methods

Simultaneous quantification of lyso-neutral glycosphingolipids and neutral glycosphingolipids by *N*-acetylation with [^{3}H]acetic anhydride

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Abstract **We describe a new method that permits quantification in the pmol to nmol range of three lyso-neutral glycosphingolipids (lyso-n-GSLs), glucosylsphingosine (GlcSph), galactosylsphingosine (GalSph), and lactosylsphingosine, in the same sample as neutral glycosphingolipids (n-GSLs). Lyso-n-GSLs and n-GSLs are initially obtained from a crude lipid extract using Sephadex G25 chromatography, followed by their isolation in one fraction, which is devoid of other contaminating lipids, by aminopropyl solid-phase chromatography. Lyso-n-GSLs and n-GSLs are subsequently separated from one another by weak cation exchange chromatography. N-GSLs are then deacylated by strong alkaline hydrolysis, and the *N*-deacylated-GSLs and lyso-n-GSLs are subsequently *N*-acetylated using [^{3}H]acetic anhydride. An optimal concentration of 5 mM acetic anhydride was established, which gave >95% *N*-acetylation. We demonstrate the usefulness of this technique by showing an ~40-fold increase of both GlcSph and glucosylceramide in brain tissue from a glucocerebrosidase-deficient mouse, as well as significant lactosylceramide accumulation.** The application and optimization of this technique for lyso-n-GSLs and lyso-GSLs will permit their quantification in small amounts of biological tissues, particularly in the GSL storage diseases, such as Gaucher and Krabbe's disease, in which GlcSph and GalSph, respectively, accumulate.—Bodennec, J., S. Trajkovic-Bodennec, and A. H. Futerman. **Simultaneous quantification of lyso-neutral glycosphingolipids and neutral glycosphingolipids by *N*-acetylation with [^{3}H]acetic anhydride. *J. Lipid Res.* 2003.** 44: **1413–1419.**

Supplementary key words glucosylceramide • galactosylceramide • lactosylceramide • Gaucher disease • Krabbe's disease

Lyso-neutral glycosphingolipids (lyso-n-GSLs), including glucosylsphingosine (GlcSph) and galactosylsphingosine (GalSph), occur in small but significant quantities in biological tissues, and a number of roles have been ascribed to them, including modulation of enzyme activities (1–5) and modulation of calcium release from intracellular stores (6, 7). GalSph has also been shown to inhibit cytokenesis (8) and to act as an agonist of an orphan G protein-coupled receptor (9). Lyso-n-GSLs accumulate at high levels in the sphingolipidoses, and GlcSph and GalSph may be involved in disease pathogenesis in Gaucher and Krabbe's disease, respectively (10–18).

The most common method for lyso-n-GSL quantification is derivatization of the free amino group by *o*-phthalaldehyde (19), followed by separation by high-performance liquid chromatography (HPLC). However, resolution of GalSph from GlcSph is often difficult using this method (20), although better resolution can be achieved by modification of chromatographic conditions (18). GalSph can also be quantified after derivatization with 4-fluoro-7-nitrobenzofurazan (15, 17, 21), and recently, electrospray ionization-tandem mass spectrometry has also been used for GalSph quantification (22). Other methods include derivatization with dansyl chloride (23), fluorescamine (24), or dimethylaminoazobenzene sulphonyl chloride (25). However, there are limitations with each of these methods, including processing of multiple samples, multiple analytical steps, lack of sensitivity, and use of expensive equipment.

We now describe a new method for lyso-n-GSL quantification, and quantification of glycosphingolipids (GSLs) after their *N*-deacylation, involving derivatization with [^{3}H]acetic anhydride, which has previously been used to quantify sphingosine (26), sphingosine-1-phosphate (27), and sphingosylphosphorylcholine (28), but has not been optimized for quantitative determination of lyso-n-GSLs (28). We have optimized various parameters, including purification of lyso-n-GSLs and neutral GSLs (n-GSLs) by solid-phase extraction, conditions of derivatization, extent of recovery, and resolution of the *N*-acetylation products by

Abbreviations: C2-*N*-GSL, *N*-acetyl-GSL; GalSph, galactosylsphingosine; GlcSph, glucosylsphingosine; GSL, glycosphingolipid; LacCer, lactosylceramide; LacSph, lactosylsphingosine; lyso-n-GSL, lyso-neutral glycosphingolipid; n-GSL, neutral glycosphingolipid.

