methods

Direct quantitation of psychosine from alkaline-treated lipid extracts with a semi-synthetic internal standard

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Abstract **Psychosine is an important bioactive sphingolipid metabolite and plays an essential role in the pathogenesis of Krabbe's disease. Herein, we extended shotgun lipidomics for the characterization and quantitation of psychosine in alkaline-treated crude lipid extracts by using neutral loss scan of 180 amu (i.e., galactose) in the positive-ion mode. Specifically, we semi-synthesized *N*,*N*-dimethyl psychosine and used it as an internal standard for quantitation of psychosine. After characterization of the fragmentation patterns of psychosine and the selected internal standard and optimization of the experimental conditions, we demonstrated that a broad linear dynamic range for the quantitation of psychosine and a limit of detection at a concentration of low fmol/μl were achieved using this approach. The developed method is generally simpler and more efficient than other previously reported methods. Multiple factors influencing quantitation of psychosine were extensively examined and/or discussed. The levels of psychosine in diabetic mouse nerve tissue samples were determined by the developed methodology. Collectively, the developed approach, as a new addition to the shotgun lipidomics technology, will be extremely useful for understanding the pathways/networks of sphingolipid metabolism and for exploring the important roles of psychosine in a variety of physiological and pathological conditions.**—Jiang, X., K. Yang, and X. Han. **Direct quantitation of psychosine from alkaline-treated lipid extracts with a semi-synthetic internal standard.** ***J. Lipid Res.*** **2009.** 50: **162–172.**

Supplementary key words Alzheimer's disease • electrospray ionization mass spectrometry • diabetes mellitus • Krabbe's disease • shotgun lipidomics • sphingolipid metabolism • sphingolipidome

Psychosine (i.e., galactosylsphingosine) is an important bioactive sphingolipid metabolite that has been shown to be linked with the activation of a G protein-coupled receptor, i.e., TDAG8, a member of the sphingosine-1-phosphate receptor superfamily (1). Recent studies have shown that psychosine can induce cell apoptosis, cytokine activation, phospholipase activation, peroxisomal dysfunction, and altered calcium homeostasis, among others (1–6). It is known that psychosine plays an essential role in the pathogenesis of Krabbe's disease (i.e., globoid cell leukodystrophy), which is an autosomal recessive disorder caused by a generalized deficiency of the lysosomal enzyme galactosylceramidase (7). The underlying biochemical mechanism of this disease is probably due to the psychosine-mediated toxic effects via mitochondria (e.g., inhibition of the electron transport chain, dispassion of mitochondrial membrane potential, releasing of cytochrome c, etc.). However, the role(s) of psychosine in the pathophysiology of Krabbe's disease have not yet been fully elucidated, because research has been partially hindered by the lack of a simple, sensitive, and specific method for quantitative analysis of this low-abundant metabolite in biological samples.

Multiple methodologies have previously been developed and employed for the quantification of psychosine present in biological samples. Previous methods were largely based on chromatography, either TLC or HPLC, with or without chemical modifications (8–14). The drawbacks of these methods include the usage of radioactive reagents, the labor intensiveness of the procedures, relatively low sensitivity of detection, or the lack of the structural information.

A novel method based on electrospray ionization (ESI) tandem mass spectrometry (MS/MS) has recently been developed. In that method, Whitfield and colleagues (15) have employed lactosylsphingosine as an internal standard in sample preparation in which a cationic solid-phase extraction cartridge was used to isolate the analytes from 100 mg of tissue samples. A C18 solid-phase extraction cartridge was employed to desalt the samples prior to the MS analysis through direct infusion in the positive-ion mode. Either precursor ion scanning of *m/z* 282 or

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Abbreviations: CID, collision-induced dissociation; ESI, electrospray ionization; MS, mass spectrometric or mass spectrometry; NL, neutral loss.

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multiple-reaction monitoring of the pairs from the molecular ions of psychosine and lactosylsphingosine to *m/z* 282 was performed for identification and/or quantitation. This method is much simpler, more sensitive, and more specific than those that have been previously developed based on chromatographic separation. However, lactosylsphingosine, which is used as an internal standard in the method, might differentially be recovered during sample preparation. Moreover, substantially different fragmentation after collision-induced dissociation (CID) between psychosine and lactosylsphingosine is apparently present, which could introduce severe systematic experimental error.

Recently, we have developed an approach to globally analyze individual lipid molecular species of a biological sample directly from a lipid extract, now known as shotgun lipidomics (16–19). This analytical platform has very recently been extended to the analyses of low-abundant lipid classes of the sphingolipidome after treatment of lipid extracts with lithium methoxide followed by cleaning and recovering of sphingolipidome through liquid-liquid partition (20). This approach has now been termed shotgun sphingolipidomics. Herein, we extended shotgun sphingolipidomics for the characterization and quantitation of psychosine in crude lipid extracts after treatment with lithium methoxide. In this methodology, we employed an analog of psychosine (i.e., *N,N*-dimethyl psychosine that was semi-synthesized from psychosine) as an internal standard. Quantitative analysis of psychosine was achieved by using neutral loss (NL) scan of 180 amu (corresponding to galactose) in the positive-ion mode.

In the study, we demonstrated that a broad linear dynamic range for the quantitation of psychosine with *N,N*-dimethyl psychosine as an internal standard and a limit of detection at low fmol/μl concentration were present. The developed method for quantitation of psychosine is generally much simpler and more efficient than any other previously developed method. Multiple factors that might influence the quantitation of psychosine, including ion suppression, CID conditions, extraction efficiency, potential overlapping with other molecular species, and the possible presence of d20:1 psychosine, which is isomeric with the selected internal standard, were extensively examined and/or discussed. The levels of psychosine in multiple biological samples including cortex, cerebellum, spinal cord, and brain stem from diabetic mice and controls at different ages were determined by the developed methodology. With this extension, shotgun lipidomics not only allows us to analyze the major lipid classes, but also to analyze many of the minor lipid classes [e.g., psychosine, sphingoid-based-1-phosphates (21), sphingosine (20, 22), and ceramide (23)] all from one lipid extract of a biological sample (e.g., approximately 10 mg tissue sample). Accordingly, this developed method, as a new addition to the shotgun lipidomics technology, will be extremely useful for understanding the pathways/networks of altered sphingolipid metabolism in a disease state (e.g., Alzheimer's disease) and for exploring the important roles of psychosine in a wide range of physiological and pathological conditions.

MATERIALS AND METHODS

Materials

Psychosine, formaldehyde (37 wt % solution in water), and sodium cyanoborohydride (95%) were obtained from Sigma-Aldrich (St. Louis, MO). Bovine brain cerebrosides and bovine brain sulfatides were obtained from Matreya, Inc. (Pleasant Gap, PA). Synthetic *N*-heptadecanoyl sphingomyelin and *N*-lauroryl ceramide were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All solvents used for synthesis, sample preparation, and MS analysis were obtained from Burdick and Jackson (Honeywell International, Inc., Burdick and Jackson, Muskegon, MI). Formic acid (85%), acetic acid, and ammonium hydroxide (28–30%) were purchased from Thermo Fisher Scientific (Pittsburgh, PA). The stock solutions of psychosine and the synthesized *N,N*-dimethyl psychosine were prepared in chloroform-methanol (1:1, v/v) and stored under nitrogen at −20°C.

