Development and Validation of a Liquid Chromatography–Tandem Mass Spectrometry Method for the Identification and Quantification of JWH-018, JWH-073, JWH-019, and JWH-250 in Human Whole Blood

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Abstract

A sensitive and specific method for the quantification of JWH-018, JWH-073, and JWH-250 and the qualitative identification of JWH-019 in whole blood was developed and validated. Samples fortified with JWH-018-d_9 and JWH-073-d_9 underwent liquid–liquid extraction and were analyzed by liquid chromatography–positive ion electrospray ionization-tandem mass spectrometry. Two transitions were monitored for all analytes except JWH-250, for which there was only one available transition. JWH-019 did not meet the stringent requirements for quantitative analysis, and thus this method is only appropriate for the qualitative identification of this compound in whole blood. The linear range was 0.1–20 µg/L for all quantitative analytes. The maximum average within- and between-run imprecision was 7.9% and 10.2%, respectively, and all controls quantified within 8.2% of target concentrations. Process efficiency, a measurement that takes into effect extraction efficiency and matrix effect, was ≥ 32.0% for all quantitative analytes; similar results were obtained for the deuterated internal standards. All analytes were stable at room, refrigerated, and frozen temperatures for at least 30 days. The method was used to quantify JWH-018 and JWH-073 in a blood specimen collected from a person known to have used an herbal incense blend containing these substances.

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Introduction

First developed to study the cannabinoid receptor system, various synthetic cannabinoid agonists are now being used recreationally as an alternative to cannabis (1,2). Marketed as "herbal incense" or "legal highs" under a wide variety of names, including Spice, Yucatan Fire, Smoke, Sence, Skunk, Space, K2, K2 Citron, K2 Blonde, K2 Strawberry, K2 Pink, K3, K4, and many others, the chemical agonist compounds are being sprayed onto inert plant substrates and smoked. There are numerous different synthetic cannabinoids with varying degree of selectivity and affinity for cannabinoid CB1 and CB2 receptors and thus having different therapeutic and abuse potentials. Figure 1 includes the chemical names, structures and common abbreviations of the synthetic cannabinoids discussed in this paper.

In late 2008, German researchers evaluated seven herbal incense products and identified three synthetic cannabinoids: JWH-018, and the C-6 and C-7 homologues of CP-47,497, along with the endogenous cannabinoid receptor ligand oleamide (3). In early 2009, the U.S. Customs and Border Patrol reported that it had identified HU-210, a Drug Enforcement Administration (DEA) Schedule 1 drug, in seized herbal incense products from five different shipments into the U.S. (3). Subsequently, Uchiyama et al. (4) analyzed 46 different herbal blends purchased on the illegal drug market in Japan and identified JWH-018, CP-47,497-C-6, and C-7 homologues, oleamide, and JWH-073 in the products. Recently, Dresen et al. (5) described the chemical composition of 140 smokeable herbal products and documented further diversity of active synthetic chemicals.

HU-210 is a DEA schedule 1 drug and the DEA recently used emergency schedule powers to move to control JWH-018, JWH-073 and several other compounds as Schedule 1 drugs (6). Individual states and municipalities have passed legislation banning specific compounds or the general class of drug, in some cases as broadly as cannabinoid agonists, rather than specific chemicals or structural analogues. As more states institute legal restrictions on the sale and use of these substances, analytical methods to detect and quantitate them in a range of plant materials and biological tissues and fluids will be needed. Methods have been published detailing the identification of synthetic cannabinoids in botanical material using gas-chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and LC–high-resolution accurate MS (7,8). Methods for the analysis of the compounds and their metabolites in biological matrices has been limited. Teske et al. (9) reported a method for the identification and quantitation of JWH-018 in human serum by LC–MS–MS, and two other groups have subsequently employed LC–MS–MS to identify metabolites from in vivo and in vitro experiments (10,11).

