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One-step synthesis of carbon-13-labeled globotriaosylsphingosine (lyso-Gb3), an internal standard for biomarker analysis of Fabry disease

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Abstract

Globotriaosylsphingosine (lyso-Gb3) is a well-established biomarker for diagnosis and prognosis of Fabry disease. This biomarker is measured in biological samples by liquid chromatography-tandem mass spectrometry using an internal standard. The ideal internal standard is a variant of lyso-Gb3 substituted with heavy isotopes, but the total synthesis of such a compound is very labor intensive. In this report, we describe a simple, one-step synthesis of lyso-Gb3 labeled with carbon-13 in all of the galactosyl carbons.

Keywords

Fabry disease; lysosomal storage disease; biomarker; tandem mass spectrometry; lyso-Gb3

Introduction

Fabry disease is a treatable lysosomal storage disease caused by deficiency of the lysosomal enzyme alpha-galactosidase A involved in the breakdown of sphingolipids. Newborn screening for Fabry disease is typically done by measurement of residual alpha-galactosidase A enzymatic activity in dried blood spots by tandem mass spectrometry [1] or fluorometry [2]. Deficiency of alpha-galactosidase A leads to a buildup of globotriaosylceramide and its hydrolysed form lacking the fatty acyl chain (globotriaosylsphingosine, lyso-Gb3). Lyso-Gb3 is measured by liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in the presence of an internal standard [3]. Most assays use a lyso-Gb3 analog (lyso-Gb3 with a glycine acyl linked to the amino group) as an internal standard [4]. However, in any LC-MS/MS assay it is always better to use a structurally-identical, but isotopically substituted, internal standard so that the internal standard and analyte of interest exactly co-migrate during LC. In this way suppression of

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ionization in the electrospray source of the mass spectrometry by multiple components in the sample will be identical for analyte and internal standard (usually not true if analyte and internal standard elute from the LC column at different times). Also, any losses of lyso-Gb3 due to sample handling are best mimicked by use of a structural identical internal standard. Although the synthesis of heavy isotopic lyso-Gb3 has been reported, the multi-step synthesis makes it impractical for worldwide distribution [5]. In this study we describe a simple one-step synthesis of carbon-13-labeled lyso-Gb3.

Experimental Methods

Materials.

UDP-Galactose labeled with carbon-13 uniformly in all galactose carbons was obtained from Omicron Biochemicals (Cat NTS-005), and lactosyl-sphingosine was from Avanti Polar Lipids (Cat. 860542). Sodium taurocholate was from Carbosynth (Cat. FS45308). A stock solution of labeled UDP-Galactose was prepared in 20 mM sodium acetate buffer, pH 5.5 and stored at -20°C . Stock solutions of lactosyl-sphingosine and sodium taurocholate were prepared in methanol and stored at -20°C . The plasmid for bacterial expression of α -1,4-galactosyltransferase C from *Neisseria meningitidis* (LgtC) was obtained as a generous gift from Prof. Stephen G. Withers (Univ. of British Columbia) [6].

Preparation of LgtC.

An overnight culture of *E. coli* (BL21, DE3), harboring the pET29a(+) plasmid containing the LgtC-25 gene in Luria broth was diluted 100-fold into Luria broth containing kanamycin (50 $\mu\text{g}/\text{mL}$). The culture was shaken at 37°C until the OD600 reached 0.6, then isopropylthiogalactoside was added to a final concentration of 0.5 mM, and the culture was continued for 6 h. Cells were harvested by centrifugation at $3000 \times g$ for 20 min at 4°C [6].

All subsequent steps were done on ice or in a room at 4°C . The cell pellet from a 1 L culture was re-suspended in 30 mL of cold binding buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 10% (v/v) glycerol, 10 mM β -mercaptoethanol, 0.5 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche Cat. 11873580001), pH 8.0). Protease inhibitors and β -mercaptoethanol were added to buffer just before use. The suspension was magnetically stirred on ice for 15–20 min. The suspension was probe sonicated on ice (6–8 cycles of 20 on-off). The lysate was centrifuged at $15,000 \times g$ for 20 min at 4°C .

