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New extraction method of THC and its metabolites, 11-OH-THC and THC-COOH, in plasma

Nouvelle méthode d'extraction du THC et ses métabolites, 11-OH-THC et THC-COOH dans le plasma

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Abstract – Objectives: A liquid/liquid extraction technique on solid support of Δ^9-tetradydrocannabinol (THC), 11-hydroxy-Δ^9-tetradydrocannabinol (11-OH-THC) and 11-nor-Δ^9-tetradydrocannabinol-9-carboxylic acid (THC-COOH) in plasma was developed in order to be assayed by high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS). **Methods:** The samples were extracted by liquid/liquid extraction over solid support of an extraction cartridge. The extracts were thereafter dried down and injected into the HPLC-MS/MS system set with a positive electrospray mode using a Waters XTerra MS C18 3.5-µm 2.1 × 150 mm column. **Results:** The extraction recovery levels were 66%, 70% and 71% for THC, and 75%, 93% and 101% for 11-OH-THC at concentrations of 2.5, 5 and 10 ng/mL, respectively. They were 86% and 78% for THC-COOH at concentrations of 5 and 10 ng/mL. The limits of detection (LOD) were 0.09, 0.08 and 0.91 ng/mL for THC, 11-OH-THC and THC-COOH, respectively. The limits of quantification (LOQ) were 0.16, 0.15 and 3.24 ng/mL for THC, 11-OH-THC and THC-COOH, respectively. The inter-series incertitude CV determined for concentrations of 1, 2.5 and 10 ng/mL were 12.1%, 12.0% and 6.4% for THC, 14.5%, 11.1% and 7.2% for 11-OH-THC, and 14.9%, 26.2% and 11.3% for THC-COOH. **Conclusion:** The novel extraction method for THC, 11-OH-THC and THC-COOH developed in this work is rapid, sensitive and specific. It may be a valuable tool for predictive toxicology, high-throughput metabolism and pharmacokinetic studies of cannabinoids.

Key words: THC, THC-COOH, 11-OH-THC, extraction, HPLC-MS/MS, cannabis

Résumé – Objectifs : Une technique d'extraction liquide/liquide sur support solide du Δ^9-tetradydrocannabinol (THC), 11-hydroxy-Δ^9-tetradydrocannabinol (11-OH-THC) et de l'acide 11-nor-Δ^9-tetradydrocannabinol-9-carboxylique (THC-COOH) dans le plasma est développée afin d'être dosés par chromatographie liquide haute pression couplée à une spectrométrie de masse en tandem (HPLC-MS/MS). **Méthodes :** L'échantillon est extrait par extraction liquide/liquide sur support solide, puis séché et injecté dans le système HPLC-MS/MS en mode electrospray positif et sur une colonne Waters XTERRA MS C18 3,5 µm 2,1 × 150 mm. **Résultats :** Les rendements d'extraction sont de 66 %, 70 % et 71 % pour le THC, 75 %, 93 % et 101 % pour le 11-OH-THC aux concentrations de 2,5, 5 et 10 ng/mL et de 86 % et 78 % pour le THC-COOH aux concentrations de 5 et 10 ng/mL. Les limites de détection (LOD) sont de 0.09 ng/mL pour le THC, 0,08 ng/mL pour le 11-OH-THC et 0,91 ng/mL pour le THC-COOH. Les limites de quantification (LOQ) sont de 0,16 ng/mL pour le THC, 0,15 ng/mL pour le 11-OH-THC et 3,24 ng/mL pour le THC-COOH. Les CV d'incertitude inter-séries, déterminés aux concentrations de 1, 2,5 et 10 ng/mL, sont de 12,1 %, 12,0 % et 6,4 % pour le THC, 14,4 %, 11,1 % et 7,2 % pour le 11-OH-THC et de 14,9 %, 26,2 % et 11,3 % pour le THC-COOH. **Conclusion :** La technique d'extraction du THC, 11-OH-THC et THC-COOH dans le plasma développée dans cette étude est rapide, sensible et spécifique. Il s'agit d'un outil utile pour les études de toxicologie prédictive, de métabolisme et de pharmacocinétique des cannabinoïdes.

Mots clés : THC, THC-COOH, 11-OH-THC, extraction, HPLC-MS/MS, cannabis

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1 Introduction

Cannabis is the most widely consumed illicit drug all over the world [1,2]; it can be administered orally or via inhalation. Cannabis has an agonist effect on CB1 receptors inducing behavioral effects such as euphoria, altered time perception and reduced concentration, as well as physical relaxation and reduced motor coordination. In some cases, cannabis consumers may develop panic reactions and paranoia [3,4].

