Analysis of Cannabinoids from Biological Specimens by LC/MS/MS

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1 INTRODUCTION

Marijuana, obtained from the Cannabis sativa plant, is a commonly abused illicit drug. It is typically dried and smoked. Δ^9-Tetrahydrocannabinol (THC) is the primary psychoactive component of marijuana. 11-hydroxy-Δ^9 -tetrahydrocannabinol (THC-OH) is a major active metabolite of THC. 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) is a major inactive metabolite of THC.

2 SCOPE

Analyses	☒ Screening ☒ Confirmation ☐ Quantitation
Matrices	Blood, Urine, Serum, Plasma
Analytes	THC, THC-OH, THC-COOH
Personnel	This document applies to authorized personnel who perform the described tasks, singly or in combination.

3 PRINCIPLE

Biological specimens are commonly assayed for the presence of THC, THC-OH, and THC-COOH. Specimens are mixed with an internal standard solution containing the deuterated analogs of the analytes of interest. Blood specimens are prepared via protein precipitation using cold acetonitrile. Urine specimens are prepared by hydrolysis with a strong base, followed by neutralization with acid. Prepared samples are extracted using solid phase extraction (SPE). Extracts are analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) in the multiple reaction mode (MRM).

4 SPECIMEN CRITERIA

This procedure uses a biological fluid such as: blood, serum, plasma, or urine. Typically, 0.5 mL of matrix is used. Dilution of samples due to limited specimen volume or suspicion of high drug and metabolite concentrations is acceptable.

5 EQUIPMENT

5.1 Equipment

A. Pipettors
B. Vortexer
C. Heating block
D. Heated evaporator with nitrogen
E. Centrifuge
F. Positive pressure solid phase extraction manifold

5.1.1 Column

A. HPLC Column: Xterra MS C18 (5cm x 3mm x 5µm) or equivalent
B. Guard Column

5.2 Consumables

A. Disposable test tubes (silanized glass)
B. Pipette Tips
C. Autosampler vials
D. CEREX PolyChrom THC solid phase extraction columns (6 mL)

5.3 Instruments

A. Sciex 6500+ QTRAP Mass Spectrometer
B. Shimadzu HPLC

5.4 Software

Component	Software	Version
Operating System	Microsoft Windows	10 Enterprise 2016 LTSB
Mass Spectrometer	Analyst	1.7.1
Data Analysis	MultiQuant	3.0.3

5.5 Chemicals/Reagents

Storage/stability determined by manufacturer unless otherwise noted.

5.5.1 Purchased

A. Acetic Acid	ACS grade
B. Acetonitrile	Optima grade
C. Ammonium Hydroxide	Reagent grade
D. Ethyl Acetate	Optima grade
E. Formic Acid	Optima or LC/MS grade
F. Glacial acetic acid	ACS grade
G. Hexane	HPLC grade
H. Methanol	Optima grade
I. Potassium hydroxide	Reagent grade
J. Sodium acetate trihydrate	Reagent grade
K. Water	Deionized and Optima grade

5.5.2 Prepared

A. Mobile Phase 1 (Aqueous) 0.1% Formic Acid in Water:
Combine 500 mL deionized water and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least two weeks.
B. Mobile Phase 2 (Organic) 0.1% Formic Acid in Acetonitrile:

	Combine 500 mL acetonitrile and 0.5 mL formic acid and mix well. Store in glass at room temperature. Stable for at least two weeks.
C. 0.1 M Sodium Acetate Buffer (pH 7.0):	
	To a 250-mL volumetric flask, add 3.4 g sodium acetate trihydrate and 200 mL deionized water. Mix well and adjust to 6.5<pH<7.5 by slow addition of 1 N hydrochloric acid. Bring to volume with deionized water. Store refrigerated in glass. Stable 3 months.
D. 11.8 N Potassium Hydroxide (Hydrolysis Reagent) (KOH):	
	To a 100-mL Nalgene volumetric flask add 66 g potassium hydroxide and 50 mL deionized water. Mix well to dissolve and bring to volume with deionized water. Store at room temperature in Nalgene container. Stable 1 year.
E. Water:Acetonitrile:Ammonia (90:10:1):	
	Combine 90 mL deionized water, 10 mL acetonitrile and 1 mL ammonium hydroxide and mix well. Prepare fresh.
F. Hexane:Ethyl Acetate:Acetic Acid (88:10:2):	
	Combine 88 mL hexane, 10 mL ethyl acetate and 2 mL acetic acid and mix well. Prepare fresh.

5.6 Standards/Controls

Storage/stability determined by manufacturer unless otherwise noted.

