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ORIGINAL ARTICLE

Development and Validation of an Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry Method for the Determination of MAM-2201 Metabolites in Human Urine

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ABSTRACT

Background. Although synthetic cannabinoids (SCs) are controlled and banned in many countries, they remain prevalent and continue to pose a serious public health problem due to severe intoxications and fatal cases. Their analytical detection is challenging because SCs undergo extensive metabolism, and urine rarely contains parent compounds. Therefore, forensic laboratories need analytical methods to identify and quantify synthetic cannabinoid metabolites.
Objectives. This study aimed to develop and validate a targeted UHPLC-MS/MS method to determine MAM-2201 metabolites in human urine.
Methods. Urine samples were hydrolyzed with β-glucuronidase and extracted via solid-phase extraction using Bond Elut Plexa PCX cartridges. Analysis was performed using a UHPLC–MS/MS system in positive electrospray ionization (ESI) mode with MRM detection on a C18 column.
Results. The method demonstrated good linearity (0.5-45 ng/mL, 0.75-45 ng/mL; $r^2 \geq 0.996$), minimal matrix effects, high recovery, and acceptable accuracy and precision (<15% RSD). LODs were 0.25 and 0.5 ng/mL, and LLOQs were 0.5 and 0.75 ng/mL, respectively. No carryover was observed, and analytes were stable under the tested conditions.
Conclusions. The developed UHPLC-MS/MS method provides a sensitive, simple, and rapid approach for reliable determination of MAM-2201 metabolites in human urine. It is suitable for forensic and toxicological applications.

Keywords. MAM-2201 N-(4-hydroxypentyl); MAM-2201 N-pentanoic acid; Synthetic cannabinoids; Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry (UHPLC-MS/MS).

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BACKGROUND

Since the late 2000s, many countries, including European Union member countries, the United States, and Georgia, have actively implemented legislative measures to control and ban synthetic cannabinoids in order to reduce their consumption and associated intoxications and fatal cases.[1-4] Despite legislative control measures, synthetic cannabinoids remain popular among young users and continue to pose a serious public health concern due to their severe toxic effects.[5-8] From an analytical perspective, detecting them in biological samples remains challenging because synthetic cannabinoids undergo extensive metabolism, and researchers rarely detect the parent compounds.[9]

Like other synthetic cannabinoids, 4'-methyl-AM-2201 (MAM-2201) acts as a potent full agonist at cannabinoid receptors, exhibiting high affinity for CB1 receptors, which are mainly located on neuronal presynaptic terminals, and CB2 receptors, which are primarily expressed on peripheral immune cells.[9,10] MAM-2201 undergoes extensive hepatic metabolism mainly through Phase I reactions on the N-pentyl side chain.[11,12] The primary metabolic pathways include ω- and ω-1 hydroxylation, producing monohydroxylated metabolites such as MAM-2201 N-(5-hydroxypentyl) and MAM-2201 N-(4-hydroxypentyl), which are further oxidized to the corresponding carboxylic acid metabolite, MAM-2201 N-pentanoic acid (**Fig.1**).[11,12]

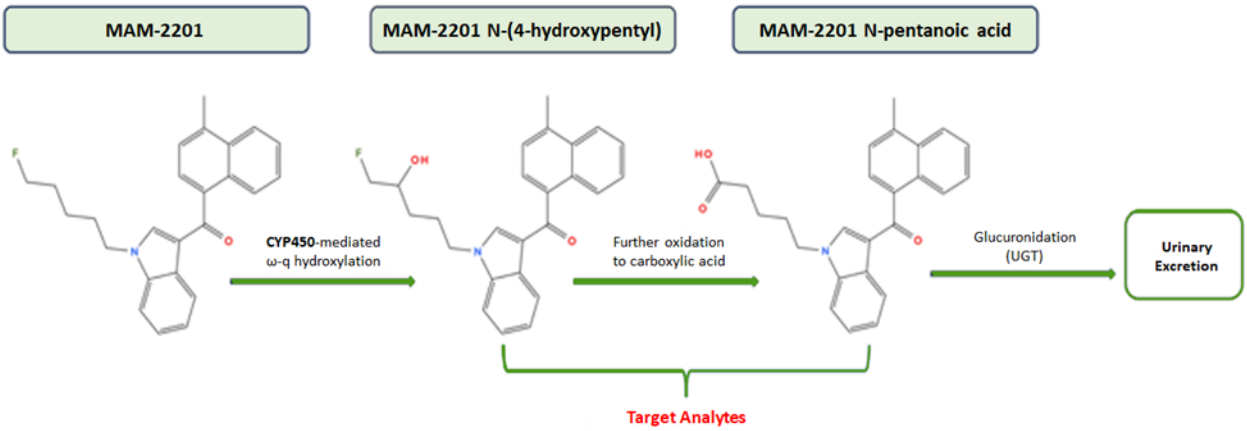
Additionally, oxidative defluorination and minor hydroxylation on the indole ring have been reported.[11] Phase I metabolites are mainly excreted in urine as glucuronide conjugates.[12]