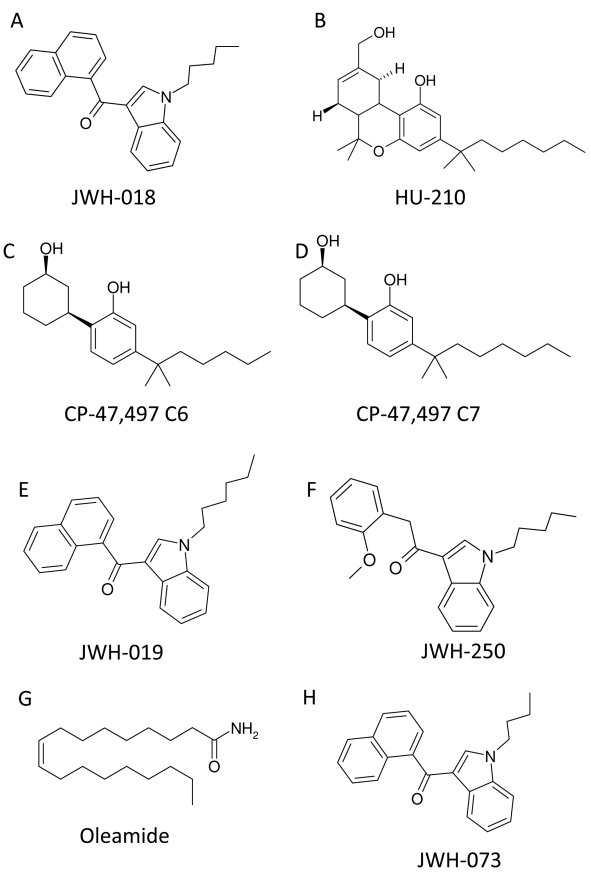

Figure 1. 1. Chemical structure and common name for (1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone (A); 3-(1,1'-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol (B); 2[(1S,3R)-3-hydroxycyclohexyl]-5-(2-methylheptan-2-yl)phenol (C); 2[(1S,3R)-3-hydroxycyclohexyl]-5-(2-methyloctan-2-yl)phenol (D); (1-hexyl-1H-indol-3-yl)-1-naphthalenyl-methanone (E); 1-(1-pentyl-1H-indol-3-yl)-2-(2-methoxyphenyl)-ethanone (F); oleamide (G); and (1-butyl-1H-indol-3-yl)-1-naphthalenyl-methanone (H).

Although current legislation largely focuses on JWH-018 and JWH-073, the rate with which replacement compounds may reach the market emphasizes the need for analytical procedures which include not only currently abused synthetic cannabinoids, but also other analytes with abuse potential. This was demonstrated in Germany where, 4 weeks after JWH-018 was banned, JWH-073 began appearing in herbal incense products (12). Additionally recent trends in the chemical composition of synthetic cannabinoid compounds (5,13), and monitoring of activity in online internet drug user forums, have prompted the development of this LC–MS–MS method for the identification and quantification of JWH-018, JWH-073, JWH-019, and JWH-250 in blood.

Experimental

Chemicals and reagents

JWH-018 was purchased from Toronto Research Chemicals (North York, ON, Canada). JWH-019, JWH-073, JWH-250, and JWH-018-d_9 were obtained from Cayman Chemical (Ann Arbor, MI), and JWH-073-d_9 was purchased from Chiron (Trondheim, Norway). HPLC-grade ethyl acetate, 95–97% formic acid, and 99+% sodium chloride were obtained from Sigma Aldrich (St. Louis, MO). HPLC-grade methanol (MEOH, HPLC grade) and sodium bicarbonate were purchased from Thermo Fisher Scientific (Waltham, MA), and glass-distilled hexane was from EMD Chemicals (Gibbstown, NJ). Ultra-pure water was produced in-house using a Solution 2000 Water Purification system (Aqua Solutions, Jasper, GA). Drug-free whole blood containing EDTA sodium was purchased from Biological Specialty (Colmar, PA).

Instrumentation

LC–MS–MS analyses were performed using a Waters Premiere tandem MS with an electrospray ionization (ESI) source, interfaced with a Waters Acquity ultra-performance autosampler and UPLC pump (Waters, Milford, MA).

Preparation of calibrators, internal standard, and controls

Individual 100 ng/µL stock standards solutions of JWH-018, JWH-019, JWH-073, and JWH-250 were made in methanol. These individual stock standards were used to create mixed stock standard solutions containing 2, 0.2, and 0.01 ng/µL JWH-018, JWH-019, JWH-073, and JWH-250 in methanol. These stock standards were spiked into 200 µL blank whole blood to prepare calibrators at the following concentrations: 0.1, 0.2, 0.5, 2.0, 10, and 20 µg/L. Mixed quality control (QC) standards containing all four analytes were prepared separately in methanol at concentrations of 10 and 1 ng/µL and used to prepare high and low concentration quality control (HQC = 15 µg/L and LQC = 0.3 µg/L) standards in whole blood. The QC standards were stored in snap-cap conical tubes in 300-µL aliquots. A stock JWH-018-d_9 and JWH-073-d_9 internal standard solution (IS) was prepared at 100 ng/µL in methanol and

further diluted with methanol to a working concentration of 0.10 ng/µL. All stock and working standards were stored below –10°C.