Preparation of *N,N*-dimethyl psychosine

To a solution of psychosine (20 mg, 0.0436 mmol) and formaldehyde (37 wt % solution in water, 70 μl, 0.87 mmol) in methanol (2 ml), sodium cyanoborohydride (54 mg, 0.87 mmol) was added. The pH value of the solution was adjusted to 6 by dropwise addition of acetic acid. The final solution was stirred at room temperature for 1 h. The solvents in the reaction were evaporated under a nitrogen stream. The residue was dissolved in 200 μl of chloroform-methanol (2:1, v/v) and loaded on a silica gel column. The reaction product, *N,N*-dimethyl psychosine, was eluted with chloroform-methanol-water (2:1:0.15, v/v/v), and 17 mg of *N,N*-dimethyl psychosine was obtained (85% yield). The identity and the purity of this compound were determined by NMR spectroscopy and MS.

Preparation of lipid extracts from brain tissue samples

Male mice (C57BL/6, 1 and 4 months of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). Diabetes in 4-month-old wild-type mice was induced by a single intravenous injection (in the tail vein) of streptozotocin (165 mg/kg body weight in 0.1 ml of 0.1 M citrate buffer, pH 4.5) as described previously (24). All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science, 1996) and were approved by the Animal Studies Committee at Washington University. Mice were euthanized by asphyxiation with carbon dioxide. Brain tissues were dissected, immediately dried with Kimwipes padding, and freeze-clamped at the temperature of liquid nitrogen. Wafers were pulverized into a fine powder with a stainless steel mortar and pestle. Protein assays on the fine powders (approximately 10 mg) were performed after further homogenization in 10× diluted PBS (500 μl). Therefore, the final lipid content was normalized to the protein content.

Each of the homogenized tissue sample solutions (see above) was diluted with 2 ml of 5% ammonium hydroxide and individually transferred into a disposable glass culture test tube. *N,N*-dimethyl psychosine (10 pmol/mg protein) (used as an internal standard for quantitation of psychosine) was added. Lipids in the homogenized solution were extracted with 4 ml of 1:1 chloroform-methanol, and the aqueous phase was further extracted twice with chloroform. The solvent of the combined crude extract from each sample was evaporated under a nitrogen stream. The residue was treated with lithium methoxide (1 M) in MeOH (200 μl) as described previously (20). The reaction mixtures were quenched with water (2 ml) and washed with diethyl ether (2 ml) and hexane (2 ml × 2). The aqueous phases were extracted with 4 ml of chloroform-methanol (1:1). The chloro-

form phases (low-phase) were collected, and the aqueous phases were re-extracted with chloroform (2 ml) twice. The solvents of the combined chloroform extracts of each sample were evaporated under a nitrogen stream. Each individual residue was then re-suspended in 4 ml of chloroform-methanol (1:1) and re-extracted against 1.8 ml of 10 mM ammonium formate containing 5% ammonium hydroxide aqueous solution (pH 11). The combined chloroform extract was dried under a nitrogen stream. Each individual residue was reconstituted in a volume of 0.1 ml/mg protein (based on the original protein content of the samples) in chloroform-methanol (1:1). The lipid extracts were finally flushed with nitrogen, capped, and stored at −20°C for ESI/MS/MS analysis (typically analyzed within 1 week). Each lipid solution was further diluted approximately 50-fold with chloroform-methanol-isopropanol (1:2:4, v/v/v) containing formic acid (0.1%, v/v) immediately prior to direct infusion and lipid analysis by ESI/MS/MS.

ESI MS/MS analysis of psychosine and *N,N*-dimethyl psychosine

A triple-quadrupole mass spectrometer (TSQ Quantum Ultra, Thermo Fisher Scientific, San Jose, CA) equipped with a TriVersa NanoMate (Advion BioSciences, Ithaca, NY) and operating under an Xcalibur software system was utilized as previously described (17, 25, 26). Briefly, an offset voltage on the ion transfer capillary was set to 158 V. The heater temperature along the ion transfer capillary was maintained at 150°C. The sheath gas and auxiliary gas (nitrogen) pressure were set to 0 psi. The diluted lipid extract solution was directly infused into the ESI source with the nanomate device. The nanomate system consists of a 96-well plate, a rack of 384 disposable tips, and a nanoESI D-chip with nozzles at the diameter of 4.1 μm. In the current study, 10 μl of the diluted sample solution was delivered to the back plane of the nanoESI D-chip. A spray voltage of 1.2 kV and gas pressure of 0.3 psi were used. The delivery of samples was controlled by ChipSoft v. 4.7.1 software (Advion Biosciences) as previously described (27).

Typically, a 1 min period of signal averaging from 0.8 s per scan between *m/z* 400 and *m/z* 600 or as indicated in the profile mode was employed for each MS spectrum. For ESI/MS/MS analysis, the collision gas (argon) pressure was set at 1.0 mTorr, and a collision energy as indicated in the product ion mode and NL mode was utilized for psychosine and *N,N*-dimethyl psychosine. Ratiometric comparison of peak intensities from NL scan of 180 amu (i.e., galactose) was used for identification and quantification of psychosine. Typically, a 5 min period of signal averaging from 0.8 s per scan in the profile mode was employed for each MS/MS.

Miscellaneous

Protein concentration was determined with a bicinchroninic acid protein assay kit (Pierce, Rockford, IL) using BSA as a standard. Data from biological samples were normalized to the protein content, and all data are presented as the mean ± SD of multiple separated sample preparations from different animals. Statistical differences between mean values were determined by using Student's *t*-test and $P < 0.05$ as significant.

RESULTS AND DISCUSSION

Preparation of *N,N*-dimethyl psychosine as an internal standard for quantitation of psychosine

A suitable internal standard for quantitative analysis of psychosine in biological samples should be as nearly identical as possible to psychosine in chemical and physical properties (e.g., extraction recovery, ionization efficiency, fragmentation pattern, etc.), as previously discussed (18). Therefore, a stable isotopic labeled analog is always the best choice as an internal standard. Owing to the high cost of the chemical synthesis of a compound of this type, it is prohibitive to make it easily available. In the current study, we explored the utilization of a derivative analog of psychosine, i.e., *N,N*-dimethyl psychosine, as an internal standard.