Therefore, this study aimed to develop a qualitative and quantitative ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) analytical method for the major urinary metabolites of the synthetic cannabinoid MAM-2201 in human urine.

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FIGURE 1. Proposed metabolic pathway of MAM-2201



Explanations: CYP450-mediated ω-1 hydroxylation of the N-pentyl side chain to form MAM-2201 N-(4-hydroxypentyl), followed by further oxidation to MAM-2201 N-pentanoic acid. These metabolites are primarily excreted in urine following glucuronide conjugation.

METHODS

Chemicals and reagents

All organic solvents were high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific (Pittsburgh, PA). We purchased ethyl acetate, Ammonium hydroxide, methanol, acetonitrile, and formic acid from Fisher Scientific. Reference standards of the synthetic cannabinoid metabolites MAM-2201 N-(4-hydroxypentyl) and MAM-2201 N-pentanoic acid were purchased from Cayman Chemical. We also purchased AB-CHMINACA M4 and AB-CHMINACA M6 from Cayman Chemical. β-Glucuronidase (300,000 units/g) was purchased from Sigma-Aldrich (ST. Louis, MO, USA).

Sample preparation and solid-phase extraction

We used Bond Elut Plexa PCX cartridges (30 mg, 3 mL, 45 µm; Agilent Technologies, USA) for solid-phase extraction, which retained the target analytes through mixed-mode interactions. We selected AB-CHMINACA M4 and AB-CHMINACA M6 as structurally related internal standards to quantify MAM-2201 N-(4-hydroxypentyl) and MAM-2201 N-pentanoic acid. Stock standard solutions of MAM-2201 N-(4-hydroxypentyl) and MAM-2201 N-pentanoic acid metabolites were prepared in methanol at a concentration of 1000 ng/mL and stored at −20 °C. We prepared working standard solutions by serial dilution of the stock solutions in methanol to obtain concentrations of 50, 100, and 200 ng/mL. We prepared initial calibration standards by spiking drug-free urine with appropriate volumes of the working solutions to obtain final concentrations of 0.25, 0.5, 0.75, 1.5, 5, 15, 30, and 45 ng/mL. We prepared quality control (QC) samples independently in drug-free urine from separate stock solutions at three concentration levels. For MAM-2201 N-(4-hydroxypentyl), QC levels were 0.5, 5, and 15 ng/mL, whereas for MAM-2201 N-pentanoic acid, QC levels were 0.75, 5, and 15 ng/mL. Internal standard working solutions of AB-CHMINACA M4 and AB-CHMINACA M6 (400 ng/mL) were prepared in methanol.

Urine samples (200 µL) were fortified with 10 µL of each internal standard working solution (nominal concentration 20 ng/mL in the original urine) and mixed with 600 µL of 0.1 M acetate buffer to adjust the pH to 5.0. We performed enzymatic hydrolysis by adding 30 µL of β-glucuronidase (Helix pomatia, 300,000 U/g) and incubating the samples at 37 °C for 1 h. During method development, we optimized hydrolysis conditions (enzyme concentration, incubation time, and temperature) by evaluating incubation parameters and selecting those that yielded the highest analytical response for the target metabolites after enzymatic treatment. We quantified metabolites after enzymatic hydrolysis; therefore, the reported concentrations represent total metabolite concentrations. Glucuronide reference standards were not available during method development. We processed all calibration standards and QC samples using the same sample preparation procedure as the urine samples.

