JOTCS A
Journal of the Turkish Chemical Society
Section: A
Chemistry
TURKISH CHEMICAL SOCIETY

Simultaneous determination of 4F-MDMB BINACA, a new synthetic cannabinoid, and its metabolites in human blood samples by LC-MS/MS

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Abstract: Methyl 2-(1-(4-fluorobutyl)-1H-indazole-3-carboxamide)-3,3-dimethylbutanoate), also referred to as 4F-MDMB BINACA (**M0**), is a recently introduced synthetic cannabinoid (SC) that was identified in herbal blends submitted to the Istanbul Narcotics Laboratory of Council of Forensic Medicine (CFM), in March 2019. A sensitive analytical method was developed to be able to detect and quantify 4F-MDMB BINACA (**M0**) and its two metabolites, 4F-MDMB BINACA {3,3-dimethylbutanoic acid ((S)-2-(1-(4-fluorobutyl)-1H-indazole-3-carboxamido)-3, 3-dimethylbutanoic acid)} (**M1**), and 4F-MDMB BINACA-*N*-4-hyroxybutyl (methyl (S)-2-(1-(4-hydroxybutyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate) (**M2**) in blood samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The samples were prepared using a solid-phase extraction method. The method validation was performed in terms of linearities, limits of detection (LODs), limits of quantification (LOQs), recoveries, matrix effects, process efficiencies, accuracies, and precisions, was also applied to six blood samples from cases of autopsy in the CFM, Istanbul.

Keywords: Synthetic cannabinoid, LC-MS/MS, validation.

Submitted: July 16, 2020. **Accepted:** October 06, 2020.

Cite this: Yeter O. Simultaneous determination of 4F-MDMB BINACA, a new synthetic cannabinoid, and its metabolites in human blood samples by LC-MS/MS. JOTCSA. 2020;7(3):827–32.

DOI: https://doi.org/10.18596/jotcsa.770427.

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INTRODUCTION

SCs are produced to imitate the effects of THC (Tetrahydrocannabinol, the major alkaloid of cannabis) in illegal laboratories and marketed as legal marihuana. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), they are the largest New Psychoactive Substance (NPS) group and they have been traded by street names such as "K2", "Bonsai", and "Spice" since 2004 and labeled with "research chemical", "not for human consumption", and "fertilizer" to circumvent the laws since 2004 (1). SCs are often highly potent substances and have been reported to have additional negative effects. Although the pharmacokinetic and pharmacodynamic properties of synthetic cannabinoids are not fully known, most synthetic cannabinoids are strong CB1 agonists, and their affinity for cannabinoid receptors is known to be higher than cannabis, thereby producing longer-lasting, stronger side effects. Among the acute effects of SC, agitation, anxiety, confusion, hypertension, sedation, psychosis, hallucination, and tachycardia have been reported. Fatal and/or nonfatal SC intoxication of cases have been reported (2–10).

4F-MDMB BINACA (**M0**) is a newly appearing synthetic cannabinoid in the drug market. This compound is structurally similar to 5F-ADB (5F-MDMB PINACA), differing by the removal of one-carbon (-CH_2) linkage from the carbon tail of the molecule. After the first report of 4F-MDMB BINACA to the Early Warning System of the EMCDDA, it was added to the European information system and database on new drugs (EDND) in November 2018 (11). Although no detailed information about the toxicological effect of 4F-MDMBBINACA (**M0**), drug-

users report it that (**M0**) causes SCRA (synthetic cannabinoid receptor agonist)-like effects (12).

Krotulski et al. identified (**M0**) in herbal samples. They also detected (**M0**) and/or its metabolites in human blood and urine samples collected from toxicology cases (13). The metabolism of (**M0**) was reported by Haschimi et al. They identified in vivo and in vitro metabolites of (**M0**) using authentic samples of human urine and an assay of pooled human hepatic microsomes (pHLM) (14).

After the identification of the (**M0**) in an herbal sample, analyzed by Istanbul Narcotics Laboratory of the CFM, a sensitive analytical method is needed to identify (**M0**) in human blood specimens to monitor its consumption. The main objective of the study was to develop and validate a liquid chromatography-tandem mass spectrometric method for simultaneous detection and quantification of (**M0**) and its metabolites, namely 4F-MDMB BINACA 3,3-dimethylbutanoic acid (**M1**), and 4F-MDMB BINACA-*N*-4-hydroxybutyl (**M2**) in blood samples (Figure 1). This method was also applied to the postmortem blood samples taken from cases of autopsy submitted to the CFM.

