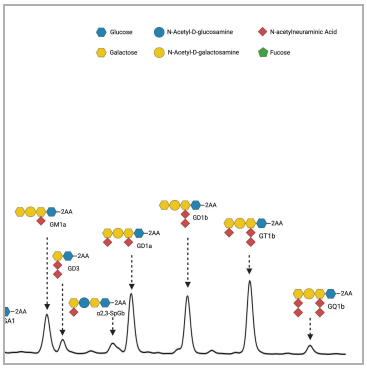

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Analysis of glycosphingolipids from human cerebrospinal fluid

Forked from Analysis of glycosphingolipids from human plasma

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[5]Wellcome Trust Investigator in Science.

Reuben Bush

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 cerebrospinal fluid 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.

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

Protocol status: Working
We use this protocol and it's working

Created: Oct 30, 2022

Last Modified: Feb 20, 2023

PROTOCOL integer ID: 72040

Keywords: Glycosphingolipids, Glucosyl Ceramide, HPLC, Oligosaccharide Analysis, ASAPCRN

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
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 START INSTRUCTIONS

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

GSL preparation from cerebrospinal fluid

1 Use 100 µL cerebrospinal fluid for GSL quantification.

2 Add 100 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).

Safety information

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

4 Leave at 4 °C for 16:00:00 16h

5 Vortex.

6 Separate into two phases: by adding 0.2 mL PBS and then 0.2 mL chloroform.

7 Vortex.

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

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

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

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

12 Add upper phase to lower phase and vortex.

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

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

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

16 Wash column with 3 x 1 mL water.

17 Elute GSLs into a new tube with:

17.1 2 mL chloroform/methanol (98:2). Push through first 0.5 mL. You can use syringe with adapter that fits into top of column.

17.2 2 mL chloroform/methanol (1:3).

17.3 1 mL methanol.

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

GSL digestion with EGC'ase I

16h

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

20 When about 150 µL sample remaining, transfer to 1.5 mL screw-cap tube.

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

22 Rinse sample tube with 200 µL chloroform, 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 200 µL C:M 2:1, vortex, dry down under a very slow stream of nitrogen.

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

Note

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

26 Add 90 µL enzyme/buffer to each sample and vortex:

26.1 rEGCase : 5 µL enzyme (stored in freezer) plus 85 µL buffer per sample.

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

27 Incubate at 37 °C for 16:00:00. 16h

> **Note**
>
> rEGCaseI: recombinant Endoglycoceramidase I was custom synthesised by GenScript.

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

28

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

Dissolve 2AA in labelling buffer first with vortexing.

29 Add 2AA in buffer to sodium cyanoborohydride and vortex.

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

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

31 Incubate in oven at 80 °C for 01:00:00, vortexing at 00:30:00. 1h 30m

32 Allow to cool to Room temperature.

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

34 Transfer from screw-cap tube to 15 mL tubes.

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

36 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

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

38 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.

39 Elute with 600 µL water into new tubes.

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

41 Resuspend in 60 µL of 70:30 acetonitrile:water

42 For HPLC, inject 50 µL .

HPLC protocol

1h

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 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).

Note

Analysis of fluorescently labeled glycosphingolipid-derived oligosaccharides following ceramide glycanase digestion and anthranilic acid labeling
DCA Neville, V Coquard, DA Priestman, DJM te Vruchte, DJ Sillence, Raymond A Dwek, Frances M Platt, Terry D Butters
Analytical biochemistry 331 (2), 275-282, (2004).
doi: 10.1016/j.ab.2004.03.051.

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

46 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.

47 The mobile phases are acetonitrile (solvent A), de-ionised water (solvent B) and [M] 100 millimolar (mM) ammonium acetate, pH 3.85 (solvent C).

