Simultaneous quantification of glucosylceramide and galactosylceramide by normal-phase HPLC using O-phtalaldehyde derivatives prepared with sphingolipid ceramide N-deacylase

Keywords: high-performance liquid chromatography/galactosylceramide/glucosylceramide/glycosphingolipid/sphingolipid ceramide N-deacylase

Introduction

Glycosphingolipids (GSLs) are ubiquitous components of plasma membranes of eukaryotes. In vertebrates, evidence is accumulating that GSLs and/or sphingomyelin form microdomains with or without cholesterol on plasma membranes and function as modulators of various cellular activities such as adhesion, growth, and differentiation (Todeschini and Hakomori 2008). GSLs are also utilized by pathogens and toxins as cell-surface receptors, prior to invading host cells (Ilver et al. 2003).

Glycosylceramide (GlcCer) and galactosylceramide (GalCer) are a common precursor for ganglio-, lacto-/neolacto- and globo-series GSLs and sulfatide, GM4 (sialo-GalCer), and galo/neogala-series GSLs, respectively (Hirabayashi et al. 2006). The knockout and/or knockdown of GlcCer synthase (UDP-glucose:ceramide β-1,1′-glucosyltransferase, GlcT; EC 2.4.1.45) and GalCer synthase (UDP-galactose:ceramide galactosyltransferase, GalT; EC 2.4.1.45) revealed the biological relevance of GSLs synthesized from GlcCer and GalCer as a precursor (Bosio et al. 1996; Coetzee et al. 1996; Yamashita et al. 1999; Kohyama-Koganeya et al. 2004; Jemmernann et al. 2005, 2007). Furthermore, GlcCer and GalCer themselves seem to be integral to neural functions, e.g., GlcCer is involved in the axon growth of neural cells (Schwarz and Futerman 1997) and GalCer is impairment for myelin functions (Coetzee et al. 1996). However, the accumulation of GlcCer in lysosomes due to a dysfunctional acid glucocerebrosidase (aGCase, EC 3.2.1.45) leads to an inherited lysosomal storage disorder, known as Gaucher disease (GD) (Zhao and Grabowski 2006). The knockout and/or knockdown of GlcCer synthase inhibitor P4 resulted in a marked decrease in GlcCer but not GalCer, coincidentally with a significant decrease in the GlcCer synthase activity. On the other hand, GlcCer but not GalCer increased 2-fold when an acid glucocerebrosidase inhibitor CBE was injected into zebrafish embryos. Interestingly, the treatment of CHO cells with ciclosporin A increased GlcCer possibly due to the inhibition of LacCer synthase. A significant increase in levels of GlcCer in fibroblasts from patients with Gaucher disease was clearly shown by the method. The proposed method is useful for the determination of GlcCer and GalCer levels in various biological samples.

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We report here a method of simultaneously quantifying glucosylceramide (GlcCer) and galactosylceramide (GalCer) by normal-phase HPLC using O-phtalaldehyde derivatives. Treatment with sphingolipid ceramide N-deacylase which converts the cerebrosides in the sample to their lyso-forms was followed by the quantitative labeling of free NH₂ groups of the lyso-cerebrosides with O-phtalaldehyde. Using this method, 14.1 pmol of GlcCer and 10.4 pmol of GalCer, and 108.1 pmol of GlcCer and 191.1 pmol of GalCer were detected in zebrafish embryos and RPMI 1864 cells, respectively, while 22.2 pmol of GlcCer but no GalCer was detected in CHOP cells and 100 g of protein under the conditions used, which corresponds to approximately 10³ to 10⁵ RPMI cells and 5 to 80 zebrafish embryos. The present method clearly revealed that the treatment of RPMI cells with a GlcCer synthase inhibitor P4 resulted in a marked decrease in GlcCer but not GalCer, concomitantly with a significant decrease in the GlcCer synthase activity. On the other hand, GlcCer but not GalCer increased 2-fold when an acid glucocerebrosidase inhibitor CBE was injected into zebrafish embryos. Interestingly, the treatment of CHO cells with ciclosporin A increased GlcCer possibly due to the inhibition of LacCer synthase. A significant increase in levels of GlcCer in fibroblasts from patients with Gaucher disease was clearly shown by the method. The proposed method is useful for the determination of GlcCer and GalCer levels in various biological samples.