Sodium cyanoborohydride ($NaCNBH_3$) reduces a wide variety of organic functional groups with remarkable selectivity. Reaction of an aldehyde or ketone with ammonia, primary amine, or secondary amine at pH 7 in the presence of sodium cyanoborohydride leads to primary, secondary, or tertiary amines, respectively, via reductive amination of the carbonyl group (28). When psychosine was reacted with formaldehyde in the presence of sodium cyanoborohydride, it was converted into its corresponding dimethylamine derivatives in a high yield. The structure of *N,N*-dimethyl psychosine was determined by ^{1}H NMR and ^{13}C NMR as follows: ^{1}H NMR spectroscopy (500 MHz, CD_3OD): δ 0.89 (t, J = 7.8 Hz, 3H), 1.20–1.37 (m, 22H), 1.37–1.49 (m, 2H), 2.84 (s, 6H), 3.47 (dd, J = 2.5, 10.2 Hz, 1H), 3.66–3.77 (m, 1H), 3.83 (d, J = 2.5 Hz, 2H), 3.92–3.95 (m, 2H), 4.18–4.32 (m, 5H), 5.54 (dd, J = 6, 15.4 Hz, 1H), 5.83 (dt, J = 7.7, 15.4 Hz, 1H); and ^{13}C NMR spectroscopy (125 MHz, CD_3OD): δ 134.9, 130.2, 104.6, 77.0, 74.8, 72.3, 70.3, 70.0, 69.7, 64.8, 42.5, 33.3, 33.1, 30.8, 30.7, 30.6, 30.4, 30.3, 30.2, 23.7, 14.4 ppm. In comparison to psychosine, the presence of an extra singlet resonance at 2.84 ppm of six protons confirmed the structure of *N,N*-dimethyl psychosine.

ESI/MS/MS analyses of psychosine and *N,N*-dimethyl psychosine in the positive-ion mode

Psychosine and *N,N*-dimethyl psychosine contain a galactosyl moiety and a sphingosine backbone carrying an amine. They should be readily ionized as protonated molecules or adducts with alkali ions such as a sodium ion. ESI/MS/MS analyses of equimolar mixtures of psychosine and *N,N*-dimethyl psychosine (0.1 pmol/μl each) demonstrated that both psychosine and *N,N*-dimethyl psychosine could be readily ionized as the protonated ions in the presence of 0.1% formic acid in chloroform-methanol-isopropanol (1:2:4, v/v/v) (**Fig. 1A**). It was also demonstrated that the peak intensities of the protonated species were essentially identical. Sodium adducts of these species were also present in relatively low abundance, which became apparent when the increased concentration of the equimolar mixture was used in the analysis under identical experimental conditions. Therefore, the protonated psychosine and *N,N*-dimethyl psychosine were chosen for qualitative and quantitative analyses of psychosine molecular species in biological samples in the study (see next section).

It should also be pointed out that although the tertiary amine in *N,N*-dimethyl psychosine tends to be more easily protonated than the primary amine in psychosine, ESI/MS/MS analyses showed nearly identical ionization efficiency between these two compounds. For example, positive-ion ESI

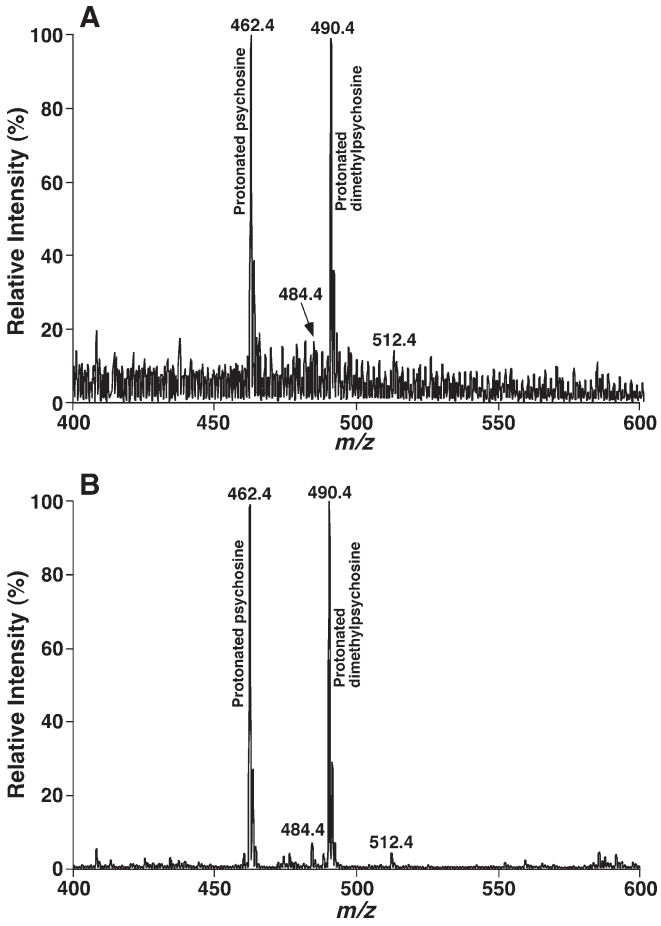


Fig. 1. Electrospray ionization mass spectrometric (ESI MS) analysis of psychosine and *N,N*-dimethyl psychosine. Positive-ion MS analyses of equimolar mixtures of psychosine and *N,N*-dimethyl psychosine at the concentration of 0.1 pmol/μl (A) and 1 pmol/μl (B), each in the presence of 0.1% formic acid, were performed through first-quadrupole scanning from *m/z* 400 to *m/z* 600.

