Supporting Information

Analysis of Cannabinoids and Their Metabolites in Human Urine

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Experimental Section

Chemicals and Materials

We bought native and isotopically labeled standards, including THC, THC-*d*3, COOH-THC, COOH-THC-*d*3, OH-THC, OH-THC-*d*3, CBD, CBD-*d*3, CBN and CBN-*d*3 from Cerilliant (Round Rock, TX, USA). We bought HPLC–grade methanol (≥99.9%), water, acetonitrile (≥99.9%), 2-propanol (≥99.9%), formic acid (≥99.5%), and sodium hydroxide solution (10N/certified) from Fisher Scientific (Fair Lawn, NJ, USA), ammonium formate (≥99%) and ammonium acetate (≥98%) and β-glucuronidase/sulfatase (*Escherichia coli*, type IX-A) from Sigma–Aldrich Laboratories, Inc. (St. Louis, MO, USA). Solid phase extraction (SPE, C18, 100mg) 96-well plate was bought from Biotage (Charlotte, NC, USA).

Standard Solution Preparation

We prepared 19 working solutions from serial dilutions of primary stock solutions with methanol and water (v/v: 60:40), and stored them in Teflon–capped amber glass vials at –24 °C. We added 50 µL of each working solution to 500 µL of blank urine using a Hamilton automated liquid–handling system (Reno, NV, USA) during sample preparation. This automatically created calibrators at 0.001, 0.002, 0.005, 0.010, 0.020, 0.0625, 0.125, 0.250, 0.650, 1.25, 2.50, 5.0, 10, 25, 50, 100, 250, 500 and 800 ng/mL. Primary standard solutions from different lot numbers were used to prepare QC samples. We prepared three working solutions by diluting appropriate volumes of the primary solutions with methanol and water (v/v: 60:40).

Ultra-High Pressure Liquid Chromatograph (UHPLC)

The UHPLC system consisted of a DGU-20A degasser, two LC-30AS pumps, a SIL-30AC autosampler, and a CTO-20AC column oven (Shimadzu Corp, Columbia, MD, USA).

Determination of Extraction Recoveries and Matrix Effects.

We used three sets of samples at low, medium and high concentrations (0.025, 25 and 500 ng/mL, respectively) to determine the optimized extraction recoveries and matrix effects.(Wei *et al.*, 2014) In the first set, 12 blank urine samples were fortified with native and deuterated internal standard solutions at the beginning of the sample preparation. In the second set of 12 blank urine samples, spiking solutions were added immediately before LC injection. The third set of 12 samples were prepared by spiking native and internal standard solutions in methanol and water (v/v: 50:50). Sample extraction recovery was estimated by dividing the average peak areas of set 1 by those of set 2 and then multiple by 100. The matrix effect was estimated by dividing the average peak areas of set 2 by those of set 3 and subtracted from 1 and then multiple by 100.

Analytical Specificity

The analytical specificity in this method was assessed by following measures: (1) The use of MS/MS allowed a means of monitoring ion transitions specific to each analyte; (2) target native analyte should co-elute with the corresponding isotope labeled internal standard analog (ISTD); (2) Bothe native analyte and ISTD should elute at the specific retention time; (3) native analyte should have specific ratios of the quantitation

transition's response to the confirmation transition's response to confirm the analyte determined in unknown samples. The quantitation and confirmation ion transitions for each analyte are presented in Table S-2.

Data analysis and software

Analyst software (version 1.6.2) was used for data acquisition and quantitation. Calibration curves were constructed using peak area ratios of analytes to corresponding internal standards for each batch via linear least-squares regression with a 1/*x* weighting factor.

Table S–1: UHPLC gradient Elution program.

Time	Module	Event	Parameter
0.01	System Controller	start	-
1.20	Pumps	%B	50
2.20	Pumps	%B	75
2.80	Pumps	%B	75
3.50	Pumps	%B	96
4.20	Pumps	%B	96
4.21	Pumps	%B	50
6.00	System Controller	Stop	

Table S–2: Multiple reaction monitoring (MRM) transitions and mass spectrometry settings.

