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Quantification of AB-CHMINACA Metabolites in Urine by Validated Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry Method

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ABSTRACT

Background: Synthetic cannabinoids (), chemically classified as psychoactive substances, target the endocannabinoid system by binding to CB1 and CB2 receptors. Unlike Δ^9-tetrahydrocannabinol (Δ^9-THC), they act as full agonists at these receptors and generally exhibit significantly greater potency and efficacy. Despite being marketed as "Herbal blends," SCs are associated with severe intoxications and fatalities. Due to rapid metabolic transformation, the parent SCs are not detectable in urine samples, so analytical methods must rely on the identification and quantification of their metabolites.

Objectives: The objective of this study was to develop a targeted UHPLC-MS/MS method for the quantitative analysis of AB-CHMINACA's metabolites in urine.

Methods: Urine samples were hydrolyzed with β-glucuronidase and extracted through liquid-liquid extraction using ethyl acetate-isopropanol ammonium hydroxide. UHPLC analysis–MS/MS in positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM) detection was performed on a C18 column.

Results: The method demonstrated good linearity (2-100 ng/mL, r^2 >0.995), high recovery (~92%), minimal matrix effects, and acceptable accuracy and precision (≤11.8% RSD). LOD and LLOQ were 1 and 2 ng/mL, respectively, with no carryover and sufficient stability.

Conclusions: The developed UHPLC-MS/MS method provides a sensitive, simple, and rapid approach for the reliable identification of AB-CHMINACA metabolites in urine, demonstrating its suitability for forensic and clinical toxicology applications.

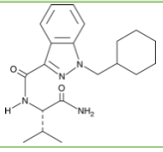
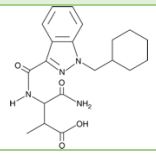
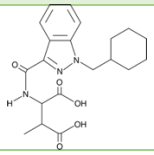
Keywords: AB-CHMINACA; metabolites; synthetic cannabinoids; ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS); validation.

BACKGROUND

Synthetic cannabinoids (SCs) are a diverse group of recently developed psychoactive compounds that act as full agonists at both central and peripheral cannabinoid CB1 and CB2 receptors, with higher affinity for these receptors than the partial agonist tetrahydrocannabinol.[1-3] Their metabolites also retain varying degrees of biological activity, functioning as agonists, inverse agonists, or neutral agonists, which makes the toxic effects caused by SCs consumption more complex and difficult to manage.[4-7] In addition to CB1 and CB2 receptors, synthetic cannabinoids interact with non-cannabinoid targets.[7,8] Their pharmacological effects may be mediated through vanilloid receptors (TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, TRPM8) and G protein-coupled (metabotropic) receptors (GPR55, GPR3, GPR6, GPR12, GPR18, and GPR19).[1,9,10] These receptors are widely expressed in the central nervous system and peripheral tissues, modulating multiple intracellular signaling pathways independently of CB1 and CB2 receptors, which also explains the diverse toxicity profile of synthetic cannabinoids.[1]

Indazole-3-carboxamides represent one of the most prevalent and pharmacologically potent structural subclasses of synthetic cannabinoids,[11] characterized by an indazole core bearing a carboxamide linkage at the 3-position, an amide linked side chain, and an N-1 alkyl or cycloalkyl substituent.[11,12] Among them, AB-CHMINACA has emerged as a prominent compound due to its high CB1 receptor full agonist activity, partial CB2 agonism, and frequent detection in forensic casework, leading to its classification as a Schedule I substance by the Drug Enforcement Administration (DEA).[13,14] The chemical structures and properties of AB-CHMINACA and its metabolites are summarized in Table 1.