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TLC. The suitability of this method for use with biological tissues was demonstrated by quantification of GlcSph and glucosylceramide (GlcCer) in brains of an animal model of Gaucher disease (29).

MATERIALS AND METHODS

Materials

Sephadex G-25 (superfine), galactose oxidase (*Dactylium dendroides*, 220 U/mg), 1-β-D-GlcSph, 1-β-D-GalSph, sphingosylphosphorylcholine, glucosylceramide (GlcCer), and galactosylceramide (GalCer) were from Sigma. D-lactosyl-β1-1'-D-*erythro*-sphingosine (LacSph) and lactosylceramide (LacCer) were from Avanti Polar Lipids (Alabaster, AL). An n-GSL mixture (containing ceramide monohexosides, ceramide dihexosides, ceramide trihexosides, and globosides) was from Matreya (Pleasant Gap, PA). Aminopropyl (LC-NH2, 100 mg), and weak cation exchange (LC-WCX, 100 mg) solid-phase extraction cartridges were from Supelco (Bellefonte, PA). Silica gel 60 TLC plates and sodium tetraborate were from Merck (Darmstadt, Germany). NaB[^{3}H]$_4$ (specific activity of 25 Ci/mmol) and [^{3}H]acetic anhydride (specific activity of 9.7 Ci/mmol) were from Amersham (Little Chalfont, UK). All solvents were of analytical grade and were purchased from Biolab (Jerusalem, Israel).

Animals

A breeding pair of the *Gba* mouse model of Gaucher disease (29) was obtained from Jackson Laboratories (Bar Harbor, ME) and bred in the Experimental Animal Center of the Weizmann Institute of Science. Individual embryos were genotyped (30). Cortices were removed from individual embryos on embryonic day 17 and lipids extracted as described below.

Lipids

D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph were synthesized by oxidation of GalCer and LacCer, respectively, by galactose oxidase, reduction of the resulting aldehyde by NaB[^{3}]H$_4$ (31), alkaline hydrolysis [100°C, 3 h in butanol:10 M KOH (9:1; v/v)] (32) to *N*-deacylate the GSLs, and purification by Unisil column chromatography and TLC (33), to give a final specific activity of 15 Ci/mmol. [6-^{3}H]GalCer and [6-^{3}H]LacCer were synthesized identically except that they were not *N*-deacylated. C2-GlcCer (*N*-acetyl-glucosylsphingosine), C2-GalCer (*N*-acetyl-galactosylsphingosine), and C2-LacCer (*N*-acetyl-lactosylsphingosine) standards were synthesized by *N*-acetylation of the respective lyso-n-GSLs with acetic anhydride as described below.

Extraction and purification of lyso-n-GSLs

Cortices (~50 mg of tissue) from Gba mouse brains were homogenized in 5 ml chloroform-methanol (2:1; v/v) and aliquots removed for protein determination (34). After 2 h at room temperature, homogenates were centrifuged (20 min/700 g_{av}) and the pellet reextracted in 5 ml of methanol for 1 h. After further centrifugation, the methanol extract was combined with the initial chloroform-methanol extract and dried under N_2. The crude lipid extract was resuspended in 1 ml of chloroform-methanol-water (60:30:4.5; v/v/v). A schematic diagram of the methods described in the subsequent sections is given in **Fig. 1**.