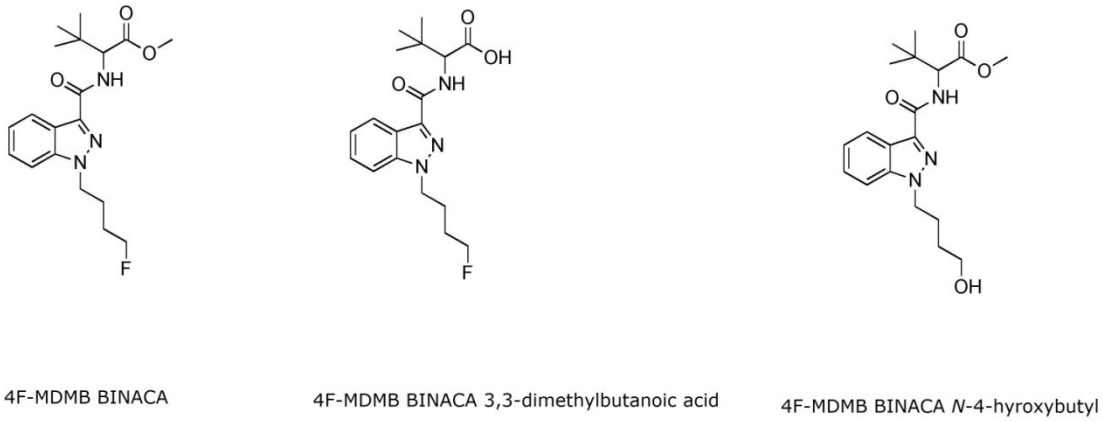

Figure 1: Chemical structure of 4F-MDMB BINACA (**M0**) and its two metabolites, (**M1**) and (**M2**).

MATERIALS AND METHODS

Chemicals and reagents

4F-MDMB BINACA (**M0**), 4F-MDMB BINACA 3,3-dimethylbutanoic acid (**M1**), and 4F-MDMB BINACA *N*-4-hyroxybutyl (**M2**) metabolites were procured from Cayman Chem. (AnnArbor, Michigan, USA). All the organic solvents and water were of LC-MS grade; they were provided by Merck (Darmstadt, Germany) and formic acid (≥98.0%) and ammonium acetate (≥99.0%) were used in the chromatographic analysis, supplied by Sigma-Aldrich (Steinheim, Germany); lastly, OASIS HLB cartridges were obtained from Waters (Milford, MA, USA).

Liquid chromatography-tandem mass spectrometry

LC-MS/MS system consisted of an ultra high-performance liquid chromatography (Shimadzu Nexera X2 LC-30AD) coupled with Shimadzu 8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The analytes were separated using an Agilent Poroshell 2.7-µm (150 × 4.6 mm) column (Agilent, CA, USA)at 40 °C using 15 min gradient elution with 0.6 mL/min flow rate. The mobile phase consisted of 5 mM ammonium acetate containing 0.1% formic acid in water (mobile phase A) and methanol (mobile phase B). The initial mobile phase composition was 10% B (0-0.3 min), increasing to 80% B (0.3 to 3 minutes), increasing to 95% B (3 to 7 minutes), held constant at 95% B for 4 min (7 to 11 minutes), decreasing back to 10% B (11 to 11.1 minutes) and held constant at 10 % (11.1-15 min). All analytes were analyzed using the positive ESI-multiple reaction monitoring (MRM) mode, with the following source parameters: heating gas: 250 °C, heat block temperature:400 °C, interface temperature: 300 °C, heating and drying gas flow: 10 L/min and nebulizing gas flow: 3 L/min. The MRM transitions with the corresponding collision energies for all the analytes and IS are presented in Table 1.

Table 1: LC-MS/MS parameters for (M0), (M1), (M2), and AB PINACA-d_9.

Analytes	Parent ions (m/z)	Product ions (m/z)	Collision energy (eV)
4F-MDMB-BINACA	364.00	219.20*	-27
		145.10	-42
		304.20	-17
4F-MDMB-BINACA 3,3-dimethylbutanoic acid	350.00	219.10*	-25
		145.10	-40
		304.20	-15
4F-MDMB BINACA *N*-4-hyroxybutyl	362.00	145.10*	-40
		217.10	-25
AB PINACA-d_9	340.00	224.20	-28
		295.30	-16
		146.10	-40

*Quantitative ion.

Standard solutions

The main analyte stock solutions of (**M0**), (**M1**), and (**M2**) were prepared at 1000 µg/mL in methanol. The working solutions, at 0.1–500 ng/mL concentration, were prepared by proper dilution from the primary stock solutions in a daily manner. The IS solution was 500 ng AB PINACA-d_9/mL methanol. All standard and IS solutions were stored at -20 °C and waited for 20 min at ambient temperature before use.

