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Analysis of glycosphingolipids from human plasma

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ABSTRACT

Interest in the role of cellular glycosphingolipids (GSLs) in health and disease led to us developing a sensitive method to analyse the full complement of GSL structures present in mammalian cells, fluids and tissues. The original qualitative method we developed was published in 2004 and measured the oligosaccharides selectively released from glycosphingolipids using a ceramide glycanase enzyme derived from the medicinal leech. We have now updated and refined this protocol with the focus on achieving sensitive and reproducible quantitation of GSLs in control and patient plasma samples. The method uses the fluorescent compound anthranilic acid (2-AA) to label oligosaccharides prior to analysis using normal-phase high-performance liquid chromatography. The labelling procedure is rapid, selective, and easy to perform. With the inclusion of a 2AA-labelled chitotriose calibration standard, it is possible to obtain accurate and reproducible molar quantities of individual GSL species.

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KEYWORDS

Glycosphingolipids, Glucosyl Ceramide, HPLC, Oligosaccharide Analysis

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GUIDELINES

This protocol requires the use of some hazardous materials. As such, users must be appropriately trained and hazardous materials stored, used, and disposed of in accordance with your institution's health and safety policies, and approved laboratory policies, risk assessments and codes of practice.

MATERIALS TEXT

Acetonitrile 1.00030

Gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur
https://www.sigmaaldrich.com/catalog/product/mm/100030?lang=en&region=GB&gclid=EAIaIQobChMI1JyZu_Xa8AIVcwUGAB10lgVLEAAYASAAEgJHmfD_BwE

Anthranilic acid
A89855 Sigma-Aldrich
Reagent grade, ≥98%
https://www.sigmaaldrich.com/catalog/product/sial/a89855?lang=en&region=GB

Boric acid
ReagentPlus®, ≥99.5%
B0252 Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigald/b0252?cm_sp=Insite-_-caSrpResults_srpRecs_srpModel_boric%20acid%20reagent%20plus-_-srpRecs3-1

Chloroform
Suitable for HPLC, ≥99.8%, amylene stabilized
34854-M Sigma-Aldrich
https://www.sigmaaldrich.com/catalog/product/sial/34854m?lang=en&region=GB

Discovery® DPA-6S SPE Tube
https://www.sigmaaldrich.com/GB/en/search/dpa-6s?focus=products&page=1&perPage=30&sort=relevance&term=DPA-6S&type=product_name

Gibco™ DPBS, no calcium, no magnesium
14190094
https://www.thermofisher.com/order/catalog/product/14190094#/14190094

Glycosphingolipid standards
https://www.matreya.com/Departments/Product-Categories/Glycosphingolipids.aspx

Kinesis SPE Columns: TELOS® C18(EC) 100mg/1ml SPE Columns
https://kinesis.co.uk/kinesis-spe-columns-telosr-c18-ec-100mg-1ml-spe-columns-210-100m-001t.html

Ludger - BioQuant 2AA Labelled Chitotriose Standard

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Cat. #: BQ-CAA-CHI-01 Batch: B37I-02
https://www.ludger.com/docs/products/bq/bq-caa-chi/bq-caa-chi-01-b37i-02-cofa.pdf

Ludger - 2-AA Labeled Glucose Homopolymer Ladder
Cat. # CAA-GHP-30
https://www.glycoprofiling.com/docs/products/caa/ludger-caa-ghp-30-guide.pdf

Methanol
34860 Sigma-Aldrich
Suitable for HPLC, ≥99.9%
https://www.sigmaaldrich.com/catalog/product/sigald/34860?lang=en&region=GB&cm_sp=Insite-_-caSrpResults_srpRecs_srpModel_methanol%2034860-_-srpRecs3-1

rEGCase I: recombinant Endoglycoceramidase custom-synthesized by GenScript
https://www.genscript.com/

Sarstedt 1.5 ml Micro tubes with screw cap and seal
Product reference number 72.692
https://www.sarstedt.com/en/products/laboratory/screw-cap-micro-tubes-reaction-tubes/screw-cap-micro-tubes/product/dmsarproducts/specificationPdf/Product/72.692.005/

