 was predominantly detected. This peak was shifted by 84 mass units by acetylation of the hSM fraction, indicating that two hydroxy groups are present in the structure of hSM (Fig. 2a middle and bottom). The *N*-acyl residue of the hSM was identified by GC-MS (Fig. 2b) as a fatty acid methylester. A single peak was detected in the total ion chromatography. The mass spectrum obtained at the top of the peak (Fig. 2b lower panel) coincided with that of the methylester of the standard α-hydroxy hexadecanoic acid (h16:0-me) (Fig. 2b upper panel). An intense ion observed in these mass

spectra, *m/z* 227, is assignable to be a fragment ion, $[M-59]^+$. This is a characteristic fragment ion derived from the methylester of α-hydroxy fatty acid as shown in Fig. 2b upper [21]. From these results, it was concluded that hSM with α-hydroxy hexadecanoyl residue (*d*18:1/h16:0) was the predominant molecular species present in mouse skin. These data also indicated that isolated SM and hSM fractions in our study did not contain significant contaminants of each other. The fact that *d*18:1/h16:0 is the predominant molecule of the hSM fraction of mouse skin is consistent with the results reported by Kitano et al. [14] and Uchida [13].

Determination of hSM by GC

The SM fraction (SM+hSM) obtained from mouse skin was subjected to their fatty acid analysis by GC for determination of each species. The peak of the methylester of α-hydroxy hexadecenoic acid (h16:0) was detected at 10.6 min as shown in Fig. 3a. There is no other detectable hFA peak in the chromatogram. Based on ratio of peak area between each peak and the methyl hexadecanoate added as an internal standard, the amount of each molecular species of SM was determined (Fig. 3b). We found that SM with h16:0 in mouse skin was 13 ± 1 nmol/μmol skin phospholipid. The ratio of the hSM in total SM was calculated to be 10 mol%. These data were comparable to those obtained in conventional quantification of isolated phospholipids as described above.

Determination of hSM by LC-MS/MS

The ions selected for first (Q1) and second (Q3) detections of SM molecules in our MS/MS system were $[M+H]^+$ and $[phosphorylcholine]^+$, respectively (Fig. 4a). Chromatograms of typical molecular species of SM detected in the analysis of LC-MS/MS of total lipid extracts

of mouse skin are shown in Fig. 4b. We prepared three standard curves to correct the differences in detection efficacies between different SM species (Fig. 4c) using synthetic 12:0 SM (*d*18:1/12:0), 16:0 SM (*d*18:1/16:0), 24:1 SM (*d*18:1/24:1) and isolated h16:0 SM (*d*18:1/h16:0) from mouse skin. We found that detection efficacies of 12:0 SM, 16:0 SM and 24:1 SM were almost the same in our analytical system, whereas the detection efficacy of h16:0 SM was about one thirds that of 12:0 SM (slope: 0.31). Based on these results, we quantified hSM after correction for the difference in the detection efficacy. The results of quantification of SM species in mouse skin (a) brain (b), liver (c) and intestinal mucosa (d) are shown in Fig. 5. Owing to the high sensitivity, we detected minor species of SM, such as *d*18:1/16:1, *d*18:1/17:0 (*d*17:1/18:0), *d*18:1/18:1, *d*18:1/26:0, and *d*18:1/26:1, which are undetectable in GC analysis. It should be mentioned that our assignment of each peak is based on assumption that *d*18:1 sphingosine is major species in animal tissues [22]. However, significant amount of *d*17:1 sphingosine has been known to exist in skin [23]. Thus, the peak assigned as 17:0 SM in skin is considered to contain significant amount of *d*17:1/18:0 SM in addition to *d*18:1/17:0 SM. The amount of hSM in skin was found to be 16 ± 4 nmol/μmol skin phospholipid by the LC/MS analysis. This value is comparable to those obtained by a conventional phospholipid quantification method or FA-based quantification in GC, as shown above. On the other hand, the ratio of hSM in total SM was calculated to be 18 mol% in skin. This value was slightly higher than those obtained by the other methods. We found hSM in intestinal mucosa. The molecular species of hSM in the mucosa was *d*18:1/h16:0, and its amount was determined to be 0.8 ± 0.4 nmol/μmol phospholipid. The molecular profile of SM of mouse intestinal mucosa, which shows abundance in *d*18:1/16:0, is consistent with the report of Bouhours and Guignard [24] except for detection of hSM. The inability to detect hSM in their report in 1979 would be ascribed to differences between the detection sensitivity of GC and LC/MS.