5.6.1 Purchased

5.6.1.1 Internal Standards

Purchased from Cerilliant or another approved supplier

THC-d3	0.1 mg/mL
THC-OH-d3	0.1 mg/mL
THC-COOH-d3	0.1 mg/mL

5.6.1.2 Controls

Purchased from Cerilliant, Lipomed or another approved supplier

THC	1.0 mg/mL
THC-OH	0.1 mg/mL
THC-COOH	0.1 mg/mL

5.6.1.3 Matrix

A. Negative Control Urine:	
	Synthetic urine (Surine) may be purchased from Dyna-Tek; alternatively, blank urine may be obtained in-house. Store refrigerated or obtain fresh.
B. Negative Control Blood:	
	Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or prepared in-house from an appropriate blank specimen. Store frozen, refrigerated or obtain fresh. In-house Negative Control Blood is stable for at least 2 years when frozen.

5.6.2 *Prepared*

5.6.2.1 *Internal Standards*

A. Internal Standard Working Solution

Analyte (0.1 mg/mL stock)	Aliquot (mL)	Final Conc. (µg/mL)
1. THC-d3	0.025	0.25
2. THC-OH-d3	0.025	0.25
3. THC-COOH-d3	0.125	1.25
i. Add components to		10 mL volumetric flask
ii. QS	10	Methanol (Optima)
Store in glass at <0ºC. Stable for at least 1 year.		

5.6.2.2 *Controls*

A. THC Control Working Solution

Analyte (1.0 mg/mL stock)	Aliquot (mL)	Final Conc. (mg/mL)
1. THC	0.1	0.100
i. Add components to		Vial
ii. Add	0.9	Methanol (Optima)
Store in glass refrigerated or <0ºC. Prepare fresh.		

B. High Control Solution

Analyte	Solution Conc (mg/mL)	Aliquot (mL)	Final Conc. (µg/mL)
1. THC	0.100	0.050	0.5
2. THC-OH	0.100	0.050	0.5
3. THC-COOH	0.100	0.250	2.5
i. Add components to			10 mL volumetric flask
ii. QS		10	Acetonitrile (Optima)
Store in glass at <0ºC. Stable for at least 6 months.			

C. Low Control Solution

Analyte	Solution Conc (mg/mL)	Aliquot (mL)	Final Conc. (µg/mL)
1. High Control Solution		1.0	
THC	0.5		0.05
THC-OH	0.5		0.05
THC-COOH	2.5		0.25
i. Add components to			10 mL volumetric flask
ii. QS		10	Acetonitrile (Optima)
Store in glass at <0ºC. Stable for at least 6 months.			

5.6.2.3 System Suitability

A. System Suitability Sample

Analyte	Solution Conc (mg/mL)	Aliquot (mL)	Final Conc. (µg/mL)
1. High Control Solution		0.1	
THC	0.5		0.05
THC-OH	0.5		0.05
THC-COOH	2.5		0.25
i. Add components to			Autosampler vial
ii. Add		0.45	Acetonitrile (Optima)
iii. Add		0.45	Water (Optima)
Store in refrigerated/cooled autosampler tray. Stable for at least one week.			

6 PROCEDURE

	Step	Note	Reference/Lot
	A. Samples		
	1. To labeled silanized tubes add:		
☐	i. 0.5 mL of biological fluid		
	B. Control(s)		
	1. Prepare Negative Control(s)		
☐	i. Blood		
☐	ii. Urine		
	2. Prepare Positive Control(s)		
	i. Low Control Working Solution		
☐	1. Blood (3/15 ng/mL) : Add 30 µL		
☐	2. Urine (20 ng/mL): Add 40 µL		
	ii. High Control Working Solution		
☐	1. Blood (33/165 ng/mL) : Add 33 µL		
☐	2. Urine (200 ng/mL): Add 40 µL		
	C. Internal Standard(s)		
☐	1. Add 20 µL of Internal Standard Working Solution		
☐	2. Vortex		
☐	3. Allow samples to stand for 15 minutes		
	D. Pre-Treatment		
	1. Blood		
☐	i. Add 2.0 mL cold acetonitrile drop-wise while vortexing (30s)		
☐	ii. Centrifuge 3000 rpm for 3 minutes		
☐	iii. Transfer supernatant to labeled test tube		
☐	iv. Add 4 mL deionized water		
☐	v. Vortex		
	2. Urine		
☐	i. Add 0.075 mL 11.8 N potassium hydroxide		
☐	ii. Incubate in heating block for ~15 minutes at ~60°C		

☐	iii. Remove from heating block and let cool for ~5 minutes		
☐	iv. Add 0.075 mL glacial acetic acid		
☐	v. Vortex		
☐	vi. Add 5.0 mL 0.1M sodium acetate buffer		
☐	vii. Vortex		
☐	viii. Verify pH 4.5-6.5		
	☐ a. Adjust with glacial acetic acid or 11.8N potassium hydroxide		
	E. Extract (SPE)		
☐	1. Load samples onto CEREX THC SPE columns		
☐	2. Load with positive pressure		
☐	3. Wash with 1 mL Water:Acetonitrile:Ammonia (90:10:1)		
☐	4. Dry each column for 15 min under full pressure		
☐	5. Elute with 2 mL ethyl acetate by gravity		
☐	6. Dry each column for 10 min at 40°C		
☐	7. Elute with 2 mL Hexane:Ethyl Acetate:Acetic Acid (88:10:2) by gravity into the same tube		
	F. Concentrate		
☐	1. Evaporate to dryness under nitrogen at 40°C		
	G. Reconstitute		
☐	1. Add 50 µL of acetonitrile to tube 2. Vortex and transfer to ALS vial 3. Add 50 µL of water to same tube 4. Vortex and transfer to same ALS vial 5. Add 50 µL of water to same ALS vial		
	H. Instrumental Analysis		
☐	1. LC/MS: analyze 25 µL i. Analyze LC/MS performance standard prior to batch analysis		
	ii. Mobile Phase 1 (Aqueous)		
	iii. Mobile Phase 2 (Organic)		
	iv. LC Column		