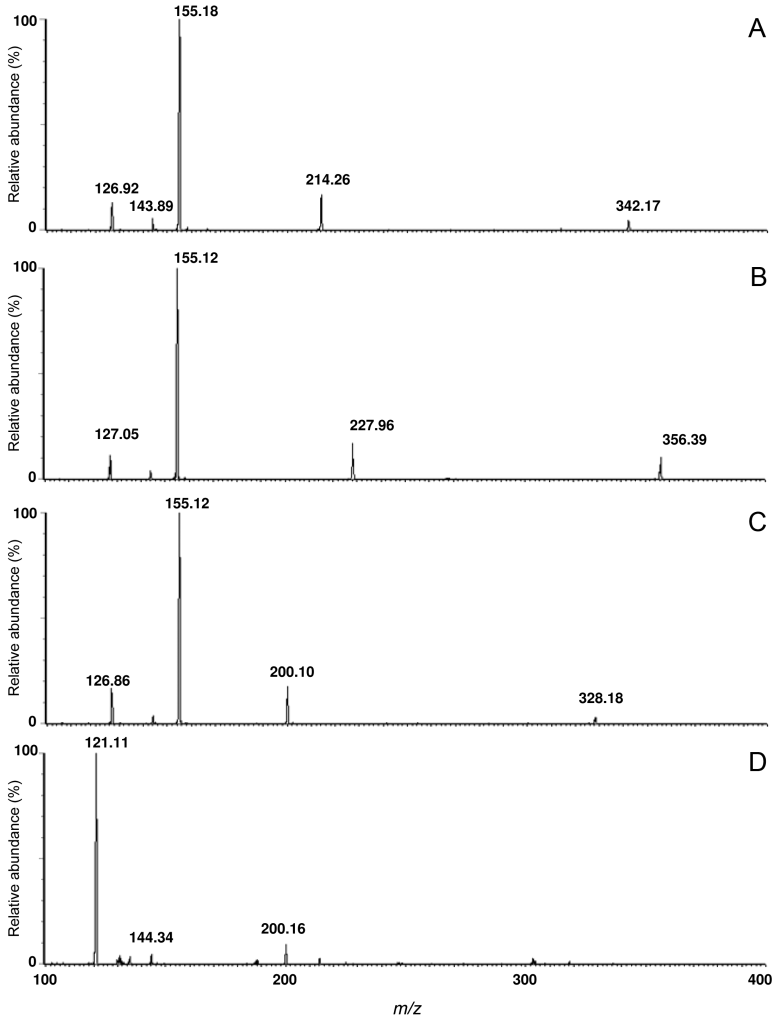

Figure 2. Product ion spectra of the protonated molecular ion of JWH-018 (A); JWH-019 (B); JWH-073 (C); and JWH-250 (D) obtained at a collision energy of 45.

Table I. Retention Times and Monitored Transition for the Analysis of JWH-018-d_9, JWH-018, JWH-019, JWH-073-d_9, JWH-073, and JWH-250 in Whole Blood by LC–MS–MS

Analyte	Retention Time (min)	Quantifier Transition	Qualifier Transition
JWH-018-d_9	3.12	351 → 155	351 → 127
JWH-018	3.13	342 → 155	342 → 127
JWH-019	3.30	356 → 155	356 → 127
JWH-073-d_9	2.92	337 → 155	337 → 127
JWH-073	2.94	328 → 155	328 → 127
JWH-250	2.93	336 → 121	none

Sample preparation

Twenty-five microliters of working IS solution was added to 200-µL aliquots of calibrators, QC, blank blood, and subject samples, along with 200 µL saturated sodium bicarbonate solution and briefly vortex mixed. Two-hundred microliters of saturated sodium chloride was added, and samples were vortex mixed prior to the addition of 3 mL extraction solvent (99% hexane/1% ethyl acetate). Samples were mixed for 20 min on a rotating mixer then centrifuged for 5 min at 3500 rpm. The upper organic layer was transferred to a clean, labeled tube and evaporated to dryness under nitrogen at 40°C. Samples were reconstituted with 200 µL 50% deionized water/50% methanol, transferred to autosampler vials, and 10 µL injected into the LC–MS–MS.

LC–MS–MS procedure

Chromatographic separation was achieved with an Acquity UPLC HSS T3 (100 mm × 2.1 mm, 1.8 µm) column coupled with a VanGuard HSS T3 1.8-micron guard column (Waters). Column temperature was maintained at 45°C, and gradient elution with a flow rate of 500 µL/min was employed. An initial mixture of 40% mobile phase A (1% formic acid in water) and 60% mobile phase B (1% formic acid in methanol) was decreased to 5% mobile phase A over 2.5 min. This mixture was held for 2.35 min before returning to the initial conditions. Total run time was 3.85 min.