Lipids were washed on a Sephadex G-25 column (35), which was preferred to strong cation exchange chromatography (23), as lyso-n-GSLs and n-GSLs were recovered in the same fraction with high recovery. This method (35) has been used to purify GlcSph from Gaucher brains (11), and was now modified to give optimum recovery of lyso-n-GSLs and n-GSLs. A polypropylene

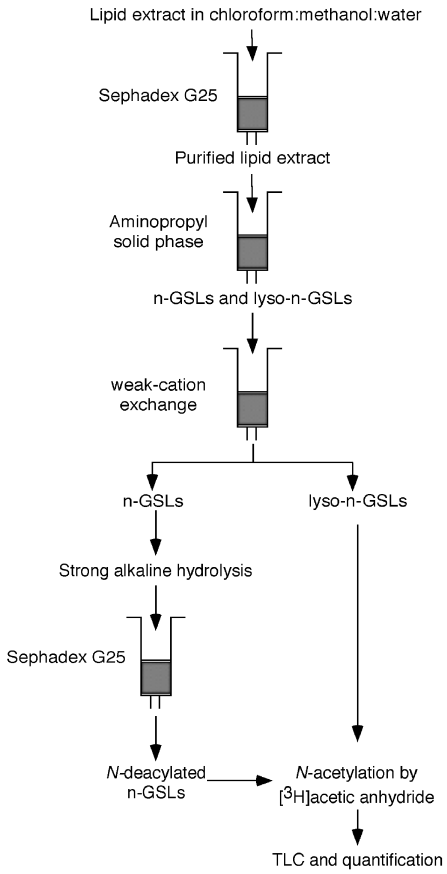

Fig. 1. Schematic diagram of the method used to purify neutral glycosphingolipids (n-GSLs) and lyso-neutral glycosphingolipids (lyso-n-GSLs) for quantification using [^{3}H]acetic anhydride. See text for further details.

tube (1 × 6.5 cm) was loaded with Sephadex G-25 in chloroform-methanol-water (60:30:4.5; v/v/v) to a bed volume of 1 ml, the column washed with the same solvent, the crude lipid extract applied, and lyso-n-GSLs and n-GSLs recovered by eluting with 3 ml of chloroform-methanol-water (60:30:4.5; v/v/v) (see **Fig. 2A, B**). The eluate was dried under N_2 and resuspended in 200 μl chloroform-methanol (23:1; v/v). The extent of recovery of lyso-n-GSLs from the Sephadex column was determined by applying D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph (20,000 dpm) with increasing amounts of GalSph and LacSph directly to the column, eluting, and TLC on a borated TLC plate [(36) and see Fig. 2A].

Lyso-n-GSLs and n-GSLs were subsequently purified by aminopropyl solid-phase extraction as recently described (36, 37). Samples were dried under N_2 prior to their separation by weak cation exchange column chromatography.

Weak cation exchange columns (100 mg) were preconditioned using 1 ml hexane, followed by 2 ml of 0.5 M acetic acid in methanol, 4 ml methanol, and finally 4 ml hexane. Lyso-n-GSLs and n-GSLs were resuspended in 200 μl chloroform and loaded on the column. N-GSLs were eluted using 3 ml of chloroform-methanol (9:2.5; v/v) (Fraction 1, see Fig. 2C), and lyso-n-GSLs subsequently eluted in 2 ml of 1 M acetic acid in methanol (Fraction 2, see Fig. 2D). The extent of recovery of n-GSLs in Fraction 1 was determined by applying [6-^{3}H]GalCer and [6-^{3}H]LacCer (20,000 dpm) with increasing amounts of GalCer and LacCer directly to the column, eluting, and TLC using chloroform-methanol-water (65:25:4; v/v/v) as the developing solvent. The extent of recovery of lyso-n-GSLs in Fraction 2 was determined using D-[6-^{3}H]Gal-