Sodium acetate trihydrate
S1304 Sigma-Aldrich
Meets USP testing specifications
https://www.sigmaaldrich.com/catalog/product/sial/s1304?lang=en&region=GB&gclid=CjwKCAjwtJ2FBhAuEiwAIKu19jp2DL5CW1Ke85Y0Ksb5boyV-5zKyUkzvg3uVVuZXV3abjGZbVruJhoCescQAvD_BwE

Sodium cyanoborohydride
Reagent grade, 95%
156159 Sigma-Aldrich
https://www.sigmaaldrich.com/catalog/product/aldrich/156159?lang=en&region=GB

TSKgel® Amide-80 HPLC Column
https://www.sigmaaldrich.com/GB/en/product/supelco/813071

SAFETY WARNINGS

This protocol requires the use of some hazardous solvents, reagents and chemicals. Refer to the Safety Data Sheets (SDS) provided by supplier and applicable Control of Substances Harmful to Health (COSHH). The correct personal protective equipment must be worn, and incidents reported in line with your institution's policy and procedures.

BEFORE STARTING

Check that you have the required reagents, solvents, chemicals, equipment and PPE.

GSL preparation from plasma

1 Use **50 µl** plasma for GSL quantification.

2 

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Add **150 µl** de-ionised water to make the volume up to **200 µl** in a 1.5 ml screw-cap tube.

3

Add **0.8 mL** of chloroform/methanol (1:2, v/v) to give (C/M/W 4:8:3 final).

Chloroform and methanol are very toxic, and methanol is flammable. Refer to the Safety Data Sheets.

4

Leave **Overnight** at **4 °C** .

5 Vortex.

6 10m

Centrifuge at 16,000 x g for **00:10:00** at **Room temperature** .

7

Transfer supernatant (about **1 mL**) to new tube and separate into two phases: by adding **0.2 mL** PBS and then **0.2 mL** chloroform.

8 Vortex.

9 10m

Centrifuge at 16,000 x g for **00:10:00** at room temperature.

10 Remove very carefully the lower phase to a new tube and retain the upper phase.

11 Dry down the lower phase under a stream of (oxygen-free) nitrogen in heating block (**42 °C**).

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12 When dry, re-suspend the lower phase in **20 µl** chloroform/methanol (1:3).

13 Add upper phase to lower phase and vortex.

14 Pre-equilibrate C18 columns (telos, Kinesis, UK) with 4 x **1 mL** methanol and 3 x **1 mL** deionised water.

15 Load lower/upper phase mix onto column, let drip through gravity flow.

16 

Rinse sample tube with **1 mL** water, apply to column to wash.

17 

Wash column with 3 x **1 mL** water.

18 Elute GSLs into a new tube with:

18.1 **2 mL** chloroform/methanol (98:2). Push through first **0.5 mL** . You can use syringe with adapter that fits into top of column.

18.2 **2 mL** chloroform/methanol (1:3).

18.3 **1 mL** methanol.

19 

Vortex and leave **Overnight** at **4 °C** or carry on to enzymatic digestion.

GSL digestion with EGC'ase I 16h

20 

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Vortex (5 mL , C18) and dry down samples under a stream of nitrogen in heating block (42 °C).

21 When about 150 µl sample remaining, transfer to 1.5 mL screw-cap tube.

22 

Rinse sample tube with 200 µl C:M 2:1, vortex and combine with the rest of the sample in the screw-cap tube.

23 Dry down, under a slow stream of nitrogen in heating block (42 °C).

24 Re-suspend in 50 µl C:M 2:1, vortex, dry down under a very slow stream of nitrogen.

NB It is essential that ALL the sample is dried in the bottom of the tube.

25 

Add 90 µl enzyme/buffer to each sample and vortex:

25.1 rEGCase : 4 µl enzyme (stored in freezer) plus 86 µl buffer per sample.

25.2 rEGCase buffer (stored at 4 °C): 0.6 % Triton in [M]50 Milimolar (mM) sodium acetate pH 5.2.

