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RESEARCH PAPER

Quantitative analysis of Δ^8- and Δ^9-tetrahydrocannabinol metabolites and isomers: a rapid assay in urine by LC-MS/MS

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Abstract

An increasing number of cannabis-related products have become available and entered the market, particularly those containing cannabidiol (CBD) and Δ^8-tetrahydrocannabinol (Δ^8-THC). Analytical methods for cannabinoids in urine have been described extensively in the literature. However, methods providing good resolution for distinguishing interferences from THC positional isomers are needed. The aim of this project was to develop and validate a liquid chromatography with tandem mass spectrometry (LC-MS/MS) method to quantitate a broad panel of cannabinoids in authentic urine specimens. The method was optimized to quantitate Δ^8-THC and Δ^9-THC, 11-OH-Δ^8-THC and 11-OH-Δ^9-THC, Δ^8-THC-COOH and Δ^9-THC-COOH, CBD, 7-COOH-CBD, CBG, and CBN, and validated with the guidance of the American Academy of Forensic Sciences Standards Board (ASB) Standard 036. The validated assay was then used to evaluate urine samples collected over various time points from female patients ($N = 69$) enrolled in a study assessing prevalence of marijuana/CBD use during pregnancy from November 2022 to May 2024. Δ^8- and Δ^9- isomers were chromatographically resolved and successfully separated. For all analytes, the lower limit of quantitation (LLOQ) was determined to be 10 ng/mL, and the upper limit of quantitation (ULOQ) was 1000 ng/mL. In the authentic samples, the most frequently detected analyte was Δ^9-THC-COOH, with a median concentration of 278 ng/mL ($n = 38$). Δ^9-THC and 11-OH-Δ^9-THC were detected with a median concentration of 42.4 ng/mL ($n = 5$) and 65.7 ng/mL ($n = 34$), respectively. Δ^8-THC-COOH was detected in $n = 3$ specimens, with a median concentration of 25.5 ng/mL. The study provided a rapid assay for the analysis of cannabinoids in urine.

Keywords Cannabinoids · Urine specimens · Filtration · Analytical method optimization · LC-MS/MS

Introduction

Cannabis is considered one of the most widely used substances worldwide. Its increased popularity and availability is due to factors such as changes in the drug policies, perceived risks associated with its use, and cultural tolerance [1].

Cannabis contains several different chemical compounds, with Δ^9-tetrahydrocannabinol (Δ^9-THC) being the primary psychoactive constituent and often the focus of cannabis research and legal debate. The pharmacological effects of THC are a consequence of the binding to the G protein–coupled central cannabinoid receptor CB1 that occurs primarily in the brain, cerebellum, hippocampus, basal ganglia, cerebral cortex, and also throughout the periphery including the heart, bladder, lungs, thymus, uterus, spleen, and gastrointestinal tract [2, 3]. THC is also a partial agonist of the peripheral cannabinoid receptor CB2, which is primarily

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expressed by cells of the hematopoietic system and modulates immune function and bone mass [4]. As a result of the interactions, cannabis can induce euphoria, impair cognition and memory, alter time perception, and cause sedation and dysphoric reactions such as psychosis and panic attacks [5]. Additional effects include vasodilation and tachycardia, conjunctival reddening, dry mouth, appetite stimulation, and respiratory depression [6].

Despite the increased accessibility and use of medical cannabis in recent years [7], the controversies regarding evidence of clinical benefits and potential harms and the legal and ethical considerations associated are still widely debated [8]. In the United States (US), cannabis was widely utilized as a patent medicine until the federal restriction of cannabis use and cannabis sale occurred in 1937 [9]. Subsequently, legal penalties for possession increased and prohibition under federal law occurred with the Controlled Substances Act of 1970 [10].

Recently, some states in the US have legalized the possession and personal use of cannabis for medical and/or recreational purposes. In the US, hemp production was legalized in 2018 by the Agriculture Improvement Act (2018 Farm Bill). Hemp was defined as containing < 0.3% Δ^9-THC by dry weight. This resulted in an increasing number of unregulated hemp-based products containing cannabidiol (CBD) that became available and entered the market [11]. CBD elicits its pharmacological effects without any significant intrinsic activity on CB1 and CB2 receptors and has been thoroughly tested in humans in numerous controlled experimental studies and clinical trials for multiple sclerosis, neuropathic pain, schizophrenia, bipolar mania, social anxiety disorder, insomnia, Huntington's disease, and epilepsy [12]. Another outcome of the legalization of hemp has been the growing trend to isolate or synthesize isomers of Δ^9-THC [13] and the proliferation of associated products containing Δ^8-tetrahydrocannabinol (Δ^8-THC) [14].

Δ^8-THC is a psychotropic compound and a positional isomer of Δ^9-THC that has primarily been derived from hemp [15]. Most cannabis strains produce minimal amounts of Δ^8-THC. However, the chemical conversion of CBD may promote the synthesis of Δ^8-THC [16]. Δ^8-THC shows lower potency than Δ^9-THC although it exhibits similar psychoactive properties [17, 18].

Recently, Δ^8-THC has rapidly risen in popularity among consumers of cannabis products and can easily be found where cannabis-related products are sold in the form of concentrates, flowers, and edibles [19, 20].