Before extraction, we sequentially conditioned the SPE cartridges with 1 mL methanol, followed by 1 mL ultrapure water. The hydrolyzed urine samples (approximately 850 µL) were then directly loaded onto the conditioned cartridges at a controlled flow rate of approximately 1 mL/min. The cartridges were washed with 1 mL water, then 1 mL methanol: water (40:60, v/v) to remove matrix components and potential interferences while retaining the analytes on the sorbent. The cartridges were then dried under vacuum for 5 min. The retained analytes were eluted with 1 mL of a mixture of ethyl acetate, methanol, and

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ammonium hydroxide (75:23:2, v/v/v). We evaporated the eluates to dryness under a gentle stream of nitrogen at 40 °C, then reconstituted the residues in 200 µL of the initial mobile phase composition for LC–MS/MS analysis.

Instrumental analysis for the determination of MAM-2201 metabolites in urine

We used an Agilent 1290 Infinity UHPLC system coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with an ESI interface for chromatographic separation and detection. The UHPLC system consisted of a binary pump, an autosampler, and a thermostatically controlled column oven. Analytical conditions are summarized in **Table 1A**. Chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column (4.6 x 100 mm, 3.5 µm) maintained at 30 °C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient elution was performed at 0.8 mL/min over a 5-minute analytical run. The gradient program started with 10% solvent B and increased linearly to 70% within 2.5 min. The mobile-phase composition returned to the initial conditions by 5.0 min, followed by 3 min of column re-equilibration, for a total cycle time of 8 min. **Table 1B** presents the gradient elution program. The retention times of MAM-2201 N-pentanoic acid and MAM-2201 N-(4-hydroxypentyl) were 2.00 and 2.17 min, respectively.

TABLE 1. UHPLC-MS/MS conditions and MRM parameters for the determination of MAM-2201 metabolites

A. Optimized UHPLC-MS/MS instrumental parameters

UHPLC conditions	LC system	Agilent Infinity 1290 liquid chromatograph
	Column	Zorbax Eclipse Plus C18 (4.6 x 100 mm, 3.5 µm)
	Column Temperature	30.0 °C
	Autosampler Temperature	8.0 °C
	Mobile phase A	0.1% formic acid in water
	Mobile phase B	0.1% formic acid in acetonitrile
	Elution	Gradient elution
	Flow rate	0.8 mL/min
	Equilibration Time	3.0 min
	Injection volume	5.00 µL
	LC system	Agilent Infinity 1290 liquid chromatograph
MS conditions	Mass spectrometer	Agilent 6460 triple quadrupole MS
	Ionization source	Positive electrospray ionization (ESI+) mode
	Acquisition mode	Multiple reaction monitoring (MRM)
	Drying Gas Temp	300 °C
	Drying Gas Flow	6.5 L/min
	Capillary	4000 V
	Sheath Gas Flow	6.5 L/min
	Sheath Gas Temp	300 °C
	Nebulizer	40.0 psi
	Dwell	200 ms
	Quadrupole Resolution	Unit (Q1 and Q3)
	Fragmentor	100 V
	Cell Accelerator Voltage	4 V
	Polarity	Positive
	Collision energy	Optimized for each MRM transition

B. Gradient elution program used for chromatographic separation of MAM-2201 metabolites

Time (min)	% A	% B	Flow (mL/min)
0.0	90	10	0.8
1.0	90	10	0.8
1.5	70	30	0.8
2.0	50	50	0.8
2.5	30	70	0.8
3.5	80	20	0.8