26  16h

Incubate at 37 °C for 16:00:00 .

rEGCasel: recombinant Endoglycoceramidase I was custom synthesised by GenScript.

2AA labelling of glycans released from GSLs 1h 30m

27

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Labelling mix: 30 mg/mL 2AA in labelling buffer and 45 mg/mL sodium cyanoborohydride
Labelling buffer: 4% sodium acetate and 2% boric acid in methanol

Dissolve 2AA in labelling buffer first with vortexing.

28 

Add 2AA in buffer to sodium cyanoborohydride and vortex.

Sodium cyanoborohydride is very toxic. Refer to the Safety Data Sheet.

29 

Add **310 µl** labelling mix to the **90 µl** sample (digest) in **1.5 mL** screw-cap tube.

30 

1h 30m

Incubate in oven at **80 °C** for **01:00:00** , vortexing at **00:30:00** .

31 Allow to cool to **Room temperature** .

32 

Add **1 mL** acetonitrile:water (97:3).

33 Transfer from screw-cap tube to **15 mL** tubes.

34 Rinse screw-cap tube with 2 x **1 mL** acetonitrile:water (97:3) and add to **15 mL** tube.

35 Pre-equilibrate 50 mg Discovery® DPA-6S SPE Tube (supplied by Sigma-Aldrich) with:

1 mL acetonitrile

2 x **1 mL** water

2 x **1 mL** acetonitrile

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36 Apply samples (**3.4 mL**) to equilibrated DPA-6S columns, let drip through gravity flow.

37 

Wash columns with acetonitrile:water (95:5) → add **1 mL** into the **15 mL** tubes to wash and add to columns, and then add 3 x **1 mL** acetonitrile:water (95:5) directly onto the columns.

38 

Elute with **600 µl** water into new tubes.

39 

Add **60 µl** : **140 µl** (sample:acetonitrile) to HPLC vials and vortex.

40 For HPLC, inject **5 µl** - **50 µl** .

41 DPA-6S eluate is stable stored at **-20 °C** .

HPLC protocol 1h

42 Purified 2AA-labeled oligosaccharides are separated and quantified by normal-phase high-performance liquid chromatography (NP-HPLC) as previously described (Neville *et al.*, 2004, see the reference below).

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Analytical biochemistry 331 (2), 275-282, (2004).
doi: 10.1016/j.ab.2004.03.051.

43 The HPLC system consists of a Waters Alliance 2695 separations module and an in-line Waters 2475 multi λ-fluorescence detector set at Ex λ360 nm and Em λ425 nm.

44 The solid phase used is a 4.6 × 250 mm TSK gel-Amide 80 column maintained at **30 °C** (Anachem, Luton, UK).

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45 The chromatographic flow rate is 0.8 mL/min, and run time was 60 min. The total run time is **01:00:00** . 1h

Time (min)	0	6	35	37	39	41	42	54	60
Flow (mL/min)	0.8	0.8	0.8	0.8	0.8	0.8	1.2	1.2	0.8
% Solvent A	71.6	71.6	52.8	23.0	23.0	71.6	71.6	71.6	71.6
% Solvent B	8.4	8.4	27.2	57.0	57.0	8.4	8.4	8.4	8.4
% Solvent C	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0

Gradient conditions for Normal Phase HPLC. All chromatography was controlled and data were collected and processed using Waters Empower software.

46 The mobile phases are acetonitrile (solvent A), de-ionised water (solvent B) and **[M]100 Milimolar (mM)** ammonium acetate, **pH3.85** (solvent C).

