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Analysis of sphingosine, sphinganine and glucosylsphingosine from cells, animal tissues and plasma

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We use this protocol and it's working

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Grant ID: 202834/Z/16/Z

Abstract

Sphingosine (SphO), sphinganine (SphA) and glucosylsphingosine (GlcSph) are crucial molecules involved in sphingolipid metabolism. Sphingosine and sphinganine are long-chain bases that serve as building blocks for more complex sphingolipids like ceramides and glucosylceramides (GlcCer). Glucosylsphingosine comprises of a sphingoid base with glucose attached.
Here we describe a method which can measure sphingosine, sphinganine and glucosylsphingosine in cell lines, animal tissue and plasma. The extracted sphingosine, sphinganine and glucosylsphingosine
are reacted with Phthaldialdehyde (OPA) reagent, in the presence of the thiol compound mercaptoethanol, to form a fluorescent derivative which can be measured by reverse phase HPLC. With the inclusion of OPA-labelled C20 sphingosine or OPA-labelled C20 glucosylsphingosine standards, it is possible to obtain molar quantities of these molecules.

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

Acetone for HPLC, ≥99.8%
34850-M Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sial/34850m

Acetonitrile for HPLC, gradient grade, ≥99.9%
34851 Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigald/34851

Bicinchonic Acid solution
B9643-1L Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigma/b9643?srsltid=AfmBOoq6n40M013YTRhV9rEDobVTfCL49_Z9ZPBpcNM3KWP8Pu7Zcnx7

Boric acid
194810 MP Biomedicals, LLC
https://www.mpbio.com/uk/boric-acid?queryID=27e466009c404622801dbc590258b64b&objectID=52001&indexName=MpBio_Production_Store_uk_products

Chloroform Stabilized with Amylene, for HPLC, ≥99.8%
C/4966/17 Fisher Scientific
https://www.fishersci.co.uk/shop/products/chloroform-stabilized-amylene-hplc-fisher-chemical/10615492

Chromolith Performance RP-18e 100-4.6 HPLC column (Merck, Darmstadt, Germany)
1.02129.0001 VWR International Ltd.
https://www.avantorsciences.com/uk/en/catalog-number/1.02129.0001

Copper(II) sulfate solution
C2284 Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigald/c2284

Ethanol, ≥99.8%
32221-M Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sial/32221m

C18-glucosylsphingosine standard (1 mM in EtOH, store at -80°C)
860535 Glucosyl(ß) Sphingosine (d18:1), Avanti Polar Lipids
https://avantiresearch.com/product/860535

C20-glucosylsphingosine standard (1 mM in EtOH, store at -80°C)
860438 Glucosyl (ß) Sphingosine (d20:1), Avanti Polar Lipids

https://avantiresearch.com/product/860438

2-Mercaptoethanol, ≥99.0%
63689 Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigma/63689?srsltid=AfmBOoqw0ieNmCN9Guq7aBo2lcVdf0TOeeGQL0V-AdRfGXVe2Gh1iCOF

Methanol for HPLC grade, ≥99.9%
34860 Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigald/34860

OPA (Phthaldialdehyde), ≥99.0% (HPLC)
79760 Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigma/79760

Sodium chloride, AR
S/3160/60 Fisher Scientific
https://www.fishersci.co.uk/shop/products/sodium-chloride-certified-ar-analysis-meets-analytical-specification-ph-eur-fisher-chemical/10428420

Sodium hydroxide
06203 Sigma-Aldrich
https://www.sigmaaldrich.com/GB/en/product/sigald/06203?srsltid=AfmBOorZjlns7uY_IQmW22fbzx3HWex8Bj9AgA7MtlEDMgAdZX2UH58V

C18-sphinganine standard (1 mM in EtOH, store at -80°C)
860498 Sphinganine (d18:0), Avanti Polar Lipids
https://avantiresearch.com/product/860498

C18-sphingosine standard (1 mM in EtOH, store at -80°C)
860490 Sphingosine (d18:1), Avanti Polar Lipids
https://avantiresearch.com/product/860490

C20-sphingosine standard (1 mM in EtOH, store at -80°C)
860660 Sphingosine (d20:1), Avanti Polar Lipids
https://avantiresearch.com/product/860660

Tris base
sc-3715A Santa Cruz
https://www.scbt.com/p/tris-base-77-86-1?srsltid=AfmBOorCdFXWCa0RlGTvscM5u57kFWQMmJ_4FX1ABllZcZ2giYjad8Zp

ISOLUTE® NH2 100 mg/1 mL

#470-0010-A Biotage
https://www.biotage.com/isolute-nh2

Equipment:
Pipettes
Homogeniser
Vortex mixer
Ultrasonic water bath
Centrifuge
Sample concentrator (Heatblock with a nitrogen supply)
RP-HPLC

Troubleshooting

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

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

Cells homogenisation

1 Lyse and homogenise cell pellet (≥ 1 × 10^6 cells) in 200 µL ddH_2O through three cycles of freeze-thawing with vortexing after each cycle.