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from GalCer (Kean 1966) but is insufficient in sensitivity and reproducibility in our experience. The separation and quantification of perbenzoylated derivatives of GlcCer and GalCer by HPLC have also been reported (Kay and Ullman 1984). However, the method requires the isolation of cerebrosides by silica gel chromatography before their derivatization which is laborious and time-consuming. Furthermore, the sensitivity of perbenzoylated derivatives to a UV detector would be much lower than that of fluorescent derivatives to a fluorescent detector. Thus, a new method for the quantification of GlcCer and GalCer is still required to elucidate the functions of these simple but indispensable core GSLs. Sphingolipid ceramide N-deacylase (SCDase) is an enzyme which hydrolyzes the N-acyl linkage of the ceramide moiety of various GSLs generating lyso-forms of GSLs and fatty acids (Ito et al. 1995; Furusato et al. 2002). Although SCDase acts on ceramide at exactly the same point as ceramidase, it clearly differs in specificity in that it hardly hydrolyzes free ceramide while ceramidase does not hydrolyze GSLs. SCDase hydrolyzes GlcCer and GalCer, efficiently generating glucosylsphingosine (GlcSph) and galactosylsphingosine (GalSph) which have a free NH₂ group at the sphingoid moiety.

In this paper, we report a sensitive and reliable HPLC-based method for the quantification of O-phthalaldehyde (OPA) derivatives of GlcSph and GalSph, which are generated from the sample after treatment with SCDase. Standard GlcCer and GalCer were quantitatively determined in the range of 5 pmol to 1 nmol using normal-phase HPLC within 15 min. The method was useful for chasing the amounts of GlcCer and GalCer in zebrafish embryos, Chinese hamster ovary-derived CHOP cells, and hamster melanoma-derived RPMI 1864 cells before and after treatments with specific inhibitors for the glycosidase and glycosyltransferases. Furthermore, it clearly showed that GlcCer but not GalCer was accumulated in fibroblasts from patients with GD.

Results

Separation of OPA-GlcSph and OPA-GalSph by normal-phase HPLC

First, we examined how to separate OPA-labeled GlcSph and GalSph by HPLC. After several trials, we found that these OPA-GSLs can be separated by a normal-phase HPLC using n-hexane/isopropylalcohol/H₂O (73/26.5/0.5, v/v) as a mobile phase as described in Material and methods. Under the conditions used, OPA-GlcSph and OPA-GalSph were eluted at 6.8 min and 11.3 min, respectively (Figure 1). Both peaks were analyzed by LC-MS and determined as OPA-hexosylceramides (supplementary Figure 1).

Quantification of GlcCer and GalCer by normal-phase HPLC after deacylation of GSLs with SCDase followed by OPA-derivatization

The GlcCer and GalCer in samples were hydrolyzed by SCDase to obtain GlcSph and GalSph quantitatively. These lyso-cerebrosides were labeled with OPA and then analyzed by normal-phase HPLC. An outline of the method is shown in supplementary Figure 2.

The extent to which GlcCer and GalCer were hydrolyzed by SCDase was examined using HPLC as described above. Both cerebrosides were hydrolyzed by the enzyme in a time-dependent manner; maximum hydrolysis was obtained after 2–3 h, and a plateau of 75.1 ± 8.2% and 79.3 ± 2.7% hydrolysis was observed for GlcCer and GalCer, respectively, when 1 nmol of cerebroside was added (Figure 2A and B). Next, the relationship between the amount of cerebroside added and the release of lyso-cerebrosides by the enzyme was examined. As shown in Figure 2C and D, the hydrolysis of both cerebrosides by the enzyme proceeded quantitatively from 5 pmol to 1 nmol, indicating that the cerebroside content in this range can be determined by measuring the amount of lyso-cerebroside released. It is worth noting that linearity was maintained up to 10 nmol when GlcCer was used for the assay (R² = 0.9998), indicating that the method provides a wide-ranging assay for the determination of GlcCer (supplementary Figure 3). C6-NBD-GlcCer added as an internal standard was not hydrolyzed by SCDase possibly because of the short acyl chain (data not shown).

