Quantitative LC/MS/MS Analysis for Selected Oxylipins in Human Serum Samples from the Hepatitis B Research Network Cohort

In collaboration with Keyur Patel, University of Toronto

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Objective: Measure the levels of selected oxylipins in 60 human serum samples.

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RESULTS and Discussion

Materials

Stable Isotope Labeled (SIL) oxylipin standard solutions (listed in Table 1) were purchased from Cayman Chemical (Ann Arbor, MI). Solutions were combined in a stock SIL mixture in methanol which was further diluted with acetonitrile to a final concentration of 6.25 nM (IS Working Solution). Butylated hydroxytoluene, an antioxidant, was also added to the internal standard solution at a final concentration of 62.5 µg/mL.

Table 1: Internal Standards

Compound Name
d4 6-keto PGF1a
d4-TXB2
d4-PGF2a
d4-PGE2
d4-PGD2
d4-LTB4
d4-(±)12,13-DiHOME
d4-(±)9,10-DiHOME
d11-14,15-DiHETrE
d6-20-HETE
d4-9(S)-HODE
d8-12(S)-HETE
d8-5(S)-HETE
d7-5-KETE
d4 15-deoxy-12,14-PGJ2

Analytical standard solutions (listed in Table 2) were purchased from Cayman Chemical (Ann Arbor, MI). Solutions were combined in a stock mixture containing 1 µg of each compound in methanol which was further diluted with 1:1 acetonitrile:methanol to prepare Spiking Solutions from which Quality Control samples (QC) and calibration standards were made.

Table 2: Analytical Standards

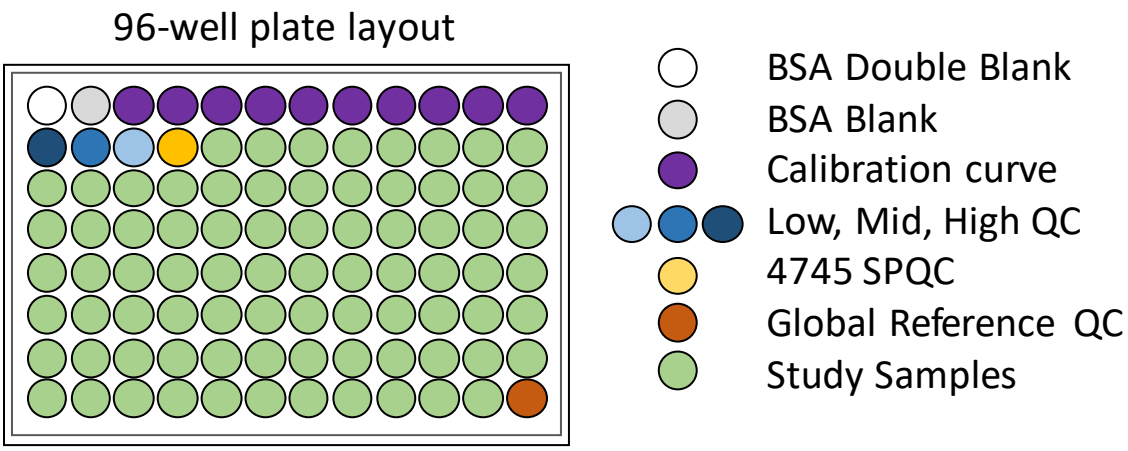
Compound Name
6-keto PGF1a
PGD2
PGE2
TXB2
11ß-PGF2a
13,14-dihydro-15-keto PGD2
13,14-dihydro-15-keto PGE2
13,14-dihydro-15-keto PGF2a
Tetranor-PGFM
12,13-DiHOME
5(S),6(S)-Lipoxin A4
(+/-) 5-iPF2a-VI
LTD4
LTE4
11-HETE
12-HETE
15-HETE
5-HETE
8-HETE
9-HETE
13-HODE
9-HODE
12(S)-HpETE
13-HpODE
15-HpETE
5(S)-HpETE
9-HpODE
14,15-DiHETrE
12(13)-EpOME
10(S),17(S)-DiHDoHE
NeuroprotectinD1
ResolvinD1
ResolvinE1
Maresin 1
ResolvinD2
17-HDoHE
±18-HEPE
11,12-DiHETrE
12(S)-HHTrE
15(S)-HETrE
9(10)EpOME
9,10-DiHOME

Calibration standards and QCs were prepared in 50 mg/mL Bovine Serum Albumin (BSA) in 50 mM ammonium bicarbonate (AmBic). Calibration standards were analyzed in duplicate bracketing the study samples and QCs. The concentrations of the calibration standards were 10, 25, 50, 100, 250, 500, 1000, 5000, 10000, and 50000 pg/mL. QC samples were prepared at three concentrations 40000, 4000, and 400 pg/mL. These were analyzed in triplicate with the study samples.