TABLE 1. Chemical structures and properties of AB-CHMINACA and the metabolites M6 and M7

	AB-CHMINACA	AB-CHMINACA metabolite M6	AB-CHMINACA metabolite M7
IUPAC name	N-[(1S)-1-(aminocarbonyl) -2-methylpropyl]- 1-(cyclohexyl methyl)-1H-indazole-3-carboxamide	4-amino-3-[[[1- (cyclohexylmethyl)-1H-indazol-3-yl]carbonyl]amino]-2-methyl-4-oxo-butanoic acid	2-(1 – (cyclohexylmethyl)-1H-indazole-3-carboxamindo)-3-methylsuccinic acid
Molecular formula	$C_{20}H_{28}N_4O_2$	$C_{20}H_{26}N_4O_4$	$C_{20}H_{25}N_3O_5$
Molecular weight (g/mol)	356.5	386.5	387.4
Structure			

Structurally derived from the AMB-CHMINACA scaffold through substitution of valinamide for the valine methyl ester,[11] AB-CHMINACA retains the high lipophilicity typical of SCs, predisposing it to extensive biotransformation.[15] Notably, indazole-containing SCs, such as AB-CHMINACA, are more susceptible to terminal hydrolysis than indole analogues, highlighting the importance of structural features in determining metabolic pathways.[7] Its high lipophilicity leads to extensive hepatic biotransformation, meaning the parent compound is rarely detectable in urine.[3,11]

Therefore, developing robust analytical methods for specific metabolites, such as M6 and M7, is critical for confirming exposure in legal and medical settings.

METHODS

Chemicals and reagents

All organic solvents, including Ethyl acetate, Isopropanol, Ammonium hydroxide, Methanol, Acetonitrile, and formic acid, were HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA). Synthetic cannabinoid AB-CHMINACA metabolites M6 (Batch No. 0463379) and M7 standard (Batch No. 0463555) were purchased from Cayman Chemical. β-Glucuronidase (90,000 units/mL, Lot No. SLBS5776) was purchased from Sigma-Aldrich.

Standards and spiked urine samples

Stock and working methanolic solutions were stored at −20°C. Selected SC metabolites were spiked into drug-free urine samples to achieve the following concentrations: 0.5, 1, 2, 10, 20, 50, 80, and 100 ng/mL.

Preparation of standards and controls

A stock standard of the AB-CHMINACA metabolites was prepared at a target concentration of 1000 ng/mL in methanol (MeOH). Working solutions of the synthetic cannabinoid metabolites were prepared by serial dilution with MeOH at concentrations of 100, 80, 50, 20, 10, 2, 1, and 0.5 ng/mL.

Sample preparation and extraction

Calibrators and QC samples were prepared by spiking blank urine with AB-CHMINACA metabolites M6 and M7. Urine samples were adjusted to pH 5.0 using 0.1 M acetate buffer and hydrolyzed at 37°C with 50 µL of β-glucuronidase (Helix pomatia). Samples (1.0 mL) were extracted through liquid-liquid extraction with ethyl acetate-isopropanol-ammonium hydroxide (85:13:2, v/v). After vortexing and centrifugation at 5000 rpm for 5 minutes, the organic phase was collected, evaporated under nitrogen at 40°C, and reconstituted in 100 µL of 0.1% formic acid in acetonitrile/0.1% formic acid in water (1:4, v/v). The extracts were transferred to autosampler vials and stored at 25°C before LC–MS/MS analysis.

Instrumental analysis for the determination of AB-CHMINACA metabolites in urine

The analysis was conducted using the Agilent 1290 Infinity Ultra-High-Performance Liquid Chromatograph (UHPLC) coupled with an Agilent 6460 Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. The LC system included a quaternary pump, a thermostatted column compartment, and an autosampler. Analytical conditions are summarized in Table 2.

