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Selective Extraction and Effective Separation of Galactosylsphingosine (Psychosine) and Glucosylsphingosine from Other Glycosphingolipids in Pathological Tissue Samples

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Abstract

To facilitate the study of the chemical pathology of galactosylsphingosine (psychosine, GalSph) in Krabbe disease and glucosylsphingosine (GlcSph) in Gaucher disease, we have devised a facile method for the effective separation of these two glycosylsphingosines from other glycosphingolipids (GSLs) in Krabbe brain and Gaucher spleen samples. The procedure involves the use of acetone to selectively extract GalSph and GlcSph, respectively, from Krabbe brain and Gaucher spleen samples. Since acetone does not extract other GSLs except modest amounts of galactosylceramide, sulfatide, and glucosylceramide, the positively charged GalSph or GlcSph in the acetone extract can be readily separated from other GSLs by batchwise cation-exchange chromatography using a Waters Accell Plus CM Cartridge. GalSph or GlcSph enriched by this

simple procedure can be readily analyzed by thin-layer chromatography or high-performance liquid chromatography.

Keywords

Galactosylsphingosine; Psychosine; Glucosylsphingosine; Krabbe disease; Gaucher disease

Introduction

Although galactosylsphingosine (psychosine, GalSph) and glucosylsphingosine (GlcSph), respectively have been shown to be elevated in the affected tissues of Krabbe and Gaucher patients [1, 2], it has been formidable to perform the analysis of these two glycosylsphingosines in pathological tissue samples due to their low abundance. The methods currently used for the analysis of GalSph require the following steps for sample preparation: (1) extraction of the tissue sample with chloroform/methanol (C/M) (2/1) and (1/1); (2) anion-exchange or cation-exchange chromatography; (3) Sep-Pak C-18 cartridge reverse phase chromatography; and (4) silicic acid column chromatography [3, 4]. Likewise, the methods for the analysis of GlcSph are basically adopted from that for GalSph determination. It has been widely recognized that the laborious sample preparation steps are the most serious stumbling blocks for the analysis of these two low abundance glycosylsphingosines in pathological tissue samples. To facilitate the analysis of GalSph and GlcSph, respectively in the affected tissues of Krabbe disease and Gaucher disease, we have developed a facile method for the selective extraction of GalSph and GlcSph from tissue samples and their effective separation from other glycosphingolipids (GSLs). The procedure involves the selective extraction of GalSph from Krabbe brain samples and also of GlcSph from Gaucher spleen samples by acetone, followed by batchwise cation-exchange chromatography using a Waters Accell Plus CM Cartridge to separate GalSph or GlcSph from other GSLs. GalSph or GlcSph enriched by this procedure can be readily analyzed by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC).

Experimental Procedures

Tissue Samples

Normal and Krabbe human brain samples were obtained from the Brain and Tissue Bank of the University of Maryland. Normal and Krabbe rhesus monkey brain samples were from the Tulane University National Primate Research Center, Covington, Louisiana. Normal and Krabbe dog brain samples were from the Krabbe dog colony maintained at the University of Pennsylvania, School of Veterinary Medicine, under NIH and USDA guidelines for the care and use of animals in research. Dogs were housed in runs, maintained on a 12-h light/dark cycle, and provided ad libitum access to standard dog chow and fresh water. Normal and twitcher mouse brain samples were obtained from Dr. Bruce Bunnell's breeding colonies at the Tulane University School of Medicine. Spleen samples from normal controls and from patients with Gaucher disease were obtained from splenectomy or autopsy samples, collected with informed consent under NIH Institute Review Board approved clinical protocols. Genotypes were performed as previously described [5]. These samples were lyophilized and kept at -80°C until used.

Chemicals

All solvents were HPLC grade and were used without further purification. The following were obtained from commercial sources indicated: galactosylceramide (GalCer), glucosylceramide (GlcCer), GalSph, GlcSph (Matreya, Pleasant Gap, PA), precoated Silica

Gel 60 TLC plates (EMD Chemicals, Inc., Gibbstown, NJ), Sep-Pak Vac 3 cc Accell Plus CM cartridge (Waters, Milford, MA).