Discussion

In this study, we quantified hSM by three methods, namely a conventional phosphorus colorimetric method, FA-based quantification by GC and LC-MS/MS. Among them, LC-MS/MS has advantageous over the other two methods in the sensitivity and simplicity of samples preparation. Although the other two methods need purification of SM from extracted lipids, they do not require expensive equipment. As shown in GC and ESI-TOF MS results, the hSM fraction isolated by 2dTLC from skin is composed almost single molecular species of *d*18:1/h16:0. Using the h16:0 SM obtained from skin, we found that detection efficacy of h16:0 SM was considerably low compared to usual SM, such as 12:0 SM, 16:0 SM and 24:1 SM. This is consistent with our observation that detection efficacy of CER with h16:0 in LC-MS/MS is approximately half that of CER with 16:0 (unpublished observation). The lower detection efficacy in a lipid with hFA is also observed in MALDI-TOF MS. We reported that detection efficacy of C1P with hFA in MALDI-TOF MS is about half that of C1P with non-hFA [16]. It seems that detection efficacy was not considered in the previous quantification of hSM by LC-MS/MS [25]. It is important to consider the detection efficacy in the mass spectrometric analysis of lipids with different number of OH groups.

One of the sources of intracellular ceramide that plays a signaling role in apoptosis and differentiation is SM. Recent reports revealed that ceramides having different *N*-acyl chains play different roles in cells [26]. For example, forcibly changing the ceramide composition in cells by overexpression or silencing the ceramide synthases shows that long-chain ceramide is pro-apoptotic, while ceramide with very long chain FA is anti-apoptotic [26]. It is also reported that ceramide with hFA potently induces apoptosis compared to that with non-hydroxylated FA [27]. In this regard, hSM detected in intestinal mucosa and skin may serve as precursor of a pro-apoptotic ceramide with high potency. C1P has been known to function as an anti-apoptotic

signal in the cells. Although we identified hC1P in mouse skin in a previous work, the physiological role of this characteristic species is unknown [16]. Research on biosynthesis and degradation of the hSM are needed for better understanding of physiological relevance of this SM species, which is uniquely present in epithelium of the skin and digestive tract. We hope the methods for analysis of hSM described here helps such research.

Acknowledgement

This work was supported by JSPC KAKENHI Grant Number JP 19K05863 (to T.T.) and Kobayashi Foundation (to T.T.). We thank Dr. Kei Yamamoto for donating animals used in this study and advise on the preparation of the manuscript.

Authorship

H.A. and R.Y. designed, performed the research and writing this manuscript. M.A., J.M., K.M. and N.K. assisted mass spectrometric analysis. J.H., R.K. and K.T. analyzed data and reviewed the manuscript. T.T. contributed as a mentor of H.A. and R.Y. and writing manuscript. All authors have approved this manuscript.

Conflict of interest

None declared for all authors

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Table 1 Phospholipid composition of mouse skin

Phospholipid	mol %
Sphingomyelin (SM)	13.1±3.6
Hydroxy-sphingomyelin (hSM)	1.7±0.4
Phosphatidylcholine (PC)	52.7±1.4
Phosphatidylethanolamine (PE)	17.0±1.9
Phosphatidylserine (PS)	5.3±1.7
Phosphatidylinositol (PI)	4.3±0.9
Lysophosphatidylcholine (LPC)	3.0±0.2
Cardiolipin (CL)	2.6±2.1
Phosphatidic acid (PA)	0.4±0.2

The isolated phospholipids in mouse skin were determined by the phosphorus colorimetric method as described in Materials and Methods. Data are presented as the mol percentage of total phospholipids. Values are mean ± S.D. from three independent experiments performed on three mice. Total skin phospholipids were quantified to be 5.9 ± 0.5 μmol/g (wet weight).