Sample preparation

All blood samples were prepared using solid-phase extraction (OASIS HLB 3 cc, 60 mg). Blood samples were added with 10 µL of the IS solution and diluted with 2 mL of water. After vortexing the samples, they were centrifuged at 5000 rpm for 10 min. The SPE was performed as follows: conditioning: 2 mL X 2 ethyl acetate, 2 mL x 2 methanol, and 2 mL X 2 distilled water, sample loading onto the cartridge, washing: 2 mL of 5% methanol (in water,v/v), drying for 10 min using nitrogen stream, and elution: 2 x 0.5 mL methanol and 2 x 0.5 mL ethyl acetate. All eluates were evaporated at 40 °C using nitrogen stream, reconstituted in 0.5 mL of the mobile phase A/B (80:20 v/v) mixture, and 5 µL of aliquot was injected to LC-MS/MS.

Validation of the analytical method

The method validation was carried out using drug-free human blood samples spiked with analytes according to international guidelines (15,16). Validation parameters were studied as follows: selectivity, linearity, detection, and quantification limits (LOD, LOQ), intra- and inter-day accuracy and precision, recovery, matrix effect, and process efficiency. Selectivity was performed by analyzing of drug-free blood from five different sources and any interferences at the retention times of analytes and IS were checked. To assess the linearity, seven calibration standards (0.05, 0.1, 0.5, 1.0, 2.5, 5.0, and 10.0 ng/mL) were analyzed in triplicate at each concentration in the blood samples.

The calibration curves of analytes were required to correlation coefficient (R^2) of 0.995 and calibrators were± 15% deviation from the nominal value. LOD and LOQ were calculated by analyzing the spiked blood samples at the lowest concentrations (n=10). The precision and accuracy of the method were determined through the analysis of low (0.05 ng/mL), medium (2.5 ng/mL), and high (10 ng/mL) level quality control samples prepared in from drug-free blood, with ten replicates per level. Inter-assay precision and accuracy were calculated from triplicates per run on five days in a consecutive manner. Recovery, matrix effect, and process efficiency were estimated at low, medium, and high concentrations (n=6) using Matuszewski's approach (17).

Application to real samples

The regional blood donation center provided blank human blood samples. Postmortem blood samples were collected from the cases of autopsy performed in CFM, Istanbul, according to 5271/87-89 (Turkish Criminal Procedure). No blood samples were taken specifically for the study. Blank and postmortem blood samples were stored at -20 °C until the time of analysis. In this analysis, postmortem blood samples of cases containing (**M0**) and/or its metabolites (**M1**, **M2**) (n=6) were used.

Table 2: Validation data of the developed method.

Parameter	4F-MDMB BINACA	4F-MDMB-BINACA butanoic acid	4F-MDMB BINACA N-4-hyroxybutyl
Intra-assay precision (RSD %)			
0.05 ng/mL	2.1	2.9	2.9
2.5 ng/mL	1.4	3.3	3.5
10.0 ng/mL	2.9	2.3	1.9
Intra-assay accuracy (%)			
0.05 ng/mL	99.6	99.4	99.6
2.5 ng/mL	91.6	98.1	95.6
10.0 ng/mL	107.1	101.3	98.6
Inter-assay precision (RSD %)			
0.05 ng/mL	2.1	8.7	7.9
2.5 ng/mL	6.4	8.5	7.7
10.0 ng/mL	3.5	5.8	3.6
Inter-assay accuracy (%)			
0.05 ng/mL	99.7	94.8	96.3
2.5 ng/mL	93.6	96.5	94.1
10.0 ng/mL	102.5	99.8	99.9
Matrix effect (%)			
0.05 ng/mL	91.1	105.2	113.7
2.5 ng/mL	100.3	102.1	107.2
10.0 ng/mL	104.9	99.7	109.4
Recovery (%)			
0.05 ng/mL	95.7	97.5	93.3
2.5 ng/mL	86.6	84.1	87.1
10.0 ng/mL	83.1	87.3	85.9
Process efficiency (%)			
0.05 ng/mL	87.2	100.1	106.1
2.5 ng/mL	86.9	85.8	93.3
10.0 ng/mL	87.2	87.0	94.6
LOD	0.02	0.05	0.02
LOQ	0.05	0.1	0.05

