



Development of a Label-Free LC-MS/MS-Based Glucosylceramide Synthase Assay and Its Application to Inhibitors Screening for Ceramide-Related Diseases

Zhicheng Fu^ψ, So Yoon Yun, Jong Hoon Won, Moon Jung Back, Ji Min Jang, Hae Chan Ha, Hae Kyung Lee, In Chul Shin, Ju Yeun Kim, Hee Soo Kim and Dae Kyong Kim*

Department of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, Seoul 06974, Republic of Korea

Abstract

Ceramide metabolism is known to be an essential etiology for various diseases, such as atopic dermatitis and Gaucher disease. Glucosylceramide synthase (GCS) is a key enzyme for the synthesis of glucosylceramide (GlcCer), which is a main ceramide metabolism pathway in mammalian cells. In this article, we developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to determine GCS activity using synthetic non-natural sphingolipid C8-ceramide as a substrate. The reaction products, C8-GlcCer for GCS, could be separated on a C18 column by reverse-phase high-performance liquid chromatography (HPLC). Quantification was conducted using the multiple reaction monitoring (MRM) mode to monitor the precursor-to-product ion transitions of m/z 588.6 \rightarrow 264.4 for C8-GlcCer at positive ionization mode. The calibration curve was established over the range of 0.625-160 ng/mL, and the correlation coefficient was larger than 0.999. This method was successfully applied to detect GCS in the human hepatocellular carcinoma cell line (HepG2 cells) and mouse peripheral blood mononuclear cells. We also evaluated the inhibition degree of a known GCS inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) on GCS enzymatic activity and proved that this method could be successfully applied to GCS inhibitor screening of preventive and therapeutic drugs for ceramide metabolism diseases, such as atopic dermatitis and Gaucher disease.

Key Words: Ceramide, Chemotherapeutic multi-drug resistance, Gaucher disease, Glucosylceramide, Glucosylceramide synthase, LC-MS/MS

INTRODUCTION

A deficiency of ordinary ceramides in the corneum is an essential etiologic factor for the dry and barrier-disrupted skin of patients with atopic dermatitis (Choi and Maibach, 2005; Imokawa, 2009; Mizutani *et al.*, 2009). It is known that ceramides are produced from sphingomyelin (SM) and glucosylceramide (GlcCer) as a form of glycosphingolipids (GSL) by enzymatic reactions of sphingomyelinase (Kolesnick, 2002) and GlcCer deacylases (Vielhaber *et al.*, 2001), respectively. Thus, in order to prevent the pathogenesis of atopic dermatitis, many pharmacological approaches have been performed to develop

an inhibitor or activator, which could regulate dermal levels of the above lipid mediators (Imokawa, 2009).

GSL are membrane components as a group of membrane lipids in which the lipid portion is embedded in the outer side of the plasma membrane with the sugar chain extending to the extracellular site (Liu *et al.*, 2004; Yoshizaki *et al.*, 2008; Wang *et al.*, 2009; Kartal Yandim *et al.*, 2013). GSL have been known to be involved in many cellular processes, including growth, differentiation, morphogenesis, sensitivity, and response to exogenous compounds (Hakomori, 2008; Kartal Yandim *et al.*, 2013). GlcCer is a crucial GSL metabolic intermediate, which serves as the precursor in the biosynthesis

Open Access <https://doi.org/10.4062/biomolther.2018.122>

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received Jul 4, 2018 Revised Aug 7, 2018 Accepted Aug 9, 2018

Published Online Sep 20, 2018

*Corresponding Author

E-mail: kimdk@cau.ac.kr

Tel: +82-2-820-5610, Fax: +82-2-825-7920

^ψPresent Address

National institute for nutrition and health, Chinese center for disease control and prevention, Beijing 100050, China

Table 1. MS parameters of C8-GlcCer and C12-GlcCer

	Q1	Q3	DP	FP	EP	CE	CXP
C8-GlcCer	588.6	264.2	110 V	340 V	6 V	40 V	6 V
C12-GlcCer	644.5	264.2	120 V	340 V	7 V	46 V	6 V

DP, Declustering Potential; FP, Focusing Potential; EP, Entrance Potential; CE, Collision Energy; CXP, Collision Cell Exit Potential.

of a variety of GSL, such as lactosylceramide (LacCer) and the remaining neutral and acidic oligoglycosphingolipids (van Meer and Holthuis, 2000; Duclos, 2001; Degroote *et al.*, 2004; Ekiz and Baran, 2010).

GlcCer is generated by glucosylceramide synthase (GCS) and converted to LacCer by lactosylceramide synthase (LCS). GCS transfers the glucose from UDP-glucose to ceramide, and is degraded both by a glucocerebrosidase in lysosomes and by a non-lysosomal glucocerebrosidase in the cytosol (Maunula *et al.*, 2007; Ekiz and Baran, 2010; Tuuf *et al.*, 2011). GCS is known to be a transmembrane protein localized in the cis/medial Golgi, with an N-terminal signal-anchor sequence and a C-terminal catalytic domain located in the cytoplasm (Abe *et al.*, 1995; Chujor *et al.*, 1998; Miura *et al.*, 1998; Zheng *et al.*, 2006; Wang *et al.*, 2009). This enzyme does not have similarity to other known glycosyltransferases. The structure of the enzyme is quite unique since all other glycosyltransferase involved in GSL synthesis are known to be localized to the luminal side of the Golgi apparatus or ER (Di Sano *et al.*, 2002; Kartal Yandim *et al.*, 2013).