Analyte	ESI mode	Precursor/ product ion (quant, confirm) [1]	Settings used in this study (volts) [2]				Optimized
			DP	CE [3]	CXP	EP	CE
OHTHC	+	331.0/(193.1, 201)	40	34/33	11	10	
	–	329.1/(268.1, 311.2)	-55	-50/-35	-16	-10	-37/-26
OHTHC-D3	+	334.1/196.1	50	35	11	8	
	–	332.1/314.2	-50	-26	-16	-10	
COOH-THC	+	345.1/(193.1, 299.2)	50	37/28	16	10	
	–	343.2/(191, 245)	-65	-54/-46	-15	-10	-45/-37
COOH-THC-D3	+	348.1/196.1	60	37	13	8	
	–	346.1/248.1	-60	-39	-16	-8	
CBD	+	315.2/(193.1, 123.1)	65	32/41	11	10	
	–	313.1/(245.1, 179)	-60	-47/-26	-16	-10	-33/-26
CBD-D3	+	318.2/196.2	50	31	13	8	
	–	316.1/248.1	-60	-33	-16	-10	
CBN	+	311.1/(208, 241)	55	40/26	11	10	
	–	309.1/(279.1, 222.1)	-60	-62/-60	-16	-10	-44/-58
CBN-D3	+	314.2/223.1	55	30	13	7	
	–	312.1/282	-60	-44	-16	-10	
THC	+	315.1/(193.1, 123.1)	65	32/41	11	10	
	–	313.2/(245.2, 191)	-60	-50/-39	-16	-10	-36/-39
THC-D3	+	318.1/196.1	60	32	12	8	
	–	316.1/248.2	-60	-37	-16	-10	

Abbreviations: ESI – electrospray ionization; DP – declustering potential; CE – collision offset energy; CXP –collision cell exit potential; EP – entrance potential.[1] two ion transitions for each native analyte (quantitation/confirmation) and one transition for labeled standard were monitored. [2] CE values under negative ESI were detuned from the optimized values to higher levels to yield lower analytical responses. [3] Collision energy: quantitation/confirmation transitions

Table S–3: Limit of detection (LOD) in terms of mass-on-column.

	LOD, ng/mL	LOD, pmol/mL	LOD, pg on-column[2]	LOD, fmol on-column[3]
Analyte	"free"/"total" [1]	"free"/"total"	"free"/"total"	"free"/"total"
OHTHC	0.008/0.017	0.024/0.051	0.08/0.17	0.242/0.514
COOH-THC	0.005/0.015	0.015/0.044	0.05/0.15	0.145/0.435
CBD	0.004/0.009	0.013/0.029	0.04/0.09	0.127/0.286
CBN	0.002/0.007	0.006/0.023	0.02/0.07	0.064/0.225
THC	0.002/0.005	0.006/0.016	0.02/0.05	0.064/0.159

Abbreviations: pmol – picomole. pg – picogram. fmol – femtomole.

[1] "Free" and "total" refer to unconjugated and the sum of conjugated and unconjugated forms, respectively.

[2] LOD in terms of pg on-column was calculated as: LOD (ng/mL)×0.01mL (injection volume) $\times 10^3 \times$pg/ng

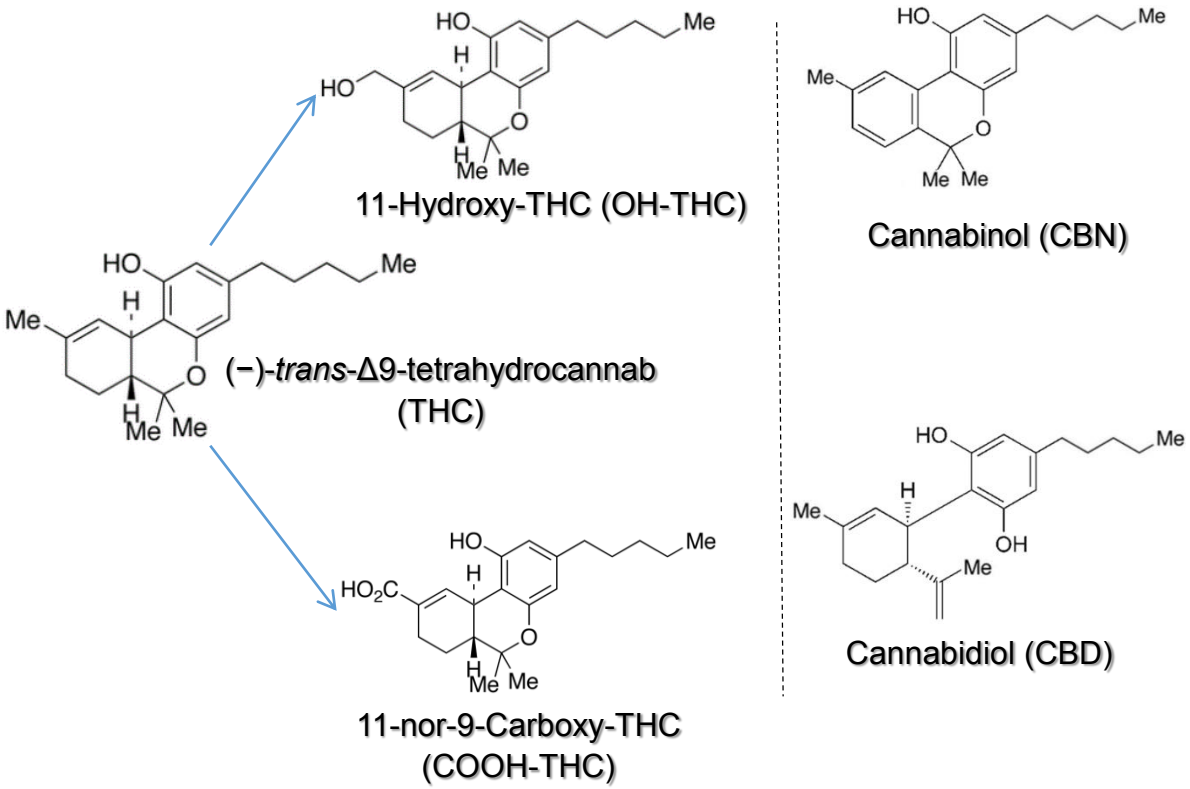
[3] LOD in terms of fmol-on-column was calculated as: LOD (ng/mL)×0.01mL (injection volume) ×(1/Molecular weight)×nmol/ng$\times 10^6 \times$fmol/nmol

