

Quantification of galactosylsphingosine in the twitcher mouse using electrospray ionization-tandem mass spectrometry

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Abstract Globoid cell leukodystrophy (Krabbe disease) is an autosomal recessive inherited neurodegenerative disorder caused by the deficiency of the lysosomal enzyme β -galactosylceramidase. The pathogenesis of the disorder has been proposed to arise from the accumulation of the cytotoxic metabolite galactosylsphingosine (psychosine). The twitcher mouse is a naturally occurring murine model of globoid cell leukodystrophy. We have developed a rapid, sensitive, and specific mass spectrometric method for determining the galactosylsphingosine concentration in the tissues of twitcher mice. Galactosylsphingosine is extracted from the tissues in methanol, isolated using strong cation-exchange and C18 solid-phase extraction chromatography, and then directly analyzed using electrospray ionization-tandem mass spectrometry. A lactosylsphingosine internal standard has been employed for quantification. The assay demonstrated significant accumulation of galactosylsphingosine in the brain, spinal cord, and kidney of twitcher mice. It is anticipated that this method may be of use in the monitoring of experimental therapies for globoid cell leukodystrophy.—Whitfield, P. D., P. C. Sharp, R. Taylor, and P. Meikle. Quantification of galactosylsphingosine in the twitcher mouse using electrospray ionization-tandem mass spectrometry. *J. Lipid Res.* 2001, 42: 2092–2095.

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Globoid cell leukodystrophy (Krabbe disease) is an autosomal recessive disorder caused by the deficiency of the lysosomal enzyme β -galactosylceramidase (EC 3.2.1.46) (1). The disease is characterized by the degeneration of oligodendroglia and progressive demyelination of the central and peripheral nervous system. Clinically, the disease manifests in early infancy and results in complete neurological dysfunction that often leads to death by 2 years of age (2).

In globoid cell leukodystrophy galactosylceramide, the primary natural substrate of the deficient enzyme does not accumulate in the tissues of affected individuals. Rather,

the pathogenesis of the disease has been proposed to arise from the storage of a cytotoxic metabolite, galactosylsphingosine (psychosine), which is also a substrate for the missing enzyme (3). The twitcher mouse is an authentic murine model of globoid cell leukodystrophy that is genetically and enzymatically equivalent to the human form of the disease and displays similar pathological features (4, 5). Studies have also demonstrated a marked accumulation of galactosylsphingosine in the brain and other tissues of twitcher mice (6–9).

A number of methods previously have been employed for the analysis of galactosylsphingosine and related compounds from tissues (6–12). These methods have typically involved the solvent extraction of galactosylsphingosine, and then isolation and derivatization prior to detection by either TLC or HPLC. In recent years, mass spectrometers have become more common in metabolic laboratories and have been applied to the study of lysoglycosphingolipids (13, 14). In this study, we describe the development of a rapid, sensitive, and specific method using electrospray ionization-tandem mass spectrometry (ESI-MS/MS) to characterize and quantify underivatized galactosylsphingosine from the tissues of twitcher mice.

MATERIALS AND METHODS

Materials and reagents

Solvents were analytical grade. Galactosylsphingosine and lactosylsphingosine (lyso-lactosyl ceramide) were purchased from Matreya Inc. (Pleasant Gap, PA). [6-³H]galactosylsphingosine (psychosine, [galactose-6-³H]) was obtained from American

Abbreviations: ESI-MS/MS, electrospray ionization-tandem mass spectrometry; ISTD, internal standard; MRM, multiple-reaction monitoring; *m/z*, mass to charge ratio; SCX, strong cation-exchange.

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Radiolabeled Chemicals, Inc. (St. Louis, MO) and used after purification. Supelclean™ strong cation-exchange (SCX) solid-phase extraction cartridges (sodium form, 500 mg/3 ml) were purchased from Supelco (Bellefonte, PA). Bond Elut® end-capped C18 solid-phase extraction cartridges (500 mg/6 ml) were purchased from Varian, Inc. (Harbor City, CA).

Mouse tissues

A colony of twitcher mice was maintained under Animal Ethics Committee approval at the University of Sydney Veterinary School. The genetic status of individual mice was determined by PCR on DNA extracted from the clipped toe or tail at post-natal day 5. After the animals were decapitated, whole brain, spinal cord, liver, spleen, and kidney were dissected. Wet weights were measured and the tissues were then stored at -70°C until use.