With the increased availability of Δ^8-THC products, forensic toxicology laboratories have reported a higher rate of interferences affecting the identification and quantitation of Δ^9-THC [21], illustrating the need to evaluate its impact on casework and to ensure accurate reporting and prevalence of use. With this emergence of alternative cannabinoid products, analytical techniques capable of separating the Δ^8- and Δ^9- isomers and their metabolites in biological specimens are needed. Methods for detecting cannabinoids in urine samples have been described extensively in the literature. However, methods providing good resolution for distinguishing interferences from positional isomers are limited and have been reported in the literature only in recent years [11, 13, 14, 22]. Karas et al. described a validated automated extraction and confirmation method for 11-nor-Δ^8-carboxy-THC (Δ^8-THC-COOH) and 11-nor-Δ^9-carboxy-THC (Δ^9-THC-COOH) in urine by liquid chromatography with tandem mass spectrometry (LC-MS/MS) [11]. The method provided analyte separation and was used to confirm the presence of Δ^8-THC-COOH and Δ^9-THC-COOH in urine specimens presumptively positive by immunoassay ($n = 2939$). Both the analytes were found to be present together above the cutoff (15 ng/mL) in 33% of specimens, whereas Δ^8-THC-COOH was detected alone in nearly one-third of the cases. Similarly, Crosby et al. identified Δ^8-THC-COOH in postmortem urine specimens from November 2021 to mid-March 2022 [13]. The authors confirmed Δ^8-THC-COOH in 26 of 194 presumptive THC-COOH positive cases using gas chromatography with mass spectrometry (GC-MS). Due to the increase in cases containing Δ^8-THC, Reber et al. described a validated method to resolve and quantitate Δ^8-THC and Δ^9-THC in blood by LC-MS/MS and qualitatively confirm the inactive Δ^8-THC-COOH and Δ^9-THC-COOH metabolites in blood and urine [14]. The challenges to resolve and quantitate Δ^8- and Δ^9-isomers were reported by the authors.

On this basis, the aims of this project were to develop and validate an LC-MS/MS method to quantitate a broad panel of cannabinoids in authentic urine specimens, and to provide an example of a simple, rapid, and cost-effective assay for their separation and quantitation.

Materials and methods

Chemicals and reagents

Δ^8-THC and Δ^9-THC, 11-hydroxy-Δ^8-THC (11-OH-Δ^8-THC) and 11-hydroxy-Δ^9-THC (11-OH-Δ^9-THC), Δ^8-THC-COOH and Δ^9-THC-COOH, cannabidiol (CBD), 7-carboxy-cannabidiol (7-COOH-CBD), cannabigerol (CBG), and cannabinol (CBN) reference standard solutions were purchased from Cerilliant (Round Rock, TX, USA). Quality control standard solutions for Δ^8-THC and Δ^9-THC, 11-OH-Δ^8-THC and 11-OH-Δ^9-THC, Δ^8-THC-COOH and Δ^9-THC-COOH, CBD, CBG, and CBN were purchased from Cayman Chemical (Ann Arbor, MI, USA), while the quality control standard solution for 7-COOH-CBD was purchased from Cerilliant (Round Rock, TX, USA). The internal

standards 11-OH-Δ^9-THC-d_3, Δ^9-THC-COOH-d_9, CBD-d_3, 7-COOH-CBD-d_3, and CBN-d_3 were purchased from Cerilliant (Round Rock, TX, USA), while Δ^8-THC-COOH-d_3, Δ^9-THC-d_9 and CBG-d_9 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Finden BGTurbo® recombinant β-glucuronidase enzyme was acquired from KURA Biotech (Atlanta, GA, USA). LC-MS grade solvents, mobile phases, and sodium hydroxide were purchased from Thermo Fisher (Fairlawn, NJ, USA). UCT Clean Screen® FASt Extraction Columns (200 mg/3 mL) were purchased from United Chemical Technologies (Bristol, PA, USA).

Sample preparation

The working standard solutions were prepared in methanol with concentration ranges between 0.1 and 10 µg/mL. These solutions were used to yield a standard calibration curve ranging from 10 to 1000 ng/mL for all the analytes. The working internal standard mix was prepared in methanol at a concentration of 1 µg/mL to yield a final concentration of 100 ng/mL. The urine samples (500 µL) were spiked with 50 µL of the internal standard solution mix, 100 µL of Finden BGTurbo® Enzyme (KURA Biotech, Atlanta, GA, USA), and incubated at 50 °C for 30 min. A second hydrolysis at 50 °C for 10 min was performed after the addition of 50 µL of 10.0 N sodium hydroxide. Finally, samples were diluted with 500 µL of the organic mobile phase 0.1% formic acid in acetonitrile before being centrifuged at 3000 rpm for 5 min and filtered using UCT Clean Screen® FASt Extraction Columns. The eluent was collected and transferred into a 1.5 mL glass autosampler vial for analysis.

Instrument parameters

The urine samples were analyzed using a Thermo Scientific Vanquish™ Flex UHPLC System (Waltham, MA, USA) coupled with a Thermo Scientific TSQ Altis™ triple quadrupole mass spectrometer (Waltham, MA, USA). Chromatographic separation was achieved using a Waters Acquity™ UPLC BEH Shield RP18 column (2.1 × 100 mm, 1.7 µm) (Milford, MA, USA) with matching guard column. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Gradient elution at 0.5 mL/min was used for separation with the column temperature held at 20 °C. The gradient was kept at 50% B for 6 min, then increased to 68% B over 1 min and held for 2.5 min, increased to 72% B and held for 3 min, increased to 98% B and held for 3 min, then decreased to 50% B and re-equilibrated for 3 min. The injection volume was 5 µL. Additional source parameters, operating in positive mode, included ion spray voltage (4000 V), sheath gas (30 Arb), aux gas (20 Arb), sweep gas (1.2 Arb), ion transfer tube temperature (350 °C), and vaporizer temperature (280 °C). Data were acquired and analyzed using Thermo Scientific TraceFinder software (5.1). The analysis was performed in multiple reaction monitoring mode (MRM) by targeting the *m*/*z* ratio ions as reported in Table 1.

