Shotgun Lipidomics: SOP

Reagents and Internal Standards

High-performance liquid chromatography (HPLC) grade acetonitrile, dichloromethane were purchased from Sigma (St. Louis, MO) isopropanol was purchased from Optima – LC/MS, Fisher (New Jersey, NJ), methanol from – J.T. Baker. Water was obtained from a Millipore high purity water dispenser (Billerica, MA). Mass spectrometry grade lipid standards (Table 1), namely, 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine LPC (17:0/0:0), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine PC (17:0/17:0), 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine PE (17:0/17:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) PS (17:0/17:0), N-heptadecanoyl-D-*erythro*- sphingosylphosphorylcholine 17:0 SM (d18:1/17:0), cholest-5-en-3ß-yl heptadecanoate 17:0 cholesteryl ester, 1-palmitoyl-2-oleoyl-*sn*-glycerol 16:0-18:1 DG, 1-heptadecanoyl-rac-glycerol 17:0 MG, 1,2,3-triheptadecanoyl-glycerol Triheptadecanoate 17:0TAG, N-heptadecanoyl-D-*erythro*-sphingosine C17 Ceramide (d18:1/17:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphate (sodium salt) 17:0 PA, 1,2-diheptadecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) 17:0 PG), 1-heptadecanoyl-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phospho-(1'-myo-inositol) (ammonium salt) 17:0-20:4 PI.

Table 1: Internal standards used for the study:

Item #	Product #	CAS-Number	Description	MW	100pmol/uL from 1mg/mL stock
1	855676P	50930-23-9	17:0 Lyso PC	509.348	50.9uL
2	850360P	70897-27-7	17:0 PC	761.593	76.1uL
3	830756P	140219-78-9	17:0 PE	719.547	71.9uL
4	830456P	799268-52-3	17:0 PG	772.523	77.2uL
5	860517P	67492-16-4	17:0 Ceramide	551.528	55.1 uL
6	860585P	121999-64-2	17:0 SM	716.583	71.6uL
7	840028P	799268-51-2	17:0 PS	785.518	78.5uL
8	830856P	154804-54-3	17:0 PA	698.486	69.8uL
9	111001	Not available	17:0 TG	848.783	84.8uL
10	110607	5638-14-2	17:0 MG	344.293	34.4uL
11	800815C	29541-66-0	34:1DAG	594.522	29.761(2mg/mL stock)
12	110864	24365-37-5	17:0ChoE	632.8	63.2

Preparation of 100pmol/uL Stock in 1mL from 1mg/mL stock solutions:

50.6uL Lyso PC, 76.1uL PC, 71.9uLPE, 77.2uL PG, 55.1uL ceramide, 71.6uL Sm, 78.6uLPS, 69.8uL PA, 84.8uL TG, 43.4uL and MG 30ul and DAG 29.7 and 63.8 uL ChoE pipetted out from corresponding 1mg/mL stock solutions. Total 764uL +236uL Buffer (CHCl3:MeOH 2:1) =100pmol/uL

Sample preparation for mass spectrometry analysis:

All samples will be maintained at -80^{o}C until analyzed. The lipids were extracted using a modified Bligh-Dyer method. The extraction will be carried using 2:2:2 volume ratio of water/methanol/dichloromethane at room temperature after spiking internal standards lipids (17:0LPC, 17:0PC, 17:0PE, 17:0PG, 17:0 ceramide, 17:0SM, 17:0PS, 17:0PA, 17:0TG, 17:0MG and 17.0-20.4 PI). The organic layer will be collected and dried completely under nitrogen. Prior to mass spectrometry analysis, the dried extract was resuspended in 100 µL of Buffer B (10:90 ACN/IPA)) containing 10mM ammonium acetate and analyzed using LC-MS based lipidomics.

Sample extraction Protocol:

1. 50uL plasma was transferred 1.7mL eppendroff tubes.
2. 50uL Pooled human plasma and 50uL Pool of Test samples used as QC and Pooled internal standard were used for instrument performance
3. 200uL 0.15M KCl in water, 400uL Methanol, 200uL dichloromethane and 1uL acetic acid were added, mixed well.
4. Equimolar 10uL (from 100pmol/uL stock) internal standard containing 10 different lipids (17:0LPC, 17:0PC, 17:0PE, 17:0PG, 17:0 ceramide, 17:0SM, 17:0PS, 17:0PA, 17:0TG 17:0MG and 34:1 DAG) Mix and 20uL 17:0PI (LM-1802)
5. Add 200uL water and 200uL dichloromethane.
6. Vortex gently, left at RT for 5 minutes.
7. Centrifuge at 4000RPM for 5 minutes at RT.
9. Carefully collect organic layer (lower) containing lipids.
10. Dried under N2 for 1. 30 minutes.
11. Reconstitute with 100ul buffer B (85%IPA+5%H2O+10%ACN)
12. 5uL injected into MS both positive and negative mode.