47 Starting conditions are 71.6% solvent A. 8.4% solvent B and 20% solvent C at 0.8 mL/min constant flow-rate for 6 mins. **00:06:00** 6m

48 The gradient is developed from 6 min to 35 min by increasing solvent B from 8.4% to 27.2% with concomitant decrease in solvent A from 71.6% to 52.8 %. **00:29:00** 29m

49 From 35 to 37 min solvent B is increased to 57% and solvent A reduced to 23%, then maintained for 2 min up until 39 min. **00:04:00** 4m

50 Between 39 and 41 min the solvent ratios are returned to the starting conditions and then maintained for 1 min. **00:03:00** 3m

51 At 42 mins the flow-rate is increased to 1.2 mL/min and maintained for 12 min up to 54 min. **00:12:00** 12m

52 Between 54 and 60 min the flow-rate is gradually returned to 0.8 mL/min. **00:06:00** 6m

53

Individual GSL species are identified by their glucose unit values (GUs), calculated with the HPLC Empower software using a homopolymer dextran ladder (Fig. 2).

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53.1 To help identify GSL peaks in the plasma samples, compare the GUs of the peaks in the plasma samples with those in a GSL standard mix.

53.2

Prepare a mixture of commercially available authentic GSLs in a screw-cap tube:

- **50 µl** Matreya Neutral GSL mix Cat No 1505 (Glc-Cer, Lac-Cer, Gb3, Gb4) 1.0 mg/ml
- **100 µl** Matreya Ganglioside mix Cat No 1510 (Lac-Cer, GM3, GD3) 0.5 mg/ml
- **100 µl** Matreya Ganglioside mix Cat No 1511 (GA1, GM1a, GD1a, GD1b,GT1b) 0.5 mg/ml
- **30 µl** Matreya GM2 Ganglioside Cat No 1502 (GM2) 0.5 mg/ml
- **30 µl** Sigma Asialo-GM2 Cat No G9398 (GA2) 1.0 mg/ml

53.3 Dry down the mixture under nitrogen and then digest, label and clean up as done for the plasma samples in steps 25-41.

53.4 Aliquot and store at **-20 °C** .

53.5 In order to calculate molar quantities from integrated peaks in the chromatogram, inject a calibration standard containing 2.5 pmol 2AA-labelled chitotriose (Ludger) with each sample set (not shown).

Figures 1- 4: HPLC profile and sugar structures for GSLs in human plasma together with the GSL biosynthetic pathway

54

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Figure 1 GSL Biosynthetic pathway in the background together with a typical HPLC trace below. GSLs detected in the profile are highlighted in the scheme.

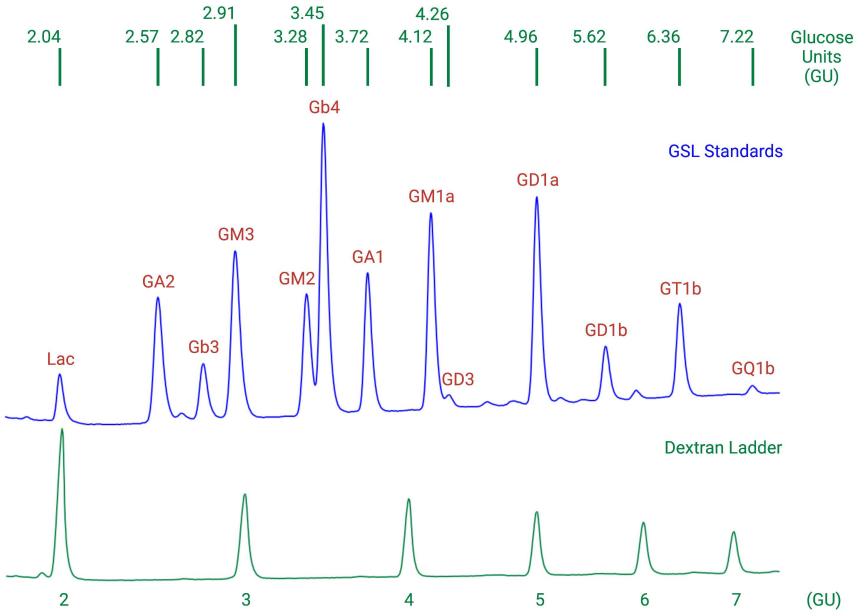

Figure 2 HPLC profiles for GSL standards and 2AA-labelled homopolymer dextran ladder. Glucose Units were calculated using the retention times of a 2AA-labelled homopolymer dextran ladder and are shown for each of the GSL standard peaks.