Previous studies have shown that GlcCer has proliferative functions in various cells (Ekiz and Baran, 2010; Kartal Yandim *et al.*, 2013). Therefore, it is important in chemotherapeutic drug resistance (Kartal Yandim *et al.*, 2013). In 1996, adriamycin resistance was reported to be related with the increased GlcCer levels in breast cancer MCF-7 cells. This effect of GlcCer on drug resistance has been observed in many other types of cancer cells, such as melanoma, leukemia, and neuroblastoma (Sietsma *et al.*, 2001; Bleicher and Cabot, 2002; Xie *et al.*, 2008). It has also been reported that a high level of GlcCer accumulates in white blood cells, especially in macrophages, in patients with Gaucher disease (Bennett and Mohan, 2013; Nagral, 2014). On the other hand, it is known that many physiological molecules can activate LCS to generate a GSL, LacCer, which can induce critical phenotypes such as cell proliferation, migration, adhesion, angiogenesis, and apoptosis (Chatterjee and Alsaedi, 2012). Recent studies of LacCer revealed that it can play an important role in pathological cardiac hypertrophy through the generation of superoxides are the activation of p44 mitogen activated protein kinase/extracellular signal-related kinase-1 (MAPK/ERK-1) in freshly cultured neonatal rat ventricular myocytes and H9C2 cells, a transformed cardiomyocyte cell line (Mishra and Chatterjee, 2014). Therefore, in order to screen pharmacological inhibitors of the related enzymes for the related diseases, it is very important to develop an assay system for GlcCer-related sensitive and specific enzymes such as GCS and LCS.

For measurement of GCS activity, several useful methods have been reported (Basu *et al.*, 1973; Hospattankar and Radin, 1981; Ichikawa *et al.*, 1996; Hayashi *et al.*, 2005). In general, GCS activity is assayed by a thin-layer chromatography (TLC)- or a high-performance liquid chromatography (HPLC)-based method using C6-4-nitrobenzo-2-oxa-1,3-dia-

zole (NBD)-Cer as an acceptor substrate. Although the NBD-labeled substrate has been successfully used in the enzyme activity detection, the distal NBD group could still introduce nonspecific interactions. Therefore, we decided to develop a label-free detection method with an LC-MS/MS readout to eliminate any reporter interference.

In this study, we report a new LC-MS/MS-based method that uses non-natural sphingolipid C8-ceramide (C8-Cer) and non-radioisotope UDP-glucose as substrates. The product, C8-glucosylceramide (C8-GlcCer), was separated by a C18 column and detected by mass spectrometer in the positive electrospray ion mode (ESI), and the intensity of C8-GlcCer was normalized by the internal standard C12-glucosylceramide (C12-GlcCer). To validate usefulness of this assay, the method was employed to determine the activities of GCS in HepG2 cell and mouse peripheral blood mononuclear cells (PBMC) lysate after incubation with a known GCS inhibitor, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). We successfully evaluated the inhibition degree of PDMP on GCS at different working concentrations to validate this LC-MS/MS-based GCS enzymatic activity assay. Furthermore, we confirmed its additional usefulness by evaluating simultaneously enzymatic activity of LCS, another enzyme involved in a cellular level of GlcCer. This method could be applied to GCS specific inhibitor screening for developing therapeutic drugs against chemotherapeutic multi-drug resistance and Gaucher disease.

MATERIALS AND METHODS

Materials

HepG2 cells (Hepatocellular carcinoma cell line) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). C57BL/6 mice were purchased from Orient Bio (Gyeonggi, Korea). All animal experiments were performed in accordance with the National Research Council's Guidelines for Care and Use of Laboratory Animals and the Guidelines for Animal Experiments of Chung-Ang University. C8-Cer, C8-GlcCer, and C12-GlcCer were purchased from Avanti Polar Lipids (AL, USA). Lecithin was obtained from Sigma (MO, USA). All other reagents were obtained at the highest available purity.

Preparation of lipid standard stock solutions and calibration standards

Each lipid was dissolved in methanol to prepare the standard stock solution to a final concentration of 1 mg/mL and stored at -80°C . This solution was further diluted in methanol with 0.1% formic acid to obtain standard solutions at several concentrations. Calibration standard samples were prepared by serial dilution with a known amount of each compound. For the internal standard (ISD) solution, C12-GlcCer was dis-