Figure legend

Fig. 1 Isolation of hSM of mouse skin lipids by TLC

Skin lipids were separated by two-dimensional TLC (a). Lipid spots observed under UV light following application of primuline were traced with pencil. Phospholipid spots were visualized with Dittmer reagents. Alkaline-treated skin lipids were separated by preparative TLC (b). Lipid bands were observed under UV light following application of primuline. Abbreviations used are as follows: VLCFA, very-long chain fatty acid (mainly C22 to C24); LCFA, long chain fatty acid (mainly C16 to C20); FFA, free fatty acid. Other abbreviations are shown in Table 1.

Fig. 2 Structural confirmation of hSM by ESI-TOF MS (a) and GC-MS (b)

The isolated SM, hSM and acetylated hSM in mouse skin were analyzed by ESI-TOF MS (a). A synthetic methylester of α-hydroxy hexadecenoic acid (h16:0-me) and fatty acid methylester prepared from hSM were analyzed by GC-MS (b).

Fig. 3 Determination of molecular species of SM of mouse skin by GC

FA methylesters prepared from the SM fraction (SM + hSM) of mouse skin were subjected to GC (a). The FA composition of SM was determined based on peak area on GC (b). Values are means ± SD from three independent experiments.

Fig. 4 Determination of molecular species of SM of mouse skin by LC-MS/MS.

Lipids extracted from mouse skin were analyzed by LC-MS/MS for determination of SM using 12:0 SM as an internal standard. Structures of molecular ion $[M+H]^+$ and fragment ion

(m/z 184) used for detection of SM are shown in a. Chromatograms of typical SM species in mouse skin are shown in b.

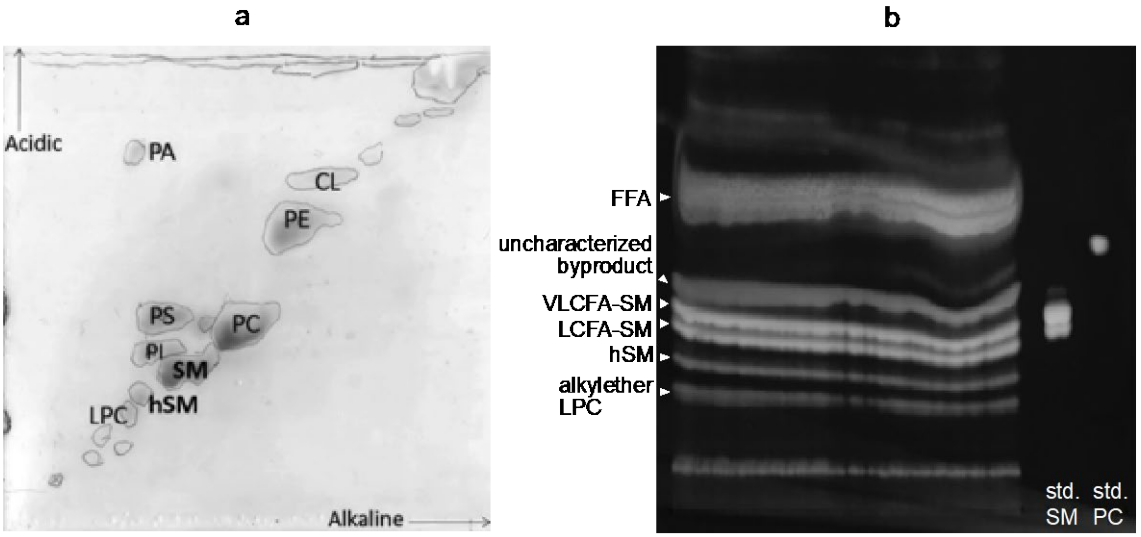
Standard curves prepared with SM mixtures consisting of different amounts of *d*18:1/16:0, *d*18:1/24:1 or hSM from mouse skin and a fixed amount of *d*18:1/12:0 SM are shown in c.