Analytical Methods

The solvent system used for the TLC-analysis of both GalSph and GlcSph was C/M/10% acetic acid (60/35/8, v/v/v) and GSLs were revealed by spraying with the diphenylamine-aniline-phosphoric acid (DPA) reagent [6]. The amino group containing biomolecules on a TLC-plate were revealed by spraying the plate with 0.1% ninhydrin in acetone and heated at 80°C for 10 min. GalSph and GlcSph were also analyzed by HPLC following the method of Merrill, et al. [7]. Standard or extracted sample in methanol was derivatized for 5 min in an equal volume of *o*-phthalaldehyde (OPA) reaction solution (1 mg of OPA was initially dissolved in ethanol then diluted to 7.5 mM in 0.4 M boric acid as sodium tetraborate, pH 10.5; 2-mercapto-ethanol was added to a concentration of 14 mM). The reaction was then diluted 1:10 into mobile phase, centrifuged briefly, and kept at 4°C until 20 µl was injected. The separation was performed on a Shimadzu Shimpak VP-ODS C-18 reverse phase column (4.6 × 150 mm) with an isocratic mobile phase (methanol:5 mM potassium phosphate pH 7.0, 90:10 v/v) at 0.5 ml/min in a 50°C column oven. Peak height from an RF-10Axl fluorescence detector (340 nm excitation, 455 nm emission) was quantified against a standard curve spanning 0.06–140 pmol GalSph or 0.2–45.5 pmol GlcSph injected. Curve fitting was calculated as a second order polynomial in Microsoft Excel. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) analyses were performed on a 4700 Proteomic Analyzer (Applied Biosystems, Framingham, MA), operated in positive ion reflectron mode. Samples dissolved in methanol were mixed 1:1 with 2,5-dihydroxybenzoic acid matrix (10 mg/ml in 50% acetonitrile) for spotting onto the MALDI target plate.

Extraction and Separation of GalSph and GlcSph from Other GSLs in Tissue Samples

Each 200 mg of lyophilized brain or spleen sample was extracted with 150 ml of acetone using a Polytron homogenizer to selectively extract GalSph or GlcSph. The extract was filtered through a Büchner funnel and evaporated to dryness. The dried residue was dissolved in 2 ml C/M (2/1) and a 2-µl aliquot of this solution was analyzed by TLC (see Fig. 1). The rest of the solution was subsequently applied onto a Waters Sep-Pak Vac 3 cc Accell Plus CM cartridge that had been equilibrated with C/M (2/1). After collecting the breakthrough fraction, the cartridge was eluted with C/M/water (30/60/8) and 2 ml/fraction was collected. Each eluate was evaporated to dryness. The breakthrough fraction and the first eluate were dissolved in 0.5 ml of C/M (2/1) while the eluates 2 through 5 were dissolved in 50 µl of C/M (2/1) and a 3 µl-aliquot of each fraction was subsequently analyzed by TLC (see Fig. 2).

Results and Discussion

The Merit of Using Acetone for the Selective Extraction of GalSph from Krabbe Brain Samples

The previously reported methods for the analysis of GalSph in pathological brain samples involved the initial use of C/M mixture to extract GalSph along with numerous other lipids [3, 4]. Although GalSph has been shown to be soluble in C/M mixture [3], its levels in pathological brain samples were found to be extremely low. Thus, it is formidable to separate GalSph from other GSLs extracted by C/M. To facilitate the analysis of GalSph, we carried out a search for a solvent suitable for selective extraction of GalSph from pathological brain samples. Among various solvents tested, we found that GalSph was quite soluble in acetone. The solubility of GalSph in acetone was estimated to be approximately 10 mg/ml at room temperature. Acetone was used as a solvent for the extraction of cholesterol from the brain in 1906 [8]. It is also well known that most GSLs are not soluble