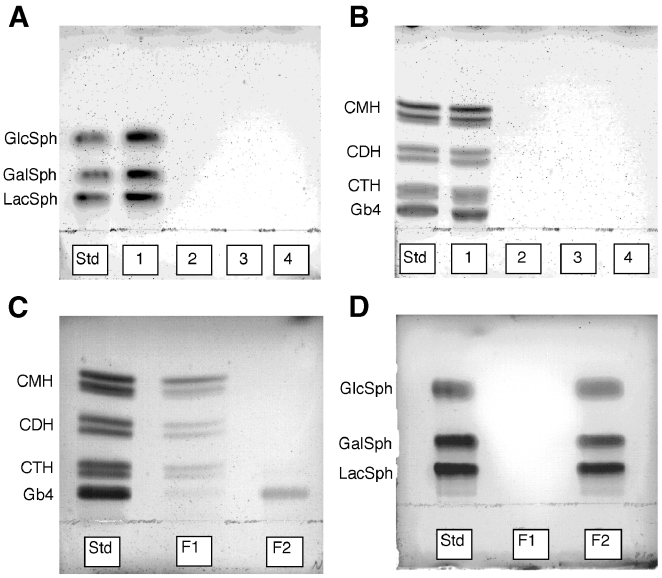

Fig. 2. Recovery of lyso-n-GSLs and n-GSLs by Sephadex G-25 chromatography and separation of n-GSLs from lyso-n-GSLs by weak cation exchange chromatography. A: Lyso-n-GSLs (15 μg each) were applied to a Sephadex G-25 column, eluted in successive fractions of 3 ml chloroform-methanol-water (60:30:4.5; v/v/v), separated by TLC using chloroform-acetone-methanol-acetic acid-water (50:20:10:15:5; v/v/v/v/v) run two-thirds the length of the plate, followed by chloroform-methanol-water-NH_4OH (25% v/v) (20:20:2:0.35; v/v/v/v) as the developing solvents, and lipids visualized using orcinol. B: N-GSLs were loaded on another Sephadex G-25 column and eluted as in (A), separated by TLC using chloroform-methanol-water (65:25:4; v/v/v), and lipids visualized using orcinol. C: N-GSLs were eluted from a weak cation exchange column (Fraction 1, F1) using 3 ml of chloroform-methanol (9:2.5; v/v), and lyso-n-GSLs eluted (Fraction 2, F2) using 2 ml of 1 M acetic acid in methanol. Lipids were separated using the same solvent system as in B, and visualized using orcinol. D: Lyso-n-GSLs (15 μg each) were separated on a weak cation exchange column as in C, and separated by TLC as in A. Std., standards; CMH, ceramide monohexosides; CDH, ceramide dihexosides; CTH, ceramide trihexosides; GalSph, galactosylsphingosine; Gb4, globosides; GlcSph, glucosylsphingosine; LacSph, lactosylsphingosine.

Sph and D-[6-^{3}H]LacSph and increasing amounts of GalSph and LacSph. The n-GSLs eluted in Fraction 1 were *N*-deacetylated for subsequent quantification by strong alkaline hydrolysis using 1 M KOH-methanol [100°C, (38)]. After 24 h, the mixture was neutralized with 10 M HCl and adjusted to a ratio of 60:30:4.5 (v/v/v) chloroform-methanol-water. *N*-deacylated-GSLs were desalted using Sephadex G-25 as above, dried under N_2, and quantified together with the original lyso-n-GSLs. Note that the use of Sephadex G-25 rather than dialysis (38) significantly increased the recovery of lyso-n-GSLs after hydrolysis, whereas C18 solid-phase extraction columns gave poor yields in the pmol range.

Derivatization of lyso-n-GSLs and *N*-deacylated GSLs, and TLC separation

Although the general procedure for derivatization of lyso-n-GSLs is based on that described for sphingosine (26), several parameters were optimized specifically for lyso-n-GSLs. Thus, both the optimal concentration of acetic anhydride and the optimal specific activity of [^{3}H]acetic anhydride (stored in a benzene solution at −80°C) were determined. This was particularly important, because traces of [^{3}H]acetic anhydride (26, 28) not removed during alkaline hydrolysis lead to high background values of ^{3}H-radioactivity. Lyso-n-GSLs, in screw-capped teflon tubes, were resuspended in 40 μl of methanolic NaOH (8 mM)-10 mM acetic anhydride in chloroform (1:1; v/v) containing 2 μCi [^{3}H]acetic anhydride. The mixture was vortexed and sonicated, and after 1 h at 37°C, the reaction was stopped by addition of 2 ml chloroform-NaOH (0.6 M) in methanol (1:1; v/v). After 1 h at room temperature, chloroform-methanol (1:1; v/v) (2 ml) was added to each tube and phase partition achieved by adding 1.8 ml of aqueous NaCl (1 M). The lower phase, containing the *N*-acetyl-GSLs (C2-*N*-GSLs), was washed twice with 1.8 ml chloroform-methanol-water (3:48:47; v/v/v), dried under N_2, resuspended twice in 2 ml toluene, and dried again under N_2; the toluene removed any remaining [^{3}H]acetic anhydride (39), which was particularly important when pmol amounts of lyso-n-GSLs were quantified. C2-*N*-GSLs were resuspended in chloroform-methanol (1:1; v/v) and separated on a 10 × 10 cm TLC plate (borated in 1% borate in methanol) using chloroform-methanol-water aqueous NH_4OH (7 M) (65:37:8:1; v/v/v/v) as the developing solvent, and C2-*N*-GSLs detected by iodine. Lipids were removed from the plates by scraping directly into scintillation vials, to which 1 ml methanol and 5 ml of Ultima Gold scintillation fluid (Packard, Downers Grove, IL) were added for counting in a Packard 2100 β-radiospectrometer.