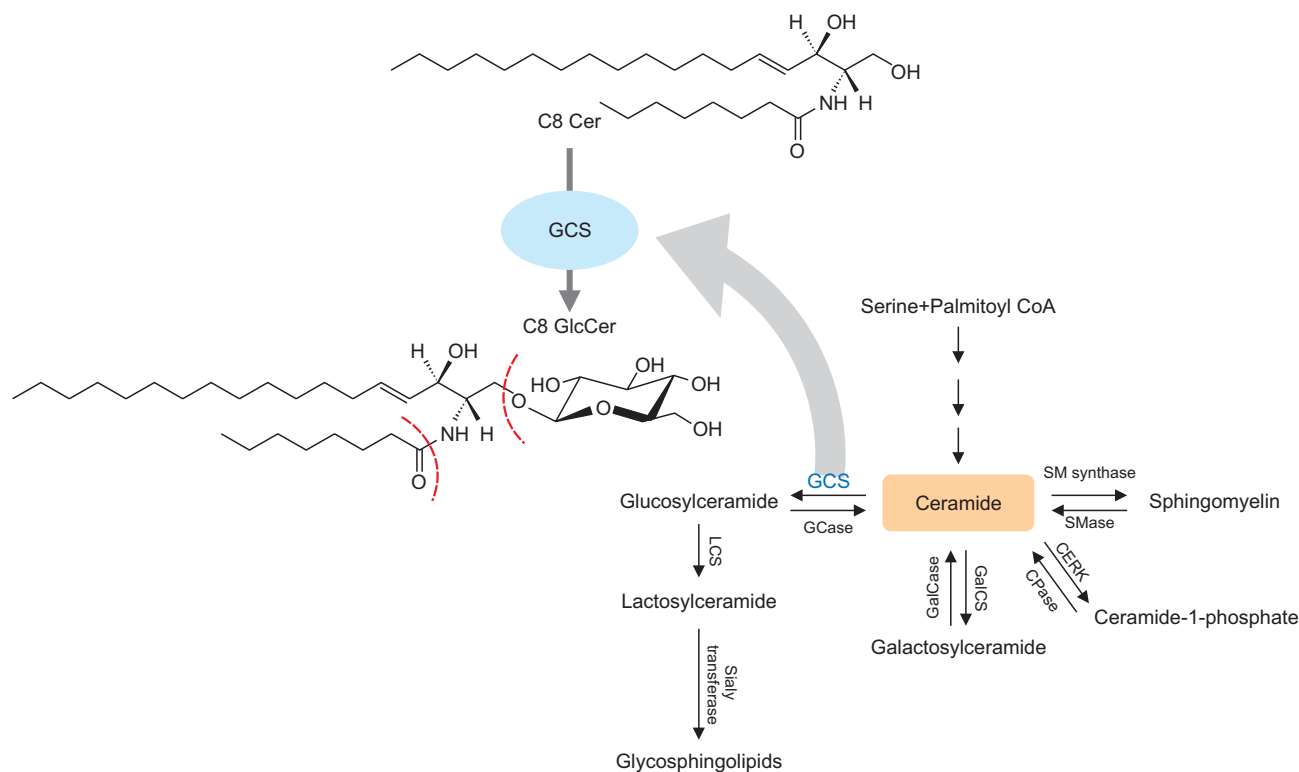


Fig. 1. Ceramide metabolic pathway related to glucosylceramide. SMase, sphingomyelinase; GCS, glucosylceramide synthase; GCSase, glucosylceramidase; LCS, lactosylceramide synthase; GalCS, galactosylceramide synthase; GalCase, galactosylceramidase; CERK, ceramide kinase; CPase, Ceramide-1-phosphate phosphatase.

solved in methanol at the concentration of 100 ng/mL. Calibration standard samples were constructed using nine concentration points.

Cell culture

HepG2 cells were cultured at 37°C, 5% CO₂ in MEM supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin in a humidified incubator.

For cell harvest, HepG2 cells were detached from the culture plate using 0.25% trypsin/EDTA and washed thrice with ice-cold PBS.

Mouse PBMC isolation

PBMC were isolated from mouse peripheral blood using the Histopaque-1077 density gradient method. Briefly, whole blood was isolated from pathogen-free male C57BL/6 mice (6-8 weeks) into a 3 mL vacutainer tube coated with EDTA. Subsequently, 1 mL of blood was suspended with 2 mL PBS and carefully layered onto 3 mL Histopaque-1077. Following, the solution was centrifuged at 400×g for 30 min at room temperature. Finally, the PBMC layered in the interface was transferred to a new 15 mL tube and washed thrice with ice-cold PBS.

Preparation of enzyme from HepG2 cells and mouse PBMC

To prepare cell lysates, harvested HepG2 cells and mouse PBMC were suspended in lysis buffer (10 mM Tris-HCl, pH 7.5, containing 1x protease inhibitor and 1 mM EDTA) and

lysed by sonication. Following, the cell lysate was centrifuged at 1,000×g, 4°C for 10 min. The supernatants were transferred to a new tube prepared for the GCS activity assay.

Protein assay

The protein concentration of each sample was measured using the bicinchoninic acid protein assay (Pierce, MA, USA), with bovine serum albumin as the standard.

LC-MS/MS-based GCS assay procedures

Acceptor substrate, 1 nmol of C8-Cer, and 130 nmol of lecithin were mixed in 0.2 mL of methanol, followed by evaporation of the solvent. Subsequently, 0.2 mL of water was added, and the mixture was sonicated to form liposomes. For the GCS enzymatic activity assay, 5 µL of 5 mM UDP-Glc, 10 µL of 10 mM EDTA, 10 µL of 25 mM of conduritol B epoxide (CBE), 10 µL of C8-Cer liposome, and 65 µL of enzyme were combined to make a total of 100 µL. Enzyme activity assays were carried out at 37°C for 1 h. For the enzyme activity assay of LCS, 0.1 mM UDP-Gal, 5 mM MgCl₂, 5 mM MnCl₂, 2.5 mM of conduritol B epoxide (CBE), 10 µL of C8-GlcCer liposome, and 65 µL of enzyme were combined. Enzyme activity assays were carried out at 37°C for 1 h. The reaction was stopped by adding 600 µL of chloroform/methanol (1:2, v/v) and 20 µL of ISD (100 ng/mL of C12-GlcCer in methanol) solutions. After vortexing for 1 min, 100 µL of water was added. The mixture was vortexed for 5 min, incubated at room temperature for 10 min, and centrifuged at 5,000×g for 5 min. After centrifugation, the supernatants were taken, and 200 µL of water and 200