Δ^9−tetrahydrocannabinol (THC) is the main psychoactive component of cannabis. Antiemetic, appetite-stimulating and analgesic effects are induced by THC binding to CB1 receptors [5].

In fact, some medicines are made of cannabis components (dronabinol, cannabidiol) and have been approved by North American and European agencies to treat multiple sclerosis-related neuropathic pain, anorexia in AIDS patients and nausea in cancer patients under chemotherapy [6,7].

Cytochrome P450 2C9, 2C19 and 3A4/5 [8–10] metabolize THC into multiple phase I metabolites. The principal metabolite of THC is 11-OH-THC; it has a psychoactive action and undergoes an additional oxidation forming 11-nor-Δ^9-carboxy-tetrahydrocannabinol (THC-COOH). Phase II metabolism reactions transform THC, 11-OH-THC and THC-COOH into glucuronide derivatives [5].

Detection of cannabis consumption is performed by identification of THC as well as its two most toxicologically relevant metabolites, 11-OH-THC and THC-COOH.

Monitoring and quantifying THC and its metabolite levels in plasma is of importance for forensic and predictive toxicology purposes. Secondly, a robust, easy and fast assay method for THC and its metabolites is very valuable for pharmacokinetic analysis in order to study the dose-response relationship of THC and its derivatives.

Many different assay methods for cannabis and its derivatives in biological matrices have been described. The first techniques that were used to detect and quantify cannabis components were gas chromatographic mass-spectrometric methods [6,7].

Liquid chromatography coupled to mass spectrometry (LC–MS) methods were first used in the 1990s and their use has increased tremendously in recent years [11–18]. The method for quantification of cannabinoids developed by Schwope *et al.* uses liquid chromatography tandem mass spectrometry (LC–MS/MS) and showed a limit of quantification (LOQ) of 1 ng/mL for THC, 11-OH-THC and THCCOOH [5].

Additionally to the latter method, König *et al.* used online solid-phase extraction for blood samples with a LOQ of 0.5 ng/mL for THC and 11-OH-THC, and 2.5 ng/mL for THC-COOH [18]. Finally, Ferreirós *et al.* developed a simultaneous LC-MS/MS method to assay THC and its metabolites in plasma after solid-phase extraction [17].

The objective of this study was to develop a robust, sensitive and rapid liquid/liquid extraction method on solid support of THC, 11-OH-THC and THC-COOH in plasma in order to be quantified by high-performance liquid-chromatography tandem mass spectrometry (HPLC-MS/MS).

The method was successfully applied to determine plasma levels of THC, 11-OH-THC and THCOOH in plasma for a patient of the hospital consuming inhaled cannabis.

2 Materials and methods

2.1 Materials and reagents

Solutions of Δ^9-tetradydrocannabinol (THC), 11-hydroxy- Δ^9-tetradydrocannabinol (11-OH-THC), 11-nor-Δ^9-tetradydrocannabinol-9-carboxylic acid (THC-COOH), THC-*d3,* 11-OH-THC-*d3* and THC-COOH-*d3* (internal standards) at a concentration of 1 µg/mL were purchased from LGC Standards (Molsheim, France) and stored at –20 °C. Working solutions were thereafter prepared in LC-MS-grade methanol (Merck, Germany). The calibrators were constructed with lyophilized human plasma (Lyotrol™ N Ref. 62373, Biomérieux, France) for control of normal levels of substrates, electrolytes and enzymes.

The migration solvents used for the liquid chromatography consisted of an ammonium bicarbonate buffer (10 mM) prepared in LC-MS-grade water (Sigma, Germany). Extraction solvents were composed of a mixture of hexane/ethylacetate 9/1 (V/V) (Merck, Germany).

2.2 Sample preparation

Calibration samples were prepared by spiking drug-free samples with standard solutions. The calibration range for THC and 11-OH-THC was performed using the following concentrations: 1, 2.5, 5 and 10 ng/mL. The calibration range for THC-COOH was obtained with the following four concentrations: 2.5, 10, 50 and 100 ng/mL. The exact volume of standard solutions of THC (1 ng/µL), 11-OH-THC (1 ng/µL) and THC-COOH (10 ng/µ L) was added to lyophilized human plasma, Lyotrol™, to reach a volume of 1mL. A control vial (blank) without THC, 11-OH-THC or THC-COOH was also included in the calibration range. All examined samples were supplemented with three deuterated internal standards, THC-*d3* (5 ng), 11-OH-THC-*d3* (5 ng) and THC-COOH-*d3* (50 ng). Samples were finally acidified by adding 200 µL of acetic acid (10%, V/V in water) prior to the extraction step.