RESULTS and DISCUSSION

Analytical Method Validation

Table 2 shows the method validation parameters. Selectivity was studied by analyzing the blank blood samples, and any interfering peaks were not detected at the retention times for the analytes and IS. 4F-MDMB BINACA (**M0**), 4F-MDMB BINACA 3,3-dimethylbutanoic acid (**M1**), 4F-MDMB BINACA *N*-4-hyroxybutyl (**M2**), and AB PINACA-d_9 (IS) were eluted at 8.0, 7.1, 7.4, and 8.0 minutes, respectively (Figure 2). The matrix-matched calibration standards were prepared in the range 0.05–10.0 ng/mL, with a coefficient of determination (R^2) that was greater than 0.995. The calibration curves were established with (1/x) linear regression model for all analytes. The intra- and inter-assay precisions and accuracies of the method were within the range of 1.4-3.5% and2.1–8.5% and 91.6–107.1% and 93.6–102.5%, respectively. The LOD and LOQ values were at the range of 0.02–0.05 ng/mL, and 0.05–0.1 ng/mL, respectively. The intra- and inter-day precision and accuracy of the method were acceptable with CV values below 10% and bias values below 10%. The recovery (83.1–97.5%), matrix effect (91.1-109.4%), and process efficiency (85.8–106.1%) are presented in Table 2. These findings suggest that the internal standard provides appropriate matrix match compensation and remarkable extraction recovery.

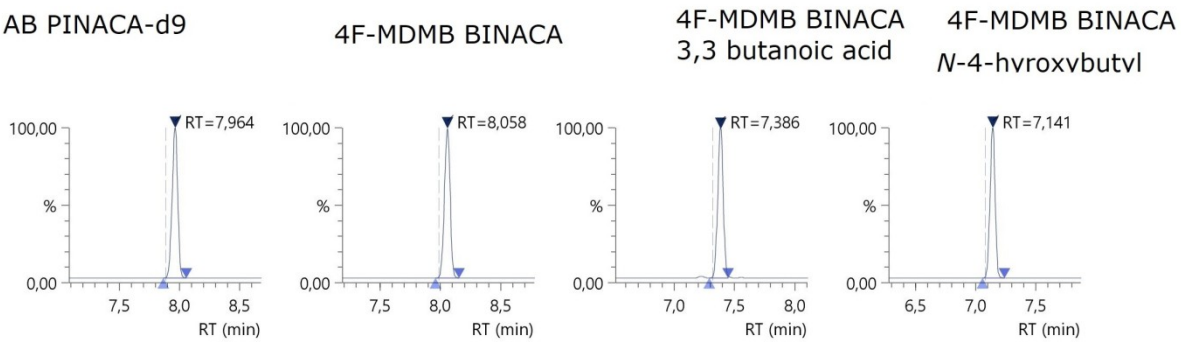

Figure 2: MRM chromatograms of (**M0**), (**M1**), (**M2**), and AB PINACA-d_9 for spiked matrix blank blood with 0.05 ng / mL concentration.

Application to real samples

The developed method was applied to the identification and quantification of (**M0, M1, and M2**) in postmortem blood samples collected from the cases of autopsy. We detected and quantified the (**M0**) and/or its metabolites (**M1**) and (**M2**) and in blood samples taken from six cases of autopsy. All of the cases were male and aged from 21 to 39 (mean: 31); (**M0**) was detected in 3 of 6 blood, with the concentration ranged from 0.10 to 2,90 ng/mL (mean: 0.42 ng/mL).(**M2**) was detected in 1 of 6 blood samples (0.21 ng/mL). (**M1**) was detected in all blood samples with a range of 0.12-9.05 ng/mL and a mean of 3.15 ng/mL. In this study, detecting and quantification of (**M0**) and its two metabolites (**M1, M2**) in postmortem blood samples is reported.

A few reports were published about 4F-MDMB BINACA and its metabolites and their identification in biological samples. Krotulski et al. reported the identification of (**M0**) and/or its metabolites in blood and urine samples (13). According to their study, (**M1**) was the most significant metabolite in blood and urine addition to (**M2**). (**M1**) was found to be a sensitive and specific urinary marker (14). However, blood concentration levels of (**M0**) and its metabolites were not reported in either of these studies.

CONCLUSIONS

The detection of (**M0**) in postmortem cases indicates a significant worrying alarm about the emergence of this substance. A sensitive LC-MS/MS method was developed and validated for the detection and quantitation of (**M0**), and its two metabolites (**M1**), and (**M2**) in the blood. The method was applied to six postmortem blood samples collected from the cases of autopsy. According to the results obtained, the method can be considered to be sensitive, reliable, and suitable for the analysis of postmortem blood samples. To the best of the author's knowledge, this is one of the first reports of quantification of the (**M0, M1, M2**) in postmortem blood samples.

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