Development of a chiral LC-MS/MS approach for measuring metabolites of the synthetic cannabinoids JWH-018 and AM2201

Kathryn A. Seely[1], Amy L. Patton[1], Matthew Trass[2], Art Miranda[2], Michael Schwartz[3], Paul D. Kennedy[4], Gregory W. Endres[4], William E. Fantegrossi[5], Matthew McMullin[6], Barry K. Logan[6], and Jeffery H. Moran[1,5]

[1]Akansas Public Health Laboratory, Arkansas Department of Health, Little Rock, AR 72205 , [2]Phenomenex, Torrance, CA 90501, [3]National Center for Environmental Health/Centers for Disease Control and Prevention, Atlanta, GA 30333,[4]Cayman Chemical Co., Ann Arbor, Michigan, 48108, [5]Department of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205, [6]NMS Labs, Willow Grove, PA 19090

Abstract

Herbal mixtures labeled as “K2” or “Spice” are often marketed as legal marijuana substitutes to circumvent existing regulations or avoid detection in standard drug screens. These products commonly contain the synthetic cannabinoids JWH-018 and AM2201, both aminoalkylindoles and potent cannabinoid receptor agonists. With reports now indicating that 1 in 9 high school students experiment with synthetic cannabinoids and several medical reports specifically linking human injury and death to JWH-018 and AM2201, public health officials are increasingly concerned about abuse trends associated with these emerging cannabinoids. Unfortunately, little is known about the metabolism and toxicology of these new drugs, but several clinical investigations identify the (ω)-hydroxyl, (ω)-carboxyl, and (ω -1)-hydroxyl metabolites as primary biomarkers. These metabolites are also known to retain significant *in vitro* and *in vivo* pharmacological activity, which may offer a mechanistic explanation of the adverse effects associated with synthetic cannabinoid use. Since the (ω-1)-hydroxyl metabolites of AM2201 and JWH-018 are chiral molecules, analytical procedures capable of low level quantification of specific enantiomeric metabolites are required to further understand the metabolic and toxicological consequences of synthetic cannabinoid use. This study validates an LC-MS/MS approach capable of simultaneously resolving each specific enantiomer while resolving parent compounds and other isobaric metabolites. Chiral separations were achieved utilizing the LUX 3 µm cellulose-3 (150 x 2.0 mm) column and a simple acetonitrile/ammonium bicarbonate (20 mM) gradient. All metabolites of interest were resolved within 8 min and standard responses were linear from 1 to 50 ng/ml in human blood. The accuracy and precision of this new assay is similar to other clinical methods currently being used. This study further evaluated the utility of the new analytical procedure by assessing specific enantiomers in human specimens and by assaying *in vitro* reactions designed to determine the stereospecificity of neuronal cytochrome P450s (e.g. CYP2J2 and CYP2D6). Stereospecificity was observed in both clinical specimens and *in vitro* reactions using recombinant CYP2J2 and CYP2D6. Continued metabolomic studies using this comprehensive LC-MS/MS approach will yield detailed information required for understanding the toxicological consequences and public health impact of emerging drugs of abuse. (NIH-GM075893 to AR-P; APHL Innovation Award to JHM; UAMS Arkansas CCTR grant to LPJ,AR-P,JHM,CLM, & PLP)

Aim

To develop an analytical method that detects chiral metabolites of the synthetic cannabinoids JWH-018 and AM2201

Background

- Synthetic cannabinoid use is a growing public health concern.
- In Arkansas, over 70% of synthetic cannabinoid products seized from 2010 – 2011 contained JWH-018 and/or AM2201.
- Cytochrome P450 enzymes produce hydroxylated metabolites of JWH-018 and AM2201. These hydroxylated metabolites retain varying degrees of biological activity at the cannabinoid type 1 receptor.
- The (ω-1)-OH metabolites of JWH-018 and AM2201 are chiral molecules. The biological implications of these chiral metabolites are unknown, especially since there is no analytical method to detect these chiral metabolites found in human specimens.

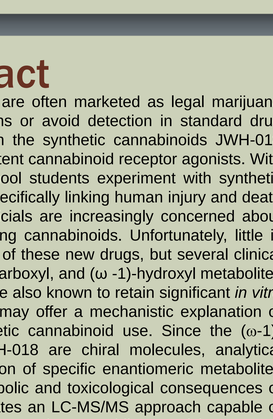

Figure 1: Metabolic oxidation of JWH-018 and AM2201. The circled compounds are chiral metabolites.