Identification of precursor and product ions and MS–MS optimization were established by direct infusion of 10 ng/µL solutions of single analytes in methanol. Mass spectral data were acquired in positive ion mode with the following optimized ESI-MS parameters: source temperature, 120°C; desolvation temperature, 500°C; cone gas flow rate, 35 L/h; desolvation gas flow rate, 800 L/h; capillary voltage, 0.50 kV; and cone voltage, 45.0 V. Figure 2 shows the product ion mass spectrum of the protonated molecular ion of each analyte and internal standard from the infusion experiments with collision energy = 45, and Table I indicates the retention time of each analyte and transitions monitored in multiple reaction monitoring mode. Two transitions were monitored for JWH-018, JWH-019, and JWH-073, and the ion ratios were monitored and required to be within ±30% of the average ratio of the calibrators analyzed in the same analytical batch as the samples. Despite analysis using a variety of collision energies, only one viable transition was identified for JWH-250. Although other transitions could be seen, the response of the transition ion was too low to be reproducible.

LC–MS–MS validation

The method was validated for sensitivity, linearity, within- and between-run imprecision, accuracy specificity, extraction efficiency, matrix effect, process efficiency, extract and sample stability, dilution integrity, carryover, and matrix-matching. Sensitivity was assessed by establishing the limit of detection (LOD) and quantification (LOQ) for each analyte. LOQ was defined as the lowest concentration of the standard curve that could be measured acceptable accuracy and imprecision as defined. For positive identification of each analyte the ratio of the qualifier transition to the quantifier transition was required to be within ±30% of the average the measured ratio from the calibration curve. The LOQ calibrator was used to calculate the LOD. To determine this concentration, the signal-to-noise was measured at the LOQ and used to calculate a concentration at which the signal-to-noise would be 3:1. Linearity was established by plotting concentration against the ratio of analyte response/internal standard response with $1/x$ weighting. The model was deemed acceptable if an un-weighted linear regression of the calculated concentrations versus the target values for three curves yielded an estimated line $y = x$. Acceptable slope ranged from 0.85 to 1.15, and acceptable intercept was –0.2*LOQ – 0.2*LOQ. If the intercept from the regression analysis was not acceptable, further evaluation was performed using EP Evaluator® Release 8 (David G. Rhoads Associates, Kennett Square PA).

Within- and between-run imprecision was determined at the LOQ, LQC, and HQC concentrations. Within-run imprecision was established using five replicates per run, over three days. Acceptable within-run imprecision was achieved if the %CV on each day, and the average over three days, was less than 15%. Between-run imprecision was evaluated by calculating the %CV of the 15 injections from the within-run imprecision experiments and was acceptable if the %CV was better than 15% at each concentration. Accuracy was determined by comparing the average calculated concentration over 15 replicates, to the target concentration. Acceptable accuracy was ±20% for the LOQ and ±15% for the LQC and HQC levels.

Specificity demonstrates that other compounds which may also be in the biological matrix will not interfere with the quantification of the analyte of interest. This is evaluated by fortifying blank matrix with high concentrations of 118 common over-the-counter and abused drugs and their metabolites and ensuring that they do not produce positive results for the analytes being determined. The following known cannabinoid agonists and antagonists were tested and found not to interfere with this assay: JWH-200, HU-210, JWH-211, WIN 55,212, JWH-015, JWH-133, CP55,940, CP-47,497 (n = 7), and CP-47,497 (n = 8). Extraction efficiency, matrix effect, and process efficiency were evaluated using five unique whole blood specimens, each analyzed in singlet, from cases which were negative for the analytes of interest. Extraction efficiency was measured by comparing the response of whole blood fortified with analyte and internal standard before and after extraction. The percent extraction efficiency was expressed as the mean analyte area of samples fortified before extraction divided by the mean area of samples fortified after extraction. Matrix effect was assessed by comparing analyte response in blank extracted whole blood fortified with QC and internal standard after extraction to the same nominal concentrations prepared in mobile phase. Process efficiency examines the overall effect of extraction efficiency and matrix effect on the quantification of analytes. It was measured by comparing the response of whole blood fortified before extraction to the response of neat samples prepared in mobile phase at the same concentration.

Analyte stability is a function of storage conditions, the chemical properties of the analyte, the matrix and the storage container. Stability was evaluated for short- and long-term storage under a variety of conditions including room temperature, refrigerated, frozen, and repeated freeze-thaw cycles. In addition the stability during sample preparation and of prepared samples on the autosampler was examined. Stability was evaluated using whole blood containing EDTA fortified at LQC and HQC concentrations and stored at ambient room temperature, 3°C, and –10°C. Duplicate LQC and HQC samples were stored and tested on day 1, day 2, day 7, day 14, and day 30. Stability during sample preparation was examined, and autosampler stability was assessed by maintaining prepared samples at room temperature and re-injecting them 27 h after initial analysis. The concentration was calculated using the original calibration curve and a newly extracted calibration curve. For all stability experiments, analytes were considered stable if the calculated concentrations were within ±20% of the mean concentration determined during the between-run imprecision experiments.