The plasma control used was purchased from LGC Standards (Molsheim, France); its reference was BTMF 3/10 C. The plasma control contains amphetamines (amphetamine 24 ng/mL, methamphetamine 24 ng/mL, MDMA 24 ng/mL, MDEA 24 ng/mL and MBDB 24 ng/mL), cocaine derivatives (cocaine 11 ng/mL, benzoylecgonine 50 ng/mL and ecgonine methyl ester 10 ng/mL), opioids (codeine 10 ng/mL, morphine 10 ng/mL and dihydrocodeine 50 ng/mL) and THC and its metabolites (THC 1 ng/mL, 11-OH-THC 1 ng/mL and THC-COOH 9 ng/mL). Precision was also tested with the plasma control.

2.3 Extraction

A liquid/liquid extraction technique on solid support was set for plasma samples; the resulting tubes with sample solutions were vortexed (HEIDOLPH, Germany) for 2 min before being dropped off on a dry cartridge ChemElut – 1 mL Unbuffered (Agilent, USA). Then the solution sample was adsorbed and distributed into a thin film over the solid support

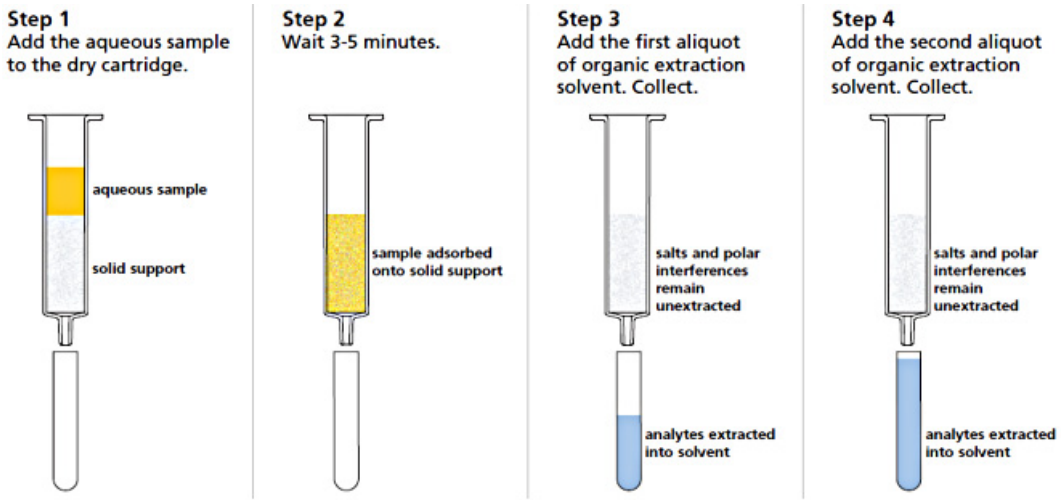

Fig. 1. Extraction procedure for THC, 11-OH-THC and THC-COOH.

of the cartridge (figure 1). After five minutes, necessary for complete adsorption, two aliquots of 2.5 mL hexane/ethyl acetate (9:1 V/V) solution were added to the cartridge, performing the extraction of the analytes from the aqueous layer. The extraction solvent containing the substances of interest was collected into centrifugation tubes and vortexed (HEIDOLPH, Germany). Finally, the extract was dried down using a drier centrifuge (RC1010 JOUAN, France) and the residues were re-dissolved by adding 150 µL of methanol. Ten microliters of the final solutions were then injected into the HPLC-MS/MS system.

2.4 LC-MS/MS analysis

The LC-MS/MS analysis was carried out on a Waters Alliance 2695 HPLC system with an XTerra C18 pre-column and an XTerra MS C18 3.5-µm 2.1 × 150 mm column (Waters, USA). A gradient mobile phase consisting of methanol and ammonium bicarbonate 10 mM buffer prepared in LC-MS-grade water was used for compound separation. The total run time was 26 min at a flow rate of 0.15 mL/min and the temperature of the oven was set at 33 ± 3 °C. Mass spectrometry detection was performed by a Waters Quatro-MicroQuadripole tandem mass spectrometer (Waters, USA) with electrospray ionization (ESI) performed in positive mode and two-transition multiple reaction monitoring (2MRM). The source temperature was set at 120 °C and the desolvation temperature was set at 350 °C. The desolvation and nebulizing gas had their flow rates set at 600 and 50 L/h, respectively. Quantification was performed by adding the three deuterated internal standards, THC-*d3,* 11-OH-THC-*d3* and THC-COOH-*d3*. The electrospray ionization (ESI) potentials and the different selected ion recordings are summarized in Table I. Data acquisition and evaluation were carried out using MassLynx 4.1 (Waters, USA).