Isolation and extraction of galactosylsphingosine

A maximum of 100 mg of wet weight tissue was homogenized with an Ultra Turrax (IKA) T25 Basic mechanical homogenizer in 3 ml methanol. After a minimum of 1 h at room temperature, denatured solids were pelleted by centrifugation. Five nanomoles of lactosylsphingosine was added to the methanol extract supernatant as an internal standard (ISTD). Galactosylsphingosine was then isolated using a modified form of the method described by Igisu and Suzuki (11). A SCX solid-phase extraction cartridge (500 mg/3 ml) was washed with 3 ml methanol after which the methanol tissue extract was applied to the column. The column was then successively washed to waste with 5 ml methanol, 5 ml chloroform–methanol 2:1 (v/v), and 5 ml methanol. Cationic lipids were then eluted with 5 ml methanol–0.4 M CaCl_2 3:1 (v/v). This eluate was then desalted using an endcapped C18 solid-phase extraction cartridge (500 mg/6 ml) that had been primed with 5 ml methanol followed by 5 ml distilled water. The methanol–0.4 M CaCl_2 3:1 (v/v) fraction was then directly applied to the column after which the column was washed with 15 ml distilled water before elution of the galactosylsphingosine with 7.5 ml chloroform–methanol 1:2 (v/v). The chloroform–methanol 1:2 (v/v) fraction was dried under nitrogen gas with gentle heat and reconstituted in 100–200 μl methanol containing 5 mM ammonium formate.

ESI-MS/MS of galactosylsphingosine

All mass spectrometric analyses were performed by ESI-MS/MS using a Perkin-Elmer Sciex API 365 triple-quadrupole mass spectrometer with an Ionspray source and LC Tune/Multiview data system (PE Sciex, Concord, Ontario, Canada). Galactosylsphingosine and the lactosylsphingosine ISTD were identified by precursor ion scanning for mass to charge ratio (m/z) 282 in the positive ion mode. Optimization of ESI-MS/MS parameters was achieved with 1 μM solutions of galactosylsphingosine and lactosylsphingosine. Tissues and calibration samples were directly infused into the electrospray source via a fused silica transfer line using a Harvard Apparatus syringe pump at a flow rate of 10 $\mu\text{l}/\text{min}$.

Quantification of galactosylsphingosine was performed using the multiple-reaction monitoring (MRM) mode. For each quantitative measurement, the ions relating to galactosylsphingosine (m/z 462) and the lactosylsphingosine ISTD (m/z 624) were monitored for 100 ms each, and 100 consecutive scans were averaged. Galactosylsphingosine calibration curves were constructed by adding varying amounts of galactosylsphingosine standard (0–8 nmol) and a constant amount of the lactosylsphingosine (5 nmol) to the brain lipid extracts from normal mice (equivalent to 100 mg wet weight tissue), which were then carried through the entire isolation and purification procedure. The response ratio of the galactosylsphingosine to the ISTD was calculated,

and the concentration of the galactosylsphingosine was determined by comparison to a calibration curve.

RESULTS

Recovery of galactosylsphingosine from twitcher tissues

Purified [$6\text{-}^3\text{H}$]galactosylsphingosine was added to a lipid extract of a twitcher mouse brain that was equivalent to 100 mg wet weight tissue. Five nanomoles of the lactosylsphingosine ISTD was also added. The recovery of the [$6\text{-}^3\text{H}$]galactosylsphingosine through the SCX and C18 isolation steps was then calculated. Recovery data were obtained from three repeat experiments, and the recovery was estimated to be 45–60%, a finding consistent with previous reports (8, 11).

ESI-MS/MS of galactosylsphingosine

The free amino group of galactosylsphingosine ionizes very efficiently, making it highly amenable to analysis by ESI-MS/MS. In positive ion mode, collision-induced dissociation of galactosylsphingosine gave rise to a major product ion at m/z 282 and a minor product ion at m/z 264. These ions result from the loss of one or two water molecules from the sphingosine backbone, respectively, and the elimination of the galactose moiety. Lactosylsphingosine was found to fragment in an identical manner. Therefore, galactosylsphingosine and the ISTD were characterized by means of a precursor ion scan for m/z 282.