in acetone [9] and that the levels of GalSph in Krabbe human, rhesus monkey, dog and twitcher mouse brains are less than 1 mg per g wet tissue [3, 10–12]. Thus, acetone serves as a convenient solvent for the selective extraction of GalSph from tissue samples to minimize the contamination of other GSLs. We routinely extracted 200 mg of the lyophilized white matter of a brain sample with 150 ml of acetone using a Polytron homogenizer. We chose to process 200 mg of lyophilized tissue since this amount is roughly equivalent to 1 g of wet tissue. Based on the solubility, 150 ml of acetone is capable of dissolving approximately 1.5 g of GalSph. Thus, 150 ml of acetone should be more than sufficient to extract all GalSph present in 200 mg of freeze-dried pathological tissue samples. Figure 1a is an example to show that the total GSL profiles of the acetone extracts derived from the white matter of a normal and a Krabbe monkey brain samples are quite simple: only three major DPA-positive bands from the Krabbe monkey brain (K) and two major DPA positive bands from the normal monkey brain (N) were detected. Among the DPA-positive bands, one had the TLC-mobility similar to that of the standard GalSph. Interestingly, considerable amounts of GalCer and sulfatide were also extracted from both normal and Krabbe monkey samples by acetone. As shown in Fig. 1b, the DPA-positive band (lane K) corresponding to the GalSph detected in the white matter of the Krabbe monkey brain was also clearly stained by ninhydrin, indicating the presence of an amino group in this band. The faint DPA positive band (Fig. 1a, lane N) with the TLC-mobility slightly slower than that of GalSph detected in the white matter of the normal monkey brain sample appeared to be also ninhydrin positive (Fig. 1b, lane N). However, by mass spectrometry, we detected the presence of GalSph only in the acetone-extract of the white matter of the Krabbe monkey brain sample but not in the extract of the normal monkey brain sample (results not shown). These results support the merit of using acetone for the selective extraction of GalSph from Krabbe monkey brain samples. Extracting a brain sample twice with acetone did not increase the recovery of GalSph. In contrast, considerable amounts of GalCer and other positively charged materials were extracted in the second acetone extraction. These materials interfered with the Sep-Pak cartridge cation exchange chromatography. When 100 μ g of the standard GalSph were mixed with 200 mg of normal human brain and processed through the two-step procedures described under “Extraction and Separation of GalSph and GlcSph from other GSLs in Tissue Samples,” the recovery of GalSph was found to be between 65 and 75%.

Separation of GalSph from Other Lipid Materials by Batchwise Cation-Exchange Chromatography Using a Water Sep-Pak CM Cartridge

Separation of GalSph from other lipid materials in the C/M (2/1)-extract by cation-exchange chromatography using AG-50WX8 resin was first introduced by Igisu and Suzuki [4]. To simplify the cation-exchange chromatography, we used a Water Sep-Pak CM cartridge for batchwise separation of GalSph from the two major GSL-contaminants, GalCer and sulfatide, found in the acetone-extract. As described under Analytical Methods, we applied the acetone-extract dissolved in C/M (2/1) onto a Water Sep-Pak CM cartridge that had been equilibrated with C/M (2/1). After collecting the breakthrough fraction, the cartridge was eluted with C/M/water (30/60/8). Figure 2 shows the profile of the TLC analysis: GalSph in the acetone-extract of a Krabbe monkey white matter was retarded by the cartridge and well separated from other contaminants. All GalCer, sulfatide and other contaminants appeared in the breakthrough fraction and the first fraction eluted with C/M/water (30/60/8), whereas GalSph was retarded by the cartridge and appeared in the fractions 2 through 4. These fractions were pooled and designated as GalSph-enriched fraction. Under the same condition, no GalSph was detected in the corresponding fractions derived from the acetone-extract of the white matter of a normal monkey. This elution profile was very reproducible from sample to sample and we have used this procedure to analyze GalSph in the white matters of: 3 normal and 3 Krabbe monkey brains; 3 normal and 3 Krabbe dog brains; 1 normal and 3 Krabbe human brains. We also analyzed the whole brains (due to their small