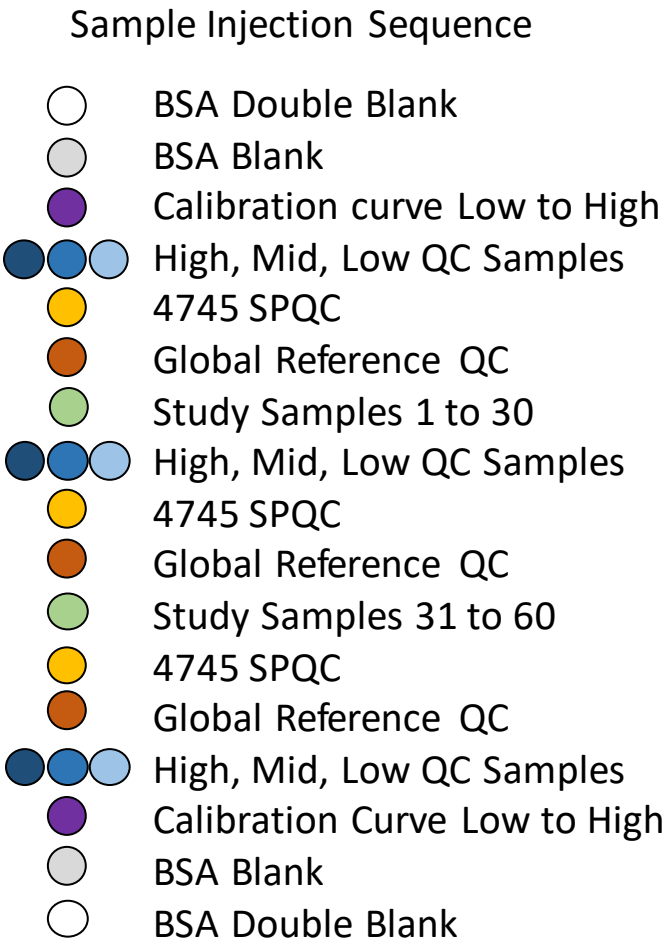
Sample Preparation

Samples were extracted by protein precipitation with acetonitrile using a Biotage (Uppsala, Sweden) PLD+ protein and phospholipid removal 96-well plate.

Figure 1: 96-well Plate Layout



Samples were thawed, vortexed, centrifuged, and kept on ice. For each blank, calibration standard, QC, and study sample 800 µL IS Working Solution were added to the appropriate well of the protein and phospholipid removal 96-well plate. 800 µL acetonitrile were added to each well to be used for double blanks. Aliquots of 200 µL blank, calibration standard, and QC sample were added to the appropriate wells. Serum samples were added in 200 µL aliquots. The extraction plate was then capped, mixed for 10 minutes at room temperature at 650 rpm then frozen for 10 minutes at -20ºC. While the extraction plate was positioned over the sample collection plate in a vacuum block, vacuum was applied for 5 minutes to elute the samples into wells containing 25 µL 80:20 methanol:glycerol. After drying in a Vacufuge set for aqueous samples at 30C samples were reconstituted in 50 µL 1:1 acetonitrile:methanol. 5 µL were injected for LC/MS/MS analysis. A QC pool of equal volumes of all samples (4745 SPQC) was extracted and analyzed in the same way as the study samples, calibration standards, and QC samples.



HPLC/MS/MS Conditions

LC separation was performed using a Waters (Milford, MA) Acquity UPLC using a Waters Acquity 2.1 mm x 10 mm 1.7 µm BEH C18 column (Waters P/N 186002352). Mobile phase A was water with 0.1% acetic acid and mobile phase B was 90:10 acetonitrile:isopropyl alcohol. The gradient program is listed in Table 3.

Table 3: LC Gradient Program

Time (min)	Flow (mL/min)	%A	%B	Temp (°C)
0	0.6	75	25	40
8.0	0.6	5	95	40
8.5	0.6	5	95	40
8.51	0.6	75	25	40
10.0	0.4	75	25	40

Samples were introduced directly into a Xevo TQ-S mass spectrometer (Waters) using negative ion Electrospray ionization operating in the Multiple Reaction Monitoring (MRM) mode. MRM transitions (compound-specific precursor to product ion transitions) for each analyte and internal standard were collected over the appropriate retention time. The MRM data were imported into Waters application TargetLynx™ for peak integration, calibration, and concentration calculations. Analytes for which analytical standards were not included were quantified against the standard curve of an analyte from the same or similar compound class.

Results Summary

Data were acquired for oxylipins from 14 compound classes: alcohols, diols, epoxides, hepoxilins, hydroxyperoxides, isoprostanes, ketones, leukotrienes, lipoxins, prostanoids, protectins, resolvins, thromboxanes, and triols. Some classes of compounds were detected in calibration standards and QCs but not in the study samples. These include lipoxins protectins, prostaglandins, and resolvins. Data for the triol compounds were not reportable due to chromatographic interferences.