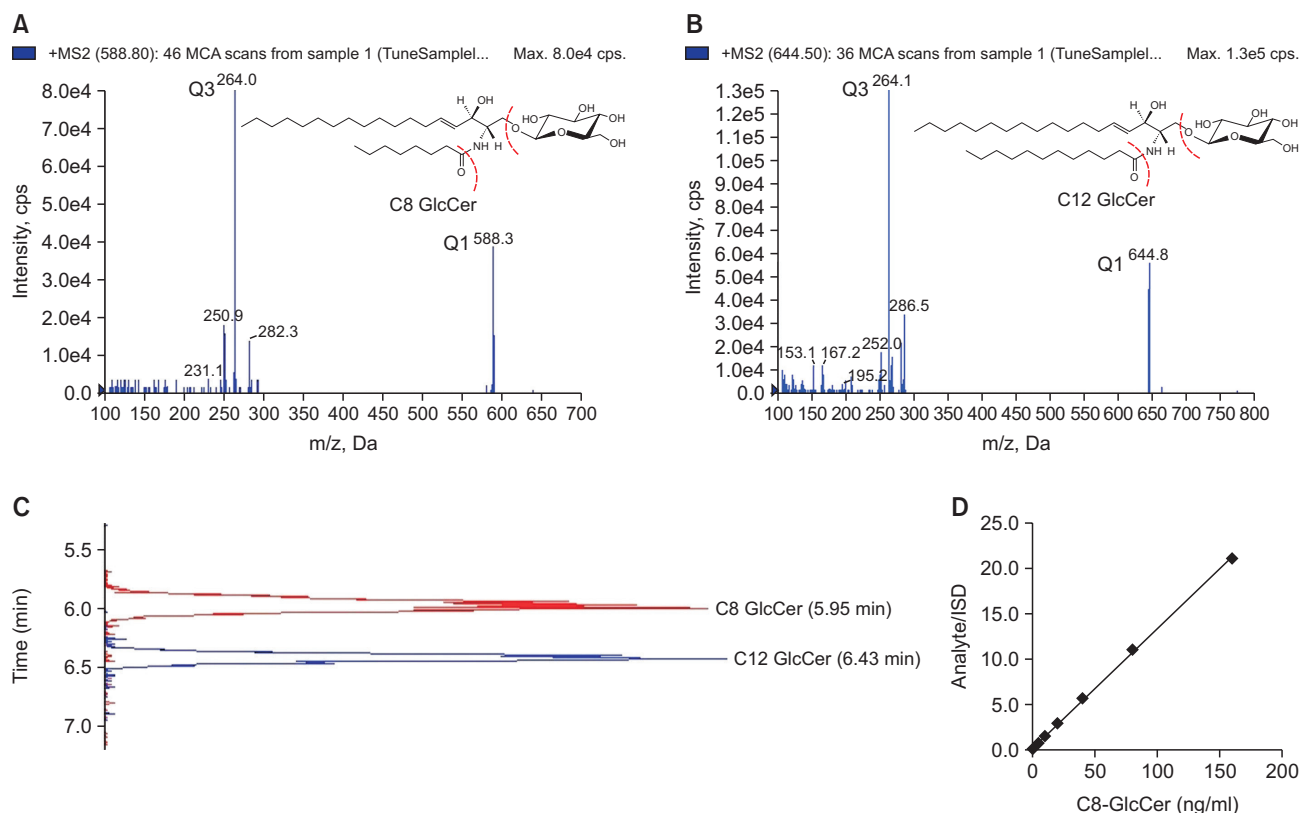


Fig. 2. Detection of C8-GlcCer and C12-GlcCer by LC-MS/MS system. C8-GlcCer (A) and C12-GlcCer (B) were detected using an API3000 mass spectrometer with an Electrospray Ion (ESI) Source (AB SCIEX) at positive ion mode. (C) Both C8-GlcCer and C12-GlcCer were eluted in 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7) at 10 ng/mL and separated on a reverse-phase C18 column using an HPLC (Agilent 1100) system with mobile A (5 mM ammonium formate (pH 4.0)/methanol, 95/5) and mobile B (2 mM ammonium formate in methanol with 0.1% formic acid/tetrahydrofuran, 7/3). (D) Different concentrations of C8-GlcCer with 10 ng/mL of ISD were loaded to the LC-MS/MS system. The linear calibration curve was obtained by plotting the peak area of Analyte/ISD against concentrations of C8-GlcCer in the range of 0.625–160 ng/mL. Details were described in the Materials and Methods section.

μL of chloroform were added. The samples were vortexed for 5 min and centrifuged at $2,000\times g$ for 5 min to separate the organic phase. The organic phase was evaporated in a speed vacuum dryer. Following, 800 μL of 10 mM NaOMe was added to the dried residue and vortexed well at room temperature for 2 h. The samples were evaporated again and dissolved in 200 μL of 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7, v/v/v).

Liquid chromatography and tandem mass spectrometry

Analytes were determined using LC-ESI/MS/MS, consisting of an HPLC (Agilent 1100, CA, USA) coupled to an API3000 mass spectrometer with an electrospray ion (ESI) source. The analytical column was an X Terra MS C18 (2.0 \times 50 mm, 3 μm , Waters Co., MA, USA). The mass spectrometer was operated in the positive ESI mode using multiple reaction monitoring (MRM). Nitrogen was used as the nebulizer gas and collision gas.

Separation was achieved using 0.1% formic acid added to methanol buffered with 2 mM ammonium formate/tetrahydrofuran (7/3, v/v) as mobile A and 5 mM ammonium formate (pH 4.0)/methanol (9/1, v/v) as mobile B. For HPLC separation, a gradient program was used at a flow rate of 200 $\mu\text{L}/\text{min}$. The initial buffer composition was 50% mobile A and 50% mobile B,

then linearly changed to 100% mobile A in 5 min and maintained for 2 min, and immediately returned to initial condition and maintained for 2 min. Each run time was 10 min. Subsequently, 10 μL of sample was injected at a flow rate of 0.2 mL/min.

The optimal mass spectrometry values were obtained by a manual tuning or automatic tuning process with each standard injected by syringe pump. Monitoring ions of each sphingolipid are shown in Table 1. The data acquisition was confirmed by Analyst[®] 1.6.2 software (AB SCIEX, MA, USA).

The calibration curves for sphingolipid standards were generated by plotting the peak area ratio (analyte/internal standard) versus the concentrations in the calibration standard samples by least-square linear regression.

RESULTS

Ceramide can be synthesized by the condensation of serine with fatty acid acyl-CoA and then converted to other bioactive lipids. Ceramide is converted to ceramide-1-phosphate, GlcCer, sphingosine, etc. by each enzyme, which can be reversibly converted back to ceramide. Among them, GCS catalyzes glucosylation of ceramide, which forms GlcCer. This is the first time to develop a sensitive and selective LC-MS/