Table S–4: Accuracy and precision of replicate analysis of five compounds in fortified urine samples by “total” method (with enzymatic-alkaline hydrolysis).

			Within-Day			Between-day		
	ESI mode	Target, ng/mL	measured	Error %	RSD %	measured	Error %	RSD %
OHTHC	+	0.050	0.0484	-3.2	8.1	0.0522	4.3	3.2
		0.100	0.103	3.0	8.0	0.103	2.8	6.1
		1.25	1.32	5.3	5.3	1.28	2.6	3.2
		25.0	27.0	7.8	4.4	27.4	9.7	3.5
	–	25.0	26.8	7.1	6.3	27.0	7.9	4.1
		100	108	7.7	1.5	105	4.5	2.1
		500	501	0.2	3.4	487	-2.6	4.7
COOH-THC	+	0.050	0.0494	-1.2	6.9	0.0504	0.8	7.7
		0.100	0.104	4.3	6.7	0.105	5.0	4.8
		1.25	1.25	0.3	7.4	1.25	-0.4	3.8
		25.0	27.2	8.8	3.0	26.6	6.6	2.6
	–	25.0	26.5	6.0	5.5	26.7	6.7	4.4
		100	106	5.7	5.7	109	8.5	4.0
		500	472	-5.6	6.1	462	-7.7	2.6
CBD	+	0.050	0.0519	3.8	9.3	0.0518	3.6	8.2
		0.100	0.096	-4.1	7.9	0.106	6.3	5.5
		1.25	1.15	-8.0	4.5	1.27	1.2	3.6
		25.0	25.2	0.8	4.7	25.2	1.0	4.4
	–	25.0	25.2	0.8	6.6	25.3	1.1	7.2
		100	105	5.0	2.0	108	7.5	3.3
		500	461	-7.8	2.6	459	-8.2	3.1
CBN	+	0.050	0.0512	2.5	7.5	0.0524	4.7	6.1
		0.100	0.094	-6.1	6.8	0.105	5.1	8.3
		1.25	1.23	-1.3	4.0	1.26	0.4	3.8
		25.0	26.0	4.0	4.5	26.6	6.2	4.1
	–	25.0	25.9	3.6	5.8	25.7	2.7	4.5
		100	100	0.3	1.6	99.9	-0.1	3.7
		500	476	-4.8	2.6	484	-3.3	3.3
THC	+	0.050	0.0465	-7.1	7.1	0.0538	7.6	8.4
		0.100	0.094	-5.6	4.8	0.105	5.1	6.2
		1.25	1.21	-3.5	5.1	1.25	-0.2	2.3
		25.0	27.0	8.2	3.4	27.0	8.1	2.9
	–	25.0	26.9	7.6	4.5	27.2	8.9	5.7
		100	104	4.3	0.6	106	6.3	2.2
		500	480	-3.9	4.0	483	-3.4	2.8

Abbreviation: ESI = electrospray ionization; RSD = relative standard deviation

Figure S–1: Cannabinoid and THC metabolites measured in this method



Additional information–S1: Complete method file for data acquisition

The instrument method file used for data acquisition in this study can be obtained upon request.

References

Wei, B., J. Feng, I.J. Rehmani, S. Miller, J.E. McGuffey, B.C. Blount and L. Wang, 2014. A high-throughput robotic sample preparation system and hplc-ms/ms for measuring urinary anatabine, anabasine, nicotine and major nicotine metabolites. Clin Chim Acta, 436: 290-297.