Quantification of GlcCer and GalCer in zebrafish embryos, human fibroblasts, CHOP cells, and RPMI 1864 cells

To quantify the amounts of GlcCer and GalCer in biological samples, all GSLs were extracted from the sample, deacylated by SCDase, derivatized with OPA, and then analyzed by normal-phase HPLC as described in Material and methods.

Fig. 1. Separation of OPA-GlcSph and OPA-GalSph by normal-phase HPLC. OPA-GlcSph and OPA-GalSph (100 pmol each) were subjected to normal-phase HPLC with n-hexane/isopropylalcohol/H₂O (73/26.5/0.5, v/v) as a mobile phase at a flow rate of 2.0 mL/min and detected using a fluorescent detector set to excitation and emission wavelengths of 340 nm and 455 nm. (A) OPA-GlcSph; (B) OPA-GalSph; (C) OPA-GlcSph and OPA-GalSph.

Details are described in Material and methods.
Fig. 2. Time course and linearity of the generation of GlcSph and GalSph from GlcCer and GalCer, respectively, by SCDase. GlcCer (A) and GalCer (B) (1 nmol) were hydrolyzed by 0.6 mU of SCDase at 37 °C for the indicated periods. Different amounts of GlcCer (C) and GalCer (D) ranging from 5 pmol to 1 nmol were hydrolyzed by 0.6 mU of SCDase at 37 °C for 4 h. Details are described in Material and methods.

Profiles of HPLC showing OPA-GlcSph (peak 1) and OPA-GalSph (peak 2) in several biological samples are shown in Figure 3. Interestingly, both OPA-GSLs were detected in zebrafish embryos (A) and RPMI cells (D) while OPA-GlcSph but not OPA-GalSph was detected in CHOP cells (B) and human fibroblasts (C) using cell lysate containing 400 μg of total protein. To verify the range of the quantification, different amounts of samples were subjected to the assay. As shown in Figure 4, linearity between the amount of sample added and the amount of OPA-GlcSph/OPA-GalSph detected was observed for both GSLs using cell lysate corresponding to 5–400 μg of total protein. It is worth noting that 4.7 pmol (Figure 4A, left) to 450 pmol (Figure 4D, left) of OPA-GlcSph and 3.8 pmol (Figure 4A, right) to 800 pmol (Figure 4D, right) of OPA-GalSph can be quantified by this method. For these experiments, peak signal-to-noise ratio is above 10. With this method, 14.1 pmol of GlcCer and 10.4 pmol of GalCer, and 108.1 pmol of GlcCer and 191.1 pmol of GalCer were detected in zebrafish embryos and RPMI.
Fig. 3. HPLC chromatograms showing the amounts of GlcCer and GalCer in zebrafish (A), CHOP cells (B), human fibroblasts (C), and RPMI 1846 cells (D). GSLs were extracted from samples, hydrolyzed by SCDase, labeled with OPA, and analyzed by normal-phase HPLC as described in Material and methods. Samples corresponding to 100 μg of total protein were subjected to the assay. 1, OPA-GlcSph (6.8 min); 2, OPA-GalSph (11.3 min).

1864 cells, respectively, using cell lysate containing 100 μg of protein. On the other hand, CHOP cells contained 22.2 pmol GlcCer per 100 μg protein of cell lysate but contained no GalCer. In the present assay, the amount of lyso-cerebrosides present in the sample could also be measured if the process using SC-Dase was omitted. However, no lyso-cerebrosides were detected in the lysates of zebrafish embryos, CHOP cells or RPMI 1864 cells when samples corresponding to 400 μg of total protein were used for the assay.

Quantification of GlcCer and GalCer with and without specific inhibitors for glycosidase and glycosyltransferases
To verify the usefulness of the proposed method, cellular levels of GlcCer and GalCer were examined with and without specific inhibitors for αGCase and GlcT. The activity of the αGCase of zebrafish embryos was found to decrease in a concentration-dependent manner when CBE, a potent inhibitor for αGCases, was injected into the embryos (Figure 5A). The cellular levels of GlcCer and GalCer in control embryos (without the inhibitor) were determined to be 20.2 pmol and 10.4 pmol, respectively, using cell lysate containing 100 μg of total protein which corresponds to 10–20 embryos. The cellular level of GlcCer increased in correlation with the decrease in enzyme activity and was 2.5 times higher when 10 mM of the inhibitor was injected into the embryos. On the other hand, the amount of GalCer was not changed when the inhibitor was injected.

