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Article

Validation of the Quantitative Determination of Tetrahydrocannabinol and Its Two Major Metabolites in Plasma by Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry According to the Total Error Approach*

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In Belgium, driving under the influence (DUI) of cannabis is prohibited and has severe legal consequences for the driver if the blood plasma concentration of Δ^9-tetrahydrocannabinol (THC) exceeds 1 μg/L. A method to quantify low concentrations of THC and its hydroxylated (THC-OH) and carboxylated (THC-COOH) metabolites in plasma was developed for DUI but also for other applications. Ultra-high-performance liquid chromatography coupled to mass spectrometry seems to be a very convenient method to combine fast chromatographic separation and good sensitivity. The method was validated according to total error approach. Chromatographic separation was achieved in a 3-min total run time. The limits of quantitation were lower or equal to 1 μg/L for all compounds. The linearity of the method was acceptable in the validated range of concentrations (from 0.5 to 50 μg/L for THC, from 0.9 to 50 μg/L for THC-OH and from 1.1 to 100 μg/L for THC-COOH). The biases were lower than 13%, and the relative standard deviations for repeatability and intermediate precision did not exceed 15%. Lower and upper β-expectation tolerance limits did not exceed the acceptance limits of 20% for concentrations higher than 2 μg/L for THC and THC-OH and higher than 4 μg/L for THC-COOH. The acceptance limits were 30% for THC and THC-OH concentrations lower than 2 μg/L and for THC-COOH concentrations lower than 4 μg/L.

Introduction

Cannabis is the most widely used illicit drug worldwide (1, 2). Δ^9-Tetrahydrocannabinolic acid (THCA-A) is the precursor of Δ^9-tetrahydrocannabinol (THC), the most psychoactive component of marijuana and hashish. When cannabis products are heated before or during consumption (i.e., smoking, vaporizing, tea, or baked products), the non-psychoactive THCA-A is converted by decarboxylation to THC (3, 4). THC is very lipophilic, and after entering the lungs, it dissolves quickly in pulmonary surfactant, enabling rapid passage into the bloodstream (5). THC is metabolized by hepatic cytochromes P450 2C9 and 2C19 to 11-hydroxytetrahydrocannabinol (THC-OH), which is also psychoactive (6, 7). It is further oxidized to 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), an inactive metabolite, mainly by CYP2C9 liver enzymes (8, 9). Since 1999, driving under the influence of cannabis has been prohibited in Belgium, and the driver can be checked first by roadside testing (saliva) and, as confirmation, by blood analysis. The analytical level considered positive for THC concentration in plasma by Belgian authorities is 1 μg/L (10).

Several methods have been described to quantify simultaneously THC and its two major metabolites THC-OH and THC-COOH in blood. Gas chromatography coupled to mass spectrometry (GC–MS) has been used for a long time and is still the reference method to quantify THC in biological matrixes (2, 7, 11–13). For a few years, liquid chromatography (LC) coupled to MS was developed for THC determination. LC–MS offers very good results with a simpler sample pretreatment, because no derivatization step is required, and with shorter chromatographic run-times (8, 9, 14–16). In 2005, the arrival of ultra-high-pressure liquid chromatography (UHPLC) coupled to tandem MS allowed even more rapid chromatographic separation and a better sensitivity (17, 18).

Materials and Methods

Chemicals and reagents

Reference materials for all compounds and internal standards were purchased from LGC Promochem (Molsheim, France). All standards had a degree of purity higher than 99%.

Hexane (analysis grade) was purchased from Lab-scan Analytical Sciences (Gliwice, Poland); methanol and water (LC–MS grade) were purchased from Biosolve (Valkenswaard, the Netherlands); ammonium bicarbonate, of at least analytical grade, was purchased from Sigma-Aldrich (Steinheim, Germany); ethyl acetate and ammonia were from VWR (Leuven, Belgium).

Stock solutions and standards

For the preparation of the internal standard (IS) stock solution, commercial solutions of THC-d_3, THC-OH-d_3, and THC-COOH-d_3 were diluted in methanol. The concentration of the IS stock solution was 1.67 mg/L for THC-d_3 and THC-OH-d_3 and 3.33 mg/L for THC-COOH-d_3.