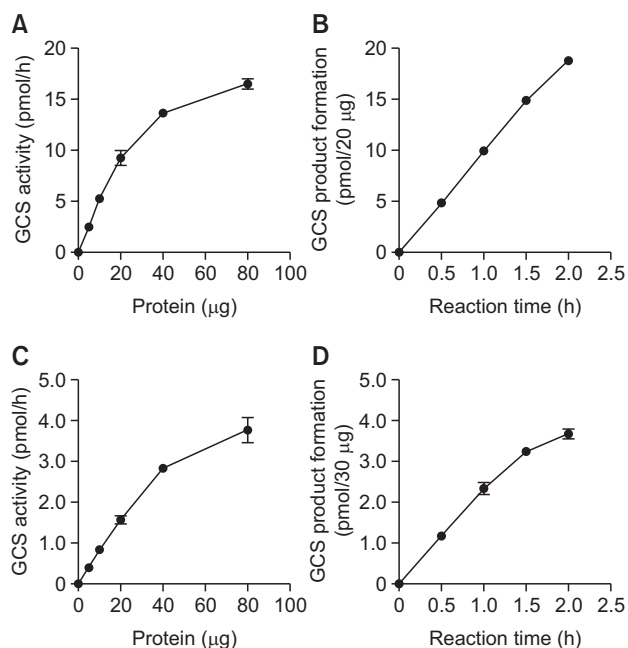


Fig. 3. Determination of GCS activities in HepG2 cells and mouse PBMC cells using different protein concentrations and incubation times. GCS activities were determined by LC-ESI-MS/MS using various amounts (0–80 μg proteins) of lysates of HepG2 cells at 37°C for 1 h (A) and using 20 μg of lysate incubated at 37°C for various times from 0.5, 1, 1.5, or 2 h (B). GCS activities were determined by LC-ESI-MS/MS using various amounts (0–80 μg proteins) of lysates of mouse PBMC cells at 37°C for 1 h (C) and using 20 μg of lysate incubated at 37°C for various times from 0.5, 1, 1.5, or 2 h (D). Each point is the average of at least three determinations with a standard deviation. Details were described in the Materials and Methods section.

MS method based on the reaction product determination to assess GCS enzymatic activity from the cell lysate. Due to the interference of endogenous sphingolipids, an excess C8-Cer was added as a substrate, and the generated C8-GlcCer was quantitatively determined (Fig. 1). In this method, C8-GlcCer and C12-GlcCer were separated by a reverse-phase LC method and quantified by ESI-MS/MS. Fig. 2 shows the precursor and product ion fragment peak. The MS parameter for each sphingolipid is shown in Table 1.

In our previous studies, we successfully separated sphingolipids at the start ratio of 80:20 (mobile A: mobile B); while in this study, we used C8-Cer as the GCS substrate. Both the substrate C8-Cer and the product C8-GlcCer have a shorter fatty acid chain, which means that they are less hydrophobic than the endogenous sphingolipids. Therefore, we reduced the hydrophobic phase at the start ratio to 50:50 (mobile A: mobile B), and both of the C8-GlcCer and internal standard were separated successfully. The retention time of each sphingolipid is shown in Fig. 2C.

To disclose the detection limits of C8-GlcCer by LC-MS/MS assay, the relationship between the amount of C8-GlcCer and peak areas were examined according to Agilent technical note, 'signal, noise, and detection limits in mass spectrometry'. It was shown less than 0.625 ng/mL of reaction product, C8-GlcCer, could be detected quantitatively by the assay using reverse-phase HPLC-ESI-MS/MS and the respective mobile

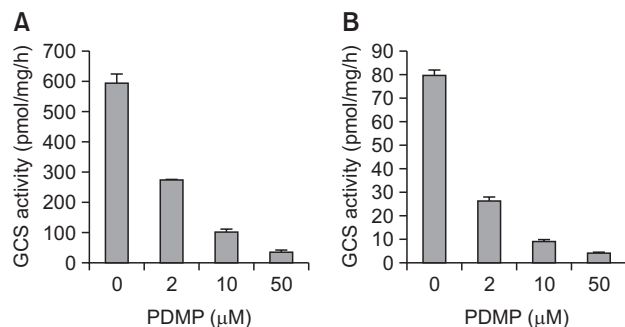


Fig. 4. The inhibition degree of PDMP on GCS enzymatic activity successfully evaluated by the LC-MS/MS-based assay system. PDMP was added to the reaction mixtures at different concentrations from 0–50 μM using 20 μg of lysate from HepG2 cells (A) or mouse PBMC (B). Reactions were carried out at 37°C for 1 h. Each point is the average of four determinations with a standard deviation. Details were described in the Materials and Methods section.

phase described above. The standard curve was linear from 0.625 ng/mL to 160 ng/mL of C8-GlcCer (Fig. 2D), providing both a sensitive and wide ranging assay for GCS.

To evaluate the assay developed in this study, we analyzed the GCS activity in HepG2 cell and mouse PBMC lysates. As shown in Fig. 3A and Fig. 3C, a linear relationship between the amount of cell lysate added and C8-GlcCer generation was observed at up to 20 μg of total protein for HepG2 cells and 40 μg of total protein for mouse PBMC. This was in the reaction mixture for the assay when the incubation was performed at 37°C for 1 h under the study conditions. The linearity lasted at least 2 h for HepG2 cells and 1.5 h for mouse PBMC when 20 μg of total protein was incubated with 1 μM substrate (Fig. 3B, 3D).

Moreover, this method was used to evaluate the inhibition degree of GCS by PDMP, which is a known GCS inhibitor. PDMP was treated at 5, 10, or 50 μM in HepG2 cell and mouse PBMC lysates and incubated with the substrate mix for 1 h. The results are shown in Fig. 4. PDMP inhibited GCS in a dose-dependent manner, nearly 90% of GCS activity was inhibited when PBMD was treated at 50 μM . These results show that the inhibition degree of GCS enzymatic activity by PDMP depends on the target enzyme. Further, this method could be possibly applied to screening of specific GCS inhibitors for development of therapeutic drugs towards chemotherapeutic multi-drug resistance and Gaucher disease.

Finally, we analyzed LCS activity, an enzyme that converts GlcCer to LacCer, in HepG2 cells (Fig. 5). The precursor and product ion fragment peak is shown in Fig. 5A and 5B. A linear relationship between the protein amount of cell lysate added and C8-LacCer generation was observed at up to 80 μg of total protein for HepG2 cells (Fig. 5C). This result indicates that the developed assay could be utilized for the simultaneous detection of GCS and LCS.