Methods

Sample Collection

- Male NIH Swiss mice were dosed i.p. with 3 mg/kg JWH-018. Blood and brain were harvested at 60 minutes after dosing.
- Human post-mortem liver and blood were collected during autopsies of two individuals who tested positive for AM2201 and JWH-018.

Extraction

- Pipette 50 µl of blood containing 10 µl of 20 µg/ml internal standard (IS) (DMSO) into 950 µl 0.1M sodium acetate pH 5.0. Using the automated SPE system (Gilson), 1 ml of sample is added to SPE cartridge (Strata-X-Drug B 33u Polymer Strong Cation, Phenomenex), washed with 1 ml sodium acetate buffer, washed a second time with a solution containing 70/30 solution of sodium acetate/acetonitrile, and eluted with 5 ml of 85/15 ethyl acetate/isopropanol. The elution is evaporated at 60°C until dry and reconstituted with 100 µl 100% ethanol.
- Liver and brain tissue is homogenized in PBS. Protein is precipitated from 200 µl homogenate containing 10 µl IS (20 µg/ml) and 800 µl acetonitrile. All samples are incubated at -40°C for 30 minutes, vortexed, and centrifuged for 10 minutes at max RPM. The supernatant is transferred to a glass test tube, evaporated until completely dry, and reconstituted with 100 µl ethanol.

LC-MS/MS

All LC-MS/MS analysis used an Agilent 1200 HPLC coupled to an AB Sciex API-4000 Q-Trap tandem mass spectrometer.

Table 1: LC Parameters

Parameter	Setting
Column Type	Phenomenex Lux 3 µm Cellulose-3
Guard Column Type	Phenomenex SecurityGuard Cartridges Lux Cellulose-3
Mobile Phase	Mobile Phase A: 20 mM ammonium bicarbonate
	Mobile Phase B: 100% acetonitrile

Table 2: LC Gradient Program

Time (min.)	% A	% B	Flow Rate (µL/min)
0	60	40	500
10	5	95	500
12	5	95	500
15	60	40	500
16	60	40	500

Table 3: MRM Configuration

	Analyte	Q1 (m/z)	Q3 (m/z)
SRM	AM2201	360	155*
			127†
	(R)-(-)-AM2201-(ω-1)-OH	376	155*
			127†
	(S)-(+)-AM2201-(ω-1)-OH	376	155*
			127†
	AM2201-(ω-1)-OH-d5	381	155*
			127†
	JWH-018	342	155*
			127†
	JWH-018-(ω)-OH	358	155*
			127†
	JWH-018-(ω)-OH-d5	363	155*
			127†
	JWH-018-(ω)-COOH	372	155*
			127†
	JWH-018-COOH-d4	376	155*
			127†
	(R)-(-)-JWH-018-(ω-1)-OH	358	155*
			127†
	(S)-(+)-JWH-018-(ω-1)-OH	358	155*
			127†
	JWH-018-(ω-1)-OH-d5	363	155*
			127†
IDA-EPI	1-12	[MH]+	80-600

Positive Ion Mode *Quantification Ion † Confirmation Ion

Results

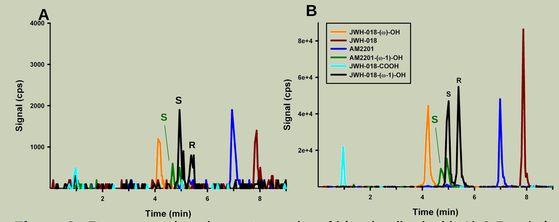

Figure 2: Representative chromatographs of blood spiked with A) 0.5 ng/ml and B) 50 ng/ml synthetic cannabinoids.

Table 5: Detection and Quantification Limits

Analyte	Mean r²	MDL* (ng/ml)	LLQ† (ng/ml)
JWH-018	0.998	< 0.5	1.74
JWH-018-(ω)-OH	0.994	< 0.5	2.57
JWH-018-COOH	0.995	< 0.5	5.38
AM2201	0.996	< 0.5	4.81
R-JWH-018-(ω-1)-OH	0.994	< 0.5	1.21
S-JWH-018-(ω-1)-OH	0.997	< 0.5	1.32
R-AM2201-(ω-1)-OH	0.996	< 0.5	7.22
S-AM2201-(ω-1)-OH	0.992	< 0.5	5.84

*MDL (minimum detection limit) estimated as less than the lowest calibrator.
†LLQ (lower limit of quantitation) estimated as 3 times the standard deviation of the mean calculated concentration of the QC Low.
Calculations for each analyte are for blood matrix (n = 4).