RESULTS

To determine the efficacy of Sephadex G-25 chromatography, lyso-n-GSLs and n-GSLs were applied to a Sephadex G-25 column and eluted with chloroform-methanol-water (60:30:4.5; v/v/v). Lyso-n-GSLs (Fig. 2A) and n-GSLs (Fig. 2B) were eluted in the first 3 ml (Fraction 1), and subsequent fractions were devoid of lyso-n-GSLs and n-GSLs. The extent of recovery of lyso-n-GSLs was determined using D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph, together with increasing amounts (80 pmol to 6 nmol) of GalSph and LacSph, respectively; 95.8 ± 9.2% of GalSph and 90.4 ± 9.6% of LacSph were recovered in Fraction 1, with <1.5% of each in Fraction 2. The recovery of [6-^{3}H]GalCer and [6-^{3}H]LacCer was also >95%. Thus, Sephadex G-25 column chromatography gives high recovery of lyso-n-GSLs and n-GSLs in one fraction with essentially no recovery of lyso-n-GSLs and n-GSLs in other fractions, in the concentration range found in normal and pathological tissues (17). Other lipids [neutral lipids, phospholipids (35), and gangliosides (11)] were also eluted in Fraction 1, and were subsequently separated from lyso-n-GSLs and n-GSLs by aminopropyl solid-phase extraction, in which lyso-n-GSLs and n-GSLs are recovered with high yield and high purity in one fraction (Fraction 3) (36, 37).

N-GSLs (Fraction 1) and lyso-n-GSLs (Fraction 2) were next separated from each other by weak cation exchange chromatography. No lyso-n-GSLs were detected in Fraction 1 (Fig. 2D), with >95% of D-[6-^{3}H]GalSph and D-[6-^{3}H]LacSph recovered in Fraction 2. Likewise, n-GSLs were essentially quantitatively recovered in Fraction 1 (Fig. 2C) (with the exception of globoside, which eluted in Fraction 2), with >96% recovery of [6-^{3}H]GalCer and [6-^{3}H]LacCer, irrespective of the initial amount applied.

We next optimized conditions for *N*-acetylation of lyso-n-GSLs. Quantitative *N*-acetylation of GlcSph (**Fig. 3A**)