Dilution integrity ensures that dilution of a specimen with a concentration higher than the highest calibrator will result in an accurate quantification. Five HQC samples were diluted 10-fold and analyzed. Dilution of specimens for analysis was considered to be acceptable if the average calculated concentration of was within ±20% of the mean concentration determined during the between-run imprecision experiments. Carryover was tested by injecting a blank sample following the highest calibrator on each of three runs and was considered negligible if the calculated concentration of the analyte was < 25% of the lowest calibrator.

Matrix matching experiments were designed to assess whether the quantification of analytes would be affected by the type of tube used to collect the blood specimen, when quantified against calibrators prepared in citrate preserved whole blood. Matrix matching was performed in whole blood containing sodium fluoride, potassium oxalate, sodium EDTA, potassium EDTA, sodium heparin, and lithium heparin to mimic the most commonly encountered collection tubes used by clinicians and medical examiners. Each tube type was tested in duplicate using two or three different blood donors. A blank whole blood from the collection tube was analyzed to ensure that the tube additive did not interfere with the analyte of interest, and aliquots of each matrix type were fortified with 0.5 and 10 µg/L of each analyte of interest. Matrix matching was considered successful if the calculated concentration was within ±20% of the target value.

Proof of method

The validated method was employed to analyze blood specimens obtained from an individual who smoked an herbal in-

cense product containing JWH-018 and JWH-073 during an Intramural Research Board approved research study. The material was tested prior to use and was found to contain both JWH-018 and JWH-073 at concentrations of 17 and 22 mg/g, respectively. One-third of a gram was placed in a pipe, but not all of the material was smoked. Blood specimens were collected in gray-topped collection tubes and stored at –20°C until analysis.

Identification of metabolites

Blood specimens collected from a known JWH-018 and JWH-073 user were analyzed to determine the major metabolites of these compounds. Samples were hydrolyzed and underwent a simple liquid–liquid extraction prior to analysis by LC–MS–MS. For these experiments analysis was performed on a Shimadzu UFLC 20AD LC (Shimadzu, Columbia, MD) and Applied Biosystems API5000 MS (Life Technologies, Carlsbad, CA).

Results and Discussion

Validation

This is the first validated LC–MS–MS method for the identification and quantification of JWH-018, JWH-019, JWH-073, and JWH-250 in human whole blood. JWH-018-d_9 was used as the internal standard for JWH-018 and JWH-250; JWH-073-d_9 was used for JWH-073. JWH-019 was examined using both deuterated internal standards; validation parameters were not acceptable with either internal standard. The method was deemed acceptable for the qualitative identification of JWH-019 but not for quantitative analysis. Table II summarizes the calibration information for JWH-018, JWH-073, and JWH-250. The slopes were acceptable in all cases, and the linear regression intercept was acceptable for JWH-073 and JWH-018. JWH-250 had to be further evaluated using EP Evaluator. Each calibrator was analyzed in triplicate, and EP Evaluator was employed to calculate the best-fit line using a proprietary Clinical Linearity algorithm. The maximum allowable systematic error (SEA) for which the data would be linear was calculated, and the linearity of the assay was acceptable if this calculated error was less than the laboratory-defined SEA for each analyte. For JWH-018, the total allowable error was set to 20% with 20% of this error allotted to systematic error, resulting in an SEA of 4.0%. The total allowable error and allotted systematic error for JWH-250 were higher, 25% each with an SEA of 6.25%, because there was not a deuterated IS for this analyte. The maximum SEA was 3.7% for JWH-018 and 5.4% for JWH-250; therefore, the calibration for both analytes was linear.

Table II. Method Calibration Verification for JWH-018, JWH-073, and JWH-250 in Whole Blood by LC–MS–MS

Analyte	Internal Standard	Slope	Intercept
JWH-018	JWH-018-d_9	0.995	0.035
JWH-073	JWH-073-d_9	1.000	0.010
JWH-250	JWH-073-d_9	0.974	0.151