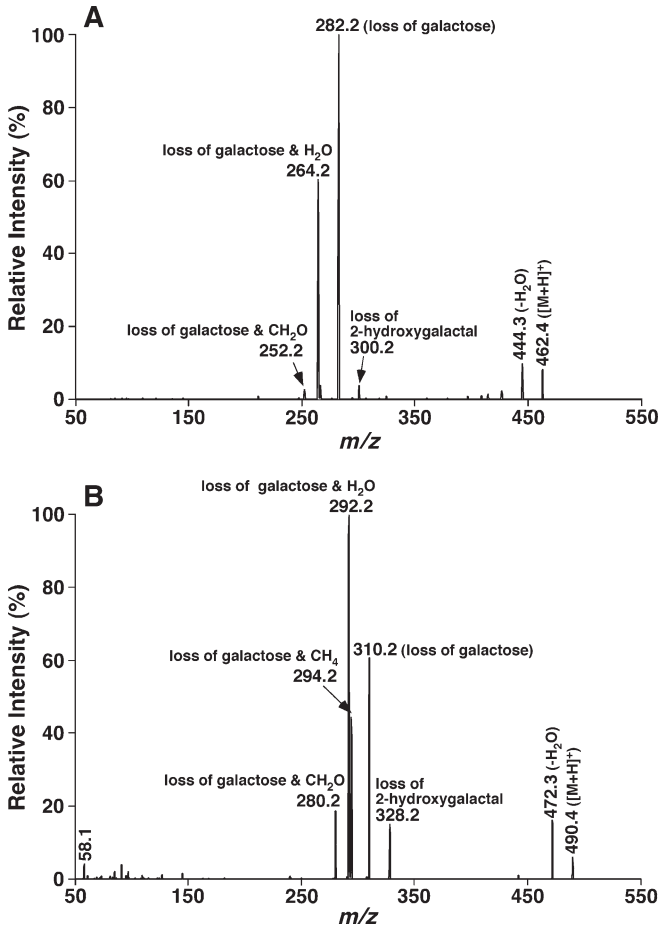
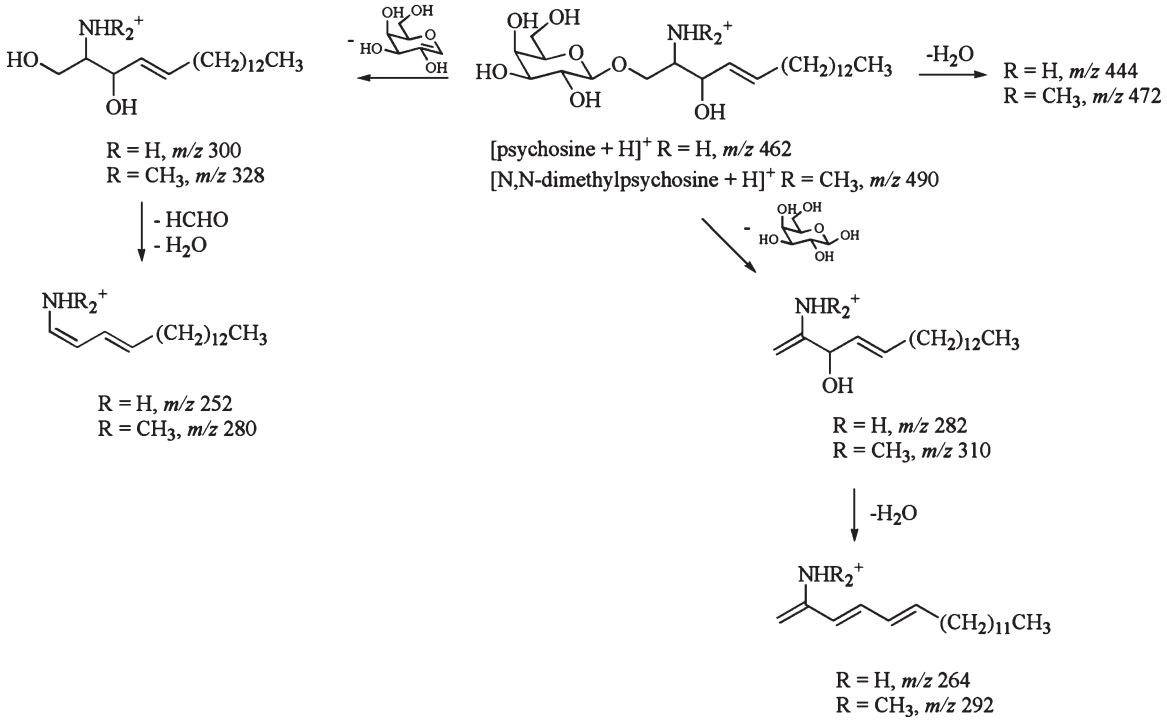


Fig. 2. Product ion ESI MS analyses of protonated psychosine and *N,N*-dimethyl psychosine. A: Product ion mass spectrum of protonated psychosine. Positive-ion tandem mass spectrometric analysis (MS/MS) of protonated psychosine (i.e., *m/z* 462.4) in the product ion scan model was performed through first quadrupole selectively passing the ion at *m/z* 462.4 and the third quadrupole scanning from *m/z* 50 to *m/z* 500, whereas collisional activation was performed in the second quadrupole as described under Materials and Methods. The collision gas pressure was 1.0 mTorr, and a collision energy of 24 eV was employed in the analyses. B: Product ion mass spectrum of protonated *N,N*-dimethyl psychosine (i.e., *m/z* 490.4) acquired as described above.

mass spectrum of an equimolar mixture of psychosine and *N,N*-dimethyl psychosine displayed essentially identical ion abundance after correction of approximately 2% difference in ^{13}C isotopic distribution between the compounds as previously described (18) (Fig. 1). This could be attributed to the fact that the intramolecular hydrogen bonding between the amine and the adjacent hydroxyl group decreases the difference in basicity between psychosine and *N,N*-dimethyl psychosine.

Characterization of psychosine and *N,N*-dimethyl psychosine by ESI MS/MS in the positive-ion mode

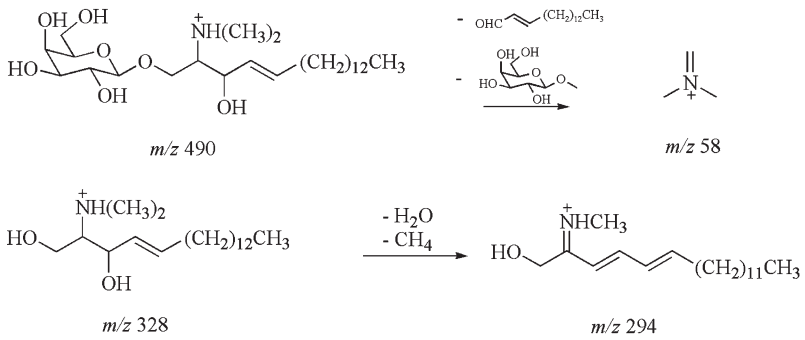
CID of the protonated psychosine and *N,N*-dimethyl psychosine yielded multiple abundant and informative product ions (**Fig. 2A, B**, respectively). The identities of these fragments were indicated in the mass spectra. Specifically, the two most abundant product ions at *m/z* 264.2 and *m/z* 282.2 from psychosine and *m/z* 292.2 and *m/z* 310.2 from *N,N*-dimethyl psychosine represent the common NL of a galactose as well as a galactose plus a water molecule from protonated molecular ions of these compounds, respectively. Other common fragments include $[M + H]^+$-H_2O, $[M + H]^+$-$C_6H_{10}O_5$ corresponding to NL of the dehydrated galactose moiety, and $[M + H]^+$-galactose–HCHO, as indicated (Fig. 2A, B). The potential pathways leading to the production of these common fragments from both protonated psychosine and protonated *N,N*-dimethyl psychosine were proposed as shown in **Scheme 1**. Two unique fragment ions at *m/z* 58 and *m/z* 294 resulted from CID of protonated *N,N*-dimethyl psychosine (Fig. 2B). The ion at *m/z* 58 was probably formed via the cleavage of C1-C2 and C2-C3 of the sphingosine backbone of protonated *N,N*-dimethyl psychosine, corresponding to NL of methylgalactoside and 2-hexadecenal (**Scheme 2**). The ion at *m/z* 294 probably arises through the secondary alcohol, forming a six-member ring following loss of an H− from C6, and undergoing a rearrangement to lose water, leaving C3-C4 and C5-C6 in conjugation with the fragment ion at *m/z* 328 result-



Scheme 1. Proposed common fragmentation pathways of protonated psychosine and *N,N*-dimethyl psychosine after low-energy collision-induced dissociation (CID).

ing from protonated *N,N*-dimethyl psychosine (Scheme 2). Next, the *N*-methyl group probably undergoes a 1, 4 elimination with the single H^- on C2 to lose methane and form a C2=N, resulting in the positive charge on the nitrogen being stabilized by conjugation with the C3-C4 and C5-C6 double bonds (Scheme 2).

