Project: Lengyel 18 hour time point WCMC Pilot & Feasibility Study

Summary Text of CERAMIDE SPE EXTRACTION & ANALYSIS PROTOCOL

Conducted in the laboratory of Dr. John W. Newman

Ceramide Extraction

Ceramides were isolated by solid phase extraction on 10 mg Waters Oasis-HLB cartridges (Milford, MA), as previously described by Luria et al (1). Prior to extraction, cartridges were washed with 1 column volume ethyl acetate followed by 2 column volumes methanol and conditioned with 2 mL of 95:5 v/v water/methanol (MeOH) with 0.1% acetic acid. The column reservoir was spiked with 5 µL anti-oxidant solution, (0.2 mg/ml solution BHT/EDTA in 1:1 MeOH:water), and 10 µL 1000nM analytical surrogates (See Table 2 below for specific compounds). Sample aliquots (250 µL media) were then introduced to the column reservoir and diluted with 1 column volume wash solution (5% MeOH, 0.1% acetic acid). Sample was allowed to gravity extract and the sorbent bed was then washed with 1 column volumes wash solution (20% methanol, 0.1% acetic acid). SPE cartridges were dried by vacuum @ -7.5in Hg for 20 min. Analytes were then eluted by gravity with 0.2 mL MeOH, followed by 0.5 mL Acetonitrile, followed by 0.5 mL Ethyl Acetate, into 2 mL autosampler vials containing 10 µL 20% glycerol solution in MeOH. Eluent was dried by vacuum evaporation for 35 min, and residues were re-constituted with 100uL of 100 nM internal standard solution containing 1-cyclohexyl ureido,3-dodecanoic acid (CUDA), in 50:50 MeOH:ACN. Vials were vortexed for 1 min to dissolve residues chilled 15 min on wet ice, and extracts were transferred to a centrifugal filter (0.1 µm Durapore, Millipore, Billerica, MA), centrifuged for 3 min at 6ºC at <4500g (rcf) and transferred to 150 uL glass inserts and into the 2 mL amber vials, and cap. Extracts were stored at -20ºC until analysis by UPLC-MS/MS. The internal standard was used to quantify the recovery of the deuterated extraction surrogates by ratio response.

Ceramide Analysis

Analytes in a 10 µL injection of extract were separated with an Aquity C_8 BEH 1.7µm 100mm x 2.1mm column utilizing a Waters Acquity UPLC (Waters, Milford, MA) with the solvent gradient described in Table 1, slightly modified from a previously published protocol (2). The autosampler was maintained at 10ºC. Resolved analytes were detected by positive mode electrospray ionization and multiple reaction monitoring on a API 4000 QTrap (AB Sciex, Framingham, MA, USA) using the following operating parameters: Curtain gas = 20.0 psi, temperature = 450 °C, IonSpray voltage = 4500.00, collision gas = high, ion source gas 1 & 2 = 40.0 psi, collision cell exit potential = 10.0 V, and entrance potential = 10.0 V. Analyte retention times, mass transitions, optimized collision and declustering potential voltages, dwell times, and analytical surrogate associations for each analyte are shown in Table 2. Analytes were quantified using isotope dilution and internal standard methodology with 5 to 7 point calibration curves ($r2 \geq 0.997$). Calibrants and internal standards were either synthesized [CUDA] or purchased from Avanti Polar Lipids Inc. (Alabaster, AL) unless otherwise indicated. Data was processed utilizing AB Sciex Analyst version 1.6.2. Surrogate recoveries can be viewed in Table 3.

(1) Luria A et al (2007). Compensatory mechanism for homeostatic blood pressure regulation in Ephx2 gene-disrupted mice. *J Biol Chem*. 282:2891-8

(2) Bielawski J et al (2009). Comprehensive quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Methods Mol Biol*. 579:443-67.

Table 1. UPLC parameters

Time (min)	A%	B%
0	90	70
2	90	80
5	60	85
5.5	60	90
13.5	20	95
13.75	20	99
14.5	5	99
14.7	5	70
15.2	90	70

Solvent A = 5 mm NH4COO 0.2% formic acid; Solvent B = 5 mm NH4COO 0.2% formic acid in MeOH, flow rate = 0.25 mL/min, column 2.1 X 100mm, 1.7 µm BEH C8 (Waters, Milford, MA), column temp = 60 °C

Table 2. UPLC/Electrospray ionization QTRAP analyte and instrument specific parameters*

Analyte	tR (min)	Transition (Da)	Declustering (V)	Collision (V)	ISTD†
1-Cyclohexyl Urea 3-Dodecanoic Acid	3.0	341.3 > 216.2	60	24	---
17:1 Sphingosine	3.4	286.4 > 268.3	40	15	CUDA
18:1 Sphingosine	3.8	300.4 > 282.4	40	21	17: 1 Sphingosine
17:1 Sphingosine-1P	3.7	366.4 > 250.3	50	23	CUDA
18:1 Sphingosine-1P	4.0	380.4 > 264.4	50	25	17: 1 Sphingosine-1P
18:0 Sphinganine-1P	4.0	382.4 > 266.4	50	25	17: 1 Sphingosine-1P
C14 Ceramide	8.8	510.7 > 492.6	50	21	C17 Ceramide
C16 Ceramide	9.5	538.8 > 264.4	55	37	C17 Ceramide
C18:1 Ceramide	9.8	564.5 > 546.4	60	24	C17 Ceramide
C17 Ceramide	9.9	552.8 > 534.5	55	24	CUDA
C18 Ceramide	10.3	566.7 > 264.4	55	37	C17 Ceramide
C18 dihydroceramide	10.6	568.7 > 266.4	85	33	C17 Ceramide
C20 Ceramide	11.3	594.4 > 576.5	55	21	C17 Ceramide
C24 Ceramide	13.3	650.9 > 264.4	55	42	C17 Ceramide
C24 dihydroceramide	13.6	652.9 > 266.4	55	42	C17 Ceramide
C25 Ceramide	11.3	664.9 > 264.4	55	45	C17 Ceramide

* - Analytes were separated under conditions described in Table I. Collision-induced dissociation was performed with nitrogen at a pressure of 2.3 mTorr.

Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

† - Internal Standards (ISTD) - Analytes were corrected for recoveries of listed surrogates.

1-Cyclohexylureido,3-dodecanoic acid (CUDA) was introduced immediately prior to analysis and used to quantify surrogate recoveries.

Table 3. Analytical surrogate recoveries

Chemical class	Compound	Mean ± SD	%RSD
Sphingosine	17: 1 Sphingosine	78.8 ± 11.9	15.1%
Sphingosine-1P	17: 1 Sphingosine-1P	57.3 ± 18.1	31.7%
Ceramide	C17 Ceramide	51.2 ± 8.96	17.5%

† -Relative standard deviation (standard deviation divided by the mean) x 100