**QuantiTation of isolevuglandin protein adducts BY LC/MS/MS**

**DAVIES LAB PROTOCOL#** PAN001-23

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**REAGENTS AND DISPOSABLES**

* MilliQ Water (18.2 mΏ-filtered);
* TRIS pH 7.5 (Trizma hydrochloride solution 1 M TrisHCl Sigma T2319-1L)
* Indomethacin, minimum 99%, TLC. (Sigma Algrich, cat. no. 17378-10G);
* Pyridoxamine dihydrochloride, minimum 98%. (Sigma Algrich, cat. no. P9380-25G); FW 241.1
* BHT (2,6-Di-*tert*-butyl-4-methylphenol - ≥99.0% (GC) (powder. Sigma-Aldrich, cat. # B1378-100G). FW 220.4
* TCEP HCl (Tris(2-Carboxyethyl) phosphine hydrochloride). PIERCE, cat. # 20490);
* Aminopeptidase M from Porcine Kidney (Calbiochem; 164598) with specific activity of approximately 65U/ml;
* Pronase Protease (Streptomyces griseus, nuclease free 100 KU) (EMD Millipore;80602-610), dissolved at 64 mg/ml in PBS pH 7.4. Make fresh each time;
* HyperSep™ Retain PEP Cartridges ThermoFisher # 60107-203
* [13C615N2]-15-E2-IsoLG-lysine-lactam std; (current stock is 0.140 ng/ul which is 0.29 pmol/ul).
* [2H11] IsoLG-protein internal standard mix (lysozyme, human serum albumin, bovine serum albumin, and chicken egg ovalbumin used in equal weight). Stock solution is 0.25 ug protein/ ul, which yields 0.439 pmol/ul of [2H11] IsoLG-Lys upon complete digestion.
* Methanol (Optima\*), Fisher Chemical, # A454-4;
* Butanol (Fisher Scientific; #A383-4)
* n-Heptane, 99% (Optima\*), Fisher Chemical, # H360-4;
* Ethyl Acetate (Optima\*), Fisher Chemical, # E196-4;
* Acetic Acid, 99.7%, HPLC Grade, Mallinckrodt Baker, # 9515-03;
* Ethyl Acetate/Methanol (1:1 vol/vol mixture);
* Methanol/Water (15:85 vol/vol mixture);
* Ethyl Ether Anhydrous (diethyl ether) (Fisher Chemical, E138-1) FW 74.12;
* BioRad protein assay dye reagent concentrate (Bio-Rad #500-0006).
* Quality Control Plasma FROM BLOOD BANK (prepared by VY 030617) Stored at -80C freezer.
* 100x Antibiotic, Anti-Mycotic solution. (Life Technologies 15240-062)
* 15 ml centrifuge tubes (Fisher Scientific #14-955-237)

**EQUIPMENT:**

* Analytical Evaporator (The Meyer N-EVAP analytical evaporator, Organomation Assoc. Inc);
* Handheld homogenizer (Polytron PT 1200E, KINEMATICA AG); (use the PT-DA 07/2 EC-C107 blade). Stored at 556RRB
* Probe Sonicator-Fischer Scientific Sonic Dismembrator Model 100 (set at at level 2) Clin Pharm Equipment room by Alan Brash lab.
* Tabletop Centrifuge (Beckman Coulter; Allegra X-12R Centrifuge);
* Water bath shaker (Precision Scientific; Dubnoff Metabolic Shaking Incubator);
* IEC HN-SII Centrifuge (4000 rpm max);
* LC/MS Waters Xevo TSQmicro
* Kinetex 2.6μ C8 100A column 50x2.1 mm (Phenomenex, 00B-4497-AN);
* Vacuum manifold for 24 cartridges.

**PROCEDURE**

**Collection of Solid Tissue Samples**

1. When available, collect approximately 0.5 g of tissue. (Usually need 1-5 mg of protein for strong signal).
2. Flash-freeze harvested plasma or tissue samples in liquid nitrogen and then immediately transfer them to -80 oC for storage until analysis.

**Day 1**

1. Prepare fresh 4 additive cocktail 100x concentrate at the beginning of each assay. (Note: Do not reuse old stocks.)

* Prepare 10 mM pyridoxamine (PM) by adding 6 mg pyridoxamine dihydrochloride to 2.4 ml MilliQ H2O.
* Prepare 10 mM indomethacin by adding 10 mg to 2.8 ml ethanol.
* Prepare 22 mM BHT by adding 12 mg for 2.4 ml EtOH.
* Prepare 500 mM TCEP by adding 60 mg for 420 ul Milli-Q H2O.
* Make 100x concentrate of additive cocktail by adding 2 ml of 100 mM PM, 2 mL of 10 mM indomethacin, 2 ml of 22 mM BHT, and 0.4 ml of 500 mM TCEP.