Ni-NTA Agarose (3 mL resin) from Qiagen (CAT 30230) was washed with purified water (Milli-Q, EMD-Millipore) and then equilibrated in binding buffer. The supernatant from the cell lysate (~30 mL) was added to the washed gel in a 50 mL plastic tube. The tube was rotated end-over-end for 1–2 h at 4°C . The tube content was poured into a 1.6×15 cm column, and eluent was collected by gravity. The column was eluted with 30 mL of binding buffer, then with 20 mL of binding buffer containing 50 mM imidazole, then with binding buffer containing 110 mM imidazole, and finally with binding buffer containing 300 mM imidazole. One mL fractions were collected. LgtC eluted in the 110 mM imidazole fraction. Aliquots of fractions were analyzed by SDS-PAGE (apparent MW ~ 29 kDa), and those containing essentially pure LgtC were combined. The solution was dialyzed at 4°C against

20 mM Tris-HCl, pH 7.5 (2 L twice over 4–5 h, then 1 L once overnight). The amount of protein was estimated using the BCA protein assay (ThermoFisher) using bovine serum albumin as a standard. The typical yield was 20 mg per liter culture (purity checked by SDS-PAGE).

Synthesis of carbon-13 labeled lyso-Gb3 (Figure 1).

Methanol was completely removed from stock solutions of lactosyl-sphingosine and sodium taurocholate to leave 1 mg lipid and 1.25 mg detergent in a glass vial. The residue was taken up in 0.5 mL of reaction buffer (20 mM HEPES, 50 mM KCl, 5 mM MnCl₂, 0.1% bovine serum albumin (Sigma Cat. A6003), 5 mM freshly added dithiothreitol, adjusted to pH 7.5 with NaOH). The buffer also contained 1.4 mg of [¹³C]-UDP-galactose. To this mixture was added ~28 µg of LgtC, and the capped vial was incubated overnight at 37°C on an orbital shaking platform (~250 rpm).

A 3 mL C18 Sep-Pak (J.T. Baker Cat. 7020–02) was conditioned with 10 mL of methanol followed by 10 mL of purified water (Milli-Q, EMD Millipore). The crude enzymatic reaction mixture was loaded onto the cartridge, which was then washed with 5 mL of water, then 5 mL of water/acetonitrile (3/1, v/v), then 5 mL of water/acetonitrile (3/2, v/v), then 5 mL of water/acetonitrile (1/4, v/v), and finally 5 mL of pure acetonitrile. Slight air pressure was used to force solvent through the cartridge, and 1 mL fractions were collected. A typical Sep-Pak elution profile is shown in Figure 2. About 80% of the lyso-Gb3 eluted in the water/acetonitrile (3/2, v/v) wash, and fractions 11–20 were combined. Solvent was removed with a vacuum, centrifugal concentrator at room temperature. This procedure provided labeled lyso-Gb3 that also contains some lactosyl-sphingosine and is suitable for most purposes (see Results). A typical yield of 0.9 mg (70 %) lyso-Gb3 was obtained (see below for quantification) using 1 mg lactosyl-sphingosine.

An alternate workup scheme was to use HPLC using a Vydac 218TP C18 column (1.0 × 25 cm, VWR Scientific, Cat. 218TP1010) at room temperature. Solvent A was 100% water with 0.1% formic acid, and solvent B was 100% acetonitrile with 0.1% formic acid (all were Optima Grade from Fisher Scientific). The crude enzymatic reaction mixture was mixed with methanol to give a methanol/water ratio of ~2:3 (v/v) and filtered through a 0.2 micron Nylon-66 cartridge filter prior to injection onto the HPLC column. The solvent program started at 30% B for 2 min, then to 45% B from 2–45 min, then to 55% B from 45–55 min, then to 100% B from 55–60 min, then hold at 100% B from 60–69 min, then to 30% B from 69–70 min, then hold at 30% B from 70–80 min. The flow rate was 6 mL/min, and 45 sec fractions were collected. A typical chromatogram is shown in Figure 3. Each fraction was diluted 100-fold and analyzed by UPLC-MS/MS for purity. Pure lyso-Gb3 fractions (fraction 49–52 eluting at 36–39 min) were combined and concentrated to dryness in a vacuum, centrifugal concentrator. A typical recovery rate after HPLC purification was 65%.