Method optimization and validation

Before the validation, the method was optimized during sample preparation to achieve the optimum recovery of the analytes by evaluating the effectiveness of the hydrolysis step at different temperatures, time intervals, and reagent ratios. Moreover, the chromatographic separation of the compounds was also studied by testing pure standard solutions at different analytical conditions (chromatographic column, mobile phase composition, gradient, column temperature). The method was then validated under the guidance of the American Academy of Forensic Sciences Standards Board (ASB) Standard 036 and in-house laboratory validation criteria. Validation parameters included selectivity; interferences from stable-isotope internal standards and common analytes; calibration model; carryover; ion suppression/enhancement; limit of detection (LOD); lower and upper limit of quantitation (LLOQ and ULOQ); accuracy (bias) and precision; dilution integrity; and processed extract stability. The carryover concentration for each analyte in the method was studied by injecting blank urine ($n = 3$) fortified with internal standards after analysis of the highest calibrator (1000 ng/mL). Carryover was considered insignificant if there was no signal at the relevant retention times exceeding 20% of the analyte signals at the LLOQ. Matrix interferences were evaluated using negative matrix samples ($n = 10$) without the addition of the internal standards. Stable-isotope internal standard interferences were assessed by analyzing blank matrix samples fortified with the internal standards and monitoring the signal of the analytes. Moreover, a single blank matrix sample fortified with the analytes at the highest calibrator concentration (1000 ng/mL) was analyzed without internal standards to monitor interferences in the mass spectra of the labeled compounds as contribution from the high analyte concentration that could impact the analyte quantitation. To evaluate interferences from other commonly encountered analytes, potential common analytes were analyzed including common over-the-counter and prescription medications and illicit drugs ($n = 77$). Ion suppression/enhancement was evaluated using three different sets of samples [23]: the first set consisted of neat standards at low and high concentrations injected a minimum of 6 times; the second set consisted of standards added at low and high concentrations to 10 varying matrix sources post-extraction; and the third set consisted of standards added at low and high concentrations to 10 varying matrix sources pre-extraction. The results from these samples were used to calculate matrix effects, recovery, and process efficiency

Table 1 Targeted *m*/*z* ratio ions for the studied compounds, and optimized LC-MS/MS parameters. Note that the mass optimization for CBG was accomplished for one qualitative ion only

Compound name	Precursor (*m*/*z*)	Product (*m*/*z*)	Collision energy (V)	Lens (V)	Retention time (min)	Internal standard
Δ^8-THC	315.5	**193.1**	27	65	11.47	Δ^9-THC-d_9
		123.1	41	65		
		135.1	24	65		
11-OH-Δ^8-THC	331.3	**201.1**	27	62	6.02	11-OH-Δ^9-THC-d_3
		271.2	22	62		
		193.1	28	62		
Δ^8-THC-COOH	345.5	**299.2**	23	67	6.79	Δ^8-THC-COOH-d_3
		193.1	30	67		
		165.1	21	67		
Δ^9-THC	315.5	**193.1**	28	65	11.27	Δ^9-THC-d_9
		123.1	42	65		
		259.2	24	65		
11-OH-Δ^9-THC	331.3	**193.2**	29	56	5.78	11-OH-Δ^9-THC-d_3
		201.1	28	56		
		271.2	22	56		
Δ^9-THC-COOH	345.5	**299.2**	23	66	7.34	Δ^9-THC-COOH-d_9
		193.1	32	66		
		119.1	35	66		
CBD	315.5	**193.1**	27	66	9.40	CBD-d_3
		123.1	41	66		
		135.1	24	66		
7-COOH-CBD	345.4	**327.2**	17	65	3.66	7-COOH-CBD-d_3
		299.2	23	65		
		193.1	31	65		
CBN	311.6	**223.2**	24	68	10.82	CBN-d_3
		293.2	21	68		
		195.1	31	68		
CBG	317.5	**193.1**	20	56	10.01	CBG-d_9
		123.0	40	56		
Δ^8-THC-COOH-d_3	348.5	**302.2**	23	67	6.72	
		196.1	30	67		
Δ^9-THC-d_9	324.5	**202.2**	28	65	11.18	
		123.0	43	65		
11-OH-Δ^9-THC-d_3	334.5	**316.2**	16	54	5.72	
		196.1	30	54		
Δ^9-THC-COOH-d_9	354.5	**336.2**	18	68	7.16	
		308.2	24	68		
CBD-d_3	318.4	**196.1**	27	64	9.37	
		123.0	41	64		
7-COOH-CBD-d_3	348.5	**330.2**	17	67	3.65	
		302.2	23	67		
CBG-d_9	326.5	**202.1**	21	59	9.94	
		123.0	41	59		
CBN-d_3	314.5	**223.1**	24	70	10.78	
		296.2	20	70		

with criteria of ± 25% determined acceptable. The LOD and LLOQ were assessed using blank matrix samples from three different sources fortified with the analyte of interest at the lowest calibrator concentration in triplicate over three runs to determine if identification, detection, precision, and bias criteria could be met for all analytes. Six non-zero calibrators were utilized and the calibration model was accomplished spanning the range of concentrations expected in day-to-day analysis. Five replicates per concentration were used in separate runs. All data points from the replicates were plotted together to establish the calibration model. The origin was not included as a calibration point. Residual plots were used to determine if the variances appeared to be equal across the calibration range with a similar degree of scatter at each concentration. Bias and precision were evaluated in triplicate at three different quality control (QC) concentrations (low QC 30 ng/mL; medium QC 200 ng/mL; high QC 400 ng/mL) for five different batches. Processed extracts were evaluated for stability at low and high QC concentrations in triplicate at different time intervals. The extracts were stored in the autosampler at 10 °C and injected immediately after preparation (t_0) and again after 24 h, 48 h, and 72 h. The sample dilution integrity was evaluated using authentic samples ($n = 3$) that had concentrations within the curve reanalyzed in triplicate after 10× dilution.