7 ANALYTICAL PARAMETERS

7.1 Shimadzu HPLC

7.1.1 Gradient

Time (min)	Mobile Phase %		Flow Rate
	1-Aqueous	2-Organic	(mL/min)
0.01	60	40	0.5
7	0	100	0.5
10	0	100	0.5
12	60	40	0.5
19.9	60	40	0.5

7.1.2 Conditions

Autosampler (°C)	15
Run Time (min)	19.9

7.2 Sciex 6500+ QTRAP Mass Spectrometer

7.2.1 Source

Mode	ESI -TurboSpray
Polarity	(+)
Resolution	Unit
Scan Type	MRM
Spray Voltage (kV)	5
Source Temperature (°C)	670
Entrance Potential	10
Curtain Gas (Nitrogen)	35
Nebulizer Gas (Nitrogen)	55
Turbo Gas (Nitrogen)	55

7.2.2 MRM

Q1	Q3	Dwell	ID	DP	CE	CXP
315.143	193.1	25	THC_1	91	31	12
315.143	259.1	25	THC_2	91	27	16
315.143	123	25	THC_3	91	41	14
315.143	135.1	25	THC_4	91	27	10
331.137	201	25	THC_OH_1	51	33	12
331.137	193	25	THC_OH_2	51	31	12
331.137	175	25	THC_OH_3	51	31	12
331.137	105	25	THC_OH_4	51	47	12
345.119	299.1	25	THC_COOH_1	121	27	20
345.119	193.1	25	THC_COOH_2	121	35	12
345.119	187	25	THC_COOH_3	121	37	18
345.119	119.1	25	THC_COOH_4	121	35	8
318.148	196.1	25	THC_d3_1	106	31	12
318.148	123	25	THC_d3_2	106	39	14
318.148	135.1	25	THC_d3_3	106	27	14
334.157	196.1	25	THC_OH_d3_1	106	33	12
334.157	201	25	THC_OH_d3_2	106	33	12
334.157	175.1	25	THC_OH_d3_3	106	31	12
348.171	302.1	25	THC_COOH_d3_1	81	27	18
348.171	196.1	25	THC_COOH_d3_2	81	37	10
348.171	119.1	25	THC_COOH_d3_3	81	35	14

Only the 345 and 348 transitions are routinely collected for the analysis of urine samples.

8 DATA ANALYSIS

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In most cases, all of the below should be met in order to identify THC or related compounds within a biological specimen:

8.1 Decision Criteria

8.1.1 Chromatography

All four ion transition peaks for the analyte of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. For low concentrations of analyte (less than 5 ng/mL), there may only be three strong transitions. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

8.1.2 Retention Time

The retention time of the peak should be within ±2% of the retention time (relative or absolute) obtained from injection of a reference standard or extracted Positive Control.

8.1.3 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or blank sample injected just prior to that sample.

8.1.4 Mass Spectrometry

Four independent MS/MS experiments are conducted for each analyte. At least two ion ratios are calculated for each analyte; the ion ratios should compare favorably to ions ratios from a reference standard or an extracted positive control. See the *Guidelines for Comparison of Mass Spectra* standard operating procedure (TOX-104) for further guidance.

8.2 Calculations

8.2.1 Software

Qualitative calculations may be performed by one or more of the following software packages:

A. Sciex
    1. Analyst
    2. Multiquant
    3. Report Builder
B. Microsoft
    1. Excel

9 Reporting

Refer to CHEM-100, TOX-100 and TOX-101.

10 Corrective Measures

Refer to Quality Control for Toxicology Examinations (TOX-101) for guidance on action steps in the event of a quality control failure.

11 Performance Characteristics

11.1 LOD

	THC	THC-COOH		THC-OH
	Blood	Blood	Urine	Blood
Limits of Detection (ng/mL):	1	2.5	5	1.0

11.2 Carryover

High analyte concentrations in samples may carryover into subsequent samples. Analysts should investigate evidence for carryover if high sample analytes loads are encountered.

12 Limitations

No significant matrix interference was identified during validation.

13 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the FBI Laboratory Safety Manual for guidance.

14 Revision History

Revision	Issued	Changes
08	02/11/2022	Document reformat. Minor additions and revision to text.