C. Multiple Reaction Monitoring (MRM) Transitions for MAM-2201 Metabolites and Internal Standards

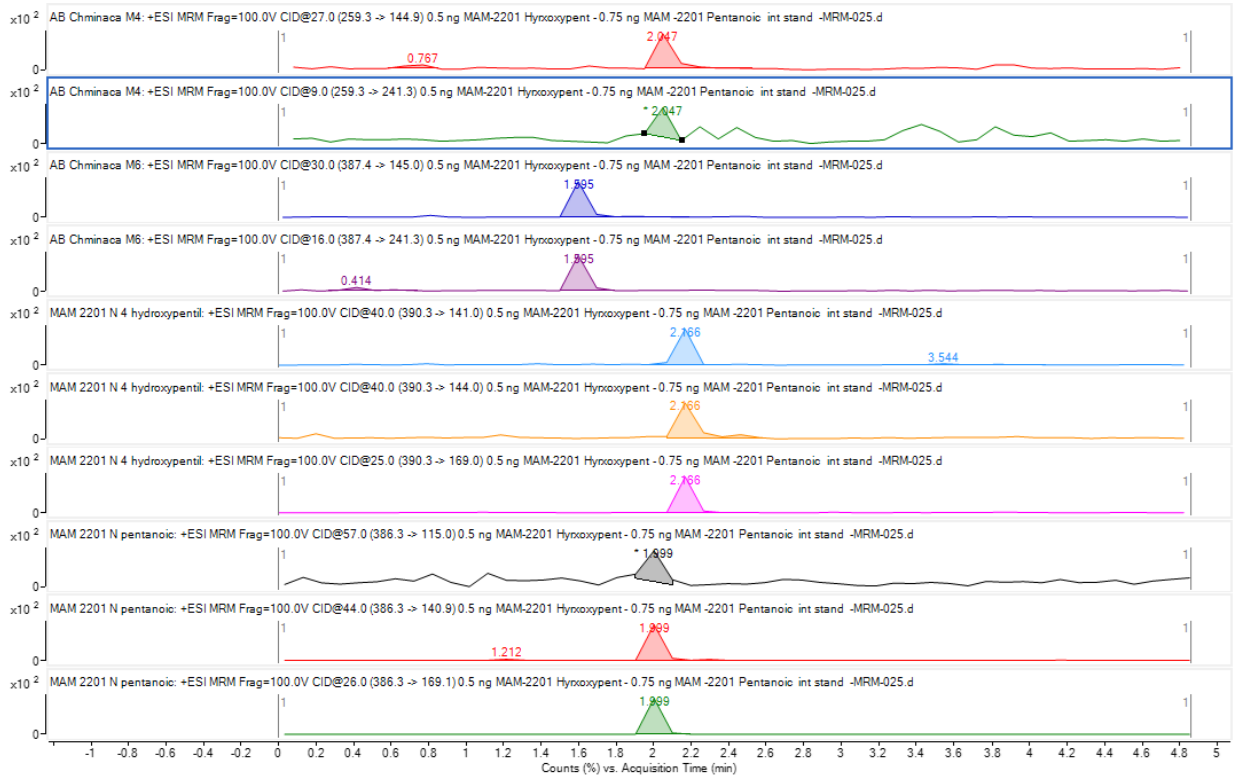
Analyte	RT (min)	Precursor ion (m/z)	Product ion (m/z)	CE (eV)	Use
MAM-2201 N-(4-hydroxypentyl) metabolite	2.17	390.3	169.0	25	Quantifier
			141.0	40	Qualifier 1
			144.0	40	Qualifier 2
MAM-2201 N-pentanoic acid metabolite	2.00	386.3	169.1	26	Quantifier
			140.9	44	Qualifier 1
			115.0	57	Qualifier 2
AB-CHMINACA M4	2.05	259.3	144.9	27	Quantifier
			241.3	9	
AB-CHMINACA M6	1.60	387.4	145.0	30	Quantifier
			241.3	16	

Abbreviations: CE, collision energy; ESI, electrospray ionization; eV, electron volt (unit of collision energy used for ion fragmentation in MS/MS); LC, liquid chromatography; MRM, multiple reaction monitoring; psi, pounds per square inch; m/z, mass-to-charge ratio; MS, mass spectrometry; UHPLC-MS/MS, Ultra-high-performance liquid chromatography - tandem mass spectrometry.