The fragmentation of sodium adducts of psychosine and *N,N*-dimethylpsychosine after CID also yielded multiple abundant and informative product ions (**Fig. 3A, B**, respectively), although relatively higher collision energy than that for their $[M + H]^+$ counterparts was required. Briefly, the ion at *m/z* 467 arose from the loss of NH_3 from $[psychosine + Na]^+$ or $NH(CH_3)_2$ from $[N,N$-dimethylpsychosine + $Na]^+$, respectively. The ion at *m/z* 203 is a galactose sodium adduct, which could be generated from $[M + Na]^+$ via the formation of a highly favored six-membered transition state between the linking oxygen and the hydrogen on the allylic secondary alcohol. Subsequent rearrangement would yield the desired product ion and two stable neutrals: hexadecenal and an amino ethylene. The ion at *m/z* 185 probably formed from the loss of water from the *m/z* 203 ion. The ion at *m/z* 157 was probably generated from the ring opening at the C5-O bond moving two electrons to the C1-O bond, yielding a carbonyl. Subsequently, the C1-C2 bond electrons could form a bond with C5, eliminating formic acid as a neutral and leaving a four-membered ring with sodium chelated to the hydroxyl groups. These pathways are schematically illustrated in **Scheme 3**. Moreover, the ion at *m/z* 102 in the CID mass spectrum of $[psychosine + Na]^+$ was probably produced from three 1,3 hydrogen shift followed by the loss of galactosyl sodium and a 1,3 diene arising from a charge remote process. Both psychosine and *N,N*-dimethylpsychosine yielded a low-abundant product ion at *m/z* 366. This fragment might be generated by charge-remote fragmentation of the sphingoid backbone and loss of an α-hydroxylacetaldehyde from



Scheme 2. Proposed pathways leading to the production of fragment ions at *m/z* 58 and *m/z* 294 resulting from protonated *N,N*-dimethyl psychosine after low-energy CID.

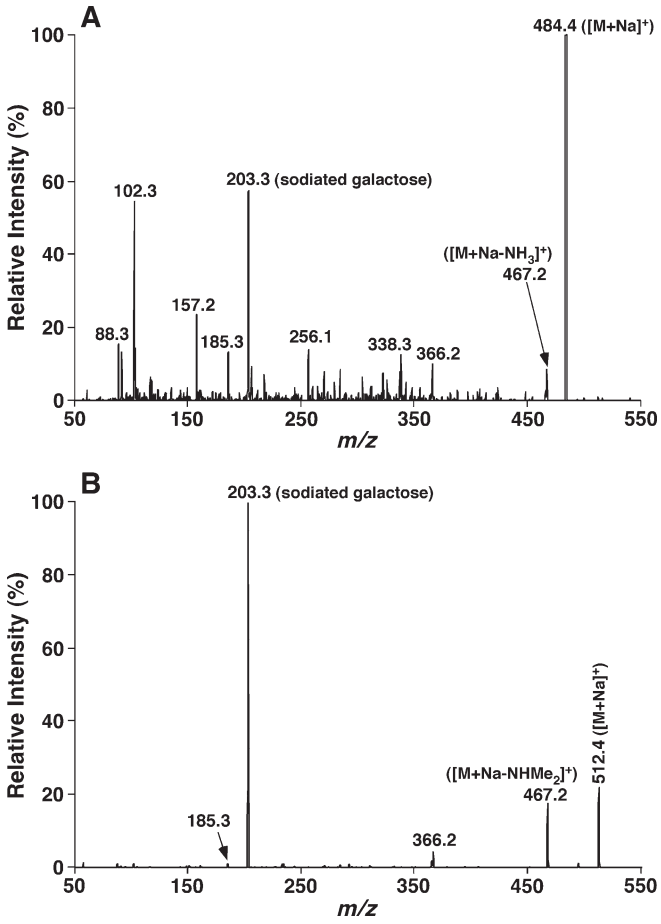


Fig. 3. ESI MS/MS analyses of sodiated psychosine and *N,N*-dimethyl psychosine. A: Product ion mass spectrum of sodiated psychosine. Positive-ion MS/MS analyses of sodiated psychosine (i.e., *m/z* 484.4 in Fig. 1B) was performed through first quadrupole selectively passing the ion at *m/z* 484.4 and the third quadrupole scanning from *m/z* 50 to *m/z* 500, whereas collisional activation was performed in the second quadrupole. B: Product ion mass spectrum of sodiated *N,N*-dimethyl psychosine (*m/z* 512.4) acquired as described above. The collision gas pressure was 1.0 mTorr, and a collision energy of 40 eV or 35 eV for sodiated psychosine or *N,N*-dimethyl psychosine, respectively, was employed in the analyses.

the galactosyl moiety after cleavage of the galactosyl ring. In the case of psychosine, 1-butene and hydrogen may be lost in the charge-remote fragmentation of the sphingoid backbone. The charge-remote fragmentation of the sphingoid backbone of *N,N*-dimethylpsychosine expulses 1-hexene and hydrogen.

Quantitation of psychosine with *N,N*-dimethyl psychosine as an internal standard

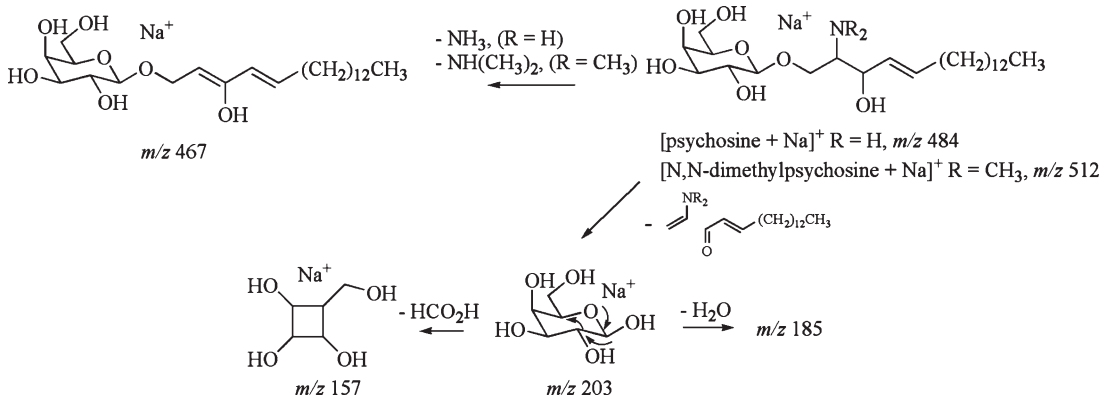
Psychosine is a class of sphingolipid metabolites present in low abundance in biological samples. Very recently, we have developed an approach to enrich the low-abundance sphingolipids via alkaline methanolysis followed by liquid-liquid extraction (20). This method allows us to enrich the sphingolipids enough for shotgun lipidomics analysis of multiple low-abundance classes of sphingolipids without chromatographic separation. Psychosine is mainly present in the brain, where the major classes of sphingolipids include sphingomyelin, cerebroside, and sulfatide. It should be pointed out that some ganglioside molecular species are also very abundant in the brain. However, the majority of the gangliosides are shown as present in the aqueous phase by using the extraction procedure employed (29).

