1. PROTOCOL FOR THE ANALYSIS OF SYNTHETIC CANNABINOIDS IN BLOOD AND URINE

PURPOSE

The following method describes the qualitative screening and confirmation of Synthetic cannabinoids in blood & urine by LC/MS/QQQ.

PLAN

A. Equipment

(1) LC/MS/QQQ with a C18 column

(2) Positive Pressure Manifold

(3) ISOLUTE SLE+ 1 ml Sample Volume part# 820-0140-CG, Biotage

(4) 12x75mm culture tubes

(5) 1.0 ml disposable transfer pipettes

(6) Heating block

(7) Sample concentrator with UHP nitrogen

(8) Vortex mixer / Multi-tube vortex mixer

B. Reagents

(1) **Deionized Water** (DI water). Label. Stable until consumed.

(2) **Ammonium formate buffer 0.01%**. Prepared by adding 0.34 g of ammonium formate and 100 µl of formic acid to 1.0 L deionized water. Stable until consumed.

(3) **Formic Acid (0.01%) in methanol**. Prepared by adding 100 µl of formic acid to 1.0 L methanol. Stable until consumed.

(4) **2% isopropanol in ethyl acetate**. Add 200 µl isopropanol to 10 ml ethyl acetate (or equivalent depending upon how much ethyl acetate is needed). Prepare fresh daily.

(5) **Ammonium Hydroxide.** Stable until consumed.

(6) **Dimethyl Formamide.** Stable until consumed.

(7) **Methanol.** Stable until consumed.

(8) **Isopropanol**. Stable until consumed.

(9) **Acetonitrile**. Stable until consumed.

(10) **Ethyl acetate**. Stable until consumed.

C. Standards: (Store refrigerated)

(1) **Prepare (as free base) or purchase individual 1.0 mg/ml stock standards in methanol or acetonitrile or dimethylformamide, depending on solubility data (provided from the manufacturer) of the following:**

AB-CHMINACA
AB-FUBINACA
AB-FUBINACA metabolite 3
AB-PINACA
AB-PINACA N-(4-hydroxypentyl) metabolite
ACHMINACA
ADB-FUBINACA
ADB-PINACA
AMB-FUBICA, [MMB-FUBICA]
APP-BUTINACA
4-cyano CUMYL-BUTINACA
EG 018
EMB-FUBINACA
5-fluoro AB-PINACA
5-fluoro AB-PINACA, N-(4-hydroxypentyl) metabolite
5-fluoro ADB, [5-fluoro MDMB-PINACA]
5-fluoro ADB-PINACA
5-fluoro-AMB
5-fluoro-AMB metabolite 7
5-fluoro-EMB-PICA
4-fluoro-MDMB-BUTICA
4-fluoro-MDMB-BUTINACA
5-fluoro-MDMB-PICA
3-fluoro NNEI
5-fluoro NNEI
5-fluoro PB-22
5-fluoro PB-22 3-carboxyindole metabolite
FUB-AMB (AMB-,MMB- FUBINACA)
FUB-PB-22
MAB-CHMINACA
MDMB-CHMINACA
MDMB-CHMICA
MDMB-FUBINACA
MDMB-4en-PINACA
MMB-CHMICA
MMB2201, [AMB-PICA], [I-AMB]
NM2201, [CBL-2201]
NNEI
PB-22
PB-22 3-carboxyindole metabolite
THJ2201, [AM2201 indazole analog], [5-fluoro THJ 018]
UR-144
UR-144 n-4-hydroxypentyl metabolite
XLR-11
XLR-11 N-(3-fluoropentyl) isomer

(2) **Purchase individual 100 µg/ml stock standards in methanol or acetonitrile (depending on the ampoule) of the following**:

d5-JWH-018 *4-hydroxypentyl*	d5-UR-144 *4-hydroxypentyl*
d5-JWH-250	d7-JWH-073
d9-JWH-210	d5-XLR-11
d9-AB-PINACA	d9-AKB48

D. Calibrators and internal standards: (Store refrigerated. Stable for 2 years)

(1) **0.1 ng/µl Synthetic Cannabinoids internal standard**: In a 10 ml volumetric flask, add 10 µl of each 100 µg/ml internal stock standards listed in C2 above. Dilute to volume with isopropanol.