The amount of GalCer in CHOP cells increased when ciclosporin A was added to the cell culture in a concentration-dependent manner (Figure 6A). Ciclosporin A was reported to inhibit the activity of P-glycoprotein which could participate in the flip-flop of GlcCer from the cytosolic-face to lumen-face of Golgi membranes (Eckford and Sharom 2005). In this study, however, we found that LacCer synthase activity was strongly inhibited by ciclosporin A (Figure 6B), via which GlcCer, the precursor of LacCer, would be accumulated. These results indicate that an increase of GlcCer in ciclosporin A-treated CHOP cells may stem from the inhibition of the activities of not only P-glycoproteins but also LacCer synthase.

P4, an analog of Cer, is a potent inhibitor for GlcT (Jimbo et al. 2000). It has been reported that the treatment of various cells with P4 decreased GlcT activity as well as the cellular level of hexosylceramide. However, numerous studies have investigated the effects of P4 on the biosynthesis of GlcCer and other GSLs using 14C-precursors such as 14C-Gal, 14C-fatty acids, and 14C-sphingosine while only a few reports have described the effects of an inhibitor for the mass content of cerebrosides. In the present paper, we found that the treatment of CHOP cells (supplementary Figure 4) and RPMI 1846 cells (Figure 7A and B) with P4 drastically decreased the activity of GlcT and concomitantly decreased the cellular amount of GlcCer after 1 day. This result indicates that the inhibitor affected the mass content as well as the biosynthesis of GlcCer, suggesting that the turnover of GlcCer is quite fast in these cell lines. On the other hand, the amount of GalCer in RPMI 1846 cells was not affected by treatment with P4 (Figure 7C), suggesting that the ceramide pool for the synthesis of GlcCer is not the same as that for GalCer in RPMI 1864 cells.

Quantification of GlcCer in fibroblasts from patients with GD
Finally, the method was used to quantify the cellular level of GlcCer in fibroblasts from healthy volunteers (controls) and patients with GD. As shown in Figure 8, the cellular amount of GlcCer in fibroblasts from the controls was about 1.0 nmol/mg protein and the value was significantly increased in the fibroblasts from the GD patients. No GalCer was detected in the fibroblasts from neither the controls (Figure 4C) nor patients (data not shown). The cellular amount of GlcCer in human fibroblasts was previously determined to be 1.2 nmol/mg protein by TLC and 0.5 nmol/mg protein by ESI-MS (Sasagasako et al. 1994; Fuller et al. 2008). These values are quite similar to the result obtained with the present method.

Discussion
Recently, several methods of quantifying sphingolipids/GSLs using specific enzymes have been developed (Wing et al. 2001;
Quantification of glucosylceramide and galactosylceramide

Fig. 4. Linearity between the amount of OPA-GlcSph/OPA-GalSph detected and amount of sample from zebrafish embryos (A), CHOP cells (B), human fibroblasts (C), and RPMI 1864 cells (D). Crude GSLs were extracted from the lysate of zebrafish embryos and cultured cells, and cellular levels of GlcCer and GalCer were determined as described in Materials and methods. Cell lysate corresponding to 25–400 μg (A–B), 5–400 μg (C), and 10–400 μg (D) of total protein was used for the assay. CHOP cells (B) and human fibroblasts (C) had no GalCer.