Fig. 5. Determination of molecular species of SM in mouse skin, brain, liver and intestinal mucosa by LC-MS/MS.

Lipids obtained from mouse skin (a), brain (b), liver (c) and intestinal mucosa (d) were extracted and analyzed by LC-MS/MS for determination of SMs using *d*18:1/12:0 SM as an internal standard. Assignment is based on assumption that *d*18:1 sphingosine is backbone of these SMs. Molecular species indicated as 17:0 in skin is considered to contain *d*17:1/18:0 species at significant level as described in the text.

Fig.1

Fig. 1


a
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SM
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Alkaline
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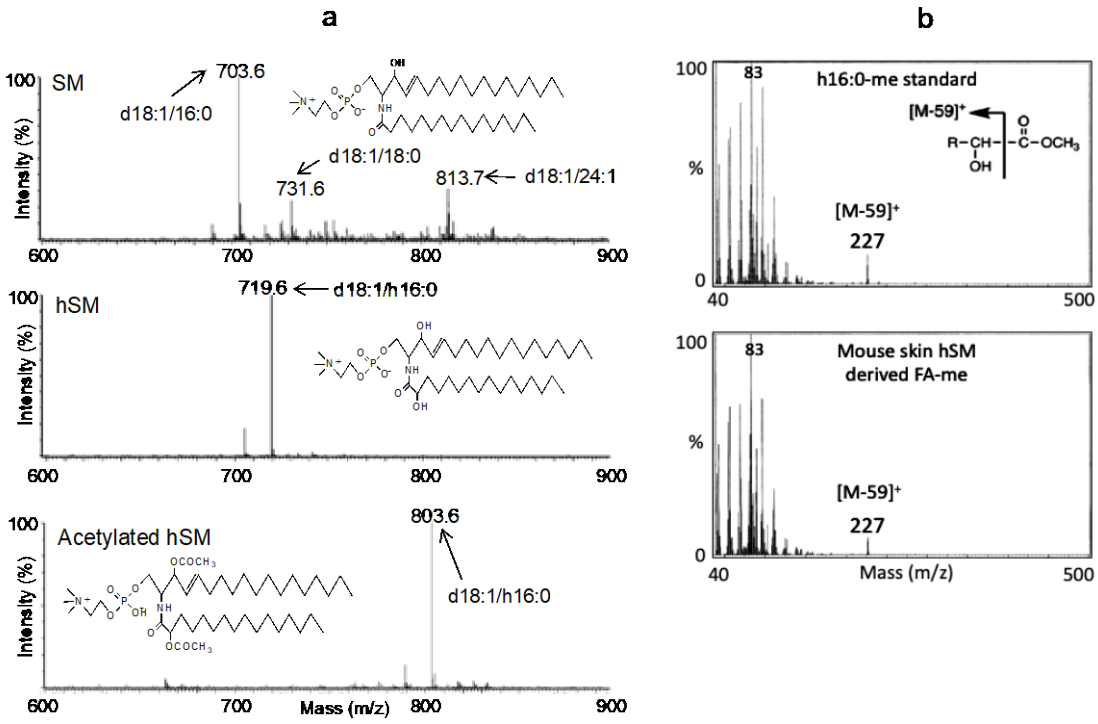
uncharacterized byproduct
VLCFA-SM
LCFA-SM
hSM
alkylether LPC
std. SM
std. PC

Fig. 2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b
100
SM
703.6
d18:1/16:0
d18:1/18:0
731.6
813.7
d18:1/24:1
Intensity (%)
600
700
800
900
hSM
719.6
d18:1/h16:0
Acetylated hSM
803.6
d18:1/h16:0
Mass (m/z)
h16:0-me standard
[M-59]+
227
%
0
40
500
83
Mouse skin hSM
derived FA-me

