Project: Lengyel 4 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 C8 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 ≥ 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.

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| **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 | | |

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| **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. | |  |  |  |  |

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| **Table 3.** Analytical surrogate recoveries | |  |  |
| **Chemical class** | **Compound** | **Mean ± SD** | **%RSD** |
| Sphingosine | 17: 1 Sphingosine | 43.6 ± 10.8% | 24.8% |
| Sphingosine-1P | 17: 1 Sphingosine-1P | 54.5 ± 16.8% | 30.8% |
| Ceramide | C17 Ceramide | 130 ± 41.9% | 32.3% |
| † -Relative standard deviation (standard deviation divided by the | | | |
| mean) x 100 |  |  |  |