Authentic samples

The validated assay was used to analyze urine samples collected over various time points from female patients ($N = 69$) enrolled in a study assessing the prevalence of marijuana/CBD use during pregnancy. Authentic specimens were obtained from three obstetrics clinics in north-central Florida following a prospective, anonymous self-report survey to assess use behaviors of medical marijuana, non-medical marijuana, and CBD from November 2022 to May 2024. The study protocol including the collection of urine samples was approved by the Institution Review Board of the University of Florida (IRB#: 202201895).

Results

Sample preparation following tandem enzyme-alkaline hydrolysis

The effectiveness of tandem enzyme-alkaline hydrolysis was evaluated by fortifying a highly concentrated authentic urine specimen in triplicate with the recombinant β-glucuronidase enzyme Finden BGTurbo® (KURA Biotech, Atlanta, GA, USA) at different temperatures (20–22 °C, 37 °C, 50 °C) and time intervals (10 min, 20 min, 30 min). A second alkaline hydrolysis with 10.0 N sodium hydroxide was evaluated as well. The response/peak area of the analytes was considered. The optimum condition for improving overall recovery of the analytes was obtained with the urine samples (500 µL) with 100 µL of the recombinant enzyme added and incubated first at 50 °C for 30 min, and subsequently at 50 °C for 10 min after the addition of 50 µL of 10.0 N sodium hydroxide.

Analytical conditions and isomeric separation

The chromatographic separation was optimized prior to validation to separate a broad panel of cannabinoids and to fully resolve the Δ^8- and Δ^9- isomers (Fig. 1). The optimization was planned by performing injections of pure standard solutions at low and high concentrations of the studied analytes within the calibration range along with testing different analytical columns, mobile phase compositions, and modifying the gradient and the column compartment temperature. A fluorophenyl phase 2.7 × 100 mm, 3.0 µm LC analytical column was evaluated along with the use of 0.1% formic acid in acetonitrile and 0.1% formic acid in methanol as the organic solvent. However, an acceptable separation of all the isomers was not achieved and mainly the symmetry and shape of the peaks for most of the analytes were not deemed optimal.

An example of the chromatography obtained for 11-OH-Δ^9-THC and 11-OH-Δ^8-THC is shown in Fig. 2. Traditional C18 stationary phase was also tested, and good chromatographic resolution for identifying the individual isomers was obtained using the slightly modified conventional C18 phase 2.1 × 100 mm, 1.7 µm analytical column held at 20 °C (Fig. 3). The narrower internal diameter provided reduced longitudinal diffusion and peak broadening, although the most evident improvements in the separation were obtained by using multi-step gradient elution. The use of 0.1% formic acid in acetonitrile as organic mobile phase was preferred over the use of 0.1% formic acid in methanol since the acetonitrile provided better resolution of the isomers.

Method validation

All analytes and their respective internal standards showed good symmetrical peak shapes with acceptable separation and signal-to-noise ratios. Isomers (Δ^8- and Δ^9-) were chromatographically resolved and successfully separated. The method validation was deemed acceptable. Carryover was not detected, as well as no interferences were observed when analyzing potential interferences from other drugs. All analytes were within ± 25% for matrix effects and extraction recovery, and ranged from 94 to 125% and 75 to 105%, respectively. Linearity for all the analytes was evaluated over 5 days using a six-point calibration curve in the range 10–1000 ng/mL. Calibration models were observed to be

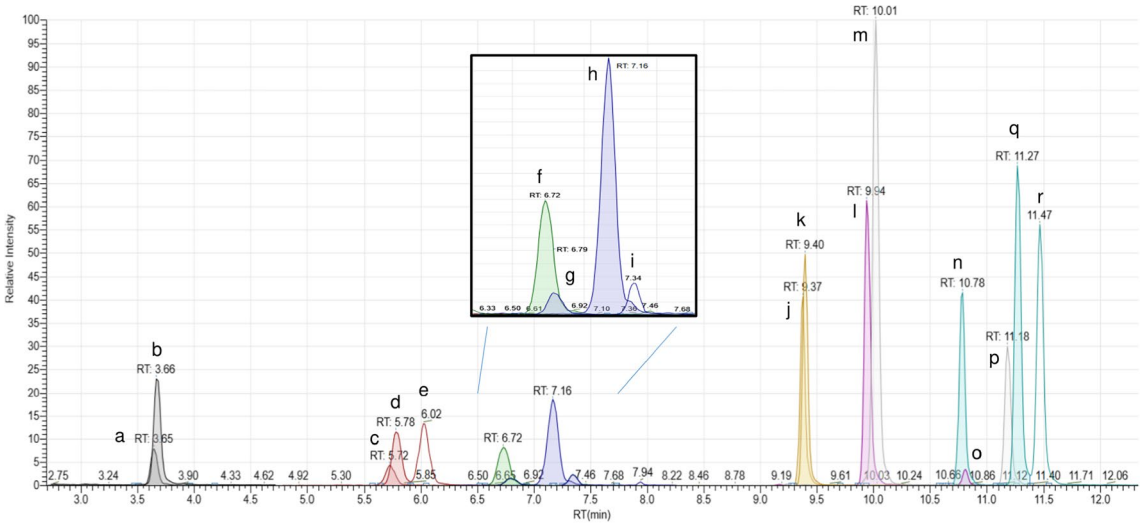

Fig. 1 Extracted ion chromatogram of the studied compounds at a concentration of 100 ng/mL obtained by using the Waters Acquity™ BEH Shield RP18 column (2.1 × 100 mm, 1.7 µm) held at 20 °C. Gradient elution at 0.5 mL/min was used for separation using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) (a, 7-COOH-CBD-d_3; b, 7-COOH-CBD; c, 11-OH-Δ^9-THC-d_3; d, 11-OH-Δ^9-THC; e, 11-OH-Δ^8-THC; f, Δ^8-THC-COOH-d_3; g, Δ^8-THC-COOH; h, Δ^9-THC-COOH-d_9; i, Δ^9-THC-COOH; j, CBD-d_3; k, CBD; l, CBG-d_9; m, CBG; n, CBN-d_3; o, CBN; p, Δ^9-THC-d_9; q, Δ^9-THC; r, Δ^8-THC)