UPLC-MS/MS was carried out using a Waters BEH C18 column (1.7 mm, 2.1 × 50 mm, Cat. 186002350) with a guard column (Cat. 186003975) held at 40 °C. The solvent gradient was 50% solvent A (100% water with 0.1% formic acid)/50% solvent B (100% acetonitrile, 0.1% formic acid) to 65% B from 0–1.5 min, then jump to 100% B and hold until 2.0 min, then jump to 50% until 2.5 min for re-equilibration. The flow rate was 0.6 mL/min. MS/MS

was carried out with a Waters Xevo TQ instrument operating in positive ion mode (MS/MS parameters given in Supplemental Material along with a typical chromatogram). UPLC solvents were Optima grade from Fisher Scientific.

Quantification of lyso-Gb3.

Unlabeled and labeled lyso-Gb3 were quantified by proton-NMR using dimethylformamide as an internal standard (a recycle delay between NMR pulses of ~10 sec was used) (spectrum shown in Supplemental Figure 1). In a typical experiment, we used 3.22 μ mole DMF and nominally 1.8 mg lyso-Gb3 in CD₃OD solvent.

Results and Discussion

The bacterial enzyme LgtC transfers a galactosyl unit from UDP-galactose to the terminal 4-hydroxyl of lactosylglycosides [6]. The enzyme is highly specific for galactosyl as the donor and lactosyl-glycosides as the acceptor [6]. Since lactosyl-sphingosine and UDP-Galactose labeled uniformly in all 6 carbons of the galactosyl-unit with carbon-13 are commercially available, we envisioned a one-step synthesis of labeled lyso-Gb3 (Figure 1). The enzymatic reaction proceeds in high yield. If chemically pure labeled lyso-Gb3 is required, the enzymatic reaction mixture is submitted to preparative HPLC to fully separate lyso-Gb3 from lactosyl-sphingosine (Figure 3). Subsequently HPLC-MS/MS analysis of the HPLC purified material showed ~0.04% lactosyl-sphingosine as a contaminant. The vendor stated that the labeled UDP-galactose is 99% enriched with carbon-13. Since the material contains 6 carbon-13 atoms, the amount of unlabeled lyso-Gb3 in the internal standard is virtually zero.

A simple solid-phase extraction method using a C18 Sep-Pak cartridge provides labeled lyso-Gb3 containing unlabeled lactosyl-sphingosine (Figure 1), with all other components removed. The absolute amount labeled lyso-Gb3 in the 2-lipid mixture can be determined by quantitative NMR in the presence of an internal standard (Methods). This material would be useful for quantification of lyso-Gb3 in biological samples by UPLC-MS/MS since the presence of lactosyl-sphingosine is of no consequence, and the absolute moles of labeled lyso-Gb3 internal standard would be known from the quantitative NMR standardization.

This simple procedure to produce labeled lyso-Gb3 will allow reference laboratories to use this as a proper internal standard for the quantification of the Fabry biomarker. This is more reliable than use of structural-related lyso-Gb3 analogs that do not exactly co-migrate with lyso-Gb3 during UPLC. The carbon-13-labeled lyso-Gb3, made as described in this study, is also expected to be available commercially in the near future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

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Figure 1.
Synthetic scheme to prepared labeled ^{13}C -lyso-Gb3.

Figure 2. UPLC-MS/MS analysis of fractions obtained from the C18 Sep-Pak partial purification of ^{13}C -lyso-Gb3.

Figure 3.
UPLC-MS/MS analysis of fractions obtained from the HPLC purification of ^{13}C -lyso-Gb3.