DISCUSSION

Ceramide, central to sphingolipid metabolism, regulates the activity of various molecular and biochemical targets involved in anti-proliferative and cellular responses (Kartal Yandim

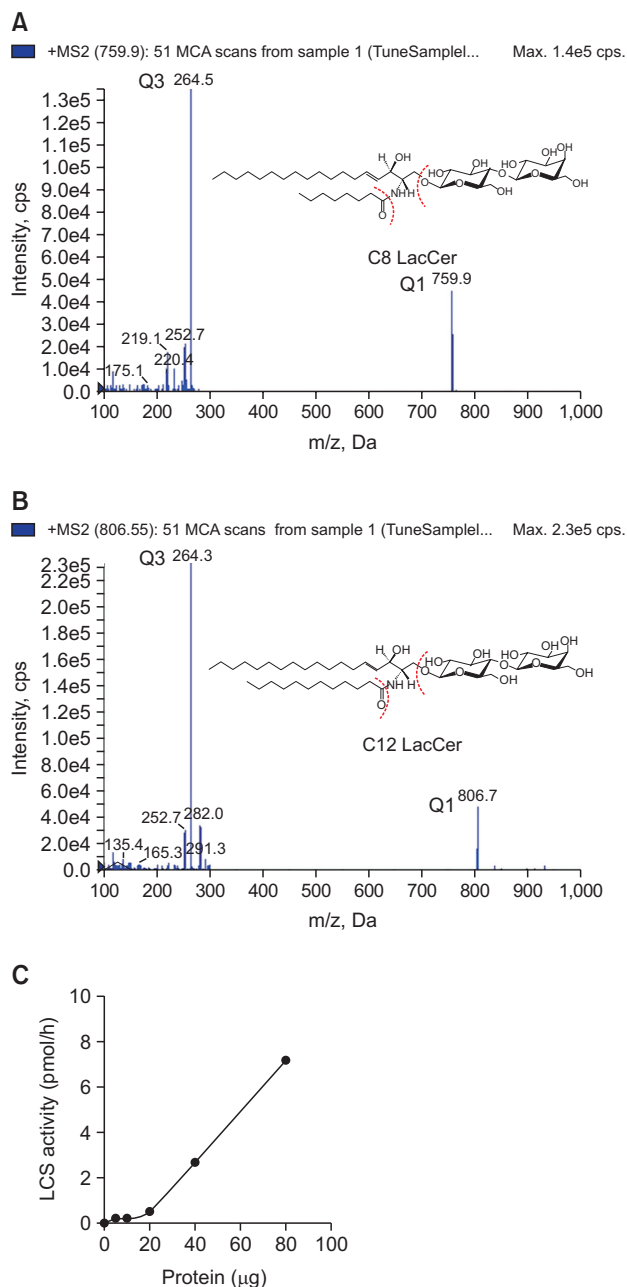


Fig. 5. Determination of LCS enzymatic activity of HepG2 cells. C8-LacCer (A) and C12-LacCer (B) were detected using an API3000 mass spectrometer with an Electrospray Ion (ESI) Source (AB SCIEX) at positive ion mode. Both C8-LacCer and C12-LacCer were eluted in 5 mM ammonium formate (pH 4.0)/methanol/tetrahydrofuran (1/2/7) at 10 ng/mL and separated on a reverse-phase C18 column using an HPLC (Agilent 1100) system with mobile A (5 mM ammonium formate (pH 4.0)/methanol, 95/5) and mobile B (2 mM ammonium formate in methanol with 0.1% formic acid/tetrahydrofuran, 7/3). (C) LCS activity was determined by LC-MS/MS using various protein amounts of lysates of HepG2 cells (0-80 µg proteins). The enzyme reaction was carried out at 37°C for 1 h. Each point is the average of four determinations. Details were described in the Materials and Methods section.

et al., 2013). Ceramide synthesis is promoted by activating sphingomyelinase or by blocking GlcCer formation (Senchenkov *et al.*, 2001). However, ceramide deficiency causes various adverse reactions. For example, ceramide deficiency in the stratum corneum causes atopic dermatitis, which is disruption of the skin barrier and dry (Choi and Maibach, 2005; Mizutani *et al.*, 2009). GCS is the major enzyme for GlcCer synthesis in ceramide metabolism that catalyzes the first reaction for ceramide glycosylation (Kartal Yandim *et al.*, 2013; Liu *et al.*, 2013). This reaction is an important step involved in the regulation of cell activity by controlling ceramide and glycosphingolipid (Liu *et al.*, 2013). Gaucher disease is caused by the lack of β -glucocerebrosidase, which acts to degrade GlcCer into ceramide and glucose. During enzyme deficiency, high levels of GlcCer are accumulated in white blood cells, especially in macrophages (Bennett and Mohan, 2013; Kartal Yandim *et al.*, 2013; Nagral, 2014). GlcCer synthesis and degradation contribute to regulation of the ceramide metabolism level, which is closely related to various diseases, including atopic dermatitis and Gaucher disease. (Kartal Yandim *et al.*, 2013). Therefore, it is very important to develop a sensitive and specific GCS activity assay method.

In a previous report on a GCS assay, lecithin-based liposomes, which contain C6-NBD-Cer as an acceptor, were used. NBD-Cer is a NBD group omega fatty acid-labeled ceramide. The labeled group may change the polarity of the molecule and may also influence the interaction between the enzyme and substrates. In this study, to rule out these unexpected influences, we used a non-labeled C8-Cer as a substrate, which does not exist naturally in any mammals. We found that GCS enzymatic activity was successfully detected using the C8-Cer/lecithin liposomes for supplying acceptor substrates.

When C6-NBD-Cer was used as a GCS substrate, thin layer chromatography (TLC) was performed to separate the product C6-NBD-GlcCer from C6-NBD-Cer, and their concentrations were first quantified by a fluorescent detector. Subsequently, a HPLC-based method was developed, and the detection sensitivity improved. However, the separation and specificity between the substrate and product on TLC and HPLC remained to be improved because both were NBD-labeled. It is difficult to rule out the substrate or other NBD-contained side contaminations in the peak area of the product.

In this study, we developed a LC-MS/MS-based GCS enzymatic activity assay to improve the assay specificity. We quantified the amount of C8-GlcCer directly from the 588.6/264.2 fragment. We used C12-GlcCer, which is a non-natural excised sphingolipid that displays physical and chemical characteristics similar to C8-GlcCer, as an internal standard to help correct variations caused by variability in the analytical procedure.

The activity of β -glucocerebrosidase, which hydrolyzes C8-GlcCer to C8-Cer, could interfere with the GCS assay if the sample contained glycosidase activity. It was reported that conduritol B epoxide (CBE), a specific β -glucocerebrosidase inhibitor, reduced effectively the β -glucocerebrosidase activity at 2.5 mM without any loss of GCS activity (Hayashi *et al.*, 2005).