sizes) of 2 normal and 3 twitcher mice. Fig. 3, (I) and (II) show the Sep-Pak CM cartridge elution profiles of the acetone-extracts of the white matters of a pair of Krabbe and normal dog brains, and that of a pair of Krabbe and normal human brains. As in the case of the Krabbe monkey brain sample shown in Fig. 2, we also detected the presence of GalSph in the GalSph-enriched fractions derived from the Krabbe dog and Krabbe human brain samples, whereas no GalSph was detected in the corresponding fractions derived from the normal brain samples. Figures 2 and 3 also show that GalSph in the GalSph-enriched fraction can be conveniently detected by TLC. It should be pointed out that Sep-Pak CM cartridge removed bulk of ninhydrin positive materials as shown in Fig. 7 for the enrichment of GlcSph. The GalSph-enriched fraction from each sample was evaporated to dryness and further analyzed by MALDI MS and HPLC to verify the presence of GalSph. As shown in Fig. 4, MALDI MS analysis clearly detected the presence of GalSph as a pair of protonated and sodiated molecular ions at m/z 462 and 484, respectively, in the GalSph-enriched fraction derived from the Krabbe monkey brain sample. These m/z values and hence the inferred molecular weight is consistent with a hexose attached to a d18:1 sphingosine base and is identical to that afforded by an authentic GalSph standard. As shown in the inset of the lower panel of Fig. 4, by magnifying the mass region for the molecular ions associated with GalSph, we have also detected m/z 462.2 and 484.2 as minor peaks at much lower intensity among other contaminants or matrix noise in the corresponding fraction from the normal brain sample, indicating the presence of a low level of GalSph in the normal monkey brain. This result is consonant with our HPLC analysis as shown in Table 1. Figure 5 shows the HPLC elution profiles for the analysis of GalSph in the GalSph-enriched fraction derived from the white matter of a Krabbe monkey brain sample. Table 1 summarizes the results of quantitative HPLC analysis of GalSph in the GalSph-enriched fractions derived from the white matters of: 3 normal and 3 monkey Krabbe brains; 3 normal and 3 Krabbe dog brains; and 1 normal and 3 Krabbe human brains. For comparison, we have also analyzed the whole brains of 2 normal and 3 twitcher mice (Table 2). It is noteworthy that the levels of GalSph in the white matter of Krabbe monkey brains were several folds higher than that found in the white matters of Krabbe dog and human brains.

Analysis of GlcSph in Spleen Samples from Patients with Gaucher Disease

In addition to the massive accumulation of GlcCer in the spleen and the liver, patient with Gaucher disease also accumulate GlcSph in these two organs [13]. The methods previously used for the analysis of GlcSph were largely adopted from the analysis of GalSph. The sample preparation also involved the use of C/M extraction followed by several tedious chromatography steps [13, 14]. We have extended our procedure based on the selective extraction of GalSph with acetone to analyze GlcSph in two normal and six human Gaucher spleen samples. Initially, we were surprised by the finding that the solubility of GlcSph in acetone was approximately 3.6 mg/ml at room temperature, which was about one-third that of GalSph (10 mg/ml). Since the levels of GlcSph in the Gaucher spleen were reported to be less than 1 mg/g wet tissues [13–16], 150 ml of acetone should be more than adequate to extract GlcSph present in 200 mg of lyophilized pathological spleen samples. As shown in Fig. 6, acetone extracted a DPA-positive band with the TLC-mobility coincided with that of GlcSph from all six Gaucher spleen samples (samples 3 through 8), but not from the two normal spleen samples (samples 1 and 2). It should be noted that acetone also extracted considerable amounts of GlcCer from the six Gaucher spleen samples. In contrast, no GlcCer was detected in the acetone-extracts of two normal spleen samples. These extracts were subsequently processed through the Sep-Pak CM cartridge as described under Analytical Methods. Fig. 7 (Ia) shows that GlcCer in the acetone-extract of a human Gaucher spleen sample emerged from the Sep-Pak CM cartridge in the breakthrough fraction and the first fraction eluted with C/M/water (30/60/8), while GlcSph was eluted in the fractions 2 through 4. Fig. 7 (Ib) shows that the DPA-positive GlcSph-bands shown in