Calibration standards and validation standards were prepared by fortifying drug-free plasma with stock solution containing all the compounds. Calibration standards were prepared to obtain final concentrations of 0.83, 2.08, 4.17, 8.33, 20.83, and 41.67 μg/L for THC and THC-OH and concentrations of 1.67, 4.17, 8.33, 16.67, 41.67, and 83.33 μg/L for THC-COOH. The calibration standards were analyzed in duplicate for three days and were used to establish the calibration curves (response function). The concentrations of the validation standards were 0.5, 1, 2, 10, and 50 μg/L for THC and THC-OH and 1, 2, 4, 20,

and 100 μg/L for THC-COOH. The validation standards were analyzed in triplicate for three days and were used to estimate the validation parameters and thus the method limits. Stock solutions for calibration and validation standards were prepared independently. An extract of drug-free plasma was also prepared for each run. Internal quality controls (BTMF) purchased from LGC Promochem (Molsheim, France) were included in each batch.

Sample pretreatment

Ten microliters of IS solution was added to 500 μL of plasma. After addition of 100 μL of a 10% acetic acid solution, sample was extracted with 5 mL of a mixture of hexane and ethyl acetate (90:10, v/v).

The aqueous phase was discarded, the organic layer was evaporated to dryness under gentle nitrogen flow at 40°C and reconstituted with 100 μL of a mixture of H_2O and methanol (80:20, v/v). Ten microliters was injected into the column.

Instrumentation

Analysis was performed on an UPLC Acquity coupled to a tandem MS (Quattro Premier XE (Waters, Zellik, Belgium). The chromatographic separation was done on an Acquity C_{18} column (1.8 μm, 50×2.1-mm i.d., Waters) equipped with an online filter at 40°C. Gradient elution was performed at a constant flow of 0.45 mL/min, using a mixture of 10 mM ammonium bicarbonate in water (pH 10) and methanol, as described in Table I.

After chromatographic separation, compounds were analyzed in the tandem MS operated in the positive electrospray mode at 1.0 kV, at a source temperature of 120°C and at a desolvatation temperature of 350°C. The collision gas flow was set at 50 L/h and the desolvatation gas flow at 800 L/h. The MS method was divided into three functions depending on the retention times of the analytes. Two transitions for each compound were monitored in the multiple reaction monitoring (MRM) mode. Ion ratio of each analyte was measured and compared to its acceptance limits to check compound identification (Table II). Acceptance limits for ion ratios are based on European Commission decision (19).

Table I
UHPLC Elution Gradient*

Time (min)	A %	B %
0.0	50.0	50.0
0.3	50.0	50.0
2.0	5.0	95.0
2.5	5.0	95.0
3.0	50.0	50.0

* A, 10 mM ammonium bicarbonate, pH 10 and B, methanol.

Method validation

According to ISO17025 and the guidelines of the French Society of Pharmaceutical Sciences and Techniques (SFSTP), the present method was fully validated using total error approach (20–22). The e-noval software V3.0 (Arlenda, Liège, Belgium) was used to compute all validation results and to build the accuracy profiles.

Results and Discussion

Elution was carried out using a segmented gradient of 3 min. Compounds were well separated, with retention times from 1.5 to 2.5 min. UHPLC offered a much shorter run-time in comparison with classical LC method (8, 16). A chromatogram of an extracted, fortified plasma sample is presented in Figure 1. The MS method was divided into three time functions, and MRM dwell times were adjusted to maximize sensitivity.

The response function is within the range of the existing relationship between the response (signal) and the concentration of the analyte in the sample (21). It was built from the calibration standards. The response function was a weighted quadratic regression for THC and a weighted linear regression for THC-OH and THC-COOH.

The linearity is the method's ability to obtain results directly proportional to the concentrations of the analyte in the sample (21). The method presents a good linearity for each compound: from 0.5 to 50 μg/L for THC, from 0.9 to 50 μg/L for THC-OH, and from 1.1 to 100 μg/L for THC-COOH.

The trueness expresses the closeness of agreement between the mean value obtained from the validation standards and the value that is accepted either as a conventional true value or an accepted reference value. Trueness is expressed in terms of relative bias (systematic error) (21). Trueness was acceptable for all compounds because the relative biases were always lower than 13%. Results are presented in Table III.