Fig. 3

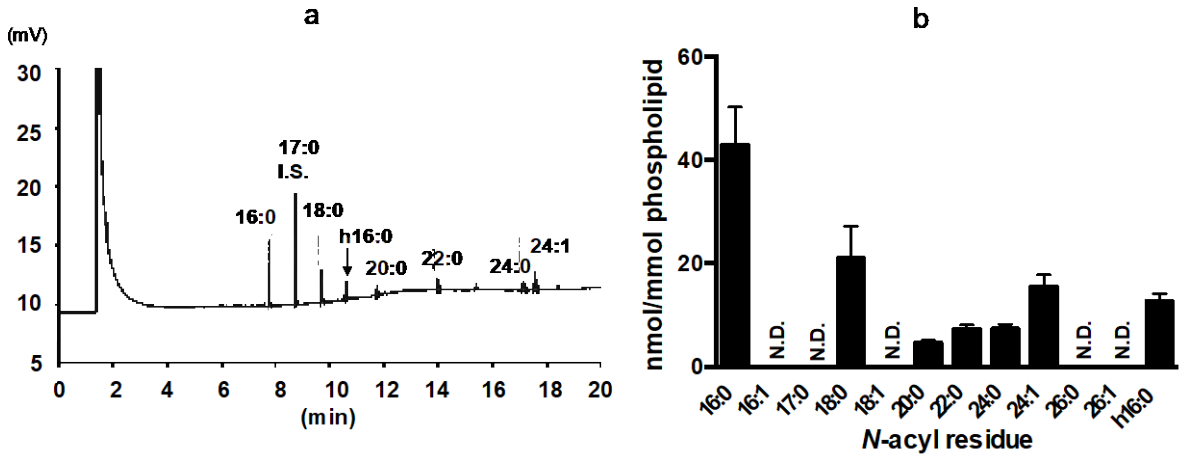

a
(mV)
30
25
20
15
10
5
0
2
4
6
8
10
12
14
16
18
20
(min)
16:0
17:0
I.S.
18:0
h16:0
20:0
22:0
24:0
24:1
b
nmol/mmol phospholipid
60
40
20
0
N.D.
N.D.
N.D.
N.D.
N.D.
16:0
16:1
17:0
18:0
18:1
20:0
22:0
24:0
24:1
26:0
26:1
h16:0
N-acyl residue