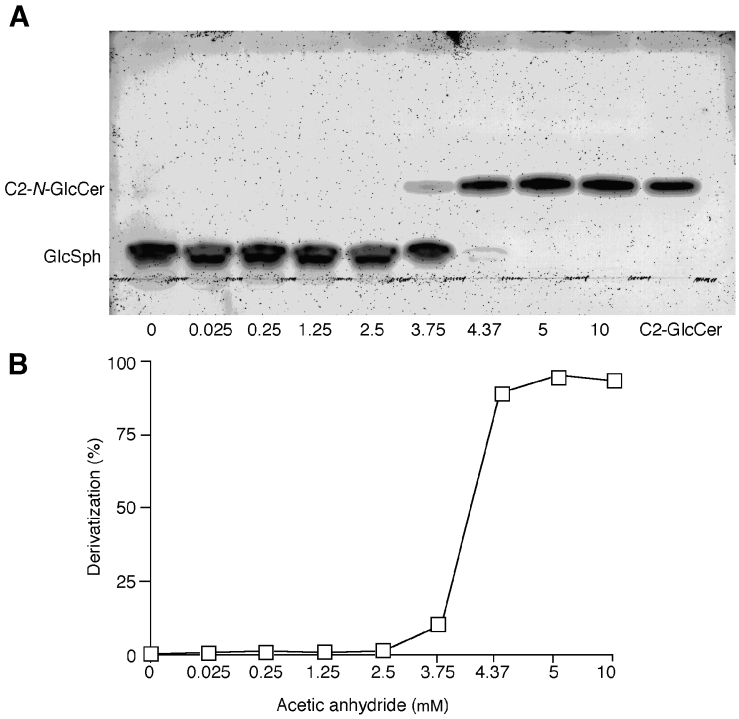

Fig. 3. Optimization of acetic anhydride concentration on lyso-n-GSL acetylation. A: GlcSph (15 μg) was derivatized with increasing concentrations of acetic anhydride. After lipid purification, lipids were separated by TLC using chloroform-methanol-water (65:25:4; v/v/v) as the developing solvent, in which GlcSph was well separated from C2-*N*-GlcCer, and lipids visualized by orcinol. B: D-[6-^{3}H]LacSph (10,000 dpm)/LacSph (2 nmol) was derivatized with increasing amounts of acetic anhydride, lipids purified and separated by TLC as in A, and *N*-acetyl-6-[^{3}H]LacSph quantified by scintillation counting.

and D-[6-^{3}H]LacSph (Fig. 3B) was achieved using 5–10 mM acetic anhydride in the reaction mixture, but at higher concentrations (i.e., mmol), some *O*-acetylation occurred, as reported for acetylation of sphingosine (26). Therefore, all subsequent *N*-acetylation reactions were performed using 5 mM acetic anhydride.

Linear and quantitative *N*-acetylation of pmol to nmol amounts of GlcSph and GalSph was obtained (**Fig. 4**). LacSph acetylation was also linear, but the percent of C2-*N*-LacCer recovered was considerably lower than that of C2-*N*-GlcCer or C2-*N*-GalCer (Fig. 4), as only ~60% of [^{3}H]C2-*N*-LacCer was recovered in the chloroform lower phase, irrespective of the amount of LacSph; in contrast, [^{3}H]C2-*N*-GalCer and [^{3}H]C2-*N*-GlcCer recovery was >95%. Some [^{3}H]acetic anhydride comigrated with C2-*N*-GlcCer, C2-*N*-GalCer, and C2-*N*-LacCer on TLC. This was particularly problematic when quantifying pmol amounts of lyso-n-GSLs, as the amount of [^{3}H]acetic anhydride (~1,000 dpm) was sufficient to interfere with lyso-n-GSL quantification. Contamination by [^{3}H]acetic anhydride could be reduced to ~200–300 dpm if the C2-*N*-GSLs were resuspended twice in toluene and dried under N_2 before TLC analysis.

C2-*N*-GlcCer, C2-*N*-GalCer, and C2-*N*-LacCer were separated from each other, and from C2-*N*-ceramide, by TLC (**Fig. 5**). Separation from C2-*N*-ceramide is important, be-

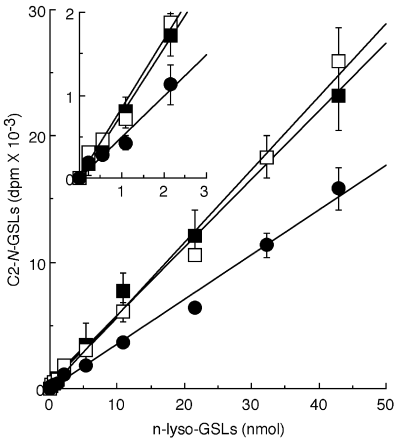

Fig. 4. Lyso-n-GSL quantification. Increasing amounts of GlcSph (filled squares), GalSph (open squares) and LacSph (circles) were *N*-acetylated using 5 mM acetic anhydride/2 μCi [^{3}H]acetic anhydride. The acetylated products were washed after adding 20 μg of the respective *N*-acetyl-GSLs (C2-*N*-GSLs) standard as a carrier, and separated by TLC as in Fig. 5. The insert shows the linearity of the method in the low nmol and pmol range. Results are means ± SD for two or three independent experiments.