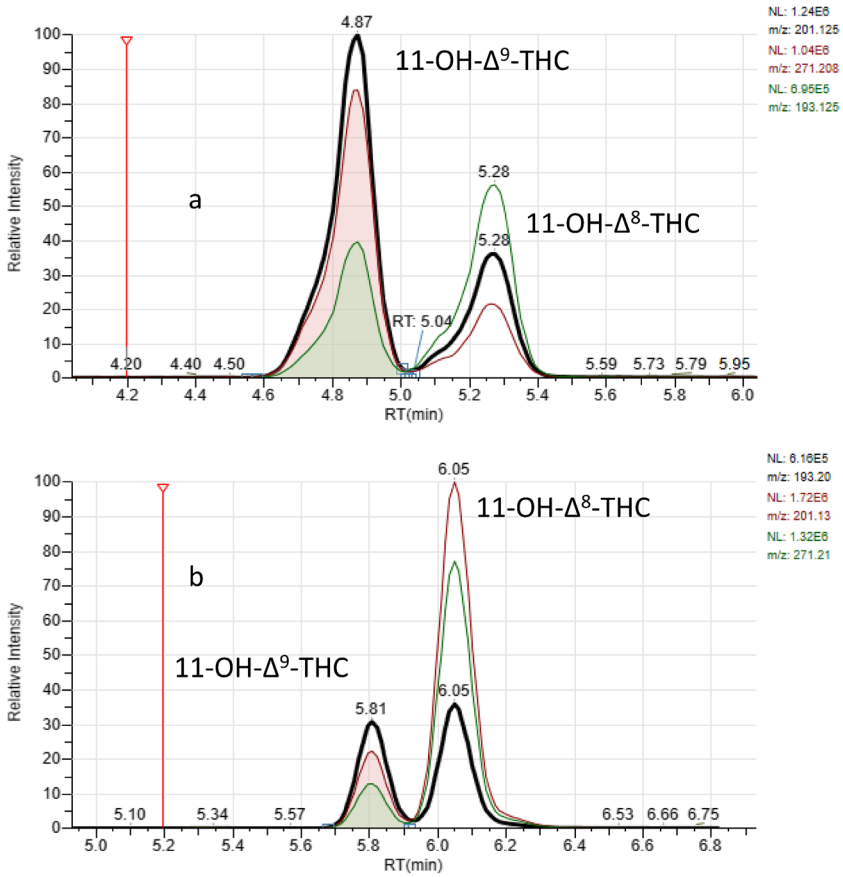

Fig. 2 Extracted ion chromatograms of 11-OH-Δ^9-THC and 11-OH-Δ^8-THC at a concentration of 500 ng/mL obtained by using the fluorophenyl phase analytical column (Restek Raptor™ FluoroPhenyl, 2.7 × 100 mm, 3.0 µm) (Bellefonte, PA, USA) (**a**), and the C18 phase analytical column (Waters Acquity™ UPLC BEH Shield RP18, 2.1 × 100 mm, 1.7 µm) (**b**) along with the use of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B)

linear with $1/\times^2$ weighting for Δ^8-THC, Δ^9-THC, 11-OH-Δ^8-THC, 11-OH-Δ^9-THC, Δ^8-THC-COOH, Δ^9-THC-COOH, CBD, 7-COOH-CBD, and CBG, and linear with $1/\times$ weighting for CBN, with a mean coefficient of determination (r^2)