Fig. 7 (Ia) were also ninhydrin-positive, indicating the presence of a free amino group in these bands. In contrast, the fractions 2–4 derived from the acetone-extract of a normal human spleen sample as shown in Fig. 7 (IIa), (IIb) were devoid of GlcSph. As in the case for GalSph determination, fractions 2–4 were pooled and designated as GlcSph-enriched fraction. GlcSph-fractions were evaporated to dryness and further analyzed by MALDI MS and HPLC. As in the case of GalSph, MALDI-MS analyses detected a pair of protonated and sodiated molecular ions at m/z 462 and 484, respectively, and verified the presence of GlcSph in the GlcSph-enriched fraction derived from the acetone-extract of a human Gaucher spleen sample (Fig. 8) but not in the corresponding fraction of a normal human spleen sample (Fig. 8). In contrast to the detection of a low level of GalSph in a normal monkey brain sample shown in Fig. 4, magnification of the mass region for the molecular ions associated with GlcSph failed to detect the presence of m/z 462 and 484 in the corresponding fraction derived from a normal human spleen (result not shown). HPLC analysis also did not detect the presence of GlcSph in the GlcSph-enriched fractions derived from 2 normal spleen samples (Table 3).

Conclusion

Three recent studies [17–19] describing improvement to the GalSph analysis still used methanol [17] or C/M extraction [18, 19] and went through multi-purification steps, such as strong cation and C18 solid phase chromatography [17]; silicic acid, strong-cation exchange and Sep-Pak C18 chromatography [18]; Sephadex G25, aminopropyl solid phase, weak-cation exchange chromatography [19] to enrich GalSph from the lipid extracts. In this report we show that acetone is a convenient solvent for selective extraction of GalSph and GlcSph from pathological tissues. We also show that GalSph and GlcSph in the acetone extract can be effectively enriched by batchwise cation exchange chromatography using Water Accell Plus CM Cartridge and subsequently analyzed by TLC and HPLC. For quantitative HPLC analysis of GalSph and GlcSph, we have chosen to express the results shown in Tables 1, 2 and 3 based on pmoles per mg dried tissue instead of per mg protein, as the protein concentration may vary depending on the method used for lipid extraction and protein determination. The variations in the levels of GalSph and GlcSph in Tables 1, 2 and 3 could be due to the different disease status of each individual subject. The purpose of this study is to introduce a convenient method for selective extraction and enrichment of GalSph and GlcSph from pathological tissue samples. Critical evaluation of GalSph and GlcSph levels in various disease states using this method will be carried out in the future.

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Fig. 1.

TLC analysis showing the GSLs extracted from 200 mg each of the lyophilized white matter of a normal monkey and a Krabbe monkey brain sample with 150 ml of acetone. **a** sprayed with the DPA reagent to reveal glycoconjugates, and **b** sprayed with ninhydrin to reveal the amino group-containing biomolecules. *N*, normal; *K*, Krabbe; *S*, standards GalCer (*upper band*) and GalSph (*lower band*) in (**a**); *S* in (**b**) shows only GalSph revealed by the ninhydrin spray. The detailed conditions are described under Analytical Methods

Fig. 2.

TLC-analysis showing the fractionation of the acetone extracts prepared from a Krabbe and a normal monkey brain white matters by Waters Sep-Pak CM cartridge. S, standard GalCer (*upper band*) and GalSph (*lower band*); BT, breakthrough fraction; Fractions 1–5 represent the fractions eluted from the cartridge by C/M/water (30/60/8). The detailed conditions are described under Analytical Methods

Fig. 3. TLC-analysis showing the fractionation of the acetone extracts prepared from the white matter of a Krabbe and a normal dog brain (I) and the white matter of a Krabbe and a normal human brain (II) by Water Sep-Pak CM cartridge. S, standard GalCer (*top band*), sulfatide (*middle band*) and GalSph (lower band); BT, breakthrough fraction; fractions 1–5 represent the fractions eluted from the cartridge by C/M/water (30/60/8). The detailed conditions are described under Analytical Methods

Fig. 4.

MALDI MS analysis of the GalSph-enriched fraction (fractions 2–4 shown in Fig. 2) from the white matter of a Krabbe and a normal monkey brain eluted from Water Sep-Pak CM cartridge. GalSph was detected as $[M + H]^+$ and $[M + Na]^+$ molecular ions at m/z 462 and 484, respectively, while identification of other signals was not attempted. In lower panel (Normal monkey brain), the mass region where the molecular ions for GalSph occurred was magnified and shown as an inset

Fig. 5. HPLC elution profiles of: **a** standard GalSph (50 pmol) and Sph (83 pmol); **b** Sph (83 pmol) plus GalSph-enriched fraction derived from 10 μ g of the white matter of a Krabbe monkey EA75; **c** Sph (83 pmol) plus GalSph-enriched fraction derived from 100 μ g of the white matter of a Krabbe monkey EA75

Fig. 6. TLC analysis showing the GSLs extracted from 200 mg (lyophilized) each of two normal human spleen (samples 1 and 2) and six Gaucher spleen (samples 3–8) with 150 ml of acetone. S, standards GlcCer (*upper band*) and GlcSph (*lower band*). The detailed conditions are described under Analytical Methods

Fig. 7.

