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ENDOCANNABINOID EXTRACTION & ANALYSIS PROTOCOL

Project: Ortiz WCMC Pilot & Feasibility Study

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), followed by 1 column volumes wash solution (20% methanol, 0.1% acetic acid). SPE cartridges were dried with vacuum @ -25psi for 20 min. Analytes were then extracted into 2 mL autosampler vials (pre-prepared with 10 µL 20% glycerol solution in MeOH) with 0.2 mL MeOH, followed by 0.5 mL Acetonitrile, followed by 0.5 mL Ethyl Acetate, by gravity. Eluent was dried by speed vacuum for 35 min, before they were re-constituted with the internal standard 1-cyclohexyl ureido,3-dodecanoic acid (CUDA) at 100 nM (50:50 MeOH:CAN), vortexed 1 min, transferred to a spin filter (0.1 µm, Millipore, Billerica, MA), centrifuged for 3 min at 6ºC at <4500g (rcf), before beign transferred to 2 mL LC-MS amber vials. Extracts were stored at -20ºC until analysis by UPLC-MS/MS. The internal standard was used to quantify the recovery of surrogate standards.

Endocannabinoid Analysis

Analytes in a 100 µL extract aliquot were separated utilizing a Waters Acquity UPLC (Waters, Milford, MA) the solvent gradient described in Table, using modifications of a previously published protocol (2). Samples were held at 10ºC. Separated residues were detected by negative 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, collision gas = high, temperature = 500 °C, 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 internal standard methods and 5 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 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 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-PGF2a-EA	75.2 ± 10.1%	21.4%
N-acylethanolamide	d8-AEA	54.7 ± 9.6%	18.3%
monoacylglygerol	d5-2-AG	62.9 ± 14%	22.5%
lipoaminoacid	d8-NA-Gly	51 ± 11.2%	23.2%
N-acylethanolamide	d4-PEA	58.6 ± 13.3%	22.4%

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