2 Measure protein concentration using the Bicinchonic acid assay (BCA) method in triplicate (repeat if triplicate values are not consistent).

3 Using determined protein concentrations, prepare samples as 200 µg protein in 100 µL ddH_2O in a 2 ml screw-cap tube on ice.

Animal tissue homogenisation

4 Homogenise tissue in ddH_2O yielding tissue concentration of 25 mg tissue (wet weight) per 1 mL ddH_2O.

5 Measure protein concentration using the Bicinchonic acid assay (BCA) method in triplicate (repeat if triplicate values are not in consistent).

6 Using determined protein concentrations, prepare samples as 200 µg - 500 µg protein in 100 µL ddH_2O in a 2 ml screw-cap tube on ice.

Plasma

7 Use 50 µL plasma and add 50 µL ddH_2O in a 2 ml screw-cap tube on ice.

Extraction of Sph and GlcSph from cells, tissue and plasma

35m

8 **Example of the experiment layout:**

	Sample/Standard name	ddH2O	Sample	Standard Master Mix	Standard
	Sample 1	50 µl	50 µl	500 µl C20 MM	-
	Sample 2	50 µl	50 µl	500 µl C20 MM	-
	Sample 3	50 µl	50 µl	500 µl C20 MM	-
	Sample 4	50 µl	50 µl	500 µl C20 MM	-

	Sample/Standard name	ddH2O	Sample	Standard Master Mix	Standard
	Sample 5, etc.	50 µl	50 µl	500 µl C20 MM	-
	Blank	100 µl	-	500 µl CHCl3:MeOH (1:2)	-
	C18 SphO	100 µl	-	500 µl C20 SphO mix	3 µl 0.1 mM C18 SphO
	C18 SphA	100 µl	-	500 µl C20 SphO mix	3 µl 0.1 mM C18 SphA
	C20 SphO	100 µl	-	500 µl C20 SphO mix	-
	C18 GlcSph	100 µl	-	500 µl C20 GlcSph mix	3 µl 0.1 mM C18 GlcSph
	C20 GlcSph	100 µl	-	500 µl C20 GlcSph mix	-

See steps 9 and 10 below on how to make up the standards and standard master mixes.

9 5 standards and a blank are needed:
- 1 mM C18 sphingosine (C18 SphO),
- 1 mM C18 sphinganine (C18 SphA),
- 1 mM C20 sphingosine (C20 Sph),
- 1 mM C18 glucosylsphingosine (C18 GlcSph),
- 1 mM C20 glucosphingosine (C20 GlcSph),
- Blank (with only $CHCl_3$:MeOH (1:2)).

9.1 Make 0.1 mM C18 SphO, 0.1 mM C18 SphA and 0.1 mM C18 GlcSph standard solutions: 50 µL stock (1 mM) + 0.5 mL EtOH.

10 Make the different C20 (master) mixes as described below in Steps 10.1 to 10.3:

10.1 For the samples make a C20 master mix **(C20 MM)** in a 15 ml tube: 6 µL 1 mM C20 Sph and 6 µL 1 mM C20 GlcSph in 10 mL CH_3Cl:MeOH (1:2) (need 500 µL MM C20 per sample, scale up if necessary).

10.2 For the Sph standards make a C20 SphO mix **(C20 SphO mix)** in a 15 ml tube: 3 µL 1 mM C20 Sph in 5 mL $CHCl_3$:MeOH (1:2) (need 500 µL MM C20 SphO per Sph standard).

10.3 For the GlcSph standards make a C20 GlcSph mix **(C20 GlcSph mix)** in a 15 ml tube:

3 µL 1 mM C20 GlcSph in 5 mL $CHCl_3$:MeOH (1:2) (need 500 µL MM C20 GlcSph per GlcSph standard).

11 For the blank and the standards put 100 µL ddH_2O in a 2 ml screw-cap tube.

12 Add 500 µL C20 MM to the sample tubes,
500 µL C20 SphO mix to the C18 SphO, C18 SphA and C20 SphO standard tubes,
500 µL C20 GlcSph mix to the C18 GlcSph and C20 GlcSph standard tubes and just
500 µL pure $CHCl_3$: MeOH (1:2) into the Blank tube.