It was demonstrated that the fragment ions resulting from the NL of either galactose (180 u) or galactose plus water (198 u) were present in the product ion mass spectra of both protonated psychosine and *N,N*-dimethyl psychosine. These product ions represented two of the most abundant and characteristic fragments (Fig. 2A, B). Therefore, NL scans of both 180 amu and 198 amu in the mass region between *m/z* 400 and *m/z* 600 in the positive-ion mode could be used to identify and quantitate psychosine in a biological lipid extract with *N,N*-dimethyl psychosine as an internal standard. It should be pointed out that the specificity of identification of a class of lipid molecular species can be markedly increased by using multiple specific NL or precursor-ion scans if present. To explore this possibility, in initial experiments, NL scans of both 180 amu and 198 amu in the positive-ion mode were acquired to analyze the equimolar mixtures of psychosine and *N,N*-dimethyl psychosine at different concentrations.

We found that the peak intensity ratios of these fragment ions from protonated psychosine and *N,N*-dimethyl psychosine in the NL mass spectra were dependent on the CID energy employed. This finding reflected the presence of differential fragmentation pathway(s) and different fragmentation efficiency between the two compounds, as shown in Fig. 2 and Schemes 1 and 2. We also found that NL scan of 180 amu was relatively more sensitive than that of 198 amu at all the CID energies examined. Therefore, an optimal CID condition for quantitation of psychosine employing the selected internal standard using NL scan of 180 u was determined, whereas NL scan of 198 amu was largely performed for identification purposes. We found that CID energy of 24 eV for NL 180 amu at a collision gas pressure (i.e., argon) of 1 mTorr was the best to meet the considerations of both sensitivity and accuracy for quantitation of psychosine. The limit of detection under these conditions by using the NL scan of 180 u was 1 fmol/μl in chloroform-methanol-isopropanol (1:2:4, v/v/v).

For quantitative analysis of psychosine with *N,N*-dimethyl psychosine as an internal standard by using a shotgun sphingolipidomics approach, a linear correlation with a broad dynamic range between the compounds has to be present and determined in the presence of other major sphingolipid classes as mentioned above. To mimic the effects of other major sphingolipids present in mouse brain on quantitation of psychosine, a mixture of *N*-heptadecanoyl sphingomyelin, bovine brain cerebrosides, bovine brain sulfatides, and *N*-lauroryl ceramide in the amounts of 3.75, 15, 7.5, and 1.2 nmol, respectively, were prepared with a variety of different molar ratios of psychosine and *N,N*-dimethyl psychosine, in which the *N,N*-dimethyl psychosine was fixed at 50 pmol.

NL scan of 180 amu from the diluted solutions of these mixtures with a fixed concentration of *N,N*-dimethyl psy-



Scheme 3. Proposed common fragmentation pathways of sodiated psychosine and *N,N*-dimethyl psychosine after low-energy CID.

chosine at 0.01 pmol/μl was acquired. **Figure 4A** shows the NL scan of 180 amu from the sphingolipid mixtures with equimolar psychosine and *N,N*-dimethyl psychosine at the concentration of 0.01 pmol/μl each. A correlation of the peak intensity ratios with molar ratios between psychosine and its derivative showed a broad linear dynamic range (>100-fold) under the conditions (Fig. 4B). The linear plot between molar ratios (x) and peak-intensity ratios (y) of psychosine to *N,N*-dimethyl psychosine was fitted through iterative change of the constant b to yield the best constant c as follows:

$$\log[(y - b)/x] = c \qquad (Eq.\ 1)$$

which is derived from the linear formula

$$y = ax + b \qquad (Eq.\ 2)$$

where $c = \log(a)$. The correlation of x and y in equation 1 is best fit with all the numbers of x equally, whereas in equation 2, it is unequivalently weighed with the numbers of x. The experimental results were best fitted to a linear correlation in a logarithm format:

$$\log(y + 0.3144) = \log(x) + 0.8031 \qquad (Eq.\ 3)$$

with a linear correlation coefficient (r^2) of greater than 0.995 from the NL scan of 180 u.

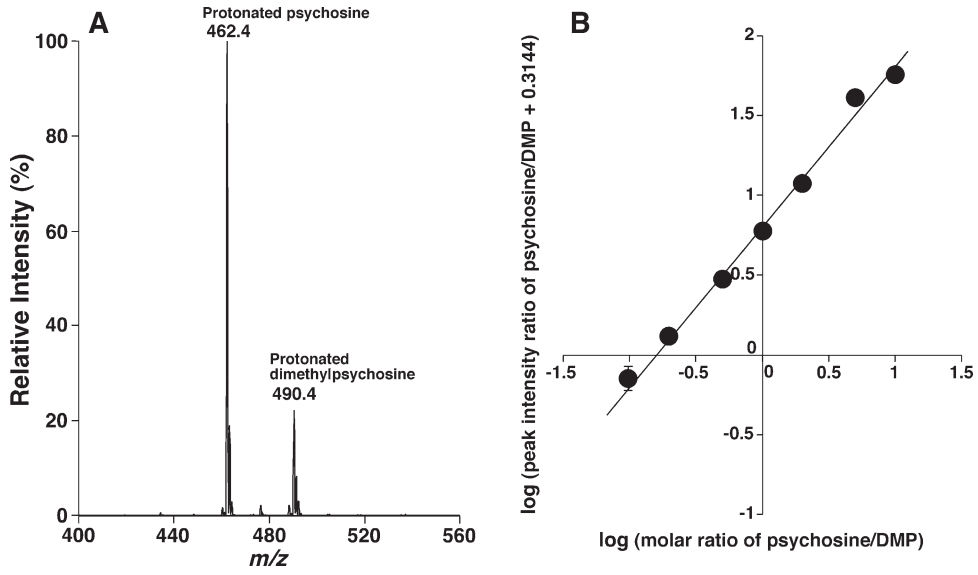


Fig. 4. Quantitative analysis of psychosine using *N,N*-dimethyl psychosine as an internal standard. Mixtures composed of different molar ratios of psychosine to *N,N*-dimethyl psychosine were separately prepared with a fixed concentration of *N,N*-dimethyl psychosine at 0.01 pmol/μl. Positive-ion MS/MS analyses through neutral loss (NL) scanning of 180 amu were performed by coordinately scanning both the first and third quadrupoles with a fixed mass difference of 180 u, respectively, whereas collisional activation was performed in the second quadrupole. The collision gas pressure was 1.0 mTorr, and a collision energy of 24 eV was employed in the NL scanning of 180 u. A: Representative NL mass spectrum of 180 u, from an equimolar mixture of psychosine and *N,N*-dimethyl psychosine at 0.01 pmol/μl each. B: Linear correlation of the molar ratios with the ratios of ion peak intensities of psychosine and *N,N*-dimethyl psychosine acquired by NL scanning of 180 u. The data points represent the means ± SD from five separate mixture preparations of each molar ratio of psychosine to *N,N*-dimethyl psychosine. Note that most error bars are within the symbols. DMP denotes *N,N*-dimethyl psychosine.