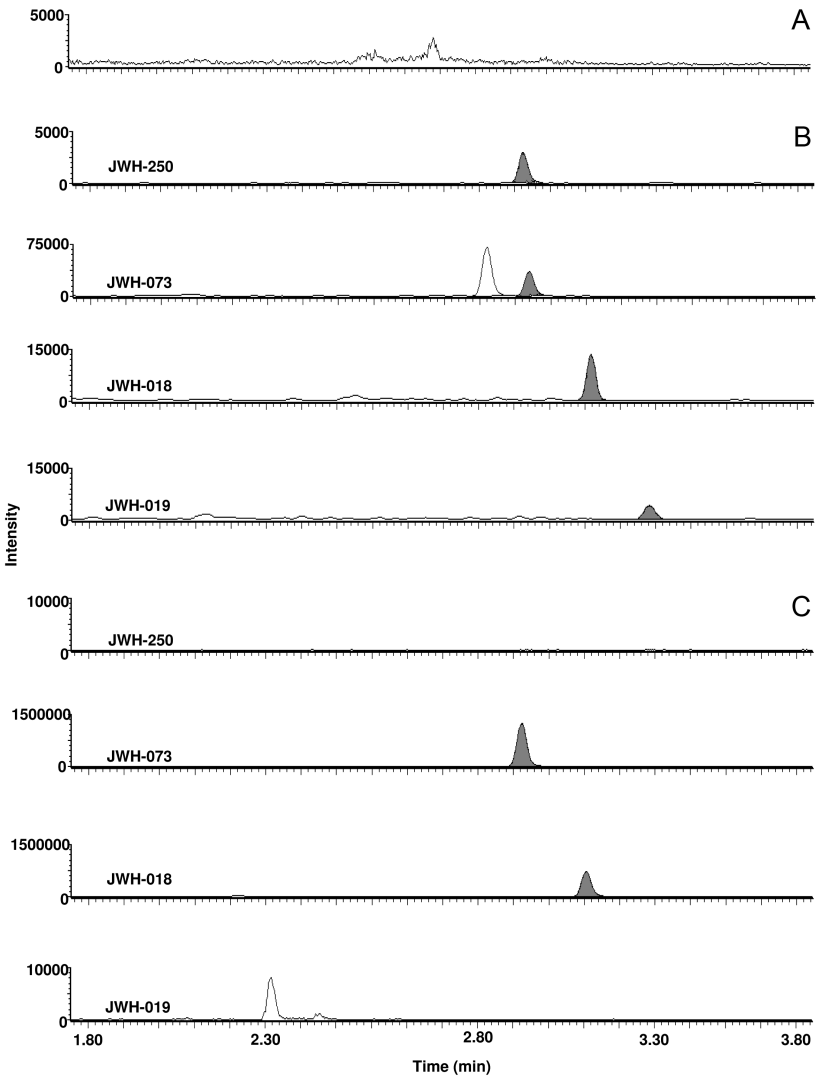

Figure 3. Total ion chromatogram of extracted blank whole blood (A). Multiple reaction monitoring chromatograms of the quantitative ion transitions for whole blood fortified with 0.1 ng/mL JWH-0250, JWH-073, JWH-018 and JWH-019 (B). Representative blood specimen from an individual who smoked an herbal incense product containing JWH-018 and JWH-073 (C).

Table III. Within-Run Precision, Between-Run Imprecision, Accuracy, Extraction Efficiency, Matrix Effect, and Process Efficiency for JWH-018, JWH-019, JWH-073, and JWH-250 in Whole Blood by LC–MS–MS

Validation Parameter	Concentration (µg/L)	JWH-018	JWH-019	JWH-073	JWH-250
Average within-run precision (%RSD)	0.1	4.3	19.3*	7.9	7.0
	0.3	5.4	11.6	4.3	1.9
	15	2.6	9.5	4.5	3.6
Between-run imprecision	0.1	7.9	35.7*	10.2	8.6
	0.3	5.9	20.6*	4.3	4.7
	15	3.2	23.0*	2.1	6.9
Accuracy (%)	0.1	99.2	112.0	99.1	104.7
	0.3	93.7	70.3*	92.2	93.6
	15	93.7	67.3*	93.0	91.8
Extraction efficiency (%)	0.3	75.5	63.6	80.8	92.8
	15	63.2	60.6	71.0	79.4
Matrix effect (%)	0.3	36.7	42.1	31.1	22.0
	15	49.4	64.7	32.2	18.6
Process efficiency	0.3	47.7	36.8	55.6	72.4
	15	32.0	21.4	48.1	64.6

* Outside method validation acceptance criteria.

Table IV. Matrix Matching for JWH-018, JWH-073, and JWH-250 in Whole Blood with a Variety of Additives by LC–MS–MS Reported as Average % of Target Concentration

Whole Blood Additive	Concentration (µg/L)	JWH-018	JWH-073	JWH-250
Sodium fluoride (n = 2)	0.5	103.1	111.8	88.5
	10	98.7	106.0	86.6
Potassium oxalate (n = 2)	0.5	96.0	102.3	87.5
	10	94.6	107.0	87.6
Sodium EDTA (n = 3)	0.5	105.3	113.5	88.5
	10	98.1	107.7	87.5
Potassium EDTA (n = 2)	0.5	109.6	104.0	94.0
	10	93.2	103.5	87.4
Sodium heparin (n = 3)	0.5	99.9	102.9	88.2
	10	92.0	97.4	84.3
Lithium heparin (n = 2)	0.5	103.1	98.4	94.5
	10	91.5	96.7	82.8

Table V. Whole Blood Concentrations from a Patient Following Smoking of an Herbal Incense Blend Known to Contain JWH-018 and JWH-073

ΔT (min)	JWH-018 (µg/L)	JWH-073 (µg/L)
19	4.8	4.2
53	1.5	1.0
107	0.6	0.3
199	0.2	0.2

The calculated LODs were 0.006, 0.009, 0.010, and 0.016 µg/L, respectively, for JWH-018, JWH-073, JWH-019, and JWH-250, and the linear range 0.1 (LOQ)–20 µg/L for all quantitative analytes. Representative MRM chromatograms for blank whole blood and whole blood fortified with the analytes at the LOQ are presented in Figures 3A and 3B.