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The mass spectrometer operated in positive electrospray ionization (ESI+) mode, with the ion source temperature set to 300 °C and the capillary voltage at 4000 V. Quantification was performed in multiple reaction monitoring (MRM) mode. **Figure 2** shows representative MRM chromatograms of urine samples spiked with MAM-2201 metabolites.

FIGURE 2. MRM chromatograms of MAM-2201 metabolites and internal standards in spiked urine



Explanations: MAM-2201 N-(4-hydroxypentyl) at 0.5 ng/mL, MAM-2201 N-pentanoic acid at 0.75 ng/mL, and the corresponding internal standards AB-CHMINACA M4 and AB-CHMINACA M6.

Metabolites MAM-2201 N-(4-hydroxypentyl) and MAM-2201 N-pentanoic acid were identified by comparison of retention times with authentic reference standards and by monitoring optimized precursor-to-product ion transitions, which are demonstrated in **Table 1C**.

Compound identification was based on retention time and MRM ion transition criteria. The retention time of analyte in unknown samples was acceptable when within ±0.1 min of the corresponding calibration standards. We considered the qualifier-to-quantifier ion ratio in unknown samples acceptable when it was within ±20% of the mean ion ratio obtained from the calibration standards and QC samples. At the LLOQ, we applied an acceptance criterion of ±30%.

We quantified each analyte using the peak area ratio to its corresponding internal standard. We calculated peak area ratios by dividing each analyte quantifier MRM peak area by the peak area of its corresponding internal standard. Specifically, we divided the peak area of MAM-2201 N-(4-hydroxypentyl) by that of AB-CHMINACA M4, and the peak area of MAM-2201 N-pentanoic acid by that of AB-CHMINACA M6. Both analytes showed symmetrical, well-resolved chromatographic peaks with no significant tailing under the optimized chromatographic conditions. We constructed calibration curves by plotting the analyte-to-internal standard peak area ratio (response variable, y) against the nominal analyte concentration (x, ng/mL) in matrix-matched calibration standards. We applied linear regression with a 1/x2 weighting factor using the equation y=ax+b, where a is the slope and b is the intercept.

Method validation

Method validation parameters were evaluated in accordance with the recommendations of the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines,[13] including assessment of selectivity, linearity, limits of detection and quantification, accuracy, precision, matrix effects, extraction recovery, process efficiency, carryover, dilution integrity, and stability.

We prepared initial calibration curves in blank urine at eight concentration levels and established the final calibration range for each analyte based on the predefined acceptance criteria. For each analytical run, we prepared and analyzed an independent set of calibration standards to generate a calibration curve. We used five calibration curves during method validation. We considered calibration standards

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acceptable when back-calculated concentrations were within ±15% of the nominal concentrations (±20% at the LLOQ). At least 75% of the non-zero calibration standards, including the LLOQ and the highest calibration level, were required to meet these acceptance criteria. We established the limit of detection (LOD) and lower limit of quantification (LLOQ) at signal-to-noise ratios of ≥3 and ≥10, respectively.

We evaluated accuracy, precision, matrix effect, and extraction efficiency at three QC concentration levels: 0.5, 5, and 15 ng/mL for MAM-2201 N-(4-hydroxypentyl), and 0.75, 5, and 15 ng/mL for MAM-2201 N-pentanoic acid.

We evaluated accuracy and precision by analyzing QC samples in five independent analytical runs on five different days, with three replicates per concentration level in each run (n=15). Acceptance criteria were 85-115% of the nominal concentrations for accuracy and ≤15% CV for precision (80-120% and ≤20% CV at the LLOQ).

We evaluated extraction efficiency and matrix effect at three QC concentration levels using pre-extraction spiked samples and post-extraction spiked samples prepared from six independent blank urine samples (n=6 per QC level). We prepared neat standard solutions in triplicate at each QC concentration level (n=3 per QC level) and used them as reference samples to calculate extraction efficiency and matrix effect. We evaluated selectivity using six independent blank urine samples. No endogenous interfering peaks were observed at the analytes' or their corresponding internal standards' retention times in any blank urine sample.

