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LC-MS/MS Analysis of Phytocannabinoids and their Metabolites in Urine, Oral Fluid and Blood

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ABSTRACT

Purpose: An LC-MS/MS analytical method was developed and verified for the quantitation of Phytocannabinoids and their metabolites in urine, oral fluid and blood for forensic use. Simple sample preparation techniques including dilute and shoot, protein crash and liquid-liquid extraction were evaluated. A Thermo Scientific™ Endura™ triple quadrupole mass spectrometer in positive and negative Electrospray mode with a Thermo Scientific™ Dionex™ Vanquish™ Horizon HPLC system was used.

Methods: 100 µL of urine, oral fluid and blood were used for the analysis of the phytocannabinoids and their metabolites. Various columns were evaluated and a Thermo Scientific™ Accucore™ C18, 50 x 2.1 mm, 2.6 µm with 0.1% Formic Acid in water and acetonitrile mobile phases achieved baseline chromatographic separation in approximately 6.5 minutes run time. Quantitative analysis was performed using scheduled reactive monitoring (SRM) transition pairs for each phytocannabinoid and metabolite and internal standard in positive and negative mode and accuracy of the analytical method was verified using pooled reference samples.

Results: Good linearity and reproducibility were obtained across the dynamic range of the phytocannabinoids and their metabolites with a coefficient of determination R^2>0.95 or better for all compounds in the various matrices. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to range from 0.25 to 2.5 ng/ml and excellent reproducibility was observed for all compounds (CV < 15%) in all matrices.

INTRODUCTION

Phytocannabinoids are a class of diverse chemical compounds that are found in the cannabis plant and there are at least 85 different kinds of which only a few have been shown to have any biological and chemical impact.

Therefore in this case, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of the 15 most common Phytocannabinoids and metabolites in urine, oral fluid and blood. The Phytocannabinoids analyzed include 11-Hydroxy-Delta9-THC, 11-Nor-9-Carboxy-Delta9-THC, 11-Nor-9-Carboxy-Delta9-THC-Glucuronide, Delta9-THC, Cannabichromene, Cannabidiol, Cannabidiolic Acid, Cannabidivarin, Cannabidivarinic Acid, Cannabigerol, Cannabigerolic Acid, Cannabinol, Cannabinolic Acid, Tetrahydrocannabinolic Acid and Tetrahydrocannabivarin. The sample preparation choices were kept simple and included dilute and shoot for urine and oral fluid and protein crash for blood as well as one step liquid-liquid extraction for all matrices and the methodologies were developed on the Endura triple quadrupole mass spectrometer in positive and negative Electrospray ionization modes with the Vanquish Horizon HPLC system with a 6.5 minute analytical gradient.

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MATERIALS AND METHODS

Standards

The following analytical reference standards and internal standards were obtained from Cerilliant Corp., Round Rock, TX-

Delta9-THC (THC):	1 mg/mL	Delta9-THC-D3:	100 μg/mL
11-Hydroxy-Delta9-THC (OH-THC):	1 mg/mL	11-Hydroxy-Delta9-THC-D3:	100 μg/mL
Cannbidiol (CBD):	1 mg/mL	Cannabidiol-D3:	100 μg/mL
Cannabinol (CBN):	1 mg/mL	Cannabinol-D3:	100 μg/mL
Cannabichromene (CBC):	1 mg/mL		
Cannabidiolic Acid (CBDA):	1 mg/mL		
Cannabidivarin (CBDV):	1 mg/mL		
Cannabidivarinic Acid (CBDVA):	1 mg/ml		
Cannabigerol (CBG):	1 mg/mL		
Cannabigerolic Acid (CBGA):	1 mg/mL		
Tetrahydrocannabinolic Acid (THCA-A):	1 mg/ml		
Tetrahydrocannabivarin (TCBDV):	1 mg/mL		
Cannabicylol (CBL):	1 mg/ml		
11-Nor-9-Carboxy-Delta9-THC (COOH-THC):		1 mg/mL	
11-Nor-9-Carboxy-Delta9-THC-D3:		100 μg/mL	
11-Nor-9-Carboxy-Delta9-THC-Glucuronide (COOH-THC-Gluc):		100 μg/mL	
11-Nor-9-Carboxy-Delta9-THC-Glucuronide-D3:		100 μg/mL	

Reagents

The following Fisher Scientific™ acids, reagents and solvents were used-

HPLC grade Water	Hexane
Methanol	Ethyl Acetate
Acetonitrile	Glacial Acetic Acid
Formic Acid	