The method validation procedure was conducted according to the guidelines published by the SFTA (*Société Française de Toxicologie Analytique*) [19]. The 2012 SFTA external plasma controls for THC and its metabolites will be presented.

3 Results and discussion

The electrospray in positive mode (ESI+) achieved good results for the three studied cannabinoids. The quantification was carried out employing the most abundant characteristic ion transitions. Table I shows the transitions and the different energies that were used. Blank samples containing only the internal standards showed no traces of the native cannabinoids (THC, 11-OH-THC and THC-COOH). The patient's spectra show the most abundant characteristic ions for the three studied compounds (figure 2).

Simple linear regression analyses were performed with calibration curves constructed from the peak signal area ratios. The calibration curves showed linear relationships for THC (r^2 = 0.998) and 11-OH-THC (r^2 = 0.991) for plasma concentrations from 0 to 10 ng/mL. The calibration curve also showed a linear relationship for THC-COOH from 0 to 100 ng/mL in plasma with a coefficient of correlation of 0.982 (Table II). The mean of 10 blanks + 3 SD (LOD) and mean of 10 blanks + 10 SD (LOQ) were used to calculate both the LOD and the LOQ. Limits of detection (LOD) of 0.09 ng/mL and 0.08 ng/mL were achieved for THC and 11-OH-THC, whereas the limit was 0.91 ng/mL for THC-COOH (Table II). The limits of quantification (LOQ) were 0.16 ng/mL for THC, 0.15 ng/mL for 11-OH-THC and 3.24 ng/mL for THC-COOH (Table II).

No interferences were observed with samples containing other drugs of abuse (*e.g.* control BTMF from LGC standards). In addition, other compounds used were tested (niflumic acid, acetaminophen, salicylic acid and ibuprofen) and no interferences were observed. Furthermore, some new synthetic cannabinomimetic drugs were tested and no interferences were observed [20].

The calculated extraction efficiencies in plasma ranged from 66 to 71% for THC, from 75 to 93% for 11-OH-THC, and from 78 to 86% for THC-COOH. The extraction method provided satisfactory efficiency for the three analyzed cannabinoids.

The extraction phase accomplishes recovery levels comparable with liquid/liquid extraction methods. Salts and polar