GCS activity has been shown to be much higher in chemotherapeutic multi-drug resistance patients (Sietsma *et al.*, 2001; Bleicher and Cabot, 2002; Xie *et al.*, 2008). High GCS activity produced a high level of GlcCer from ceramide, therefore reducing the cellular level of ceramide. Ceramide is

known to be an important sphingolipid, which plays a crucial role in the potency and effectiveness of chemotherapeutic drugs, such as doxorubicin-induced cell apoptosis. Therefore, GCS activity could determine the working concentrations of chemotherapeutic drugs to be administered. Considering the possible toxic side effect of chemotherapeutic drugs at the desired concentrations, it is very important to reduce the dosage of these drugs. Currently, several inhibitors have been developed to inhibit GCS activity, including PDMP, 1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) and, which are known to be potent competitive inhibitors of GCS as structural analogues of its natural sphingolipid substrate (Abe *et al.*, 1992; Senchenkov *et al.*, 2001). PPMP and its analogue PPPP at concentrations that maximally inhibit GlcCer synthesis have no effect on cell viability, but they sensitize cells to doxorubicin (Senchenkov *et al.*, 2001). PDMP is firstly known as a GCS inhibitor, which has been reported to suppress GCS activity and to potentiate the apoptotic effect of C6-ceramide (Vunnam and Radin, 1980; Senchenkov *et al.*, 2001). Several drugs have been used to treat Gaucher disease. For instance, macrophage-targeted enzyme replacement therapy (ERT) has long been the accepted form of treatment for Gaucher disease, but there are still several limitations in treating all aspects of this disease (Limgala *et al.*, 2016). To complement the disadvantages of ERT, oral substrate reduction therapy (SRT) was developed using GCS inhibitors. SRT minimized the accumulation of GlcCer within cells by inhibiting GCS (Limgala *et al.*, 2016; Shawky and Elsayed, 2016). Recently, some GCS inhibitors were also developed to treat Gaucher disease at a high cost (Bennett and Mohan, 2013; Nagral, 2014). In the present study, we developed a sensitive and specific LC-MS/MS-based GCS enzymatic activity assay method and validated by using PDMP as the best known GCS inhibitor (Radin, 1996). Further, this method successfully evaluated the inhibition degree of PDMP on GCS at different working concentrations, showing that this assay could be applied to a GCS-specific inhibitor screening for developing therapeutic drugs against chemotherapeutic multi-drug resistance and Gaucher disease.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This research was supported by the Chung-Ang University research Scholarship Grant to SY in 2016.

REFERENCES

- Abe, A., Inokuchi, J., Jimbo, M., Shimeno, H., Nagamatsu, A., Shayman, J. A., Shukla, G. S. and Radin, N. S. (1992) Improved inhibitors of glucosylceramide synthase. *J. Biochem.* **111**, 191-196.
- Abe, A., Radin, N. S., Shayman, J. A., Wotring, L. L., Zipkin, R. E., Sivakumar, R., Ruggieri, J. M., Carson, K. G. and Ganem, B. (1995) Structural and stereochemical studies of potent inhibitors of glucosylceramide synthase and tumor cell growth. *J. Lipid Res.* **36**, 611-621.
- Basu, S., Kaufman, B. and Roseman, S. (1973) Enzymatic synthesis of glucocerebroside by a glucosyltransferase from embryonic chicken brain. *J. Biol. Chem.* **248**, 1388-1394.
- Bennett, L. L. and Mohan, D. (2013) Gaucher disease and its treatment options. *Ann. Pharmacother.* **47**, 1182-1193.
- Bleicher, R. J. and Cabot, M. C. (2002) Glucosylceramide synthase and apoptosis. *Biochim. Biophys. Acta* **1585**, 172-178.
- Chatterjee, S. and Alsaeedi, N. (2012) Lactosylceramide synthase as a therapeutic target to mitigate multiple human diseases in animal models. *Adv. Exp. Med. Biol.* **749**, 153-169.
- Choi, M. J. and Maibach, H. I. (2005) Role of ceramides in barrier function of healthy and diseased skin. *Am. J. Clin. Dermatol.* **6**, 215-223.
- Chujor, C. S., Feingold, K. R., Elias, P. M. and Holleran, W. M. (1998) Glucosylceramide synthase activity in murine epidermis: quantitation, localization, regulation, and requirement for barrier homeostasis. *J. Lipid Res.* **39**, 277-285.
- Degroote, S., Wolthoorn, J. and van Meer, G. (2004) The cell biology of glycosphingolipids. *Semin. Cell Dev. Biol.* **15**, 375-387.
- Di Sano, F., Di Bartolomeo, S., Fazi, B., Fiorentini, C., Matarrese, P., Spinedi, A. and Piacentini, M. (2002) Antisense to glucosylceramide synthase in human neuroepithelioma affects cell growth but not apoptosis. *Cell Death Differ.* **9**, 693-695.
- Duclos, R. I. (2001) The total syntheses of D-erythro-sphingosine, N-palmitoylsphingosine (ceramide), and glucosylceramide (cerebroside) via an azidosphingosine analog. *Chem. Phys. Lipids* **111**, 111-138.
- Ekiz, H. A. and Baran, Y. (2010) Therapeutic applications of bioactive sphingolipids in hematological malignancies. *Int. J. Cancer* **127**, 1497-1506.
- Hakomori, S. I. (2008) Structure and function of glycosphingolipids and sphingolipids: recollections and future trends. *Biochim. Biophys. Acta* **1780**, 325-346.
- Hayashi, Y., Horibata, Y., Sakaguchi, K., Okino, N. and Ito, M. (2005) A sensitive and reproducible assay to measure the activity of glucosylceramide synthase and lactosylceramide synthase using HPLC and fluorescent substrates. *Anal. Biochem.* **345**, 181-186.
- Hospattankar, A. V. and Radin, N. S. (1981) A 2-phase liquid scintillation assay for glycolipid synthetases. *Lipids* **16**, 764-766.
- Ichikawa, S., Sakiyama, H., Suzuki, G., Hidari, K. I. and Hirabayashi, Y. (1996) Expression cloning of a cDNA for human ceramide glucosyltransferase that catalyzes the first glycosylation step of glycosphingolipid synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12654.
- Imokawa, G. (2009) A possible mechanism underlying the ceramide deficiency in atopic dermatitis: expression of a deacylase enzyme that cleaves the N-acyl linkage of sphingomyelin and glucosylceramide. *J. Dermatol. Sci.* **55**, 1-9.
- Kartal Yandim, M., Apohan, E. and Baran, Y. (2013) Therapeutic potential of targeting ceramide/glucosylceramide pathway in cancer. *Cancer Chemother. Pharmacol.* **71**, 13-20.
- Kolesnick, R. (2002) The therapeutic potential of modulating the ceramide/sphingomyelin pathway. *J. Clin. Invest.* **110**, 3-8.
- Limgala, R. P., Ioanou, C., Plassmeyer, M., Ryherd, M., Kozhaya, L., Austin, L., Abidoglu, C., Unutmaz, D., Alpan, O. and Goker-Alpan, O. (2016) Time of initiating enzyme replacement therapy affects immune abnormalities and disease severity in patients with Gaucher disease. *PLoS ONE* **11**, e0168135.
- Liu, Y. Y., Han, T. Y., Yu, J. Y., Bitterman, A., Le, A., Giuliano, A. E. and Cabot, M. C. (2004) Oligonucleotides blocking glucosylceramide synthase expression selectively reverse drug resistance in cancer cells. *J. Lipid Res.* **45**, 933-940.
- Liu, Y. Y., Hill, R. A. and Li, Y. T. (2013) Ceramide glycosylation catalyzed by glucosylceramide synthase and cancer drug resistance. *Adv. Cancer Res.* **117**, 59-89.
- Maunula, S., Bjorkqvist, Y. J., Slotte, J. P. and Ramstedt, B. (2007) Differences in the domain forming properties of N-palmitoylated neutral glycosphingolipids in bilayer membranes. *Biochim. Biophys. Acta* **1768**, 336-345.
- Mishra, S. and Chatterjee, S. (2014) Lactosylceramide promotes hypertrophy through ROS generation and activation of ERK1/2 in cardiomyocytes. *Glycobiology* **24**, 518-531.