Figure 1A shows the galactosylsphingosine profile from the brain of a control mouse obtained by ESI-MS/MS. Galactosylsphingosine (m/z 462) was barely detectable. The intense ion at m/z 624 corresponds to the lactosylsphingosine ISTD. In contrast, examination of the brain lipid extract from a twitcher mouse showed a marked accumulation of galactosylsphingosine (Fig. 1B). The mass spectrum was dominated by the $[\text{M}+\text{H}]^+$ ion of galactosylsphingosine. The calibration curve of galactosylsphingosine was found to be linear over the range typically encountered in control and twitcher mice tissues (0–8 nmol, regression coefficient = 0.986).

To access reproducibility of the complete assay from batch to batch, replicate analysis of a single lipid extract from a postmortem brain of a patient with globoid cell leukodystrophy was performed on 15 separate occasions. The interbatch coefficient of variation for the galactosylsphingosine concentration in the brain was 12.1%. In addition, the reproducibility of the assay within a batch was also assessed. Simultaneous analysis of the brain extract ($n = 5$) produced an intrabatch coefficient of variation of 4.1%. The reproducibility of the ESI-MS/MS was also examined. Five separate infusions of the same sample into the tandem mass spectrometry produced a coefficient of variation of 1.2%. The margin of error would be expected to be greater in control and somatic tissues where the concentration of galactosylsphingosine is extremely low. ESI-MS/MS analysis of galactosylsphingosine demonstrated a lower limit of detection of 1–5 ng/100 mg wet weight tissue.

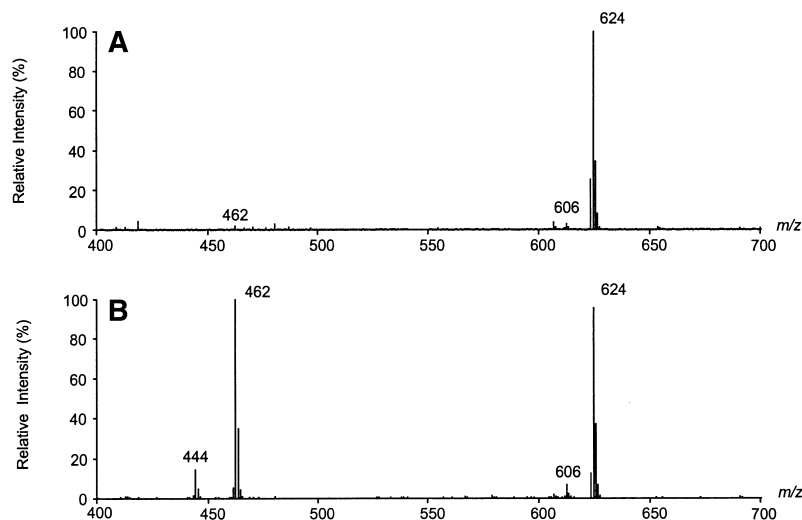


Fig. 1. Mass spectra of galactosylsphingosine from the brain of a control mouse (A) and a twitcher mouse (B) at postnatal day 40. Galactosylsphingosine (m/z 462) and the lactosylsphingosine ISTD (m/z 624) were detected in the positive ion mode by means of a precursor ion scan for m/z 282.

There was a significant accumulation of galactosylsphingosine in the liver, kidney, spleen, brain, and spinal cord of twitcher mice. A small quantity of galactosylsphingosine was also detected in the tissues of control mice. As previously reported in twitcher mice, the concentration of galactosylsphingosine was much greater in the nervous tissues than in the somatic organs (7, 9). Among the somatic organs, the kidney accumulated larger amounts of galactosylsphingosine than the liver or spleen (Table 1).

In brain and spinal cord of twitcher mice, the accumulation of galactosylsphingosine increased with age (Table 2), a finding consistent with previous studies (6, 8). The absolute concentrations of galactosylsphingosine were greatest in the spinal cord; however, interestingly, between the ages of 25 and 40 days, the rate of accumulation of galactosylsphingosine was highest in the brain of the twitcher mice (Table 2).

DISCUSSION

The determination of galactosylsphingosine in tissues of twitcher mice was originally described by Igisu and Suzuki (11). Their elegant method made use of SCX and C18 reverse-phase chromatography to isolate galactosylsphingosine after tissue lipid extraction. The galactosylsphingosine was then derivatized, chromatographed on thin-layer plates, and detected using fluorescence densito-

TABLE 1. Galactosylsphingosine concentration in somatic organs of twitcher mice

Tissue	Galactosylsphingosine Concentration ^a			
	Control		Twitcher	
	<i>ng/100 mg wet weight tissue</i>			
Liver	6	6	55	58
Kidney	7	5	94	91
Spleen	10	10	60	47

^a Samples were analyzed from two twitcher mice and two control mice at postnatal day 40.

metry. In later studies, derivatized galactosylsphingosine was detected by HPLC (7–9). To the best of the authors' knowledge, this is the first report of the quantification of galactosylsphingosine using ESI-MS/MS. This procedure is rapid, selective, reproducible, and sensitive.