We evaluated carryover by injecting a blank sample after the highest calibrator (45 ng/mL). We assessed stability at low and high QC concentrations under short-term room-temperature storage, refrigerated/autosampler storage, freeze–thaw conditions, and long-term storage.

RESULTS

We initially prepared the calibration curves using eight concentration levels (0.25, 0.5, 0.75, 1.5, 5, 15, 30, and 45 ng/mL). The 0.25 ng/mL calibration level did not meet the predefined quantitative acceptance criteria for either analyte. For MAM-2201 N-pentanoic acid, the 0.5 ng/mL level was detectable but did not meet the quantification criteria and was therefore excluded from the final calibration model. Consequently, the validated calibration ranges were 0.5–45 ng/mL for MAM-2201 N-(4-hydroxypentyl) (seven calibration levels) and 0.75–45 ng/mL for MAM-2201 N-pentanoic acid (six calibration levels), with correlation coefficients (r^2) ≥ 0.996. The limits of detection were 0.25 ng/mL for MAM-2201 N-(4-hydroxypentyl) and 0.5 ng/mL for MAM-2201 N-pentanoic acid, while the lower limits of quantification were 0.5 ng/mL and 0.75 ng/mL, respectively, with acceptable accuracy and precision (**Tab.2A**).

TABLE 2. Validation results of the UHPLC-MS/MS method for the determination of MAM-2201 metabolites in human urine

A. Calibration and sensitivity						
	MAM-2201 N-(4-hydroxypentyl) metabolite			MAM-2201 N-pentanoic acid metabolite		
Calibration range (ng/mL)	0.5 – 45			0.75 – 45		
r^2	0.997			0.996		
LOD	0.25			0.5		
LLOQ	0.5			0.75		
B. Accuracy and precision						
	MAM-2201 N-(4-hydroxypentyl) metabolite			MAM-2201 N-pentanoic acid metabolite		
	LQC	MQC	HQC	LQC	MQC	HQC
Nominal (ng/mL)	0.5	5	15	0.75	5	15
Mean (ng/mL)	0.431	4.926	16.935	0.651	4.850	17.130
Intra-day Accuracy (%)	88.4	95.0	112.9	87.9	96.0	113.2
Inter-day Accuracy (%)	86.2	98.52	113.9	86.8	97.0	114.2
Intra-day precision (CV%)	11.3	5.05	5.6	11.6	8.0	6.2
Inter-day precision (CV%)	12.4	8.0	3.3	12.5	9.09	3.5
C. Matrix effect, Extraction recovery, and process efficiency						
	MAM-2201 N-(4-hydroxypentyl) metabolite			MAM-2201 N-pentanoic acid metabolite		
Matrix effect (%)	96.79	88.96	94.81	92.64	93.70	94.66
Matrix effect CV (%)	6.16	4.72	2.68	2.5	1.38	2.15
Extraction Recovery (%)	85.46	93.72	93.85	86.6	89.99	94.4
Extraction Recovery CV (%)	5.8	6.70	1.49	3.4	2.4	1.2
Process efficiency	82.47	83.18	88.97	80.2	84.33	89.31

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TABLE 2. Validation results of the UHPLC-MS/MS method for the determination of MAM-2201 metabolites in human urine (continued)

D. Stability						
	MAM-2201 N-(4-hydroxypentyl) metabolite					
	LQC level (0.5 ng/mL)			HQC level (15 ng/mL)		
Condition	Mean (ng/mL)	% of initial	CV (%)	Mean (ng/mL)	% of initial	CV (%)
24 h RT	0.45	86.4	0.44	13.96	92.88	0.17
Autosampler (4-10 °C, 72 h)	0.48	92.5	0.63	15.76	104.87	0.49
Freeze-thaw (-20 °C ⍰ RT, 3 cycles)	0.47	89.9	3.92	15.28	101.7	2.19
Long term (-20 °C, one month)	0.47	90.87	4.35	16.49	109.7	2.17
	MAM-2201 N-pentanoic acid metabolite					
	LQC level (0.75 ng/mL)			HQC level (15 ng/mL)		
Condition	Mean (ng/mL)	% of initial	CV (%)	Mean (ng/mL)	% of initial	CV (%)
24 h RT	0.65	87.6	3.68	13.82	93.8	1.55
Autosampler (4-10 °C, 72h)	0.69	92.7	2.97	15.85	105.8	2.19
Freeze-thaw (-20 °C ↔ RT, 3 cycles)	0.68	91.2	1.98	15.34	102.5	2.32
Long term (-20 °C, one month)	0.67	90.3	3.82	16.18	108.1	2.4