48 Starting conditions are 71.6% solvent A. 8.4% solvent B and 20% solvent C at 0.8 mL/min constant 6m

flow-rate for 6 mins. 00:06:00

49 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

50 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

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

52 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

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

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

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

54.2 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

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

54.4 Aliquot and store at -20 °C.

54.5 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- 5: HPLC profile and sugar structures for GSLs in …

55

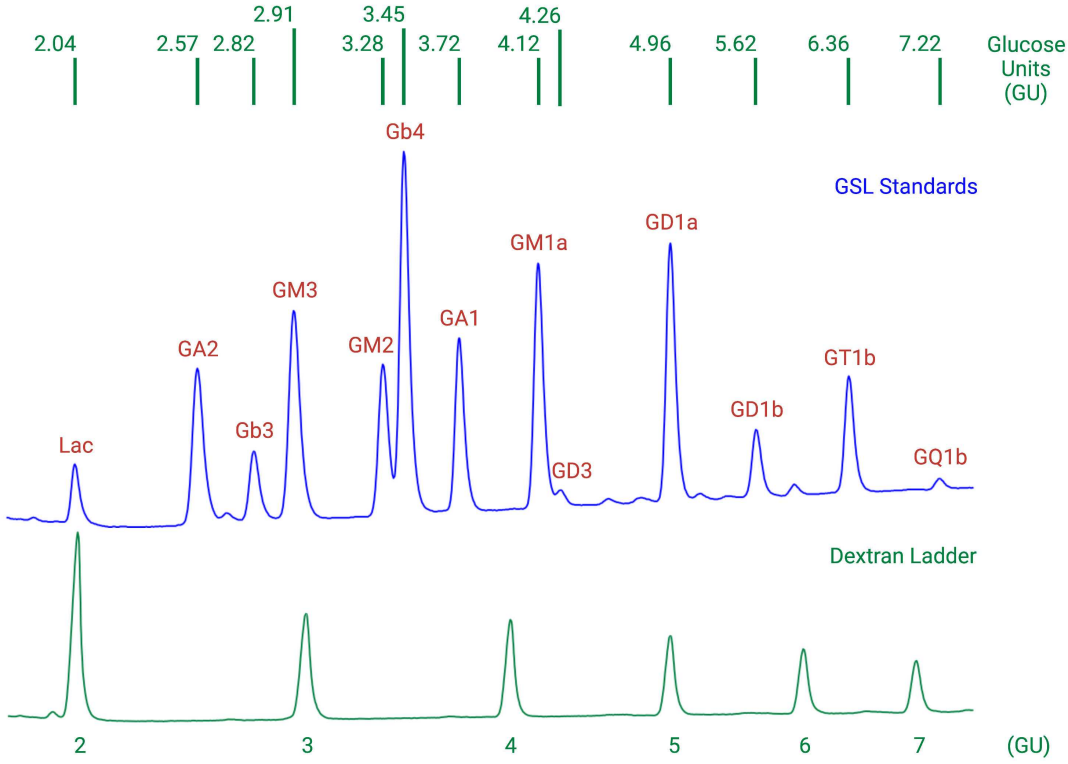

Figure 1 HPLC profiles for GSL standards and 2AA-labelled homopolymer dextran ladder. Glucose Units were calculated using the retention times of a 2AA-labelled homopolymer dextran ladder and are shown for each of the GSL standard peaks.