Within-run imprecision, between-run imprecision, recovery, extraction efficiency, matrix effect, and process efficiency were evaluated across the analytical range; results can be found in Table III. The maximum relative standard deviations (%RSD) within-run were 7.6% at 0.3 µg/L, 9.8% at 0.1 µg/L, 27.8% at 0.1 µg/L, and 11.5% at 0.1 µg/L for JWH-018, JWH-019, JWH-073, and JWH-250, respectively. Accuracy was ≥ 91.8% for all analytes except JWH-019 at all concentrations. Extraction efficiency ranged from 60.6% to 92.8%, and process efficiency was 21.4–72.4%. Matrix suppression of was noted for all analytes. JWH-018 signal was suppressed 36.7% and 49.9% at low and high concentrations, respectively; however, JWH-018-d_9 underwent a similar degree of suppression, 37.4% at low and 46.1% at high concentrations, so there was no effect on quantitative results. Similar results were observed for JWH-073 and its deuterated internal standard, both of which were suppressed by approximately 30%. Results of extraction efficiency, matrix suppression, and process efficiency experiments help explain why JWH-250 could be quantified using JWH-073-d_9 but no success was seen using either internal standard to quantify JWH-019. JWH-250 co-eluted with JWH-073-d_9 and had a similar process efficiencies. JWH-019 did not co-elute with and had higher matrix suppression and lower overall process efficiencies than either of the internal standards. Specificity was evaluated using 118 common abused and therapeutic drugs, including 9 synthetic cannabinoids not included in the assay. No positive results were produced. Stability was evaluated using LQC and HQC samples stored at ambient room temperature, refrigerated (~3°C) and frozen (~ –10°C). All analytes were stable for a minimum of 30 days under all conditions. Preparative step stability identified a step during sample preparation where a break might occur and confirmed that allowing a 1-h break after addition of the first three reagents but prior to adding the extraction solvent does not affect the quantification of the analytes. After three freeze-thaw cycles, all analytes quantified

within ±17.8% of their target value, indicating that the analytes are stable under these conditions and extracted samples reinjected after storage on the autosampler for 27 h quantified within ±13.8% using the original calibration curve and within ±18.8% when calculated against a freshly prepared curve.

Dilution integrity was confirmed by comparing the average of 5 HQC samples diluted 10-fold to the target concentration calculated by dividing the average concentration determined during imprecision experiments by 10. The average diluted concentrations were within ±3.3, 5.3, and 7.4% of the target concentration for JWH-018, JWH-073, and JWH-250, respectively. In addition, each individual diluted sample quantified within ±15.5% of target. Carryover at the highest calibrator concentration was determined for each analyte. Triplicate analysis by injecting blank matrix fortified with internal standard indicated that the maximum calculated analyte concentrations in the blank sample were ±16.0% of the LOQ for JWH-018, ±18.0% of the LOQ for JWH-073 and ±0.0% of the LOQ for JWH-250, indicating that carrying is negligible for all analytes.

The results of matrix matching experiments are summarized in Table IV. All analytes quantitated within 82.8–109.6% of target, indicating that the whole blood collection container did not impact the quantitation of analytes of interest. In addition, each tube was filled using blood from a different donor so these results support the fact that despite the potential for significant matrix suppression the method can accurately quantitative JWH-018, JWH-073, and JWH-250 in whole blood.

Extensive method validation experiments confirmed that this method is sensitive and specific for the quantitative analysis of JWH-018, JWH-073, and JWH-250. JWH-019 did not routinely meet the rigorous requirements for quantitative analysis, and thus this method is only suitable for qualitative identification of this substance.