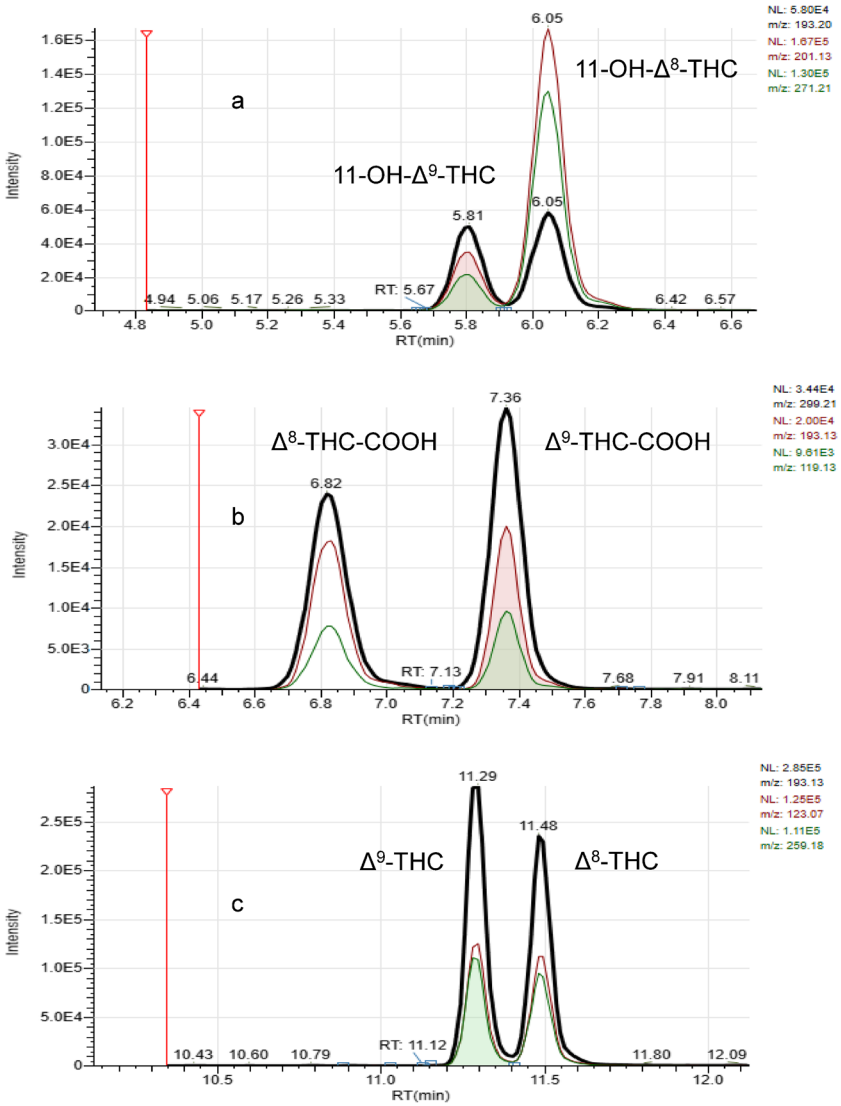

Fig. 3 Extracted ion chromatograms and baseline separation of the isomers 11-OH-Δ^9-THC and 11-OH-Δ^8-THC (**a**), Δ^8-THC-COOH and Δ^9-THC-COOH (**b**), and Δ^9-THC and Δ^8-THC (**c**) obtained by using the BEH Shield RP18 column (2.1 × 100 mm, 1.7 µm) held at 20 °C, 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B)

of 0.998 for all compounds. The LOD and LLOQ were set at 10 ng/mL, while the ULOQ was set at 1000 ng/mL. Dilution integrity results were within ± 20% of the target concentration and deemed acceptable. Precision tests for the within-run (intraday), between-run (interday), and accuracy (bias) were evaluated at three concentrations over five days in triplicate. The results for all the analytes were within the acceptable ± 20% criteria range (Table 2). The stability of the extracts that had been stored at 10 °C for 72 h in the autosampler ranged from −8.0 to 16.8%, and within ± 20% of the concentrations at t_0.

Authentic samples

Urine samples collected over various time points from female patients ($N = 69$) enrolled in a study on assessing prevalence of marijuana/CBD use during pregnancy from November 2022 to May 2024 were analyzed using the validated method (Table 3). According to the demographic and self-report surveys, the case series consisted of both positive and negative (control) urine samples. Δ^9-THC-COOH was found to be the most commonly detected metabolite ($n = 43$) and quantitated in 38 cases (55.1%), with a concentration range of 12.6–6520 ng/mL (median 278 ng/mL; mean ± S.D. 697 ± 1220). 11-OH-Δ^9-THC was identified in 37 cases and quantitated in 34 cases (49.3%), with a concentration range of 12.4–2980 ng/mL (median 65.7 ng/mL; mean ± S.D. 249 ± 555), while the parent Δ^9-THC was detected in 22 cases and quantitated in 5 cases (7.2%), with a concentration range of 12.7–76.2 ng/mL (median 42.4 ng/mL; mean ± S.D. 44.0 ± 27.1). Note that highly concentrated samples were evaluated after 10× dilution. Of the 69 samples analyzed, 3 cases (4.3%) contained Δ^8-THC-COOH at a concentration of 15.6 ng/mL, 25.5 ng/mL, and 679 ng/mL, respectively. In particular, for two cases Δ^8-THC-COOH was found along with 11-OH-Δ^9-THC and Δ^9-THC-COOH,

Table 2 LOQ, bias, and precision results from method validation studies

						Precision (%CV)					
Compound name	LLOQ (ng/mL)	ULOQ (ng/mL)	Bias (%)			Intraday			Interday		
			LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
Δ^8-THC	10	1000	−11.4	−6.9	−8.9	1.8 to 5.5	0.4 to 1.6	0.7 to 4.5	4.1	1.9	4.0
11-OH-Δ^8-THC	10	1000	−0.2	4.4	3.5	0.9 to 4.4	0.4 to 3.0	1.4 to 3.2	4.0	1.7	3.1
Δ^8-THC-COOH	10	1000	−7.7	−13.3	−15.2	3.2 to 12.3	0.4 to 6.0	0.6 to 8.7	9.6	9.3	10.1
Δ^9-THC	10	1000	−15.8	−11.6	−13.4	1.1 to 6.0	0.5 to 1.6	0.3 to 4.5	4.2	2.0	3.9
11-OH-Δ^9-THC	10	1000	−5.9	−2.4	−3.7	1.4 to 5.2	0.3 to 3.2	1.0 to 3.1	3.7	2.5	3.4
Δ^9-THC-COOH	10	1000	0.9	1.9	1.8	1.3 to 8.1	1.6 to 2.6	0.8 to 3.9	6.4	2.4	3.6
CBD	10	1000	−12.4	−10.9	−9.9	0.2 to 2.3	0.7 to 2.9	0.7 to 3.2	2.9	2.2	3.4
7-COOH-CBD	10	1000	−11.0	−19.6	−19.5	4.8 to 13.2	4.0 to 13.0	2.8 to 13.3	10.2	12.3	11.4
CBG	10	1000	18.1	−13.2	−14.8	2.5 to 4.6	1.0 to 2.8	1.7 to 5.1	2.9	3.6	5.5
CBN	10	1000	−2.8	−6.9	−7.6	6.3 to 17.2	1.2 to 4.4	1.9 to 10.2	8.8	2.6	3.2