In our method, we adapted and simplified the original isolation procedures. These improvements enable larger numbers of tissues to be prepared more rapidly than conventional procedures. We removed the need for a time-consuming postextraction rotary evaporation step, and made use of commercially available solid-phase extraction cartridges. Furthermore, we took advantage of the selectivity and sensitivity of ESI-MS/MS to detect galactosylsphingosine, eliminating the need for derivatization. This need for sensitivity is vital because even in the disease state, the absolute amount of galactosylsphingosine that accumulates in tissues is very small. For analysis, we routinely isolated galactosylsphingosine from 100 mg wet weight neural or somatic tissues for both control and twitcher mice. However, for twitcher mice brain and spinal cord, considerably smaller quantities of tissue could be reliably used.

TABLE 2. Galactosylsphingosine concentration in the central nervous system of twitcher mice

Days After Birth	Galactosylsphingosine Concentration ^a			
	Brain		Spinal Cord	
	Control	Twitcher	Control	Twitcher
	<i>ng/100 mg wet weight tissue</i>			
25	7	674	99	5484
	6	798	48	4154
30	— ^b	1313	—	5800
	—	1030	—	6093
35	—	1489	—	7092
	—	1201	—	4837
40	15	1243	122	5542
	11	1547	108	7739

^a Galactosylsphingosine was analyzed from two twitcher and two control mice of various ages.

^b Not determined.

Classical methods of lipid extraction such as the method of Folch et al. (15) are unable to partition galactosylsphingosine into either the chloroform or methanol/water phase. As a result, it is necessary to isolate galactosylsphingosine from all tissue components including other bulk lipids, using the specificity of SCX chromatography. We used commercially available SCX solid-phase extraction columns (sodium form). These cartridges avoided the problems typically associated with the preparation of ion exchange resins, such as variable packing density, pH, and stability, and were found to give highly reproducible recoveries of galactosylsphingosine from twitcher mouse tissues. For the isolation of galactosylsphingosine, it is important to apply lipid extracts to SCX columns in methanol. The presence of significant quantities of chloroform and/or water in the extraction solvents has been reported to result in considerable losses of galactosylsphingosine from SCX columns (11). Previous studies (8, 9) have typically involved homogenizing the tissues with water followed by solvent extraction with chloroform–methanol 2:1 (v/v). The water and solvents are then evaporated to dryness using a rotary evaporator. In this study, we found that galactosylsphingosine was satisfactorily extracted from twitcher tissues into methanol. Our experiments demonstrated that chloroform–methanol 2:1 (v/v) did not extract significantly more galactosylsphingosine from twitcher mouse tissues than methanol. In addition, our experiments confirmed the finding of Igisu and Suzuki (11) that the size of the total lipid sample was important for the efficient retention of psychosine on the SCX cartridge. Varying amounts of galactosylsphingosine were found to pass through the SCX column when the lipids extracted from more than 100 mg wet weight tissues were applied (data not shown).

Quantification of the galactosylsphingosine was achieved by the use of pure commercially available lactosylsphingosine as an ISTD, which was added at the extraction stage to account for any losses throughout the preparation. This analog was found to behave in a similar manner to galactosylsphingosine both throughout the extraction and purification procedure and when analyzed by ESI-MS/MS. However, due to the differences in the ionization efficiencies, it was found that the signal intensity of equimolar concentration of galactosylsphingosine was typically 1.5 times greater than that of the lactosylsphingosine ISTD. We believe that this difference arises from the longer carbohydrate moiety of the lactosylsphingosine. Ideally, a stable isotope of galactosylsphingosine would have been used as an ISTD, but this is not commercially available and is difficult to produce outside of specialist laboratories (16).

Our findings demonstrated that the highest concentrations of galactosylsphingosine were in the brain and spinal cord of the twitcher mice. The absolute concentrations of galactosylsphingosine in the neural tissues were found to be in close agreement with those determined by conventional HPLC methods (7–9). A clear increase in galactosylsphingosine accumulation in the brain and spinal cord of the mice with age was also observed. The percentage increase of galactosylsphingosine accumulation was found to be greater in brain than in spinal cord of twitcher mice (Table 2).