The precision was determined by computing the relative standard deviations (RSDs) for repeatability and intermediate precision at each concentration of the validation standards (23). They did not exceed 9% for repeatability and 15% for intermediate precision. RSDs are presented in Table III.

The uncertainty characterizes the dispersion of the values that could reasonably be attributed to the measurand. The

Table II
Retention Times and MRM Transitions of Each Analyte and Internal Standard (IS)

Compound	Ret. Time (min)	Cone Voltage (V)	MRM1 (Quantifier)	Collision Energy (V)	MRM2 (Qualifier)	Collision Energy (V)	Acceptance Limits for Ion Ratio (%) MRM1/MRM2
THC-d_3 (IS)	2.47	30	318.40 > 196.35	24	–	–	–
THC		30	315.40 > 193.35	24	315.40 > 123.25	35	78.1–117.2
THC-OH-d_3 (IS)	2.16	30	334.20 > 316.30	17	–	–	–
THC-OH		30	331.20 > 313.30	15	331.20 > 193.20	25	351.3–585.4
THC-COOH-d_3 (IS)	1.69	32	348.35 > 302.40	20	–	–	–
THC-COOH		32	345.35 > 299.40	20	345.35 > 327.40	17	102.1–153.2

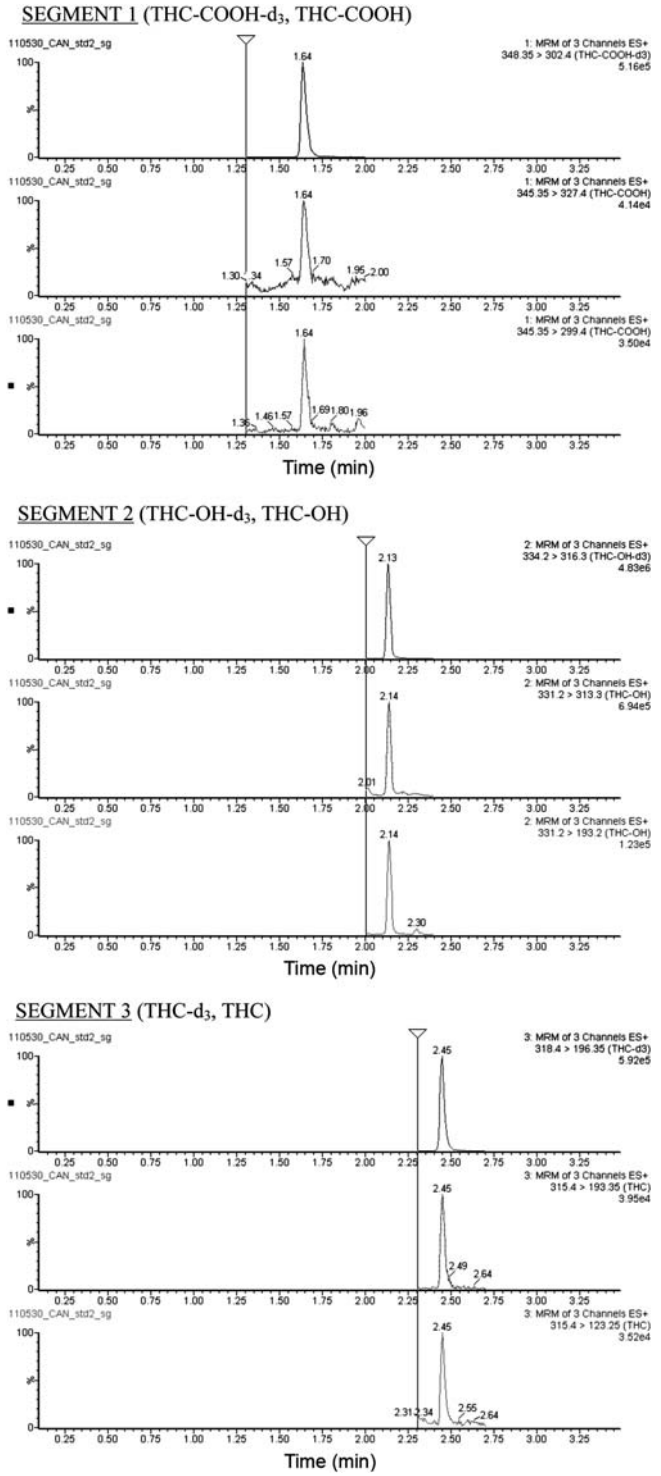

Figure 1. Chromatogram example of an extracted, fortified plasma (THC, THC-OH = 2.08 μg/L, THC-COOH = 4.17 μg/L).