TABLE 2. Optimized UHPLC-MS/MS instrumental parameters

	UHPLC-MS/MS Instrumental Parameters	
UHPLC conditions	LC system	Agilent Infinity1290 liquid chromatograph
	Column	Zorbax RRHD Eclipse Plus C18 (100 × 3.0 mm, 3.8 µm)
	Mobile phase A 80%	0.1% formic acid in water
	Mobile phase B 20%	0.1% formic acid in acetonitrile
	Elution	Gradient elution
	Flow rate	0.8 mL/min
	Injection volume	5.00 µL
MS conditions	Mass spectrometer	Agilent 6460 triple quadrupole MS
	Ionization source	Electrospray ionization (ESI), positive mode
	Acquisition mode	Multiple reaction monitoring (MRM)
	Gas Temp	300°C
	Gas Flow	7.0 l/min
	Capillary	3848 V
	Sheath Gas Flow	6.5 l/min
	Sheath Gas Temp	300°C
	Nebulizer	40.0 PSI
	Collision energy	Optimized for each MRM transition

Abbreviations: ESI, electrospray Ionization; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; PSI, pounds per square inch; UHPLC-MS/MS, ultra-high-performance liquid chromatography-tandem mass spectrometry.

Separation was performed by elution on Zorbax Eclipse plus C18 (100x3.0 mm, 3.8 µm) column, with column temperature of 30°C. The mobile phases consisted of 0.1% aqueous formic acid (A) and 0.1% acetonitrile-formic acid (B) in a 20:80 (v/v) ratio. Flow rate was 0.8 mL/min, and the total run time was 5 min.

The mass spectrometer was operated in ESI+ mode with an ion source temperature of 325°C and a capillary voltage of 550 V. Quantitative analysis was performed in MRM mode. Metabolites M6 and M7 were identified by comparison of retention times with authentic reference standards and by monitoring optimized precursor-to-product ion transitions: m/z 387.4→370.5 (CE 5 eV), m/z 387.4→241.3 (CE 16 eV), m/z and 387.4→145.0 (CE 30 eV) for M6, and m/z 388.3 → 370.4 (CE 7 eV), m/z 388.3 → 97.3 (CE 26 eV), and m/z 388.3 →145.1 (CE 32 eV) for M7. Representative MRM chromatograms of urine samples spiked with M6 and M7 at a concentration of 50 ng/mL are shown in Figures 1 and 2.

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FIGURE 1. Multiple reaction monitoring chromatogram of urine spiked with AB-CHMINACA M6 at the concentration of 50 ng/mL

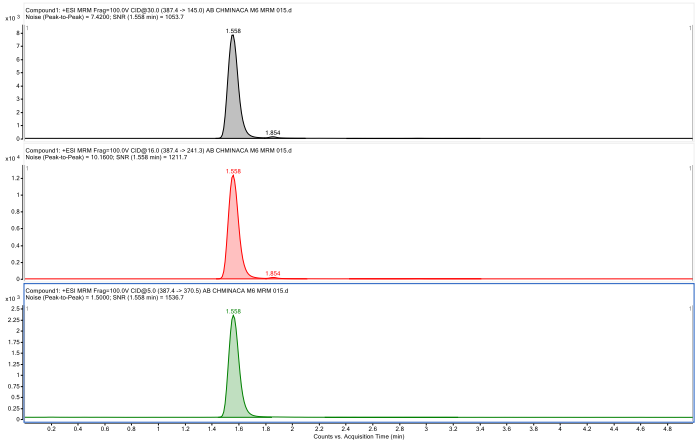
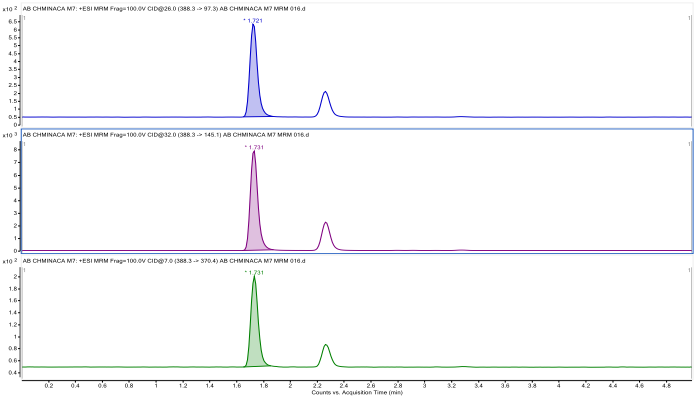


FIGURE 2. Multiple reaction monitoring chromatogram of urine spiked with AB-CHMINACA M7 at the concentration of 50 ng/mL



Method validation

The method was validated for linearity, limit of detection (LOD), lower limit of quantification (LLOQ), accuracy, precision, extraction efficiency, matrix effect, carryover, and stability.