Internal Standards and Quality Controls

The lipid stock solutions were prepared by weighing an exact amount of the lipid internal standards in a Chloroform/MeOH/H_2O as prescribed by Avanti Polar Lipids (Alabaster, AL) resulting in a concentration of about 1mg/mL and were stored at -20 °C. For analysis, the stock solutions were further diluted to 100pmol/uL by mixing of appropriate volume of each internal standard s LPC 17:0/0:0, PG 17:0/17:0, PE 17:0/17:0, PC 17:0/17:0 and TG 17:0/17:0/17:0, PG

17:0/17:0, PE 17:0/17:0, PC 17:0/17:0, and TG 17:0/17:0/17:0. 17:0-20:4 was purchased and used as such.

Two kinds of controls will be used to monitor the sample preparation and mass spectrometry. To monitor instrument performance, 10 µL of a dried matrix-free mixture of the internal standards, reconstituted in 100 µL of buffer B (90%IPA:10%ACN in 10mM NH_4OAc) was analyzed. In addition, to monitor the lipid extraction process we used either pooled plasma samples or a pool of small aliquots of test samples extracted with spiked internal standards will be run along with test samples. Internal standards will also be spiked into test samples during the extraction process.

Data Dependent LC-MS/MS Analysis

Chromatographic separation was performed on a Shimadzu CTO-20A Nexera X2 UHPLC systems equipped with a degasser, binary pump, thermostatted autosampler, and column oven (all from Shimadzu). The column heater temperature was maintained at 55oC. The injection volume was 5 µL for all analyses. For lipid separation, the lipid extract is injected onto a 1.8 µm particle 50 × 2.1 mm id Waters Acquity HSS T3 column (Waters, Milford, MA) which is heated to 55°C. Elution is performed using acetonitrile / water (40:60, v/v) with 10 mM ammonium acetate as solvent A and acetonitrile / water / isopropanol (10 : 5 : 85 v/v) with 10 mM ammonium acetate as solvent B. Column is equilibrated for 3 min before the next injection making total run time 14 min. The flow rate was 0.400uL/min. The data acquisition of each sample was performed in both positive and negative ionization modes, using a TripleTOF 5600 equipped with a Turbo V^{TM} ion source (AB Sciex, Concord, Canada). Column effluent was directed to the ESI source. The source voltage was set to 5500V for positive ionization and 4500V for negative ionization mode. The declustering potential (DP) was 60 V and source temperature was 450^{o}C for both modes. The curtain gas flow, nebulizer, and heater gas were set to 30, 40, and 45 arbitrary units. The instrument was set to perform one TOF MS survey scan (150 ms) and 15 MS/MS scans with a total duty cycle time of 2.4 s. The mass range of both mode was 50-1200 m/z. Acquisition of MS/MS spectra was controlled by data dependent acquisition (DDA) function of the Analyst TF software (AB Sciex, Concord, Canada) with application of following parameters—dynamic background subtraction, charge monitoring to exclude multiply charged ions and isotopes, and dynamic exclusion of former target ions for 9 s. Rolling collision energy was set whereby the software calculated the CE value to be applied as a function of m/z.

Mass accuracy was maintained by the use of an automated calibrant delivery system (AB Sciex, Concord, Canada) interfaced to the second inlet of the DuoSpray source. Calibrations were performed at the start of a workday or whenever ionization polarity was changed.

Pooled human plasma sample and pooled experimental sample (prepared by combining small aliquots of all client's samples) are used to control the quality of sample preparation and analysis. Randomization scheme is used to distribute pooled samples within the set. Mixture of pure authentic standards is used to monitor the instrument performance on a regular basis.

Data analysis

Lipids are identified using LIPIDBLAST package (http://fiehnlab.ucdavis.edu/projects/LipidBlast) - computer-generated tandem MS library of 212,516 spectra covering 119,200 compounds representing 26 lipid classes, including phospholipids, glycerolipids, bacterial lipoglycans and plant glycolipids. Quantification of lipids is done by Multiquant software (AB-SCIEX). Normalization of the data for different lipid classes is performed based on a set of internal standards.