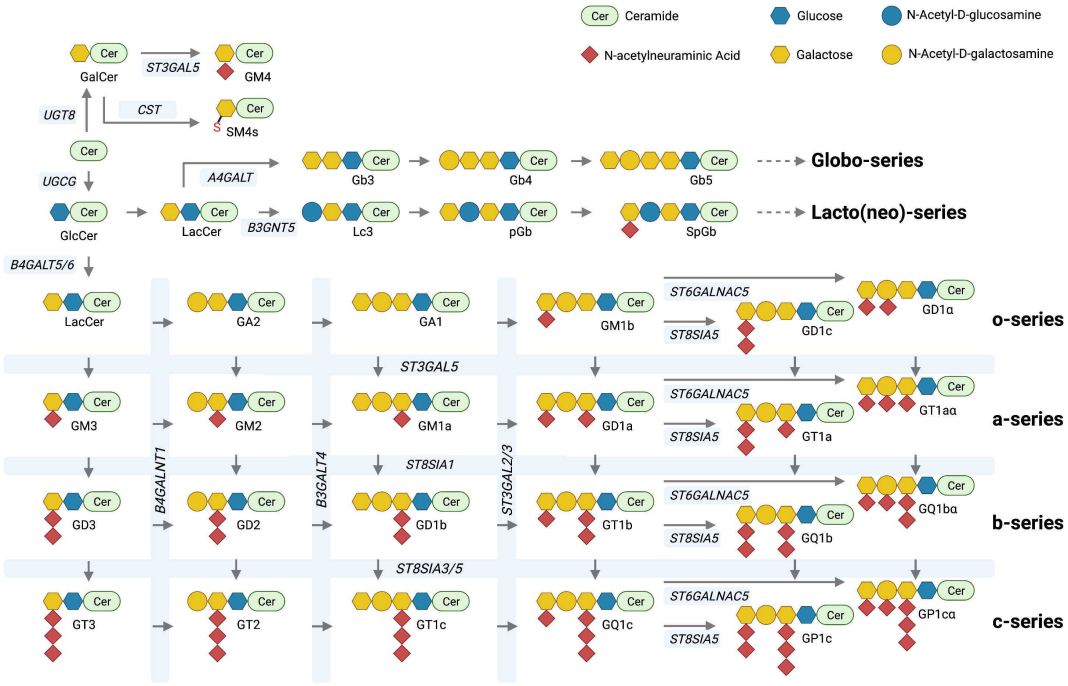

Figure 2 GSL biosynthesis. Biosynthetic enzymes genes are indicated in the blue grid. Ganglioside names are abbreviated according to Svennerholm [1]. [1] L. Svennerholm, Designation and schematic structure of gangliosides and allied glycosphingolipids, Prog Brain Res 101 (1994) XI-XIV.

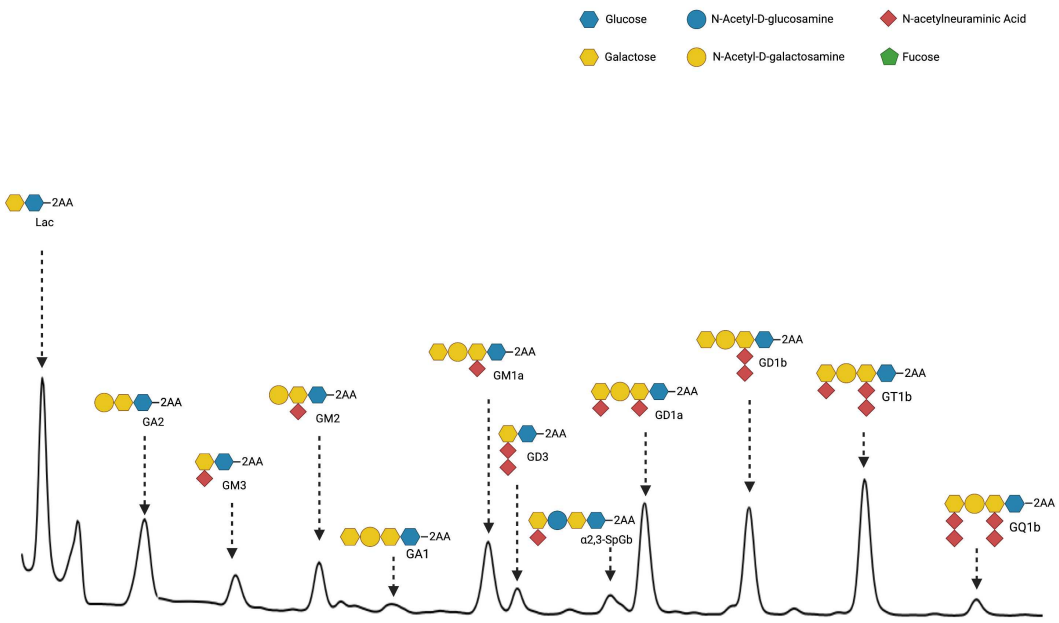

Figure 3 Typical HPLC profile for 2AA-labelled glycans released from ante-mortem human CSF glycosphingolipids with sugar structures indicated for each peak. In order to calculate values for total GSL concentrations, use the sum of the areas for all peaks from LacCer to GQ1b. GlcCer, which elutes before LacCer, is not included because it is not fully hydrolysed by EGC'ase I.