Linearity was assessed using six concentration calibrators (2, 10, 20, 50, 80, and 100 ng/mL) for each metabolite. Calibration curves were created through linear least-squares regression analysis. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were determined based on the validated calibration range. Calibrators were considered acceptable when measured concentrations fell within ±15% of their nominal values (±20% at the LLOQ).

LOD and LLOQ were determined by injecting fortified samples at decreasing analyte concentrations. The LOD was defined as the concentration producing a signal-to-noise (S/N) ratio ≥3, while the LLOQ corresponded to a signal-to-noise ratio (S/N) ≥10 with accuracy within ±20% and precision ≤20% CV.

Accuracy and precision were evaluated at four concentrations (2, 10, 40, and 80 ng/mL). Intra-day accuracy and precision were measured using six replicates in a single run, while inter-day performance was assessed over six days with four replicates per concentration. Results were considered acceptable when accuracy was within ±15% of the nominal values, and the %CV for precision did not exceed 15% (20% at LLOQ).

Extraction efficiency and matrix effect were evaluated using six blank urine sources. Three sets of samples (pre-extraction spiked, post-extraction spiked, and neat standards) were prepared at low, medium, and high concentrations.

Carryover was evaluated by injecting a blank immediately after the ULOQ sample (100 ng/mL). Carryover was considered acceptable when analyte responses in blanks did not exceed 20% of the LLOQ response and internal standard responses remained below 5% of the mean calibrator response.

Stability was assessed at room temperature for 24 h, at 4-10°C for 72 h, after three freeze-thaw cycles, and during long-term storage at -20°C for one month at both LLOQ and ULOQ levels.

RESULTS

Linearity for both metabolites (M6 and M7) was established over the concentration range of 2–100 ng/mL, with correlation coefficients (r^2) greater than 0.995. All calibrators met the preset acceptance criteria, with measured concentrations within ±15% of the nominal values (±20% at the LLOQ). The limit of detection (LOD) was 1.0 ng/mL, based on a signal-to-noise ratio of ≥3. The lower limit of quantification (LLOQ) was set at 2.0 ng/mL, satisfying the predefined accuracy and precision criteria.

For M6, intra-day accuracy ranged from 89.3% to 110.2%, while inter-day accuracy ranged from 93.2% to 108.7%. Intra-day and inter-day precision (RSD) ranged from 4.3% to 10.9% and 3.4% to 10.3%, respectively. For M7, intra-day accuracy ranged from 90.1% to 109.3%, and inter-day accuracy ranged from 93.8% to 109.5%, with intra-day and inter-day precision ranging from 4.5% to 11.8% and 3.7% to 10.5%, respectively.

Extraction efficiency was 92.4% for M6 and 91.8% for M7. Matrix effects were minimal, corresponding to 97.3% (2.7% ion suppression) for M6 and 95.8% (4.2% ion suppression) for M7. The overall process efficiency ranged from 82% to 93% for M6 and from 83% to 94% for M7, indicating efficient analyte recovery with minimal matrix interference.

For both metabolites, no significant carryover was observed. The analyte results from the subsequent blank injections were 20% of the LLOQ (2 ng/mL), and the internal standard responses remained below 5% of the mean calibrator response. Similar results were obtained with extracted blank urine samples, indicating no matrix-related carryover.