We evaluated accuracy and precision at three quality-control concentration levels for both analytes. Intra-day accuracy ranged from 88.4% to 112.9% for MAM-2201 N-(4-hydroxypentyl) and from 87.9% to 113.2% for MAM-2201 N-pentanoic acid, while inter-day accuracy ranged from 86.2% to 113.9% and 86.8% to 114.2%, respectively. Intra-day precision ranged from 5.6% to 11.3% for MAM-2201 N-(4-hydroxypentyl) and from 6.2% to 11.6% for MAM-2201 N-pentanoic acid. Inter-day precision ranged from 3.3% to 12.4% and 3.5% to 12.5%, respectively, demonstrating good repeatability and reproducibility (**Tab.2B**).

Extraction efficiency ranged from 85.4% to 93.9% for MAM-2201 N-(4-hydroxypentyl) and from 86.5% to 94.5% for MAM-2201 N-pentanoic acid. Matrix effect values ranged from 88.96% to 96.79% for MAM-2201 N-(4-hydroxypentyl) and from 92.64% to 94.66% for MAM-2201 N-pentanoic acid across the three QC levels. The corresponding coefficients of variation ranged from 2.68% to 6.16% and from 1.38% to 2.5%, respectively, indicating minimal donor-dependent matrix variability and only minor matrix influence on analyte ionization. Process efficiency ranged from 82.5% to 89.0% for MAM-2201 N-(4-hydroxypentyl) and from 80.2% to 89.3% for MAM-2201 N-pentanoic acid (**Tab.2C**). No carryover was observed after injecting the highest calibration standard.

DISCUSSION

In this study, we successfully detected and quantified the major urinary metabolites MAM-2201 N-(4-hydroxypentyl) and MAM-2201 N-pentanoic acid using enzymatic hydrolysis followed by SPE and UHPLC–MS/MS in MRM mode. Using multiple MRM transitions ensured high selectivity and reliable identification in a complex urine matrix.

Structurally related synthetic cannabinoid metabolites AB-CHMINACA M4 and AB-CHMINACA M6 were selected as internal standards. Their similar physicochemical properties and chromatographic behavior provided adequate compensation for sample-preparation and instrumental variability, as shown by satisfactory validation results.

Matrix effect was evaluated using six independent blank urine samples. Hemolyzed and high-specific-gravity urine specimens were not specifically investigated.

Enzymatic hydrolysis with β-glucuronidase has been widely used in synthetic cannabinoid metabolite analysis to cleave glucuronide conjugates and improve detection sensitivity, as shown in previous studies, including our earlier validated method.[14-17] These results align with these studies, supporting enzymatic hydrolysis to enhance detection of glucuronide-conjugated synthetic cannabinoid metabolites in urine. Solid-phase extraction with mixed-mode cation-exchange sorbents yielded high extraction efficiency and low matrix effects, resulting in clean extracts and strong analytical performance. Similarly, Jang M et al. (2015) reported SPE as a suitable technique for extracting synthetic cannabinoid metabolites from urine because it improves selectivity and reduces matrix interference.[15]

The developed UHPLC–MS/MS method demonstrated adequate sensitivity, accuracy, and precision for qualitative confirmation and quantitative determination of MAM-2201 N-(4-hydroxypentyl) and MAM-2201 N-pentanoic acid with a short analysis time, making it suitable for routine forensic toxicology applications.