2. If needed, prepare tissue homogenization buffer by adding 10 ul of 100x concentrate of additive cocktail to each ml of 1 M Tris pH 7.5 buffer (will need 3 ul of buffer per mg tissue weight.)

3. Prepare butanol/diethyl ether (1:3, vol:vol) delipidation solvent 1 by adding 50 ml of butanol to 150 ml of diethyl ether, then adding 2 ml of 100x concentrate of 4 additive cocktail. (Keep room temp.)

4. Prepare methanol/diethyl ether (1:2.5, vol:vol) delipidation solvent 2 by adding 50 ml of methanol to 125 ml of diethyl ether, then adding 1.75 ml of 100x concentrate of 4 additive cocktail. (Keep at 4C)

5. Always use a quality control plasma replicate as quality control.

6. For each tissue sample:

a. Carefully weigh tissue and place in the appropriate tube

b. Add 3 µl of ice cold homogenization buffer per mg of tissue.

c. Homogenize on ice using the hand-held homogenizer for approximately 30 seconds or until completely homogenized. Take aliquot to measure protein concentration.

SAMPLE WORK-UP

Note: Use the following volumes for each tissue:

Plasma 100 µl,

Whole tissue homogenate 100 µl,

HDL 20 – 100 µl,

Urine 100-200 µl.

1. Add 1 µl of 100x additive cocktail concentrate to each 100 l sample (or appropriate volume).
2. Add 5 µl of [2H11] IsoLG-protein internal standard mix to sample (this will yield 2.2 pmol [2H11] IsoLG-Lys) and mix well.
3. Add 900 µl of DMSO.
4. Mix well by pipetting and let stand at RT for 10 min.
5. Place 15 ml of butanol/diethyl ether/additive mix in 15 ml conical tubes.
6. Infuse the sample using 200 µl gel loading tip into 15 ml conical tube with 15 ml of butanol/diethyl ether/additives.
7. Immediately invert the sample 10 times. (Do not vortex)
8. Let stand at -20°C over night.

**Day 2**

1. Centrifuge sample at maximum speed on swing rotor centrifuge (IEC HN-SII Centrifuge) for 5 min to pellet protein.
2. Carefully aspirate the butanol/diethyl ether from the pellet using vacuum flask and tubing connected to glass Pasteur pipette.
3. To remaining pellet add 1.0 ml of 1 M TRIS pH 7.5 buffer.
4. Immediately pipette the sample to reconstitute the proteins.
5. Add 1.0 ml of DMSO.
6. Add 15 ml of -20°C cold solution of methanol/diethyl ether (1:2.5, vol:vol) containing 1x additives.
7. Immediately invert the sample 10 times. (Do not vortex)
8. Let stand at RT for 10 min.
9. Prepare proteolysis buffer by adding 200 l of 100x concentrate of additive cocktail and 200 l of 100x concentrate of antibiotic/antimycotic to 15 ml of 0.5M Tris pH 7.5 buffer.
10. Centrifuge at maximum speed on swing rotor centrifuge for 10 min to pellet protein.
11. Carefully aspirate the methanol/diethyl ether from the pellet including the walls.
12. If liquid remains on walls, use clean Kimwipe to remove residual solvent.
13. Add 1 ml of proteolysis buffer to the pellet.
14. Pipette up and down with regular pipette tip to disrupt pellet.
15. Prepare 100 mg/ml pronase stock solution in H2O (for each 4-10 mg of protein).
16. To each sample, add 10 l of pronase solution.
17. Add 5 l of [13C6 15N2]-15-E2-IsoLG-lysine-lactam std (current stock is 0.29 pmol/l so this is 1.44 pmol total in sample).
18. Incubate for 3 h at 37°C (use orbital shaker). Make sure tops are on tightly.
19. Add 5 l aminopeptidase M solution for each 1 mg of protein in sample.
20. Make sure tops are on tightly.
21. Incubate O/N (21 h) at 37°C while shaking (use orbital shaker).

**Day 3**

**Solid Phase Extraction**

1. Prepare 15% Methanol in water and Methanol/ethyl acetate (1:1 v/v) solutions.
2. Set up one HyperSep Retain PEP cartridge for each sample on the vacuum manifold apparatus.
3. Prewash cartridge with 3 ml methanol and 3 ml MilliQ water.
4. Load the pronase/aminopeptidase digested sample onto the cartridges with 2.5 ml of H2O and allow to pass through cartridge by gravity.
5. Wash with 3 ml water (by gravity).
6. Wash with 3 ml 15% CH3OH (by gravity).
7. Wash with 3 ml heptane (by gravity)
8. Wash with 3 ml 15% CH3OH (by gravity).
9. Elute IsoLG-Lys into 5 ml conical tube with 1.0 ml MeOH/EtOAc (1:1) and 1.0 ml CH3OH (allow to go through by gravity). *Best practice is to shoot samples same day. If needed samples can be stored at -20C for a few days.*
10. Dry down under nitrogen until about 50 l left (do not allow to dry completely)
11. Add 50 µl of Solvent A (0.1% acetic acid in MilliQ water.)
12. Mix well by pipetting.
13. Transfer sample to Spin-X tube.
14. Centrifuge at 10000 g for 15 minutes at RT to remove any particulate.
15. Carefully transfer fitrate to autosampler vials.