Data Return Document Descriptions

A number of documents have been added to the Express Data Repository for Project 4745 including this sample analysis summary. A link to the repository is included here. A description of the data spreadsheets follows the link.
https://discovery.genome.duke.edu/express/resources/4745/

4745 Oxylipin Data.xlsx

This is an Excel workbook containing two worksheets with the curated calculated concentration data acquired in the study. The first worksheet is titled “Oxylipin Study Data.” These are the concentration data in pg/mL acquired from the samples. Each column lists the data in pg/mg with the following headers: Row 2 lists the analyte measured. Row 3 lists the calibration reference for each analyte. (Analytical standards were not available for every analyte measured. Analytes for which an authentic standard was not used were quantified using the calibration standard for an analyte in the same or similar compound class.) Row 6 lists the compound class for each analyte. Row 7 indicates whether the data are Quantitative (Q) or Semi-quantitative (SQ). Analytes for which an authentic standard was available were measured quantitatively. Analytes for which an authentic standard was not used were measured semi-quantitatively. The results for the 4745 SPQC sample are in rows 69 through 71. Row 73 has the %CV for the 4745 SPQC sample results. It is our recommendation that if the inter-day variability of the pool sample (as measured by the %CV) for a particular analyte is greater than 25%, then the data for that analyte in the study samples should be flagged as having high variance. The average %CV for the 4745 SPQC is 23.2%, with a range from 0.5 to 146.2%.

The second worksheet contains the QC data for analytes with authentic standards. The results are listed in pg/mL.

Data_Dictionary_Oxylipin_Targets

This Excel workbook has a list of the analytes monitored in the oxylipin assay. For each analyte there is a listing of the analyte precursor, class, metabolic pathway PubChem CID, LipidMaps number, HMDB ID,

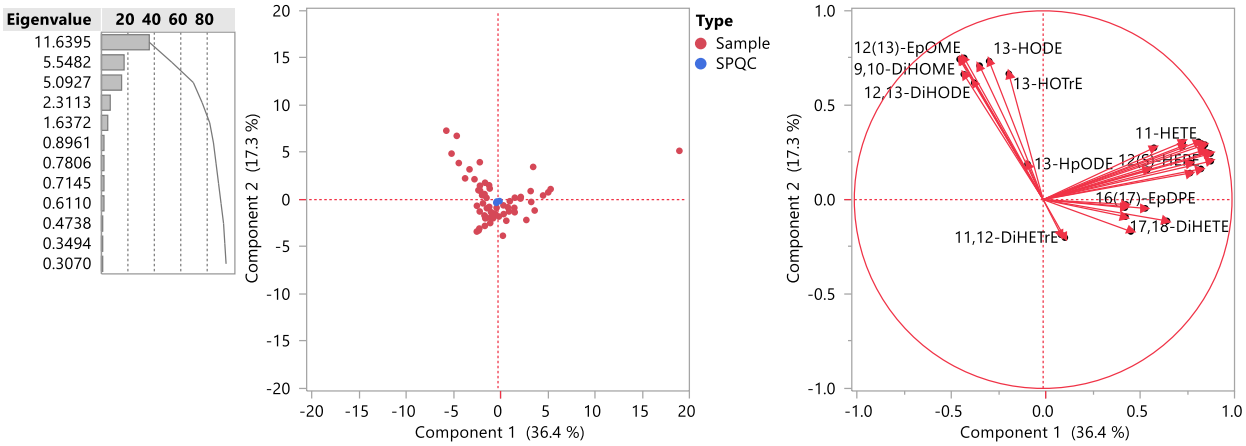
Cayman Chemical product number, and alternate compound names. This information should be useful for biological interpretation of the analytes detected in the human serum samples.

Principal Components Analysis (PCA) Plots

In order to assess general variability for the samples within each analyte class and to look for sample outliers; a Principal Components Analysis (PCA) was performed using JMP® Pro v13.0 software (SAS, Cary, NC) and the data from table ***4745 Oxylipin Data.*** Analytes with measurable concentrations for more than half of the samples were included in the analysis. For analytes for which there were missing values, missing values were replaced with the lowest calibration standard value for that analyte and the restricted maximum likelihood (REML) method was used for correlation.

The figure below shows a PCA plot for the analytes detected. Separate colors were assigned to samples (red) and the 4745 SPQC (blue) in order to evaluate variability and to check for outliers.

The results for the pools are generally clustered together centrally within the samples which implies that there is greater biological than technical variability.



Data Summary

- 36 Oxylipin analytes spanning 20 alcohols, 8 diols, 1 hydroxyperoxide, 1 thromboxane, 1 leukotriene, and 1 epoxide were reliably measured in the samples.
- Principal Components Analysis suggests that variability is low.

Recommended Steps Before Statistical Analysis

- Remove analytes with a large percentage of missing values (i.e. > 50%).
- Replace missing values ("<Lowest CS") with either the Lowest Calibration Standard value or (Lowest Calibration Standard)/2.
- Scale log2 as necessary for analytes with non-normal distribution. Common best-practice is to use one scaling method for all analytes within a study.
- Z-score normalization can be used prior to Hierarchical Clustering analysis