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Although several LC-MS/MS methods have been reported to determine synthetic cannabinoid metabolites in biological matrices, most are broad, multi-analyte methods targeting numerous compounds. In contrast, this study focused on determining two major urinary MAM-2201 metabolites using enzymatic hydrolysis, solid-phase extraction, and rapid UHPLC-MS/MS analysis. Compared with previously published methods, the developed assay requires a relatively small urine volume (200 µL), provides satisfactory extraction efficiency (85.4-94.5%) with minimal matrix effects, and achieves an analytical cycle time of 8 min, making it suitable for routine forensic toxicological applications. **Table 3** compares the analytical characteristics of the present method with previously published methods.

The present study has several limitations. The method was developed as a targeted UHPLC-MS/MS assay to qualitatively confirm and quantitatively determine two major urinary metabolites of MAM-2201 and does not provide comprehensive screening of synthetic cannabinoid metabolites. Although the method demonstrated satisfactory analytical performance using fortified human urine samples, it has not yet been evaluated with authentic forensic case samples. A single analyst developed and validated the method using one UHPLC-MS/MS system. Evaluation of inter-analyst, inter-instrument, and interlaboratory reproducibility was beyond the scope of the present study.

Overall, the results confirm that enzymatic hydrolysis combined with solid-phase extraction and UHPLC–MS/MS is an effective analytical approach for determination of MAM-2201 metabolites in urine.

TABLE 3. Comparison of the present UHPLC-MS/MS method with previously published analytical methods for the determination of synthetic cannabinoids and their metabolites in urine

Parameter	Present Study	Jang et al.[15]	Borg et al.[17]	Benvenuto et al.,[18]
Target analytes	2 major urinary MAM-2201 metabolites	37 SC metabolites from 17 parent compounds, including MAM-2201 metabolites	32 SC metabolites	7 SCs and metabolites
Matrix	Urine	Urine	Urine	Urine
Sample volume	200 µL	100 µL	1 mL	2 mL
Enzymatic hydrolysis	β-glucuronidase	β-glucuronidase	β-glucuronidase	No enzymatic hydrolysis was performed
Extraction	Mixed-mode SPE (Bond Elut Plexa PCX)	Mixed-mode SPE (Clean Screen)	LLE (chlorobutane: isopropanol, 70:30)	Protein precipitation +SPE
Instrument	UHPLC-Triple Quadrupole MS/MS	UHPLC-Triple Quadrupole MS/MS	UHPLC-Triple Quadrupole MS/MS	2D UPLC- Triple Quadrupole MS/MS
Internal standard	Structurally related internal standards (AB-CHMINACA M4 and AB-CHMINACA M6)	Deuterated JWH-018 N-(5-hydroxypentyl-d5	Isotopically labelled internal standards	JWH-018-d11
LLOQ	0.5 ng/mL (N-(4-hydroxypentyl)); 0.75 ng/mL (N-pentanoic acid)	0.25 ng/mL for MAM-2201 metabolites	0.5 ng/mL	0.005 ng/mL (AM-2201 4 hydroxypentyl metabolite)
Calibration range	0.5-45 (N-(4-hydroxypentyl)); and 0.75 – 45 ng/mL (N-pentanoic acid)	0.25 – 100 ng/mL	0.5-200 ng/mL	0.05-5 ng/mL (AM-2201 4-hydroxypentyl metabolite)
Total run/cycle time	8 min total cycle (5 min analytical run)	12 min	4 min	12 min
Recovery	85.4-94.5%	65-99%	48-104%	99% (AM-2201 4- hydroxypentyl metabolite)
vMatrix effect	88.96 – 96.79% (CV, 1.38-6.16 %)	53-110% (CV, 0.4-10.1%)	Ion suppression observed (14-76%)	8% ion suppression

CONCLUSIONS

In summary, this study describes the development and validation of a targeted UHPLC-MS/MS method for simultaneously determining the major urinary MAM-2201 metabolites, MAM-2201 N-(4-hydroxypentyl) and MAM-2201 N-pentanoic acid. The method combines enzymatic hydrolysis with solid-phase extraction, providing a simple sample preparation procedure, rapid chromatographic analysis, and satisfactory analytical performance in selectivity, sensitivity, accuracy, and precision. The validated method is suitable for routine forensic toxicology applications and provides a practical analytical approach for determining MAM-2201 metabolites in forensic laboratories.

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