**LC/MS/MS**

1. Prepare HPLC Solvent A (0.1% acetic acid in H2O) (1 ml glacial acetic acid to 1 L water.)
2. Prepare HPLC Solvent B (0.1% CH3COOH in CH3OH) (1 ml glacial acetic acid to 1 L methanol.)
3. Column is Phenomenex Kinetex 2.6μ C8 100A.
4. Set up LC instrument(Waters Xevo TSQmicro) as follows:
   1. Place solvent A and B inlet lines into appropriate solvent.
   2. Wet-prime the instrument for 3 minutes each at 8000 μl/min with the new solvents.
   3. Using PEEK tubing, connect the Kinetex 2.6μ C8 100A column from the outlet line of the HPLC to rheostat value on the LC/MS and then from rheostat value to the inlet of the electrospray ionization source. This will allow to divert the first 3 minutes off column to waste, keeping the instrument more sensitive.

Time %A %B Flow (ul/min)

0.0 70 30 250

0.5 70 30 250

6.5 0 100 250

8.0 0 100 250

8.5 70 30 250

10.0 70 30 250

MRM table

|  |  |  |  |
| --- | --- | --- | --- |
| Analyte | Precursor | Product | Collision energy |
| IsoLG-Lys (lactam adduct) | 479.3 | 84.1 | 35 |
| IsoLG-Lys (hydroxylactam adduct) | 495.3 | 84.1 | 35 |
| 12-E2-IsoLG-[13C6]Lys | 485.3 | 84.1 | 35 |
| 15-E2-IsoLG-[13C615N2]Lys | 487.3 | 90.1 | 35 |
| [2H11]IsoLG-Lys | 490.3 | 84.1 | 35 |
| PC-IsoLG-Lys | 956.3 | 184.1 | 35 |

1. Set to divert to waste for first 3 minutes, then send to ion source for 5 minutes, then divert again to waste for last 2 minutes.
2. Inject 10 ul of each sample.
3. To calculate amount of IsoLG:

IsoLG-Lys: Select the area of each double peak 479→84; RT ~4.0

[2H11]IsoLG-Lys: Select the area of each double peak 490🡪 84, these two peaks should be just slightly faster retention times than the retention time for 15-E2-IsoLG-[13C615N2]Lys internal standard seen with 487🡪 90, as the [2H] makes it just slightly more polar than [1H]. Calculate ratio of the area for 479→84 / area for 490 🡪 90.

Then amount of IsoLG-Lys in plasma sample = ratio of peak area x 2.2 pmol / 100 l

Or amount of IsoLG-Lys in tissue sample = ratio of peak area x 2.2 pmol / mg protein in 100 L sample.

**Bio-Rad Protein Assay (Microtiter Plate Protocols, Bio-Rad #500-0006)**

**Standard curve**

1. Prepare 5ml of 2 mg/ml BSA stock in PBS by adding 10 mg BSA to 5 ml PBS.
2. Prepare the following standard curve (can store at -20C).

|  |  |  |
| --- | --- | --- |
| Standard concentration | Added volume of 2 mg/ml BSA | Added volume of 1M TRIS pH 7.5 |
| 0.00 mg/ml | 0 ul | 1000 ul |
| 0.05 mg/ml | 25 ul | 975 ul |
| 0.1 mg/ml | 50 ul | 950 ul |
| 0.2 mg/ml | 100 ul | 900 ul |
| 0.3 mg/ml | 150 ul | 850 ul |
| 0.4 mg/ml | 200 ul | 800 ul |
| 0.6 mg/ml | 300 ul | 700 ul |

1. Add 10 µl of each standard into triplicate 96-well plate wells.
2. For samples, it may be necessary to dilute. If don’t have a good idea of protein concentration, it may be useful to do the following: to triplicate wells, add 10 ul of the sample, to another set of triplicate wells add 2.5 ul of the sample and then 7.5 ul of PBS to these same well (for a 4-fold dilution of sample).
3. Add 150 ul of Milli-Q water to each well.
4. Add 40 ul of BioRad protein assay dye reagent concentrate to each well.
5. Incubate for 30 to 60 minutes until color is stable.
6. Measure absorbance at 595 nm using SpectraMax instrument in the Molecular Biology Core Lab (904MRBIV).
7. Use Prism (or other similar program) to calculate unknowns from standard curve. Enter A595 values for standard and samples in the Y column and the concentration of standard in the X column. Then chose Analysis 🡪 Linear Regression and click on box “Intrapolate unknowns from standard curve”. This will give you the calculated value for your samples. Be sure to multiple by any dilution factor if necessary.

Example Run