Sample Preparation- Urine Dilution

- 100 μL of urine sample, calibrators, controls were added to 1.5 ml eppendorf tubes and 10 μL of Phytocannabinoid ISTD at 1000 ng/mL were added to each tube and vortexed briefly
- 890 μL of HPLC grade water was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators were prepared in drug-free urine (Golden West Biological, Inc, Temecula, CA)

Sample Preparation- Oral Fluid Dilution

- 100 μL of oral fluid sample (50 μL of oral fluid and 50 μL of buffer), calibrators, controls were added to 1.5 ml eppendorf tube and 5.0 μL of Phytocannabinoid ISTD at 1000 ng/mL were added to each and vortexed briefly
- 395 μL of HPLC grade water was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators and controls were prepared in negative oral fluid (Oral-Eze™ -Thermo Fisher Scientific)

Sample Preparation- Blood Protein crash

- 100 μL of blood sample, calibrators, controls added to 1.5 ml eppendorf tubes and 10 μL of ISTD at 1000 ng/mL were added to each and vortexed briefly
- 200 μL of HPLC grade Acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The supernatant was transferred to an MS vial and capped.
- All in-house calibrators were prepared in drug-free blood (Golden West Biological, Inc, Temecula, CA)

Sample Preparation- Urine and Oral Fluid Liquid-Liquid Extraction

- 100 μL of urine and oral fluid sample, calibrators, controls were added to 1.5 ml eppendorf tubes and 10 μL and 5 μL of Phytocannabinoid ISTD at 1000 ng/mL were added to each urine and oral fluid tube respectively
- 20 μL of Glacial Acetic Acid were added to each tube and vortexed briefly
- 1000 μL of Hexane:Ethyl Acetate (1:1) was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The upper organic layer was transferred to a new eppendorf tube and dried down under nitrogen at room temperature.
- The extract was reconstituted in 100 μL of water and acetonitrile (50 μL of each) and The supernatant was transferred to an MS vial and capped.

Sample Preparation- Blood Liquid-Liquid Extraction

- 100 μL blood sample, calibrators, controls were added to 1.5 ml eppendorf tubes and 10 mL of Phytocannabinoid ISTD at 1000 ng/mL were added to each blood tube
- 100 μL of Acetonitrile was added to each and vortexed briefly followed by 20 μL of Glacial Acetic Acid and the tubes were vortexed briefly again
- 1000 μL of Hexane:Ethyl Acetate (1:1) was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13000 rpm
- The upper organic layer was transferred to a new eppendorf tube and dried down under nitrogen at room temperature.
- The extract was reconstituted in 100 μL of water and acetonitrile (50 μL of each) and the supernatant was transferred to an MS vial and capped.

The calibration curves ranged from 0.01 to 1000 ng/ml in each matrix and various pooled samples were used as control material.

Data Analysis

The software used included for this method included the Thermo Scientific™ Xcalibur™ 3.1, Thermo Scientific™ TSQ Endura Tune™ 2.1 and Thermo Scientific™ Tracefinder™ 4.1

Method

HPLC Conditions-

Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Accucore C18, 50 x 2.1 mm, 2.6 μm
Column Temperature:	50 °C
Injection Volume:	20 μL DS-(Urine, Oral Fluid), 10 μL PPX-(Blood,) 10 μL LLE (All Matrices)
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50%Methanol:50%Water) 10 seconds
Mobile Phase A:	0.1% Formic Acid in Water
Mobile Phase B:	Acetonitrile
Flow Rate:	0.4 ml/min
Gradient:	0 min- 45%A:55%B 0.5 min- 45%A:55%B 5.0 min- 2%A:98%B 5.5 min- 2%A:98%B 5.6 min- 45%A:55%B
Run time:	6.5 mins

MS and Ion Source Conditions-

TSQ Endura triple quadrupole mass spectrometer

Ion mode:	Positive and Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	300 °C
Ion Transfer Tube Temperature:	225 °C
Sheath Gas:	60
Aux Gas:	25
Sweep Gas:	0
Spray Voltage:	Positive Ion (V):3500 V/ Negative Ion (V): 3500V
Q1/Q2 Resolution:	0.7 (FWHM)
Cycle time (sec):	0.5
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 secs