Fig. 4

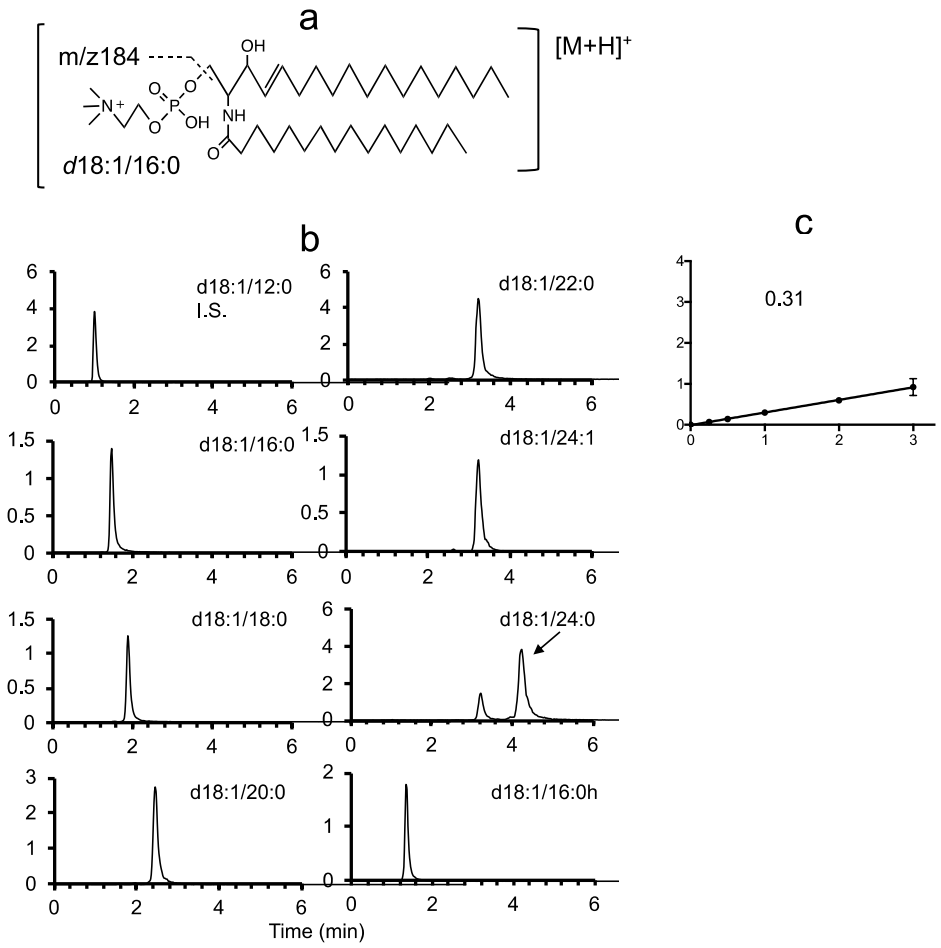
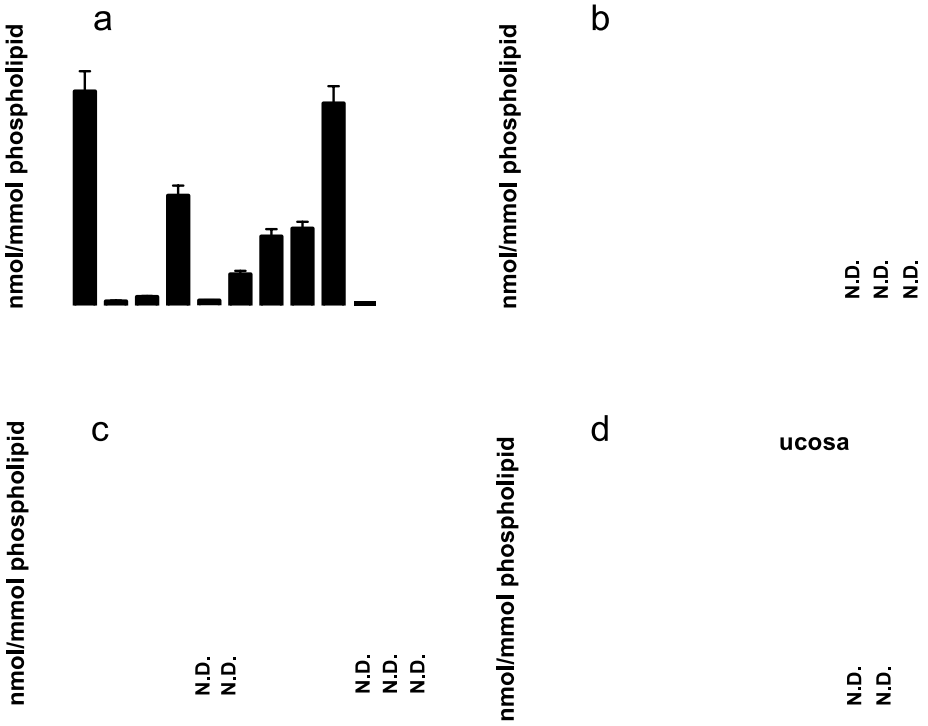

a
m/z184
[M+H]+
d18:1/16:0
b
d18:1/12:0
I.S.
d18:1/22:0
d18:1/16:0
d18:1/24:1
d18:1/18:0
d18:1/24:0
d18:1/20:0
d18:1/16:0h
Time (min)
c
0.31

Fig. 5


a
nmol/mmol phospholipid
b
nmol/mmol phospholipid
N.D.
N.D.
N.D.
c
nmol/mmol phospholipid
N.D.
N.D.
N.D.
N.D.
N.D.
d
nmol/mmol phospholipid
ucosa
N.D.
N.D.