13 Add 3 µL of 0.1 mM C18 standards according to the standard tubes labels (see experiment layout).

14 Sonicate all tubes for 00:10:00 at Room temperature . 10m

15 Add 500 µL NaCl (1 M).

16 Add 500 µL $CHCl_3$.

17 Add 100 µL NaOH (3 M).

18 Vortex and leave for 00:15:00 at Room temperature (vortex every 5 min). 15m

19 Centrifuge at 16.000 x g for 00:10:00 at Room temperature . 10m

20 Transfer the lower, organic phase very carefully to a new 1.5 ml Eppendorf tube (**keep!**), while leaving the upper phase and protein interphase (which can be discarded).

Purification of Sph and GlcSph

21 Condition the right number of ISOLUTE ®NH2 100 mg/1 mL columns with 2 x 1 mL $CHCl_3$.

22 Apply the extracted organic phase to the columns, let drip through gravity flow, discard flow-through.

23 **Elute first sphingosine and sphinganine, then glucosylsphingosine from the same column:**

23.1 To elute sphingosine and sphinganine: Apply 900 µL acetone to the column and collect the eluent in a new 1.5 ml screw-cap tube. After elution, use syringe to push to empty the column.

23.2 To elute glucosylsphingosine: Apply 900 µL acetone:EtOH (6:1) to the column and collect the eluent in a new 1.5 ml screw-cap tube. After elution, use syringe to push to empty the column.

24 Dry down the solvents under a slow stream of N_2 gas.

25 The samples are now ready for OPA-labelling (or they can be stored at -20°C for OPA-labelling next day).

OPA-labelling of Sph and GlcSph

22m

26 Solutions:
3% boric acid, pH 10.5:
dissolve 6 g boric acid in 180 mL H_2O
adjust pH with 10 M KOH to 10.5
fill up to 200 mL (Filter sterilize).

OPA-labelling solution: weigh 1 mg – 5 mg OPA in 15 ml tube covered with aluminium foil.
Dissolve OPA in EtOH (20 µl/mg OPA) (e.g. 1 mg OPA: add 20 µl EtOH),
add 2-mercaptoethanol (1 µl/mg OPA) (e.g. 1 mg OPA: add 1 µl 2-mercaptoethanol),
dilute 1:2000 with 3% boric acid (e.g. 1 mg OPA: add 2 ml 3% boric acid).

27 Prepare the OPA-labelling solution just before labelling (light-sensitive, instable).

28 Resuspend the lipids in 50 µL pre-warmed EtOH (37 °C water bath/heat block), vortex gently.

29 Add 50 µL fresh OPA-labelling solution to each sample and vortex.

30 Incubate at Room temperature in the dark for 00:20:00 . Vortex again after 10 min. 20m

31 Add 100 µL MeOH:5 mM Tris pH 7 (9:1) and vortex.

32 Centrifuge at 3000 rpm for 00:02:00 at Room temperature . 2m

33 Transfer 150 µL of the supernatant (carefully pipette from the top) to a HPLC vial with plastic insert.

34 For RP-HPLC inject 20 µL - 50 µL .

RP-HPLC 30m

35 The RP-HPLC system consists of a VWR Hitachi Elite LaChrom HPLC system with a L-2485 fluorescence detector set at Ex λ340nm and Em λ455nm.

36 The solid phase used is a Chromolith Performance RP-18e 100-4.6 HPLC column (Merck, Darmstadt, Germany).

37 The chromatographic flow rate is 2.0 mL/min, and total run time per sample is 00:30:00 . 30m

	Time	100% MeOH	100% ddH2O	100% acetonitrile	80% ddH2O/20% acetonitrile
	0.0	0.0	0.0	64.0	36.0
	2.0	0.0	0.0	64.0	36.0
	7.0	0.0	0.0	88.0	12.0

	Time	100% MeOH	100% ddH2O	100% acetonitrile	80% ddH2O/20% acetonitrile
	8.0	5.0	0.0	89.0	6.0
	22.0	100.0	0.0	0.0	0.0
	24.0	100.0	0.0	0.0	0.0
	26.0	0.0	0.0	64.0	36.0
	30.0	0.0	0.0	64.0	36.0

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

38 The mobile phases are MeOH, acetonitrile and 80% ddH_2O/20% acetonitrile mix.

39 The gradient conditions for the RP-HPLC are as described in the table above.

40 Identification of Sph-species: Individual sphingosine species are identified by their retention time in comparison to standards.

41 Approximate Retention Times:
C18 GlcSph ~ 3.80 min
C20 GlcSph ~ 6.06 min
C18 SphO ~ 6.78 min
C18 SphA ~ 8.00 min
C20 SphO ~ 9.18 min

42 Quantification: Sph species are quantified by comparison of integrated peak areas with a known amount of spiked OPA-labelled C20 sphingosine standard (Avanti Polar Lipids) or OPA-labelled C20 glucosylsphingosine standard (Avanti Polar Lipids) in each sample.

43 Results should be normalized to protein content or per ml of plasma.