Table 1. Scan Parameters- SRM Table

Compound	RT (Min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	RF Lens (V)
COOH-THC-Gluc	0.7	Negative	519.1	299.2/343.2	34/23	197
COOH-THC-Gluc-D3	0.7	Negative	522.1	302.2/346.2	35/23	189
CBDVA	1.46	Negative	329.4	217.1/311.1	27/19	183
OH-THC	1.5	Positive	331.3	193.1/313.1	25/13	123
OH-THC-D3	1.49	Positive	334.3	196.1/316.2	25/20	121
COOH-THC	1.56	Negative	343.4	245.1/291.2	28/20	187
COOH-THC-D3	1.56	Negative	346.4	248.1/302.2	27/21	188
CBDV	1.68	Positive	287.3	123.1/165.1	32/23	125
CBDA	2.27	Negative	357.5	245.1/339.1	29/20	207
CBGA	2.41	Negative	359.5	343.1/315.2	10/21	222
TCBDV	2.47	Positive	287.2	123.1/165.1	32/22	126
CBG	2.55	Positive	317.3	123.1/193.1	33/13	96
CBD	2.58	Positive	315.3	123.1/193.1	34/24	135
CBD-D3	2.57	Positive	318.3	123.1/196.1	34/23	130
CBN	3.11	Positive	311.3	223.1/293.1	20/16	139
CBN-D3	3.1	Positive	314.3	223.1/296.1	21/17	134
THC	3.43	Positive	315.2	123.1/193.1	28/23	122
THC-D3	3.42	Positive	318.2	123.1/196.1	26/24	127
CBL	3.69	Positive	315.4	81.1/235.1	29/17	145
THCA-A	3.79	Negative	357.4	245.1/313.1	32/24	256
CBC	3.84	Positive	315.3	81.1/259.1	14/12	106

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RESULTS

Table 2. Sensitivity.

Compound	Urine-DS LOD/LOQ (ng/ml)	OF-DS LOD/LOQ (ng/ml)	Blood-PPX LOD/LOQ (ng/ml)	Urine-LLE LOD/LOQ (ng/ml)	OF-LLE LOD/LOQ (ng/ml)	BLOOD-LLE LOD/LOQ (ng/ml)
COOH-THC-Gluc	1/ 2.5	NA	NA	1/ 2.5	NA	NA
CBDVA	0.5/1	1/ 2.5	0.5/1	0.5/1	0.5/1	0.5/1
OH-THC	1/ 2.5	5/5	0.5/1	0.5/1	1/ 2.5	0.5/1
COOH-THC	1/ 2.5	1/ 2.5	0.5/1	0.25/0.5	1/1	0.5/1
CBDV	1/ 2.5	2.5/5	1/1	1/1	1/ 2.5	1/ 2.5
CBDA	2.5/5	2.5/5	2.5/2.5	2.5/5	2.5/5	2.5/5.5
CBGA	1/ 2.5	1/ 2.5	0.5/1	0.25/0.5	0.5/1	0.5/1
TCBDV	1/ 2.5	2.5/2.5	0.5/1	0.5/1	2.5/2.5	1/ 2.5
CBG	0.25/0.5	0.5/1	0.5/1	0.25/0.5	1/ 2.5	0.5/1
CBD	1/ 2.5	2.5/5	0.5/1	0.5/1	1/ 2.5	0.5/1
CBN	1/ 2.5	1/ 2.5	0.5/1	0.25/0.5	1/ 2.5	0.5/1
THC	1/ 2.5	2.5/5	0.5/1	0.5/1	1/ 2.5	0.5/1
CBL	0.25/0.5	0.5/1	0.5/0.5	0.1/0.25	0.5/1	0.5/1
THCA-A	1/ 2.5	1/ 2.5	0.5/1	1/1	0.5/1	0.5/1
CBC	1/ 2.5	1/ 2.5	0.5/1	0.25/0.5	1/1	0.5/1

Linearity/Sensitivity

The linear range of the Phytocannabinoids and metabolites in each matrix was from 2.5 to 1000 ng/ml for Urine and Blood and from 5 to 1000 ng/ml for Oral Fluid. The linearity of each matrix was determined in triplicate over 3 days and the results are shown with LOD and LOQ being determined as 3:1 and 10:1 of signal to noise respectively where possible and the mean coefficient of determination (R2) > 0.99 for each matrix and the %CV for each calibration point were all <10%.