In conclusion, we described a tandem mass spectrometric method for the accurate quantification of galactosylsphingosine in the tissues of twitcher mice. It is anticipated that this method could be employed to analyze a wide range of lysoglycosphingolipids in various biological matrices, including cultured neuronal cells. In addition, this method could be further developed to analyze galactosylsphingosine in plasma, urine, or cerebrospinal fluid to monitor the efficacy of potential therapies currently being developed for globoid cell leukodystrophy. ■■

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REFERENCES

1. Suzuki, K., and Y. Suzuki. 1970. Globoid cell leukodystrophy (Krabbe's disease): deficiency of galactocerebroside β -galactosidase. *Proc. Natl. Acad. Sci. USA*. **66**: 302–309.
2. Suzuki, K., Y. Suzuki, and K. Suzuki. 1995. Galactosylceramide lipidosis: globoid cell leukodystrophy (Krabbe disease). In *The Metabolic and Molecular Basis of Inherited Disease*. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2671–2692.
3. Miyatake, T., and K. Suzuki. 1972. Globoid cell leukodystrophy: additional deficiency of psychosine galactosidase. *Biochim. Biophys. Acta*. **48**: 538–543.
4. Duchon, L. W., E. M. Eicher, J. M. Jacobs, F. Scaravilli, and F. Teixeira. 1980. Hereditary leukodystrophy in the mouse: the new mutant twitcher. *Brain*. **103**: 695–710.
5. Kobayashi, T., T. Yamanaka, J. M. Jacobs, F. Teixeira, and K. Suzuki. 1980. The twitcher mouse: an enzymatically authentic model of human globoid cell leukodystrophy (Krabbe disease). *Brain Res*. **202**: 479–483.
6. Igisu, H., and K. Suzuki. 1984. Progressive accumulation of toxic metabolite in a genetic leukodystrophy. *Science*. **224**: 753–755.
7. Kobayashi, T., H. Shinoda, I. Goto, T. Yamanaka, and Y. Suzuki. 1987. Globoid cell leukodystrophy is a generalized galactosylsphingosine (psychosine) storage disease. *Biochim. Biophys. Acta*. **144**: 41–46.
8. Shinoda, H., T. Kobayashi, M. Katayama, I. Goto, and H. Nagara. 1987. Accumulation of galactosylsphingosine (psychosine) in the twitcher mouse: determination by HPLC. *J. Neurochem*. **49**: 92–99.
9. Nozawa, M., T. Iwamaoto, T. Tokoro, and Y. Eto. 1992. Novel procedure for measuring psychosine derivatives by an HPLC method. *J. Neurochem*. **59**: 607–609.
10. Svennerholm, L., M-T. Vanier, and J-E. Mansson. 1980. Krabbe disease: a galactosylsphingosine (psychosine) lipidosis. *J. Lipid Res*. **21**: 53–64.
11. Igisu, H., and K. Suzuki. 1984. Analysis of galactosylsphingosine (psychosine) in the brain. *J. Lipid Res*. **25**: 1000–1006.
12. Orvisky, E., E. Sidransky, C. E. McKinney, M. E. LaMarca, R. Samimi, D. Krasnewich, B. M. Martin, and E. I. Ginns. 2000. Glucosylsphingosine accumulation in mice and patients with type 2 gaucher disease begins early in gestation. *Pediatr. Res*. **48**: 233–237.
13. Hara, A., and T. Taketomi. 1986. Chemical properties of heterogeneous long chain bases in lysosphingolipids by positive ion fast atom bombardment mass spectrometry and carbon-13 NMR spectroscopy. *J. Biochem*. **100**: 415–423.
14. Taketomi, T., A. Hara, K. Uemura, and E. Sugiyama. 1996. Rapid method of preparation of lysoglycosphingolipids and their confirmation by delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *J. Biochem*. **120**: 573–579.
15. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*. **226**: 497–509.
16. Duffin, G. R., G. J. Ellamas, S. Hartmann, J. M. Herbert, and D. I. Smith. 2000. Practical synthesis of [^{13}C]- and [^{14}C]-labelled glycosphingolipids. *J. Chem. Soc. Perkin Trans. 1*: 2237–2242.