Project: Ernst Lengyel 18 hour WCMC Pilot & Feasibility Study

Summary Text for ENDOCANNABINOID SPE EXTRACTION & ANALYSIS PROTOCOL

Conducted in the laboratory of Dr. John W. Newman

Endocannabinoid Extraction

Endocannabinoids 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.

Endocannabinoid Analysis

Analytes in a 10 µL injection of extract were separated with an Aquity C_{18} BEH 1.7µm 150mm 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 = 500 °C, Ionspray voltage 5500.00, 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 with5 to 7 point calibration curves ($r2 \geq 0.997$). Calibrants and internal standards were either synthesized [CUDA] or purchased from Cayman Chemical (Ann Arbor, MI) or 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) Shearer GC et al (2010). Detection of omega-3 oxylipins in human plasma and response to treatment with omega-3 acid ethyl esters. *J Lipid Res*. 51:2074-81.

Table 1. UPLC parameters

Time (min)	A%	B%
0	75	25
0.25	75	25
0.5	60	40
1.5	50	50
3	45	55
3.5	20	80
8	15	85
9	5	95
9.25	5	95
9.5	75	25
11	75	25

Solvent A = 0.1% Acetic Acid;
Solvent B = 90% Acetonitrile / 10% isopropanol flow rate = 0.25 mL/min, column 2.1 X 150mm, 1.7 µm BEH C18 (Waters, Milford, MA), column temp = 60 °C

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

Analyte	Common Abbreviation	tR (min)	Transition (Da)	Dwell (msec)	Declustering (V)	Collision (V)	ISTD†
d4-PGF2α Ethanolamide	d4-PGF2α EA	3.24	384.3 > 62.1	25	55	40	CUDA
PGF2αEthanolamide	PGF2α EA	3.25	380.3 > 62.1	25	55	41	d4-PGF2a EA
PGE2 Ethanolamide	PGE2 EA	3.26	378.3 > 62.1	25	58	38	d4-PGF2a EA
PGD2 Ethanolamide	PGD2 EA	2.9	378.3 > 62.1	25	58	38	d4-PGF2a EA
PGF2α 1-glyceryl ester	PGF2a 1G	3.04	411.3 > 301	25	55	19	d4-PGF2a EA
PGE2 1-glyceryl ester	PGE2 1G	3.07	409.3 > 317	25	55	19	d4-PGF2a EA
1-Cyclohexyluriedo-3-dodecanoic acid	CUDA	4.98	341.3 > 216	25	58	25	---
15(S)-HETE Ethanolamide	15-HETE EA	5.19	346.3 > 62.1	25	58	39	d8-AEA
11(12)-EET Ethanolamide	11(12)-EpETre EA	5.58	364.3 > 62.1	25	58	40	d8-AEA
α-Linolenoyl Ethanolamide	αLEA	6.03	322.2 > 62.1	25	72	32	d8-AEA
Docosahexaenoyl Ethanolamide	DHEA	6.42	372.3 > 62.1	25	61	36	d8-AEA
d8-Arachidonoyl Ethanolamide	d8-AEA	6.54	356.3 > 63.1	25	60	30	CUDA
Arachidonoyl Ethanolamide	AEA	6.59	348.3 > 62.1	25	65	33	d8-AEA
Linoleoyl Ethanolamide	LEA	6.66	324.2 > 62.1	25	72	31	d8-AEA
d5-2-Arachidonoyl Glycerol	d5-2-AG	6.92	384.3 > 287	20	63	19	CUDA
2-Arachidonoyl Glycerol	2-AG	6.94	379.3 > 287	20	53	19	d5-2-AG
d8-Arachidonoyl Glycine	d8-NA-Gly	6.95	370.3 > 76.1	25	79	35	CUDA
Arachidonoyl Glycine	NA-Gly	6.98	362.3 > 76.1	25	79	35	d8-NA-Gly
Dihomo-ϒ-Linolenoyl Ethanolamide	Dihomo GLA EA	7.02	350.3 > 62.1	20	65	36	d8-AEA
1-Arachidonoyl Glycerol	1-AG	7.1	379.3 > 287	20	53	19	d5-2-AG
2-Linoleoyl Glycerol	2-LG	7.11	355.3 > 263	20	52	18	d5-2-AG
d4-Palmitoyl Ethanolamide	d4-PEA	7.31	304.2 > 62.1	20	80	35	CUDA
1-Linoleoyl Glycerol	1-LG	7.32	355.3 > 263	20	52	18	d5-2-AG
Palmitoyl Ethanolamide	PEA	7.33	300.2 > 62.1	20	80	31	d8-AEA
Docosatetraenoyl Ethanolamide	DEA	7.45	376.3 > 62.1	20	66	36	d8-AEA
Oleoyl Ethanolamide	OEA	7.6	326.2 > 62.1	20	80	32	d8-AEA

* - See Table I for UPLC conditions. Collision-induced dissociation was performed with 2.3 mTorr nitrogen. 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; and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) was introduced immediately prior to analysis and used to quantify surrogate recoveries.

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

Analyte	Common Abbreviation	tR (min)	Transition (Da)	Dwell (msec)	Declustering (V)	Collision (V)	ISTD†
N-Oleoyl Glycine	NO-Gly	8.14	340.2 > 76.2	20	80	26	d8-NA-Gly
2-Oleoyl Glycerol	2-OG	8.2	357.3 > 265	50	52	18	d5-2-AG
1-Oleoyl Glycerol	1-OG	8.86	357.3 > 265	50	52	18	d5-2-AG
Stearoyl Ethanolamide	SEA	8.97	328.2 > 62.1	50	80	35	d8-AEA

* - See Table I for UPLC conditions. Collision-induced dissociation was performed with 2.3 mTorr nitrogen. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

† - Internal Standards - Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA; and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) was introduced immediately prior to analysis and used to quantify surrogate recoveries.

Table 3. Analytical surrogate recoveries

Chemical class	Compound	Mean ± SD	%RSD†
prostamide	d4-PGF2α-EA	68.8 ± 18.2	26.4%
N-acylethanolamide	d8-AEA	73.2 ± 12	16.4%
monoacylglygerol	d5-2-AG	12.1 ± 4	33.1%
N-acylethanolamide	d4-PEA	72.6 ± 10.8	14.9%

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