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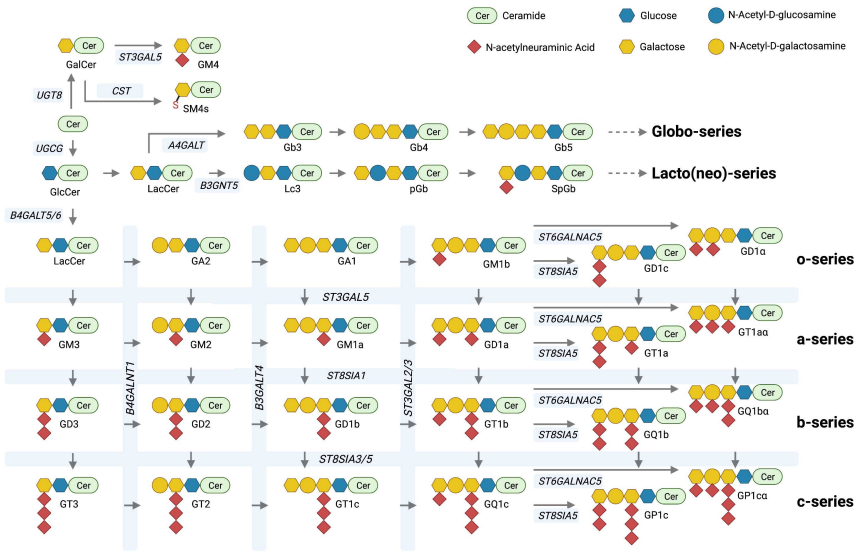

Figure 3 GSL biosynthesis. Biosynthetic enzymes genes are indicated in the blue grid. Ganglioside names are abbreviated according to Svennerholm [1]. [1] L. Svennerholm, Designation and schematic structure of gangliosides and allied glycosphingolipids, Prog Brain Res 101 (1994) XI-XIV.

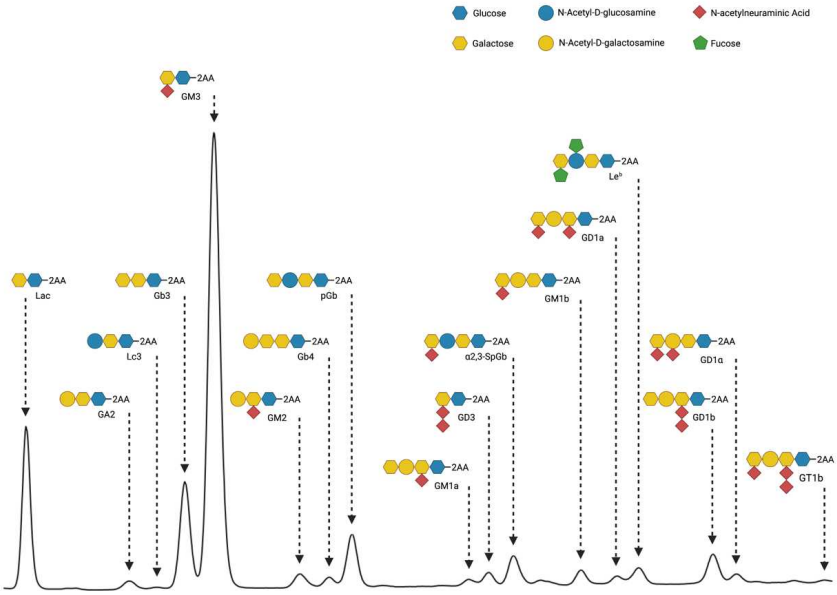

Figure 4 'Typical HPLC profile for 2AA-labelled glycans released from human plasma glycosphingolipids with sugar structures indicated for each peak. In order to calculate values for total GSL concentrations, use the sum of the areas for all peaks from LacCer to GT1b. GlcCer, which elutes before LacCer, is not included because it is not fully hydrolysed by EGC'ase I.

Figures created with BioRender.com

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