Table 4: Accuracy and Precision

Analyte	Quality Control Low (5 ng/ml) Conc. ± SD (ng/ml)	% CV	% Recovery	Quality Control Medium (50 ng/ml) Conc. ± SD (ng/ml)	% CV	% Recovery	Quality Control High (100 ng/ml) Conc. ± SD (ng/ml)	% CV	% Recovery
JWH-018	4.61 ± 0.58	12.60	92.10	49.55 ± 12.84	25.91	99.10	93.13 ± 17.37	18.65	93.12
JWH-018-(ω)-OH	4.80 ± 0.86	17.86	95.90	44.78 ± 6.90	15.40	89.55	93.78 ± 10.00	10.66	93.77
JWH-018-COOH	3.87 ± 1.79	46.32	77.40	44.70 ± 3.87	8.67	89.40	97.33 ± 11.46	11.78	97.32
AM2201	4.61 ± 1.60	34.81	92.15	51.23 ± 10.15	19.81	102.45	105.08 ± 7.71	7.34	105.08
R-JWH-018-(ω-1)-OH	4.88 ± 0.40	8.23	97.65	50.05 ± 8.70	17.39	100.10	116.53 ± 21.29	18.27	116.53
S-JWH-018-(ω-1)-OH	4.93 ± 0.44	8.95	98.65	42.75 ± 6.77	15.83	85.50	86.03 ± 14.00	16.28	86.02
R-AM2201-(ω-1)-OH	5.57 ± 2.41	43.19	111.45	40.30 ± 3.38	8.38	80.60	94.05 ± 11.97	12.73	94.05
S-AM2201-(ω-1)-OH	4.87 ± 1.95	40.01	97.30	42.73 ± 10.30	24.10	84.45	88.63 ± 6.28	7.09	88.62

Calculations for each analyte are for blood matrix (n = 4).

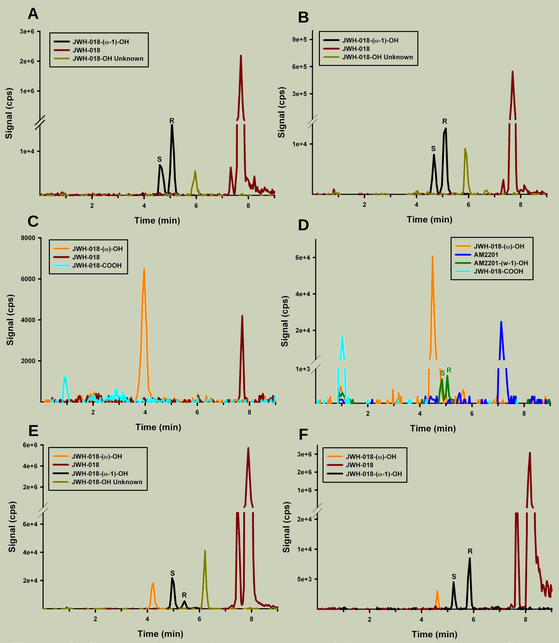

Figure 3: Representative chromatograms of A) mouse blood and B) mouse brain 60 minutes after dosing with 3 mg/kg i.p. JWH-018. JWH-018, AM2201, and metabolites in C) human liver and D) human blood. The *in vitro* metabolism of JWH-018 by two neuronal P450s E) CYP2D6 and F) CYP2J2.

Conclusions

- This LC-MS/MS method is capable of fully resolving and quantifying chiral metabolites of JWH-018 and AM2201.
- Precision and accuracy measurements are similar to previously developed clinical and forensic assays.
  - Method is still in development. Plans to improve signal-to-noise ratio and accuracy and precision include increasing sample volume and utilizing a higher concentration of organic during SPE.
- The neuronal cytochrome P450s CYP2J2 and CYP2D6 exhibit stereospecificity towards JWH-018 and AM2201 metabolism and preferentially produce specific enantiomeric metabolites of each synthetic cannabinoid.
- Tissues collected for forensic and research purposes support *in vitro* studies and indicate humans produce specific enantiomeric metabolites of JWH-018 and AM2201.
- Toxicological and pharmacological significance of these findings remain to be determined and require further pharmacokinetic and dynamic studies.

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