- Miura, T., Kajimoto, T., Jimbo, M., Yamagishi, K., Inokuchi, J. C. and Wong, C. H. (1998) Synthesis and evaluation of morpholino- and pyrrolidinosphingolipids as inhibitors of glucosylceramide synthase. *Bioorg. Med. Chem.* **6**, 1481-1489.
- Mizutani, Y., Mitsutake, S., Tsuji, K., Kihara, A. and Igarashi, Y. (2009) Ceramide biosynthesis in keratinocyte and its role in skin function. *Biochimie* **91**, 784-790.
- Nagral, A. (2014) Gaucher disease. *J. Clin. Exp. Hepatol.* **4**, 37-50.
- Radin, N. S. (1996) Treatment of Gaucher disease with an enzyme inhibitor. *Glycoconj. J.* **13**, 153-157.
- Senchenkov, A., Litvak, D. A. and Cabot, M. C. (2001) Targeting ceramide metabolism-a strategy for overcoming drug resistance. *J. Natl. Cancer Inst.* **93**, 347-357.
- Shawky, R. M. and Elsayed, S. M. (2016) Treatment options for patients with Gaucher disease. *Egypt. J. Med. Hum. Genet.* **17**, 281-285.
- Sietsma, H., Veldman, R. J. and Kok, J. W. (2001) The involvement of sphingolipids in multidrug resistance. *J. Membr. Biol.* **181**, 153-162.
- Tuuf, J., Kjellberg, M. A., Molotkovsky, J. G., Hanada, K. and Matjus, P. (2011) The intermembrane ceramide transport catalyzed by CERT is sensitive to the lipid environment. *Biochim. Biophys. Acta* **1808**, 229-235.
- van Meer, G. and Holthuis, J. C. (2000) Sphingolipid transport in eukaryotic cells. *Biochim. Biophys. Acta* **1486**, 145-170.
- Vielhaber, G., Pfeiffer, S., Brade, L., Lindner, B., Goldmann, T., Vollmer, E., Hintze, U., Wittern, K. P. and Wepf, R. (2001) Localization of ceramide and glucosylceramide in human epidermis by immunogold electron microscopy. *J. Invest. Dermatol.* **117**, 1126-1136.
- Vunnam, R. R. and Radin, N. S. (1980) Analogs of ceramide that inhibit glucocerebrosidase in mouse brain. *Chem. Phys. Lipids* **26**, 265-278.
- Wang, J., Lv, X. W. and Du, Y. G. (2009) Potential mechanisms involved in ceramide-induced apoptosis in human colon cancer HT29 cells. *Biomed. Environ. Sci.* **22**, 76-85.
- Xie, P., Shen, Y. F., Shi, Y. P., Ge, S. M., Gu, Z. H., Wang, J., Mu, H. J., Zhang, B., Qiao, W. Z. and Xie, K. M. (2008) Overexpression of glucosylceramide synthase in associated with multidrug resistance of leukemia cells. *Leuk. Res.* **32**, 475-480.
- Yoshizaki, F., Nakayama, H., Iwahara, C., Takamori, K., Ogawa, H. and Iwabuchi, K. (2008) Role of glycosphingolipid-enriched microdomains in innate immunity: microdomain-dependent phagocytic cell functions. *Biochim. Biophys. Acta* **1780**, 383-392.
- Zheng, W., Kollmeyer, J., Symolon, H., Momin, A., Munter, E., Wang, E., Kelly, S., Allegood, J. C., Liu, Y., Peng, Q., Ramaraju, H., Sullards, M. C., Cabot, M. and Merrill, A. H., Jr. (2006) Ceramides and other bioactive sphingolipid backbones in health and disease: lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy. *Biochim. Biophys. Acta* **1758**, 1864-1884.