Table 3 Quantitative data for cannabinoids in the authentic urine samples

Authentic specimens	Δ^9-THC (ng/mL)	11-OH-Δ^9-THC (ng/mL)	Δ^9-THC-COOH (ng/mL)	Δ^8-THC-COOH (ng/mL)	CBD (ng/mL)	7-COOH-CBD (ng/mL)	CBG (ng/mL)
$N = 69$	$n = 5$	$n = 34$	$n = 38$	$n = 3$	$n = 1$	$n = 2$	$n = 10$
Min	12.7	12.4	12.6	15.6	54.2	22.8	12.4
First quartile (Q1)	21.9	43.5	97.7	20.6	54.2	26.4	26.8
Median	42.4	65.7	278	25.5	54.2	30.0	49.4
Third quartile (Q3)	66.8	128	641	352	54.2	33.6	95.9
Max	76.6	2980	6520	679	54.2	37.2	233

while in only one case it was observed alone at a concentration of 15.6 ng/mL. CBG was detected in 28 cases and confirmed in 10 cases (14.5%), with a concentration range of 12.4–233 ng/mL (median 49.4 ng/mL; mean ± S.D. 75.3 ± 71.0). CBD was observed in four cases at a concentration < LLOQ, and quantitated in only one case (1.4%) at a concentration of 54.2 ng/mL. In this same case, Δ^9-THC (76.2 ng/mL), 11-OH-Δ^9-THC (2980 ng/mL), Δ^9-THC-COOH (6520 ng/mL), CBG (151 ng/mL), and 7-COOH-CBD (37.2 ng/mL) were also quantitated. Additionally, 7-COOH-CBD was detected in 3 cases (4.3%) at a concentration of 22.8 ng/mL and 37.2 ng/mL in two, and at a concentration < LLOQ in one case. The extracted ion chromatograms of a blank and authentic case are shown in Fig. 4.

Discussion

The sample preparation consisted of a simple dilution and filtration, allowing to collect the eluent directly in an autosampler vial, and making the method a rapid, simple, and inexpensive alternative for the analysis of cannabinoids in urine specimens. Although the use of a single sample dilution may limit the quantifiable range [24], this method offered appropriate sensitivity, linearity, and recovery for the considered casework and toxicology purposes.

The Δ^9-THC metabolites 11-OH-Δ^9-THC and Δ^9-THC-COOH are known to be extensively conjugated via glucuronidation [25]. Hence, alkaline, enzymatic, and/or tandem hydrolysis may be required for complete and specific analysis of cannabinoids in urine [26]. The glucuronide de-conjugation for ester Δ^9-THC-COOH is achieved with alkaline hydrolysis, while cleavage of ether Δ^9-THC and 11-OH-Δ^9-THC glucuronides usually require additional enzymatic treatments of the samples [27, 28]. Therefore, the tandem enzyme-alkaline hydrolysis was chromatographic separation was studied by testing different analytical evaluated to achieve the optimum condition for overall recovery of the analytes.

The use of LC-MS/MS for the quantitative determination of cannabinoids in biological specimens represents a valid analytical technique and an alternative to the more common GC-MS. GC-MS analysis has known limitations such as required derivatization steps to quantitate the acidic cannabinoids [24, 29, 30]. This aspect is often

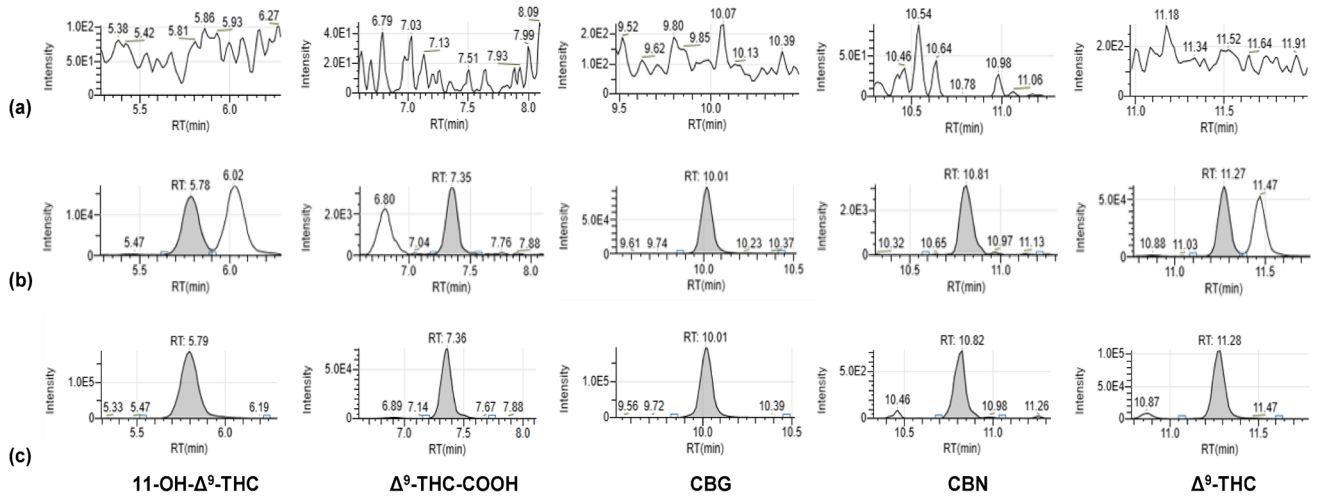

Fig. 4 Extracted ion chromatogram of a blank (**a**), the LQC (30 ng/mL) (**b**), and an authentic urine specimen tested positive for 11-OH-Δ^9-THC (405 ng/mL), Δ^9-THC-COOH (646 ng/mL), CBG (88 ng/mL), CBN (< LLOQ), and Δ^9-THC (47.8 ng/mL) (**c**)

viewed as disadvantageous and time-consuming, while LC-MS methods provide an alternative to these GC techniques [31, 32].