He et al. (2005). For the quantification of ceramide, human recombinant acid ceramidase was used to hydrolyze ceramide and the amount of sphingosine generated was determined by reverse-phase HPLC after labeling with naphthalene-2,3-dicarboxyaldehyde (He et al. 2005). This method is quantitative, specific, and sensitive but not applicable to the quantification of GSLs because the ceramidase does not hydrolyze GSLs including cerebrosides. In contrast to ceramidase, SCDase used in this study hydrolyzes GSLs and sphingomyelin (Ito et al. 1995; Furusato et al. 2002), although it acts on the ceramide moiety at exactly the same position as ceramidase, i.e., both enzymes hydrolyze the N-acyl linkage between sphingosine and fatty acid in ceramide. Using SCDase instead of ceramidase, the deacylation of GSLs and sphingomyelin could be performed quantitatively. On the other hand, microwave-mediated nonenzymatic deacylation of sphingolipids/GSLs has also been reported (Groener et al. 2007).

GSLs including gangliosides were also quantified using endoglycoceramidase (EC3.2.1.123, also known as ceramide glycanase) (Wing et al. 2001). With this method, carbohydrate moieties of GSLs were cleaved off by the enzyme and the oligosaccharides released were labeled with 2-aminobenzamide, and then subjected to normal-phase HPLC. However, endoglycoceramidase is not able to hydrolyze cerebrosides, in contrast to SCDase.

In the present study, we focused on the quantification of GlcCer and GalCer because these simple GSLs are still difficult to quantify simultaneously. However, more complex GSLs such as GM3 could not be separated by HPLC under the conditions used and require a different solvent system which might not be suitable for the separation of OPA-GlcSph and OPA-GalSph.

Shinoda et al. (1987) reported that OPA-galactosyldihydroSph (GalDHS) was eluted prior to OPA-GalSph with a linear gradient from 0 to 45% ethanol/water (100/2, v/v) in hexane by normal-phase HPLC. Thus, OPA-glucosyldihydroSph (GlcDHS) and OPA-GalDHS could be eluted prior to OPA-GlcSph and OPA-GalSph, respectively, under the conditions used in this study. Actually, a minor peak was observed prior to the peak of OPA-GlcSph when zebrafish embryos were subjected to the assay (Figure 3A), although this minor peak remains to be identified. The peak seems to be derived from GlcDHS because its intensity increased when bovine milk GlcCer, which contains a large amount of GlcDHS (Morrison and Hay 1970), was subjected to the assay.
A  

Fig. 5. Effects of CBE on aGC activity (A), and cellular levels of GlcCer (B) and GalCer (C) in zebrafish embryos. CBE was injected into one- to four-cell embryos, and the lysates of embryos at 3 days after injection were subjected to an assay of aGC activity and the quantification of GlcCer/GalCer. For the aGC assay, 10 μg of total protein was incubated at 37°C for 1 h with 2.5 μM C6-NBD-GlcCer in a 50 mM phosphate-citric buffer, pH 5.0, containing 0.25% TritonX-100 and 0.6% sodium taurocholate (Hayashi et al. 2008). For the quantification of GlcCer and GalCer, cell lysate corresponding to 100 μg of total protein was used. Details are described in Material and methods.

We found in the present study that the inhibition of GlcT with P4 resulted in a decrease in both GlcT activity and the amount of GlcCer in RPMI 1864 cells (Figure 7). Interestingly, however, the cellular content of GalCer was unchanged in the presence of P4 at a concentration of 0.05 or 2 μM. This result may indicate that the ceramide pool for the synthesis of GlcCer is different from that for GalCer in this hamster melanoma cell line. This hypothesis is consistent with reports that the molecular species of ceramide in GlcCer is quite different from that in GalCer, i.e., GalCer contains much more α-hydroxy fatty acids than GlcCer (Dehaas and Lopes-Cardozo 1995). However, analytical data on brain GSLs obtained from GalCer-synthase knockout mice somewhat contradicted this hypothesis, i.e., the GlcCer in the knockout mice contained α-hydroxyl fatty acids while that in wild-type mice did not (Coetzee et al. 1996). This result suggests the ceramide pool in mouse brain to be shared by the synthases of GlcCer and GalCer. It is worth noting, however, that the synthesis of GlcCer occurs on the cytosolic face of the Golgi apparatus while that of GalCer occurs in the lumen of the ER (Futerman and Pagano 1991). The ceramide pool for the synthesis of GlcCer and GalCer seems to vary depending on the tissues and cell type.