(2) **1 ng/µl mix Synthetic cannabinoid calibrator stock solution**: Prepare by adding 10 µl each of 1 mg/ml stock synthetic cannabinoids listed in C1 above. Dilute to 10 mL volume with acetonitrile. (10 ng/µl for carboxyindole metabolites and AB-FUBINACA metabolite 3)

(3) **0.1, 0.01, 0.001 ng/µl synthetic cannabinoid calibrators:** Prepare on day of use in a serial dilution order. (i.e. 100 µl of (2) above into 900 µl of isopropanol for the 0.1, mix and use to prepare 0.01, etc.). Discard after blood calibrators have been spiked at appropriate concentrations (see F (1) below)

E. Quality Controls: (Store refrigerated. Stable as per manufacturers expiration date)

(1) **Positive control(s): 0.12, 0.30, and 3.0 ng/ml mixed synthetic cannabinoid control**. Prepare 0.3 ng/ml control for screening batches. The other positive controls will be prepared as appropriate depending upon the synthetic cannabinoid identified. Additional controls shall be prepared when appropriate, to coincide with any limited sample volumes and/or dilution of case samples (For example: A limited sample with only 250 µl shall have a control prepared and extracted in the same manner. A sample requiring an x2 or x4 dilution shall have a control prepared likewise)

(2) **Negative Control:** Blank blood prepared in house consisting of 50% saline, 50% packed red blood cells, and 5g sodium fluoride/1g potassium oxalate (per 500 ml prepared blood) will be used as negative control and/or negative urine will be used as negative control.

F. Supported Liquid Extraction (SLE)

(1) ***Sample preparation***
Prepare in appropriately labeled 12x75 mm culture tubes as follows:

(a) ½ mL* of negative control, positive control(s), case samples, and for confirmation a set of calibrators at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/ml from calibrator stock solution above each in ½ mL of blank blood. (**Note**: This is a ½ ml sample volume extraction so prepare calibrators accordingly) Add 50µl* of internal standard to each tube.

*In case samples where a limited amount of sample is received use the same fraction of internal standard as the sample, as an example ¼ mL of blood and 25 µl of internal standard

(b) Add 200 µl of 4% ammonium hydroxide and deionized water prepared fresh daily (i.e. per 1.0 ml of water add 40 µl NH_4OH and vortex).

(c) Finally add 50 µl of isopropanol and vortex each tube until thoroughly mixed.

(2) ***Sample Application***
Apply sample to top of column bed with a disposable transfer pipette. Pulse pressure to begin absorption, let sit for 20-30 minutes or until the sample is 80% to the bottom of the sorbent bed (visually wetted).

(3) ***Elution***

(a) Elute into labeled sillanized microvials with approximately 2 ml of 2% isopropanol in ethyl acetate by gravity only or less than 1 ml/min.

(b) Evaporate the extracts under nitrogen to dryness

(c) To the microvials add 50 µl of methanol and 50 µl of H_2O, crimp cap and vortex.

G. Data Acquisition and Analysis:

(1) Perform Autotune or checktune if not previously done (weekly).

(2) Purge LC pump lines of any bubbles. At equilibrium the pump pressure should reside near 160-200psi. Binary ripple should not exceed 1%. Let LC stabilize before analysis.

(3) Perform needle rinse.

(4) The trays for samples are P1 in front and P2 in back. They have rows a-f, columns 1-9. Run a performance standard to check retention time. A batch shall include a positive controls(s), negative control and LC solvent blanks prior to case samples. For samples requiring dilution add the appropriate sample multiplier in the sequence table. Load samples onto autosampler according to sequence and have it verified by another analyst before or after analysis but prior to unloading.

(5) The transitions are fixed and derived from Method Optimizer. Retention times and transition ratios can be set by an unextracted standard or calibrator. Retention times should be set prior to running multiple samples.

(6) Analyze using the appropriate method on LC/MS/QQQ.

H. Results and Acceptability:

(*for screening*)

(1) Identification of analyte in positive control

(2) Negative control < 25% of area count of cutoff calibrator

(3) Retention time within 5% as stored or set from an unextracted standard or calibrator

(4) Transition ratios within 20% as stored or set from an unextracted standard or calibrator

(5) Quantitation ≥ 0.1/1.0/10 ng/ml (lowest calibrator for each analyte)

(*for confirmation*)

(1) Calibration R≥0.99 and calibrators within 20% of set value.

(2) Positive controls within 20% of target concentration.

(3) If the above two criteria are not met the analyte may be reported qualitatively.

(4) Negative control < 25% of area count of cutoff calibrator.

(5) Retention time within 5% as stored or set from an unextracted standard or calibrator.

(6) Transition ratios within 20% as stored or set from an unextracted standard or calibrator.

(7) Chromatographically acceptable i.e. peak purity ≥90%.

(8) Blank prior to sample < 25% area count of cutoff calibrator.

(9) Quantitation ≥ 0.1/1.0/10 ng/ml (lowest calibrator for each analyte).

(10) Results will be truncated and documented in case notes to two significant figures.