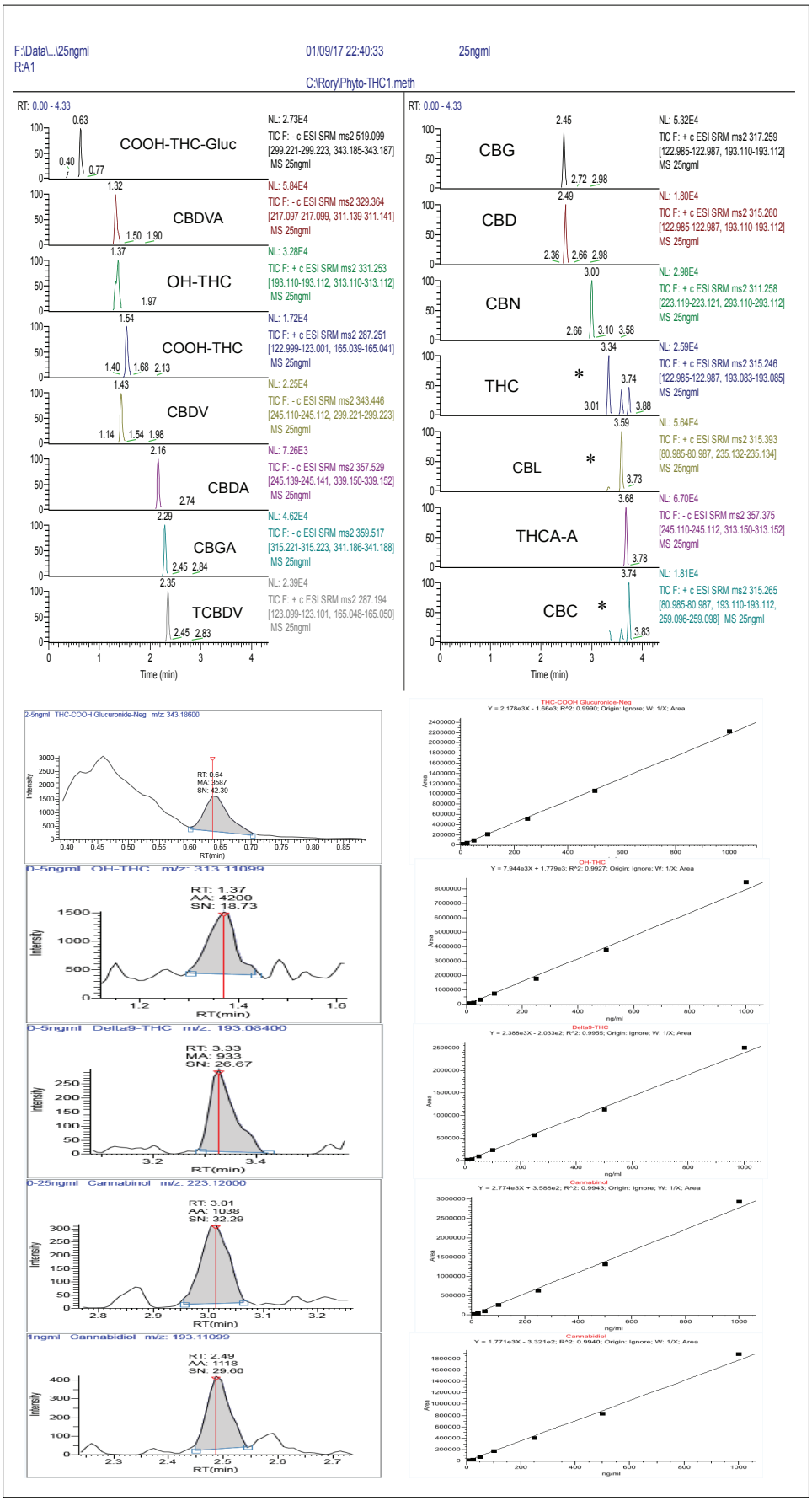
Accuracy

The accuracy was determined by the analysis of pooled sample control material as the percentage deviation from the targeted mean and the results were <10% for all levels in each matrix. The urine pooled control material concentrations were 15, 55 and 125 ng/ml. The oral fluid pooled control material were 8, 24 and 72 ng/ml. The blood pooled control material were 5, 25 and 100 ng/ml . Therefore, the analytical method can achieve the required accuracy for the analysis of the Phytocannabinoids and metabolites in urine, oral fluid and blood.

Precision/Specificity

The intra–assay precision (%CV) of the Phytocannabinoids and metabolites in each matrix were determined by extracting and quantifying three replicates of the pooled sample control material. The inter-assay precision was determined over 3 consecutive days and was found to have a %CV <10% for each Phytocannabinoids for the three levels of pooled sample control material respectively in urine, oral fluid and blood. Therefore, the analytical method can achieve the required precision for the analysis of the Phytocannabinoids and metabolites in urine, oral fluid and blood.

Figure 1: Chromatograms



CONCLUSIONS

- Baseline separation of the Phytocannabinoids and metabolites with good LOD/LOQ being achieved in three different matrix types in positive and negative ionization mode
- Simple sample preparation of three matrixes (urine, oral fluid and blood) achieved desirable LOD/LOQ with further work to be carried out to fine tune the techniques to obtain more sensitive results while maintaining ease of use and low cost
- Excellent linearity of calibration curves with acceptable accuracy, precision and reproducibility in positive and negative mode was achieved in all matrices <10% for %CV and the sample preparation techniques and analytical methodologies will be further verified

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