TLC-analysis showing the fractionation of the acetone extracts prepared from a human Gaucher spleen (I), and a normal human spleen (II) by Water Sep-Pak CM cartridge. In both cases, chromatograms **a** were sprayed with the DPA reagent to reveal glycoconjugates and chromatograms **b** were sprayed with ninhydrin to reveal the amino group-containing biomolecules. *GS*, GlcSph; *BT*, breakthrough fraction; *S*, sphingosine (*Sph*); fractions 1–4 represent the fractions eluted from the cartridge by C/M/water (30/60/8). The detailed conditions are described under Analytical Methods

Fig. 8. MALDI MS analysis of the GlcSph-enriched fraction (fractions 2–4 shown in Fig. 6) from a Gaucher and a normal human spleen eluted from Water Sep-Pak CM cartridge. GlcSph was detected as $[M + H]^+$ and $[M + Na]^+$ molecular ions at m/z 462 and 484, respectively, while identification of other signals was not attempted

Table 1
HPLC analysis of GalSph in the white matter of normal and Krabbe monkey, dog, and human samples

	Normal		Krabbe			
	Sample ID	Age (years)	GalSph pmol/mg dried tissue (mean ± SD)	Sample ID	Age (years)	GalSph pmol/mg dried tissue (mean ± SD)
Monkey	N-M018	Stillborn	2.07 ± 0.12	K-C180	0.52	688.26 ± 113.13
	N-M036	12.38	0.72 ± 0.34	K-EA75	1.67	225.48 ± 5.19
	N-F218	5.16	1.34 ± 0.07	K-V539	0.44	235.98 ± 6.99
Dog	N-I-80	1.96	0.26 ± 0.01	K-269	0.75	36.72 ± 5.64
	N-4774	2.32	0.18 ± 0.03	K-277	0.28	21.77 ± 4.96
	N-C84BL	1.87	0.39 ± 0.02	K-326	0.44	26.21 ± 5.73
Human	N-1547	1.72	1.18 ± 0.05	K-575	1.39	8.23 ± 0.52
				K-1163	39.29	3.72 ± 0.83
				K-1699	1.75	4.17 ± 0.95

Table 2
HPLC analysis of GalSph in the whole brain of normal and twitcher mice

Sample ID	Normal		Sample ID	Twitcher	
	Age (days)	GalSph pmol/mg dried tissue (mean $\pm$ SD)		Age (days)	GalSph pmol/mg dried tissue (mean $\pm$ SD)
N-98	21	0.17 $\pm$ 0.05	K-96	21	3.2 $\pm$ 0.54
N-90	60	0.08 $\pm$ 0.03	K-91	29	8.55 $\pm$ 4.12
			K-219	34	17.2 $\pm$ 1.26

Table 3
HPLC analysis of GlcSph in spleen from normal controls and patients with Gaucher disease

Control samples		Gaucher samples					
Sample ID	GlcSph pmol/mg dried tissue (mean $\pm$ SD)	Sample ID	Gaucher type	Age (years)	GBA genotype	Wt of spleen(kg)	GlcSph pmol/mg dried tissue (mean $\pm$ SD)
1	Undetectable	3	1	40	N370S/c.84GG	6.3	259 $\pm$ 13
2	Undetectable	4	1	3	RecNciI/N352L	NA	113 $\pm$ 10
		5	2	0.7	IVS2 + 1/L444P	0.1	168 $\pm$ 55
		6	3	13	RecNciI/R463C	3.1	539 $\pm$ 8
		7	3	12	c.84GG/R463C	4.1	523 $\pm$ 3
		8	3	16	IVS2 + 1/R463C	3.1	419 $\pm$ 49