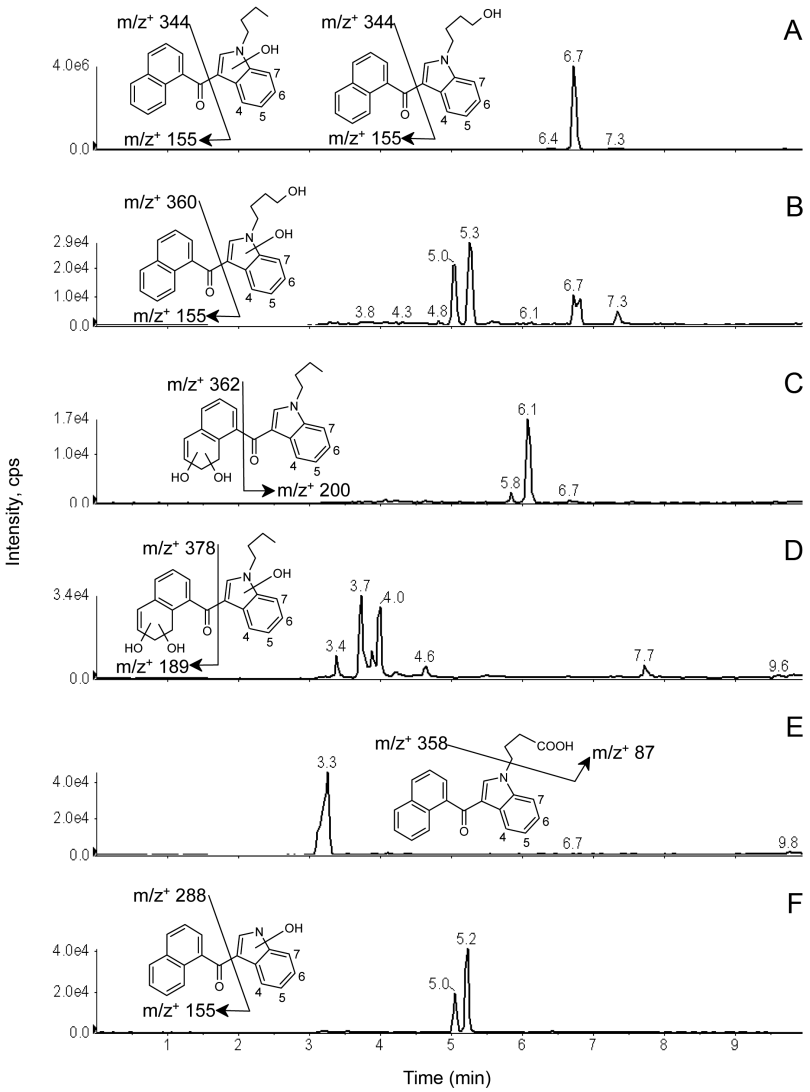

Figure 4. Representative extracted ion chromatograms of diagnostic transitions of proposed JWH-073 metabolites in blood: mono-hydroxy-JWH-073 (A); di-hydroxy-JWH-073 (B); reduced di-hydroxy JWH-073 (C); tri-hydroxy JWH-073 (D); *N*-dealkyl-JWH-073 (E); and carboxy-JWH-073 (F).

Proof of method

The validated method was used to analyze blood specimens collected from an individual who had smoked an herbal incense blend known to contain JWH-018 and JWH-073. Blood was collected at 19, 53, 107, and 199 min after dosing. Figure 3c is a representative MRM chromatograms from the blood specimen collected 19 min after dose administration, and Table V summarizes the results from this patient.

Identification of metabolites

Figure 4 depicts the MRM chromatograms of potential JWH-073 metabolites as detected in a blood specimen collected 1 h post drug administration. Based on the monitored diagnostic transitions preliminary identification of the metabolites included mono-hydroxy, dihydroxy, and tri-hydroxy compounds along with mono-hydroxy-N-dealkylated and carboxy metabolites. The precursor ion of mono-hydroxy JWH-073 has an *m/z* of 344. Monitoring the transition 344 → 155 (Figure 4A) reveals that the hydroxylation must occur on the indole side of the molecule. Attempts were made to elucidate the location of hydroxylation of the mono-hydroxy metabolite by comparing the mass spectrum from the patient specimen to spectra from reference standards obtained from Cayman Chemicals. No signification concentrations of compounds 4, 5, 6, or 7 were detected. Based on this information and the fragmentation pattern it appears as if one of the major

mono-hydroxy metabolites involves hydroxylation on the carbon adjacent to the nitrogen on the five member ring. Hydroxylation of the side-chain also occurs as verified by comparison to a reference standard. As can be seen in Figures 4B, 4C, and 4D, there are multiple possibilities for the di-hydroxylation and tri-hydroxylation of JWH-073. At this time, the exact location of the hydroxyl groups has not been identified. *N*-Dealkyl-mono-hydroxy JWH-073 and carboxy-JWH-073 are depicted in Figures 4E and 4F, respectively, along with their diagnostic transitions.

Conclusions

The reported analytical method provides a sensitive and specific method for the quantification of JWH-018, JWH-073, and JWH-073. Because of significant matrix effects and the lack of an appropriate labeled internal standard, the quantification of JWH-019 was not possible; however, it can be reported qualitatively. In the fast-changing world of synthetic cannabinoids, it is essential to be able to develop sensitive and specific methods for compounds currently being abused and those which may be abused in the future.

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