Next, multiple factors that might influence accurate quantitation of psychosine using *N,N*-dimethyl psychosine as an internal standard were examined. We first examined the potential difference of the extraction efficiency between the two compounds, because a minimal difference of the extraction efficiencies of the compounds is essential for quantitative analysis. Because the reductive amination of psychosine afforded N,N-dimethyl psychosine resulted in a minimum change in hydrophobicity of amines, the recoveries of psychosine and N,N-dimethyl psychosine were very similar. After examining the Bligh and Dyer extraction (29a) against a neutral, basic, or acidic aqueous phase, it was found that extraction under the basic conditions with 5% (v/v) ammonium hydroxide gave the best extraction efficiency in a recovery of ~95–104% (**Table 1**), whereas their extraction efficiencies were only ~11–21% under the acidic condition with 5% (v/v) acetic acid. To facilitate the separation of chloroform from basic aqueous phase during extraction of the lithium methoxide-treated biological samples, the presence of some ion strength in the aqueous phase was helpful. It was found that application of 10 mM ammonium formate containing 5% ammonium hydroxide (pH 11) as aqueous phase in final re-extraction yielded the best recovery. The overall recoveries of various amounts of psychosine and *N,N*-dimethyl psychosine in the sample preparation after treatment with lithium methoxide under basic conditions were tabulated (Table 1).

It should be recognized that no chromatographic separation is performed in the shotgun sphingolipidomics approach, although intrasource separation/selective ionization is conducted and the NL scans of 180 amu (galactose) and 198 amu (galactose plus water) are not entirely specific to psychosine in the mass region. Therefore, it is important to determine potential interferences from other components present in the biological samples by using NL scans of 180 amu and 198 amu in the positive-ion mode. In theory, any components containing mono-hexose might produce neutral fragments of 180 amu after CID and interfere with quantitation. In practice, no isobaric species that potentially interfere with either psychosine or *N,N*-dimethyl psychosine are in brain tissues, although there are many abundant sphingolipid molecular species containing at least one hexose (e.g., galactocerebrosides, glucocerebrosides, and ceramide polyhexosides). This is due to the marked differences of the mass/charge of psychosine and *N,N*-dimethyl psychosine from other sphingolipid species containing hexose. This consideration was further verified with samples prepared from other organs, such as liver and heart, in which only minimal amounts of psychosine are present, as well as samples from brain tissues in which the internal standard was not added. However, it should be emphasized that we could not exclude the possibility that other isobaric species that could yield an NL of 180 amu or 198 amu were present in other sample sources (e.g., plants, bacteria, etc.) and could interfere with accurate (14) quantitation of psychosine by using shotgun lipidomics.

TABLE 1. Overall extraction recoveries of psychosine and *N,N*-dimethyl psychosine in the sample preparation

Amounts of Material Added	Psychosine	*N,N*-dimethyl Psychosine
fmol/μl	%	
25	95 ± 5	105 ± 7
50	99 ± 7	95 ± 6
100	97 ± 3	100 ± 5

The recovery of psychosine was determined by calculation of the intensity ratio of psychosine to *N,N*-dimethyl psychosine, which was added right before mass spectrometric (MS) analysis after lithium methoxide treatment and extraction as described in Materials and Methods. The recovery of *N,N*-dimethyl psychosine was determined by post-lithium methoxide treatment-and-extraction addition of psychosine as internal standard. Data represent the means ± SD of at least three separate preparations.

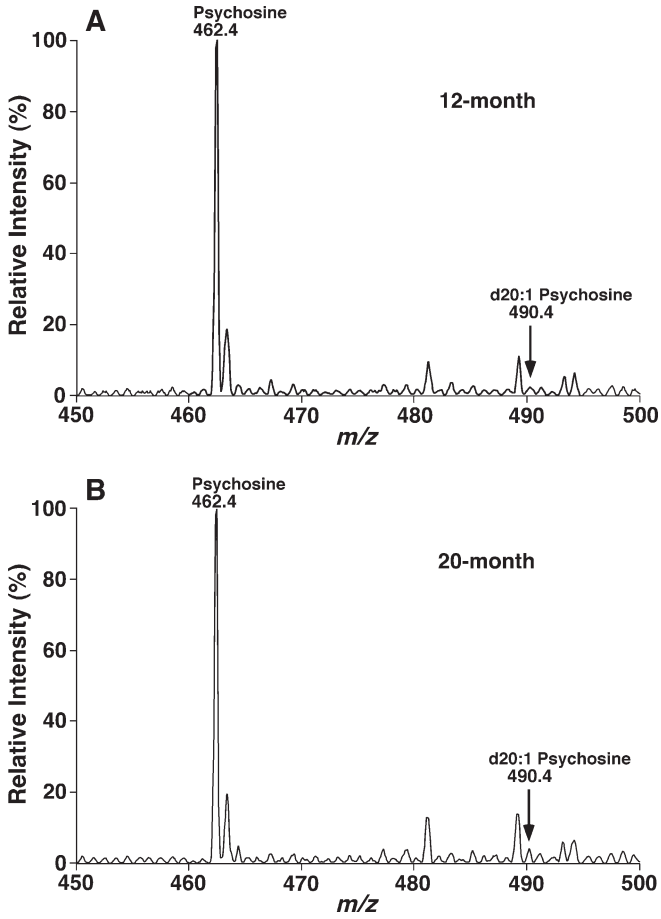


Fig. 5. ESI MS/MS analysis of lipid extracts from spinal cord of mice at different ages in NL scan mode. Total lipids of mouse spinal cord were extracted by using a modified procedure of Bligh and Dyer against 10 mM ammonium formate containing 5% ammonium hydroxide aqueous solution (pH 11) without addition of the selected internal standard. Each lipid extract was separately treated by alkaline methanolysis as described under Materials and Methods. MS/MS of lipid extracts of murine spinal cord at 12 months of age (A) and 20 months of age (B) were acquired using an NL scan of 180 u. The collision gas pressure was set at 1.0 mTorr, and a collision energy of 24 eV was employed in the analyses. A 5 min period of signal averaging of the scans acquired at a scanning rate of 500 u/s in the profile mode was employed for each MS/MS. The arrow indicates the putative ion peak of d20:1 psychosine.

Finally, it was recognized that the selected internal standard is an isomer of a low-abundance endogenous psychosine (i.e., d20:1 psychosine), the content of which usually increases with age. To examine the effects of the presence of d20:1 psychosine on the quantitation of psychosine molecular species using *N,N*-dimethyl psychosine as an internal standard, we performed MS/MS analysis of lipid extracts of different brain regions of mice aged 1 to 20 months without addition of *N,N*-dimethyl psychosine. The NL scans of 180 amu in the mass region of interest consistently showed low background levels of d20:1 psychosine in all examined brain regions. **Figure 5** displays the NL scans of 180 amu acquired with lipid extracts from spinal cord (which contains the highest amount of psychosine among neuronal tissues) of mice at 12 and 20 months of age. Therefore, the effects of the presence of d20:1 psychosine on the quantitation of psychosine using *N,N*-dimethyl psychosine as an internal standard are minimal and can be considered negligible. However, we emphasize that for quantitation of psychosine from an unfamiliar sample, a pre-scanning for the potential existence of a non-negligible amount of d20:1 psychosine should always be performed. Furthermore, we anticipate that *N,N*-dimethyl psychosine will be replaced in future studies with other suitable analogs as internal standards once they are commercially available.