GD is a genetic GSL storage disorder characterized by a decrease in aGC activity and the accumulation of GlcCer in lysosomes. However, the accumulation of GlcCer is not obvious except in laden tissue macrophages (Jmoudiak and Futerman 2005). Fujiwaki et al. found that the hexosylceramide/sphingomyelin ratio in pericardial fluid, peritoneal fluid, and serum from GD patients was increased compared to normal levels using delayed extraction-matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE-MALDI-TOF-MS), although they did not determine the species of hexosylceramide (Fujiwaki et al. 2002). This result is well consistent with our observation that the level of GlcCer in fibroblasts from...
Fig. 7. Effects of P4 on GlcT activity (A) and cellular levels of GlcCer (B) and GalCer (C) in RPMI 1864 cells. Cells were incubated at 37°C for 24 h with P4 at the concentrations indicated. For the quantification of GlcCer and GalCer, cell lysate corresponding to 150 μg of total protein was used. For GlcT activity assay, 25 μg of total protein was incubated at 37°C for 1 h with 1.0 μM C6-NBD-Cer and 0.5 mM UDP-Glc in the 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA (Hayashi et al. 2005). Details are described in Material and methods.

GD patients was significantly increased compared to that in fibroblasts of healthy volunteers (Figure 8.).

The recent trend for the quantification of GSLs is to use mass spectrometry with electro-spray ionization and/or matrix-assisted laser desorption ionization (Merrill et al. 2005). However, mass spectrometry needs expensive equipment and specialized techniques and is not suitable for the quantification of GlcCer and GalCer in crude extracted lipids.

In summary, we developed a sensitive, simple, and reproducible method of quantifying GlcCer and GalCer, in which these GSLs were deacylated by SCDase, labeled with OPA, and analyzed by normal-phase HPLC with a fluorescent detector. This method is useful for the quantification of GlcCer and GalCer in various biological samples including fibroblasts from patients with GD. Thus, it should facilitate basic and clinical research concerning GSLs.

Material and methods

Materials

Chinese hamster ovary-derived CHOP cells and hamster melanoma-derived RPMI 1864 cells were kindly provided by Dr. K. Nara (Fukushima Medical School, Fukushima, Japan) and Dr. Ogura (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), respectively. C6-NBD-Cer was purchased from Invitrogen. C6-NBD-GlcCer was purchased from Sigma-Aldrich (St. Louis, MO). CBE and lecithin were obtained from Wako Pure Chemical Industries (Osaka, Japan). GlcCer (bovine milk), GalCer (bovine brain), GlcSph (bovine) were purchased from Matreya, and GalSph (bovine brain) was from Alexis Biochemicals. Ciclosporin A and P4 were purchased from Wako and Matreya, respectively. All other reagents were of the highest purity available.

Preparation of SCDase and definition of enzyme units

Recombinant Shewanella SCDase was prepared as described (Furusato et al. 2002). Pseudomonas SCDase was purchased from Takara Bio. Co. (Shiga, Japan). Both enzymes can be used for the deacylation of GSLs. One enzyme unit was defined as the amount capable of catalyzing the release of 1 μmol of lyso-GM1a/min from GM1a under conditions as follows. The reaction mixture comprising 10 nmol GM1a and an appropriate amount of enzyme in 20 μL of 25 mM sodium acetate buffer, pH 5.5, containing 0.8% Triton X-100 was incubated at 37°C for 30 min.

Cell culture and inhibitor treatment

CHOP and RPMI 1864 cells were grown at 37°C in an α-minimal essential medium supplemented with 10% fetal bovine

Fig. 8. Level of GlcCer in fibroblasts from healthy volunteers and patients with GD. The fibroblasts were cultured for 8 h. Cell lysate corresponding to 50 μg of total protein was subjected to the quantification of GlcCer. The statistical difference between controls and GD patients was calculated using Student’s t-test. The number of samples was five for the volunteers and six for the patients with GD.
serum, 100 μg/mL streptomycin, and 100 units/mL penicillin in a humidified incubator containing 5% CO₂. Fibroblasts from patients with GD and healthy volunteers were prepared and cultured as described (Hayashi et al. 2008). Appropriate amounts of cells were preincubated in 2 mL of medium using a 6-well dish. After 24 h, the cells were treated with inhibitors (cyclosporin A or P4) at the concentration indicated. The cells were harvested after predetermined periods.