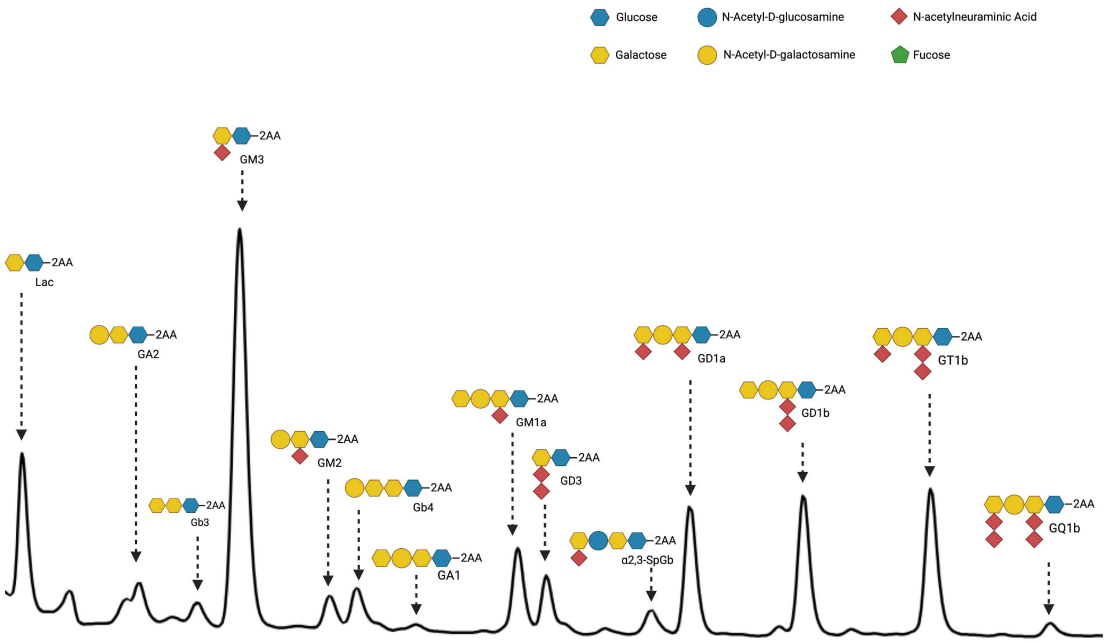

Figure 4 Typical HPLC profile for 2AA-labelled glycans released from post-mortem human CSF glycosphingolipids with sugar structures indicated for each peak. In order to calculate values for total GSL concentrations, use the sum of the areas for all peaks from LacCer to GQ1b. GlcCer, which elutes before LacCer, is not included because it is not fully hydrolysed by EGC'ase I.

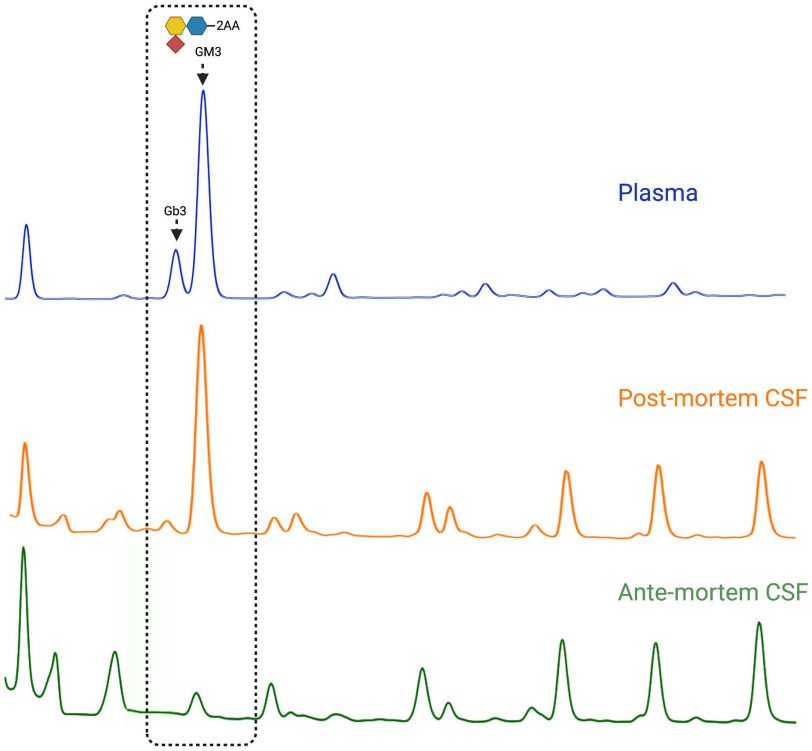

Figure 5 Overlay of typical HPLC profiles for 2AA-labelled glycans released from human: plasma, post-mortem CSF, and ante-mortem CSF. Due to breakdown of the blood-cerebrospinal fluid barrier, post-mortem CSF is characterised by significant increases in GSLs that are prevalent in plasma, notably, GM3 and Gb3.

Note

Figures created with BioRender.com