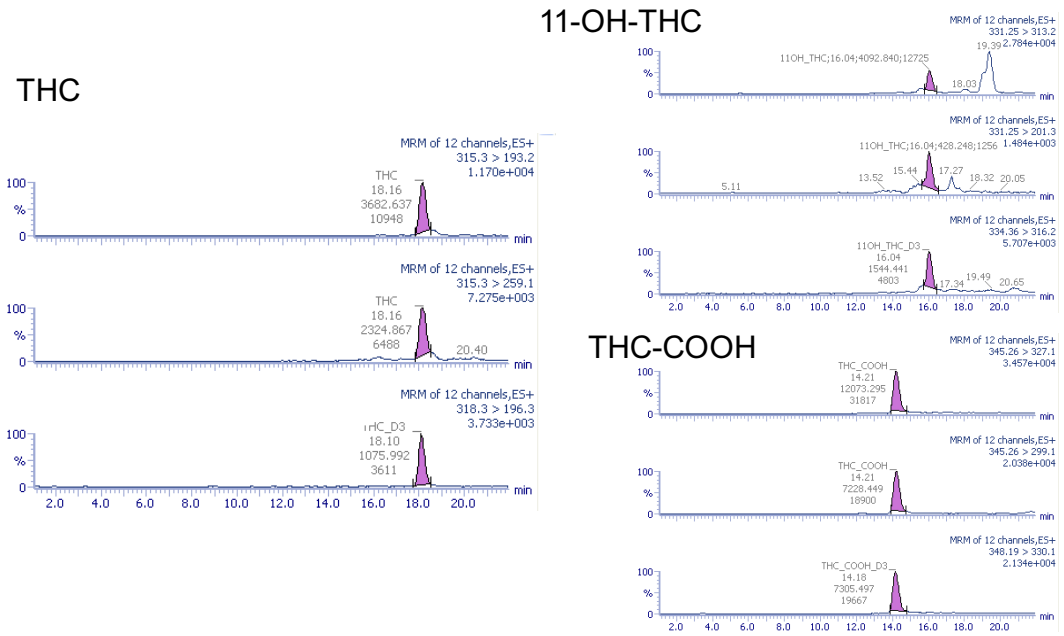

Fig. 2. Plasma analysis for a patient consuming inhaled cannabis: ES+ mass spectra for THC (*m/z* 315.3), 11-OH-THC (*m/z* 331.25), THC-COOH (*m/z* 345.26), THC-*d3* (*m/z* 318.30), 11-OH-THC-*d3* (*m/z* 334.36), and THC-COOH-*d3* (*m/z* 348.19).

Table I. LC-MS/MS parameters for THC, 11-OH-THC and THC-COOH.

	THC *(m/z: 315.3)*	*THC-d3 (m/z: 318.3)*	11-OH-THC *(m/z: 331.25)*	*11-OH-THC-d3 (m/z: 334.36)*	THC-COOH *(m/z:345.26)*	*THC-COOH-d3 (m/z: 348.19)*
Quantifier ion (*m/z*)	193.2	196.3	313.20	316.2	327.1	330.1
Confirmation ion (*m/z*)	259.1		201.30		299.1	
Cone voltage (V)	38	38	26	26	32	26
Collision energy (eV)	23	23	15	15	21	21

Table II. Validation results for THC, 11-OH-THC and THC-COOH in plasma.

Analytes	Concentration ng/mL	Mean (ng/mL)	Extraction recovery (%)	CV (%) Intra-series (n = 6)	CV (%) Inter-series	Linearity r^2	LOQ	LOD
THC	BTMF 1.0 (n = 45)	0.8			12.1	0.998	0.16	0.09
	2.5 (n = 3)	2.4	66	3.4	12.0			
	5.0 (n = 3)		70					
	10.0 (n = 3)	10.2	71	5.8	6.4			
11-OH-THC	BTMF 1.0 (n = 45)	1.1			14.5	0.991	0.15	0.08
	2.5 (n = 3)	2.2	75	4.6	12.2			
	5.0		93					
	10.0 (n = 3)	9.8	101	1.9	7.2			
THC-COOH	BTMF 1.0 (n = 45)	8.9			14.9	0.982	3.24	0.91
	2.5 (n = 3)	2.9		10.4	26.2			
	5.0 (n = 3)		86					
	10.0 (n = 3)	9.8	78	3.0	11.3			

interferences remained unextracted, as well as the gravity flow preventing the formation of emulsions.

The technique presented in this study was simple to handle and reproducible. It allows good recovery, and appropriate sensitivity and specificity, with reduced interference from the sample matrix. Methods to obtain the three compounds from the same sample matrix are often demanding and time-consuming; they require multiple liquid/liquid extractions. These liquid/liquid extractions need strict sample preparation and a significant amount of solvent. For THC, 11-OH-THC and THC-COOH, good accuracy and precision were obtained (Table II). The presented method proved to be reliable, selective and accurate for the three moieties. The LOQ and LOD levels achieved in the method presently developed are sufficient for clinical use. Indeed, the LOD that was determined is sensitive enough regarding the usual plasma levels of these

cannabinoids in cannabis consumers [2]. The external evaluation quality control from the SFTA used to assess the cannabis control in plasma was satisfactory. Therefore, this method could be well suited for the identification and quantification of THC, 11-OH-THC and THC-COOH.

In conclusion, this paper describes an HPLC-MS/MS method as well as a novel and simple liquid/liquid extraction technique for the detection and quantification of THC, 11-OH-THC and THC-COOH in plasma. This procedure achieved a high specificity and sensitivity and can be used as an alternative to more time-consuming existing methods. The use of two characteristic ions, one for quantification and another one for confirmation, allowed an improvement in the specificity and sensitivity of the method. The ratio of the two product ions was used to assess the confirmation. Also, the LOD and LOQ thresholds were satisfactory for THC, 11-OH-THC and THC-COOH, which makes this method suitable for forensic and predictive toxicology studies as well as pharmacokinetic analysis of a large range of structurally similar cannabinoids [20]. In our laboratory, we have been using this extraction technique for more than 3 years and have applied it to more than 1000 plasma samples. The analyses were very satisfactory. Furthermore, we have applied this technique to blood samples including controls on blood (LGC Standard) and the results were correct (data not published).

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