**Injection of CBE**

Zebrafish embryos at one- to four-cell stages were injected with approximately 1.4 mL of 0.5 mM or 10 mM CBE. The embryos were cultured in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄) at 28°C for 3 days.

**Protein assay**

The amount of protein was measured using the bicinchoninic acid protein assay (Pierce, USA) with bovine serum albumin as the standard (Smith et al. 1985).

**Enzyme assays**

The assays of synthase activities for GlcCer (GlcT) and LacCer were performed as described (Hayashi et al. 2005). For the assay of LacCer synthase, 50 pmol of C6-NBD-GlcCer and 6.5 nmol of lecithin were mixed in 100 μL of ethanol, and then the solvent was evaporated. Ten microliters of water was added and the mixture was sonicated to form liposomes. Fifty microliters of mixture containing 100 μM UDP-Gal, 5 mM MgCl₂, 5 mM MnCl₂, 10 μL of C6-NBD-GlcCer liposome, and 20 μL of cell lysate corresponding to 25 μg of total protein in the 50 mM HEPES buffer, pH 7.5, containing 10 μg/mL of aprotinin, leupeptin, and pepstatin A. The assay was carried out at 37°C for 1 h and the reaction was stopped by adding 200 μL of chloroform/methanol (2/1, v/v) and mixing well. The lower phase was withdrawn after centrifugation at 15,000 rpm for 5 min. After the lower phase had dried up, lipids were dissolved in 120 μL of methanol and 150 μL of water were added to the lower phase after the partition was estimated using standard GalSph. The recovery was no less than 90% after three extractions (supplementary Figure 6). The ethanol solution and OPA reagent (0.1 mL of ethanol containing 10 mg of OPA, 20 μL of 2-mercaptoethanol, and 9.9 mL of 3% (w/v) boric acid buffer, pH 10.5) were preincubated at 70°C for 20 min. The OPA reagent (15 μL) was added to the ethanol solution and the mixture was kept at 70°C for 60 min. The efficiency of labeling of GalSph with OPA was not changed in the presence or absence of crude lipids extracted from CHOP cells corresponding to 400 μg of protein (supplementary Figure 5B). The supernatant was transferred to a glass vial after centrifugation at 15,000 rpm for 10 min. An aliquot of sample (15 μL) was injected into an HPLC column using an auto-sampler (Hitachi L-2200).

**HPLC analysis**

The OPA-derivatized sample with internal standard was analyzed using a normal-phase column (Intersil SIL 150A-5, 4.6 × 250 mm, GL Science, Japan). OPA-GlcSph and OPA-GalSph were eluted at 6.8 min and 11.3 min, respectively, with hexane/isopropylalcohol/H₂O (73/26.5/0.5, v/v/v) as a mobile phase at a flow rate of 2.0 mL/min and detected using a fluorescent detector (Hitachi L-7840) set to excitation and emission wavelengths of 340 nm and 455 nm. However, under these conditions, the internal standard was not detected. To quantify the internal standard, an aliquot of sample was injected into the column and eluted with hexane/isopropylalcohol/H₂O (44:55:1) at the same flow rate. C6-NBD-GlcCer was eluted at 3.5 min as detected by a fluorescent detector set to excitation and emission wave lengths of 470 nm and 530 nm (Hayashi et al. 2005). Each peak was assigned by comparing retention times with those of standards. The amounts of GlcCer and GalCer in the samples were estimated with a standard curve based on known amounts of OPA-GlcSph.

**Supplementary Data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.
Quantification of glucosylceramide and galactosylceramide

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Conflict of interest statement
None declared.

Abbreviations
aGC, acid β-glucocerebrosidase; Cer, ceramide; Gal-Cer, galactosylceramide; GD, Gaucher disease; Glc-Cer, glucosylceramide; GlcT, UDP-glucose:ceramide β-1,1′-glucosyltransferase (GlcCer synthase); HPLC, high-performance liquid chromatography; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; OPA, O-phthaldialdehyde; SC'Dase, sphingolipid ceramide N-deacylase.

References