Quantitation of psychosine in lipid extracts of various biological samples

The developed approach was employed to directly quantitate psychosine contents in lipid extracts from spinal cord and brain tissues (including cortex, cerebellum, and brain stem) of mice at both 1 and 5.5 months of age (**Table 2**). **Figure 6** shows a series of representative ESI MS/MSs of psychosine in lipid extracts from spinal cord, cortex, cerebellum, and brain stem acquired by using NL scanning of 180 amu of 1-month-old mice. The results from mice at 1 month of age are in good agreement with those from other studies using different methods (12, 14), although determination of the psychosine content in brain samples of 5.5-month-old mice was not found in the literature. Intriguingly, the distribution of psychosine closely paralleled the expression levels of sphingosine-1-phosphate receptors (30–33), indicating a correlation of psychosine with its receptor distribution.

It has been well recognized that diabetes mellitus is a major risk factor for dementia, including Alzheimer's disease (34–38). We suspect that the altered psychosine content may contribute to the development of dementia owing to the role of psychosine in signal transduction as discussed earlier. In the study, we determined the potential variation of psychosine in brain tissue samples from mice

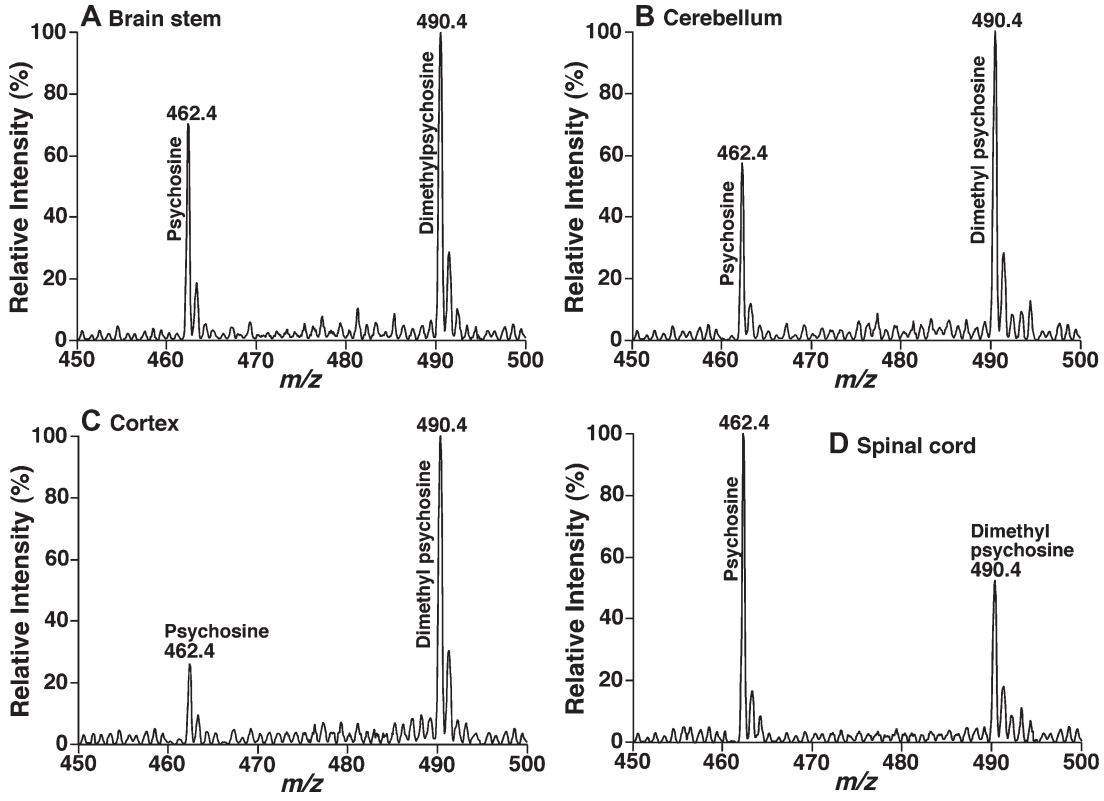


Fig. 6. ESI NL MS/MS of psychosine in lipid extracts of mouse spinal cord, cortex, cerebellum, and brain stem. Total lipids of brain stem, cerebellum, cortex, and spinal cord of mice at 1 month of age were extracted by using a modified procedure of Bligh and Dyer against 10 mM ammonium formate containing 5% ammonium hydroxide aqueous solution (pH 11). Each lipid extract was separately treated with alkaline methanolysis as described under Materials and Methods. MS/MS of lipid extracts of mouse brain stem (A), cerebellum (B), cortex (C), and spinal cord (D) were acquired using NL scanning of 180 u. The collision gas pressure was set at 1.0 mTorr, and a collision energy of 24 eV was employed in the analyses. A 5 min period of signal averaging of the scans acquired at a scanning rate of 500 u/s in the profile mode was employed for each MS/MS.

TABLE 2. Quantitative analysis of psychosine in the lipid extracts from brain samples of wild-type and diabetic mice

Brain Tissue	Wild-type (1 month)	Wild-type (5.5 months)	Diabetes (5.5 months)
		pmol/mg protein	
Spinal cord	3.79 ± 0.11	12.47 ± 0.85	13.46 ± 0.45
Cortex	0.87 ± 0.09	4.19 ± 0.17	4.05 ± 0.20
Cerebellum	1.29 ± 0.12	4.44 ± 0.39	4.47 ± 0.23
Brain stem	1.59 ± 0.14	7.36 ± 0.34	7.31 ± 0.15

Total lipids of each biological sample were extracted by using a modified Bligh and Dyer method in the presence of 10 mM ammonium formate containing 5% ammonium hydroxide in the aqueous phase (pH 11) as described under Materials and Methods. Part of each lipid extract was subjected to alkaline methanolysis as previously described (20) prior to MS analysis. Positive-ion tandem mass spectrometric analyses of psychosine in the lipid extracts were performed by coordinately scanning the first and third quadrupoles with a mass difference of 180 amu in the mass range of interest, whereas collisional activation was performed in the second quadrupole with activation energy of 24 eV. Data represent the means ± SD of at least four separate animals.

in the diabetic state induced by streptozotocin treatment for 6 weeks. Therefore, the age of the euthanized mice was 5.5 months. We found that streptozotocin-induced diabetes did not cause altered content of psychosine in mouse nerve tissue samples (Table 2).

Summary

We synthesized and characterized an analog of psychosine, which can be used as an internal standard for quantitation of psychosine. Moreover, we extended shotgun sphingolipidomics to the quantitation of psychosine present in crude lipid extracts treated with alkaline methanolysis. Ratiometric comparison of ion peak intensities between psychosine and *N,N*-dimethyl psychosine in the mass spectra of NL scanning of 180 amu in the positive-ion mode was conducted for quantitation. A broad linear dynamic range for the quantitation of psychosine and a limit of detection at a concentration of low fmol/μl were demonstrated using this approach. The developed approach is generally much simpler and more efficient than other previously reported methods. A potential limitation of this method is the possible interference of hexose-containing lipid molecular species possessing an identical *m/z* to psychosine or *N,N*-dimethyl psychosine. However, such interference owing to overlapping hexose-containing lipid molecular species in mammalian samples is considered negligible. Accordingly, we anticipate that this methodology will be very useful in a variety of physiological and pathophysiological studies related to sphingolipidomics.

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