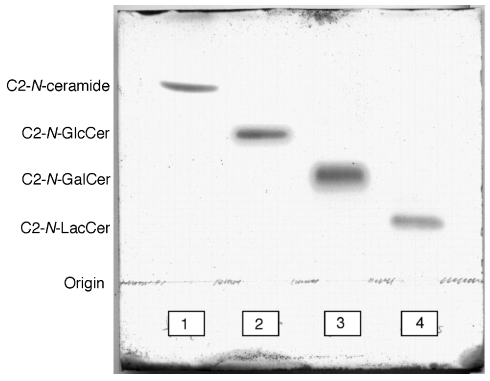

Fig. 5. TLC separation of C2-*N*-GSLs. Sphingosine (Lane 1), GlcSph (Lane 2), GalSph (Lane 3), and LacSph (Lane 4) (10–15 μg each) were individually *N*-acetylated, purified, separated by TLC on a borated TLC plate using chloroform-methanol-water-ammonia (7 M) (65:37:8:1; v/v/v/v) as developing solvent, and visualized with orcinol. In order to detect C2-*N*-ceramide, the TLC plate was also carbonized (180°C, 5–10 min).

cause sphingosine is partially recovered in the same fraction as lyso-n-GSL upon both aminopropyl solid-phase (37) and weak cation exchange (40) chromatography, rendering essential an efficient separation of the *N*-acetylated product of sphingosine, C2-*N*-ceramide, from *N*-acetylated lyso-n-GSL.

Finally, n-GSLs and lyso-n-GSLs were quantified in brain tissue from the glucocerebrosidase-deficient (Gba) mouse. In $Gba^{+/+}$ mice, 5.0 ± 1.7 and 0.049 ± 0.02 nmol of GlcCer and GlcSph/mg of protein, respectively, were detected. GlcCer and GlcSph accumulated ~40-fold in $Gba^{-/-}$ compared with $Gba^{+/+}$ mouse brains (**Table 1**). No changes were detected in levels of GalCer or GalSph, but LacCer accumulated ~4-fold in $Gba^{-/-}$ brains, and LacSph could be detected in $Gba^{-/-}$ but not $Gba^{+/+}$ brains.

DISCUSSION

In the current study, we describe a new method for simultaneous quantification of lyso-n-GSLs and n-GSLs from biological tissues. This technique, although requiring a significant number of chromatographic steps before and after *N*-acetylation using [^{3}H]acetic anhydride (Fig. 1), is a significant improvement over previous methods, inasmuch as it allows processing of multiple samples and quantification of a number of lyso-n-GSLs and n-GSLs in the same sample, is highly sensitive, and does not involve the use of specialized and expensive equipment. Concerning sensitivity, lyso-n-GSLs can be detected in the pmol range, similar to HPLC procedures (17, 20, 21), but in contrast to HPLC, our method also permits detection of amounts in the high nmol range, allowing determination of lyso-n-GSLs (which normally occur in the pmol range in biological tissues) and n-GSLs (which occur in the nmol range) in the same sample. Moreover, the C2-*N*-GSL products of acetylation are stable over a period of weeks at −20°C [in benzene-methanol (1:1; v/v)], in contrast to the *o*-phthalaldehyde (21) and 4-fluoro-7-nitrobenzofurazan (21) derivatives, which are relatively unstable.