The chromatographic separation was studied by testing different analytical conditions and parameters. For the method optimization, the negative electrospray ionization mode was evaluated versus the positive mode. Although the negative mode is the primary choice when analyzing for compounds having hydroxyl and/or carboxylic groups [33], during development it was noticed that the positive mode provided more sensitivity than the negative mode, making it preferential for analysis. Different analytical columns and elution gradients were evaluated and further optimized for improved analyte retention and selectivity. Given the performances reported in the literature in providing good selectivity and retention of cannabinoids and the isomers [11, 34], a fluorophenyl phase LC column (2.7 × 100 mm, 3.0 µm) was tested. The fluorophenyl chemistry is commonly suggested when limited retention and selectivity are observed on a C18 phase analytical column for the separation of challenging isomers. However, difficulties emerged especially in adapting the gradient to ensure optimal separation of the compounds. Moreover, where separation was acceptable, fronting and asymmetrical peak shapes were shown as seen in Fig. 2a. Acceptable resolution was achieved with the C18 phase column. The C18 stationary phase is one of the most well-known for liquid chromatography applications in providing good performance for analysis of a wide variety of compounds. Given the popularity of the use of this phase, this type of column is usually available in most laboratories equipped with LC-MS systems, and methods utilizing this chemistry for the separation of the THC isomers are reported in the literature [14, 35, 36]. Preliminary test injections showed better symmetry and peak shape of the analytes (Fig. 2b), hence the decision to continue optimization of the gradient with this type of column.

The ambiguity of the legislation concerning cannabis-related products has emphasized the importance and the need for the detection and separation of Δ^8- and Δ^9-THC isomers in routine casework. However, analytical methods for Δ^9-THC and its metabolites are not always appropriately optimized to fully resolve the isomer peaks [14]. Recently, authors have dealt with the topic of separation of Δ^8- and Δ^9-isomers, but there is limited literature which includes data on the separation of 11-OH-Δ^9-THC and 11-OH-Δ^8-THC [37]. In Reber et al. [14], 11-OH-Δ^9-THC was initially included in the method and a co-eluting peak was observed by targeting the *m/z* ion ratio 193.3 that was presumptively caused by the interference of 11-OH-Δ^8-THC. However, the authors reported that certified reference material for 11-OH-Δ^8-THC was not available at the time of method development, and further characterization was required. Recently, Sempio et al. developed and validated a method for the simultaneous quantitation of 13 cannabinoids and metabolites by LC-MS/MS including the THC major metabolites and isomers [38]. The assay was used to analyze human plasma samples (n = 534) collected as part of a double-blind, placebo-controlled clinical trial for the use of Δ^8-tetrahydrocannabivarin (Δ^8-THCV) [39]. The authors tested different chromatographic columns and stationary phases, aiming to separate the isoforms of THC and THCV. Optimum baseline separation for the 11-OH-THC isomers was not achieved. Therefore, the acceptance criteria for validation of 11-OH-Δ^8-THC were not deemed acceptable. The validated LC-MS/MS method described in the present work allowed for the quantitation of a broad panel of cannabinoids in urine specimens and complete chromatographic separation of THC isomers and metabolites.

The authentic urine samples included in this work were collected and submitted to the forensic toxicology laboratory for analysis in the context of a study on assessing the prevalence of marijuana/CBD use during pregnancy. Cannabis is the most frequently used drug during pregnancy and merging data from obstetrics care suggest an increasing number of patients using marijuana and cannabidiol (CBD) for relief of pregnancy-related symptoms [40]. To assess use behaviors of medical marijuana, non-medical marijuana, and CBD, anonymous self-report surveys are often used, although this approach may not always provide an accurate and reliable evaluation [41]. Hence, the importance of the laboratory analysis in assisting and determining cannabis exposures during pregnancy. It is worth noting that quantitative values in urine are typically not toxicologically relevant, as the drugs or metabolites that are detected in this matrix may not be associated with a pharmacological effect. However, the use of urine for the quantitation of cannabinoids was deemed appropriate in determining cannabis exposures during each trimester of pregnancy and at delivery.

The limitations of this study may be the specific study population considered, as it was not reflective of the general public, and the scarce, or not always available demographic information and history for some of the participants.

Conclusions

Due to the number of cannabis-related products that have become available and increase in recreational use, a rapid sample preparation along with an LC-MS/MS assay was developed and validated to quantitate a broad panel of cannabinoids in urine specimens. The sample run time of the described assay, comparable to similar methods previously reported in the literature, was required to achieve effective chromatographic separation and resolution for all the Δ^8- and Δ^9- isomers and metabolites. The results demonstrate the importance of developing and optimizing methods for the proper separation and accurate identification of THC isomers in future casework.

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Jennifer L. Hoyer: resources; supervision; writing—review and editing.

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Deepthi S. Varma: investigation; project administration; resources; visualization.

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Kay Roussos-Ross: funding acquisition; project administration; resources; supervision.

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Declarations

Research involving human participants and/or animals Authentic urine specimens were obtained from the obstetrics clinics following a prospective, anonymous self-report survey to assess use behaviors of medical marijuana, non-medical marijuana, and CBD. The study protocol including the collection of urine samples was approved by the Institution Review Board of the University of Florida (IRB#: 202201895).

Conflict of interest The authors declare no competing interests.

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