Both metabolites demonstrated adequate stability in urine under all tested conditions. Measured concentrations remained within 85-115% of nominal values after 24 h at room temperature (M6: 91.2-112.8%; M7: 91.4-110.8%), 72 h at 4 10°C (M6: 92.6-111.7%; M7: 92.8-111.5%), three freeze-thaw cycles (M6: 89.5-111.9%; M7: 90.1-111.8%), and one month of storage at -200C (M6: 92.4-113.2%; M7: 92.5-113.1%; n=6). Method validation results are summarized in Table 3.

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TABLE 3. Summary of Method Validation Results for AB-CHMINACA metabolites M6 and M7

Validation parameter	M6 Metabolite	M7 Metabolite	Acceptance Criteria
Linearity range, ng/mL	2-100	2-100	-
Correlation coefficient r^2	>0.995	>0.995	≥0.99
LOD, ng/mL	1.0	1.0	S/N≥3
LLOQ, ng/mL	2.0	2.0	Accuracy & precision±20%
Intra-day accuracy, %	89.3-110.2	90.1-109.3	85-115
Intra-Day Precision, RSD, %	4.3-10.9	4.5-11.8	≤15
Inter-Day Accuracy, %	93.2-108.7	93.8-109.5	85-115
Inter-Day Precision, RSD, %	3.4-10.3	3.7-10.5	≤15
Extraction efficiency, %	92.4	91.8	Consistent
Matrix effect, %	97.3 (ion suppression 2.7 %)	95.8 (ion suppression 4.2 %)	Within±15
Process efficiency, %	82-93	83-94	80-120
Carryover, %	Not observed	Not observed	<20
Stability (24 h), %	91.2-112.8	91.4-110.8	±15% of the nominal value (85%-115% of initial concentration)
Stability (4–10°C 72 h), %	92.6-111.7	92.8-111.5	
Stability (freeze-thaw cycle), %	89.5-111.9	90.1-111.8	
Stability (-20°C, one month), %	92.4-113.2	92.5-113.1	

Abbreviations: LLOQ, lower limit of quantification; LOD, limit of detection; RSD, relative standard deviation.

DISCUSSION

Indazole-3-carboxamides represent one of the most prevalent and potent subclasses of synthetic cannabinoids, with AB CHMINACA frequently encountered in forensic casework.[3,7,15] Due to its strong CB1 receptor agonism and extensive metabolism, detection of urinary metabolites is essential for confirming exposure.[8,9]

In this study, the metabolites M6 and M7 were successfully identified and quantified using enzymatic hydrolysis, liquid-liquid extraction, and UHPLC–MS/MS analysis in MRM mode. The use of multiple precursor-to-product ion transitions ensured high analytical specificity and reliable confirmation. Rapid chromatographic separation combined with efficient extraction demonstrates the suitability of this method for routine forensic toxicology analysis of AB-CHMINACA exposure.

The dynamic nature of the new psychoactive substances market requires continuous adaptation of toxicological screening techniques.[7,15] The validated UHPLC–MS/MS approach addresses the challenge posed by the rapid metabolism of AB-CHMINACA, in which the parent compound is often undetectable in urine. Targeting metabolites M6 and M7 is consistent with the metabolic pathway of indazole carboxamides, which undergo terminal hydrolysis and produce abundant urinary carboxylic acid metabolites.[3,11]

The method showed adequate analytical sensitivity (LLOQ 2 ng/mL), high recovery, and minimal matrix effects, supporting reliable detection in urine samples. Stability studies confirmed that both metabolites remained stable under common laboratory storage conditions, further supporting the method's applicability in routine forensic laboratories.

CONCLUSIONS

Overall, the developed method provides accurate and reliable detection and quantification of AB-CHMINACA metabolites (M6 and M7) in human urine and represents a practical analytical approach for forensic and clinical toxicology investigations. The method exhibited robust analytical performance, including high selectivity, low detection limits, and reproducible quantification, supporting its use for both routine screening and confirmatory analysis in suspected cases of synthetic cannabinoid exposure.

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