The usefulness of this technique was demonstrated by determination of levels of three GSLs and their lyso-derivatives in mouse (this study) and human brain tissue (41), which gave values similar to other methods. Upon derivatization of GlcSph with 4-fluoro-7-nitrobenzofurazan followed by HPLC, 1.4 nmol GlcSph/mg of protein was detected in the whole brain of $Gba^{-/-}$ mice (17), whereas our analyses yielded 1.96 nmol GlcSph/mg of protein in $Gba^{-/-}$ mouse cortices. This small difference is presumably due to different levels of GlcSph accumulation in specific brain regions. Indeed, one of the advantages of our technique is the possibility of analyzing lyso-n-GSL levels in tissue amounts as low as 1 mg. This will permit correlation of lyso-n-GSL accumulation with the extent of disease pathology in specific brain areas in GSL storage diseases, such as Gaucher and Krabbe's disease, in which key pathogenic roles have been ascribed to lyso-n-GSLs (14). Moreover, the TLC system developed in our study permits quantification of GlcSph and GalSph in the same sample, whereas their separation by HPLC has often proved difficult (20). Finally, and importantly, our method also allows determination of the parent n-GSLs in the same samples and during the same analysis. Thus, we report that both GlcSph and GlcCer increase by ~40-fold in the $Gba^{-/-}$ compared with the $Gba^{+/+}$ mouse, which may have implications for understanding the metabolic relationship between these two lipids. This kind of analysis was not previously possible using the same sample, due to the different techniques required for analysis of each lipid. For instance, no simultaneous quantification of GlcSph and GlcCer has been reported in the Gba mouse brain (17, 29, 42), although, using TLC densitometry, accumulation of both GlcSph and GlcCer was reported in brains from Gaucher patients (11), but the lack of sensitivity of TLC densitometry did not permit analysis of multiple lyso-n-GSLs or n-GSLs in small brain regions.

Use of the technique reported herein will allow systematic determination of levels of lyso-n-GSLs and n-GSLs in defined tissues, and in defined regions within the same tissue, in both animal models and human tissues of GSL storage disease samples, as well as their quantification in nonpathological tissues. By way of example, in addition to quantification of GlcSph and GlcCer, we also observed an ~4-fold accumulation of LacCer in the $Gba^{-/-}$ mouse brain, similar to that observed (36) upon treatment of rat hippocampal neurons with the glucocerebrosidase inhibitor, conduritol-B-epoxide, and similar to that reported in the cerebral cortex of neuronopathic forms of Gaucher disease (11). However, we were unable to confirm the increase in globotriaosylceramide and tetraglycosylceramides in the cerebral cortex of neuronopathic Gaucher patients (11), because the extent of globoside recovery after weak cation exchange chromatography was poor (see Fig. 2C), and in addition, strong alkaline hydrolysis could also cause *N*-deacetylation of the gangliotriosylceramide (asialo-GM2), which occurs at low levels in brain (43). However, removal of the acetamido group of *N*-acetylgalactosamine by alkaline hydrolysis is incomplete (38) and reacetylation would therefore occur at different extents for the *N*-acetylgalactosamine moiety and the free amino

TABLE 1. N-GSL and lyso-n-GSL quantification in *Gba* mouse cortex

N-GSLs and lyso-n-GSLs	$Gba^{+/+}$	$Gba^{-/-}$
	nmol/mg of protein	
LacCer	14.7 ± 2.2	60.4 ± 6.8
GalCer	0.8 ± 0.13	1.1 ± 0.2
GlcCer	5.0 ± 1.7	209 ± 20.3
LacSph	0.0	0.29 ± 0.09
GalSph	0.015 ± 0.01	0.01 ± 0.008
GlcSph	0.049 ± 0.02	1.96 ± 0.21

GalCer, galactosylceramide; GalSph, galactosylsphingosine; GlcCer, glucosylceramide; GlcSph, glucosylsphingosine; LacCer, lactosylceramide; LacSph, lactosylsphingosine; lyso-n-GSL, lyso-neutral glycosphingolipid; n-GSL, neutral glycosphingolipid. N-GSLs and lyso-n-GSLs were quantified in cortices from E17 mouse embryos. Results are means ± SD for four independent analyses. Note that values were corrected for loss of material during the whole procedure by using radioactive standards, with 55% recovery of D-[6-^{3}H]LacSph and D-[6-^{3}H]LacCer, and 75% recovery of D-[6-^{3}H]GalSph and D-[6-^{3}H]GalCer.

group of the sphingoid base, rendering this method unsuitable for asialo-GM2. Irrespective of the different extents of acylation, C2-*N*-GA2 will remain at the origin of the TLC plate using the developing solvents described in Fig. 5.

In summary, the technique reported herein should prove useful for the simultaneous, systematic, and highly-sensitive quantification of at least three lyso-n-GSLs and their parent GSLs in a range of biological tissues over a wide range of different concentrations.

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