expanded uncertainty represents an interval around the results where the unknown true value can be observed with a confidence level of 95%. The relative expanded uncertainties (%) are obtained by dividing the corresponding expanded uncertainties with the corresponding introduced concentrations. Values for each analyte are presented in Table III.

Table III

Trueness, Precision, Uncertainty of Measurement, Lower and Upper Limit of Quantitation for Plasma Determination of THC, THC-OH, and THC-COOH by UHPLC–MS–MS ($n = 9$)

	Target Conc. (μg/L)	THC	THC-OH	Target Conc. (μg/L)	THC-COOH
Trueness	0.5	−4.29	8.97	1.0	−7.69
Relative bias (%)	1.0	12.61	12.78	2.0	10.45
	2.0	0.70	0.32	4.0	−0.77
	10.0	10.60	7.02	20.0	9.49
	50.0	1.65	2.59	100.0	4.60
Intraassay precision	0.5	5.44	4.80	1.0	8.46
Repeatability	1.0	5.29	2.15	2.0	5.17
(RSD%)	2.0	6.17	3.59	4.0	5.31
	10.0	3.55	2.83	20.0	4.29
	50.0	3.66	2.02	100.0	3.34
Interassay precision	0.5	9.43	14.17	1.0	12.83
Intermediate precision	1.0	8.22	6.50	2.0	8.96
(RSD%)	2.0	6.17	3.59	4.0	6.22
	10.0	3.83	2.94	20.0	5.32
	50.0	5.82	2.25	100.0	4.80
Uncertainty	0.5	21.15	32.42	1.0	28.60
Relative expended	1.0	18.32	14.88	2.0	20.11
uncertainty (%)	2.0	13.00	7.57	4.0	13.51
	10.0	8.19	6.25	20.0	11.61
	50.0	12.99	4.84	100.0	10.61
LOD (μg/L)		0.2	0.3		0.3
LLOQ (μg/L)		0.5	0.9		1.1
ULOQ (μg/L)		50.0	50.0		100.0

The total error evaluates the ability of the method to produce accurate results. Thus, the total error estimation of a procedure is fundamental to assess the validity of the method. Total error is the sum of trueness and precision, and it is clearly a good indicator of results accuracy. The accuracy expresses the closeness of agreement between the value found and the value that is accepted either as a conventional true value or an accepted reference value (22, 23).

The accuracy profile is obtained by joining the extremes of the 87.5% interval, that is, the interval that will contain 87.5% of the future individual results. The acceptance limits were ± 20% for concentrations higher than 2 μg/L and ± 30% for concentrations lower than 2 μg/L for THC and THC-OH; they were set at ± 20% for concentrations higher than 4 μg/L and at ± 30% for concentrations lower than 4 μg/L for THC-COOH. As shown in Figure 2, the relative upper and lower β-expectation tolerance intervals did not exceed the acceptance limits for each compound in the dosing range.

The intersections between the accuracy profile and the acceptance limits define the lower limit of quantitation (LQL) as well as the upper limit of quantitation (UQL) (22, 23). The limit of detection (LOD) is the smallest quantity of the substance that can be detected, but not accurately quantified in the sample; it is one-third of the limit of quantitation. LOD, LQL, and UQL of the three compounds are presented in Table III. The LQL was lower than 1.1 μg/L for each compound, which is better than LQL generally obtained by GC–MS technology.

The selectivity is the method's ability to measure unequivocally and to differentiate the analyte(s) in the presence of components which may be expected to be present (22, 23). An extract of drug-free plasma (from different sources) was prepared for each run, and samples spiked with possibly interfering compounds (6 different amphetamines, cocaine and its main metabolite benzoylecgonine, and 27 opiates) were

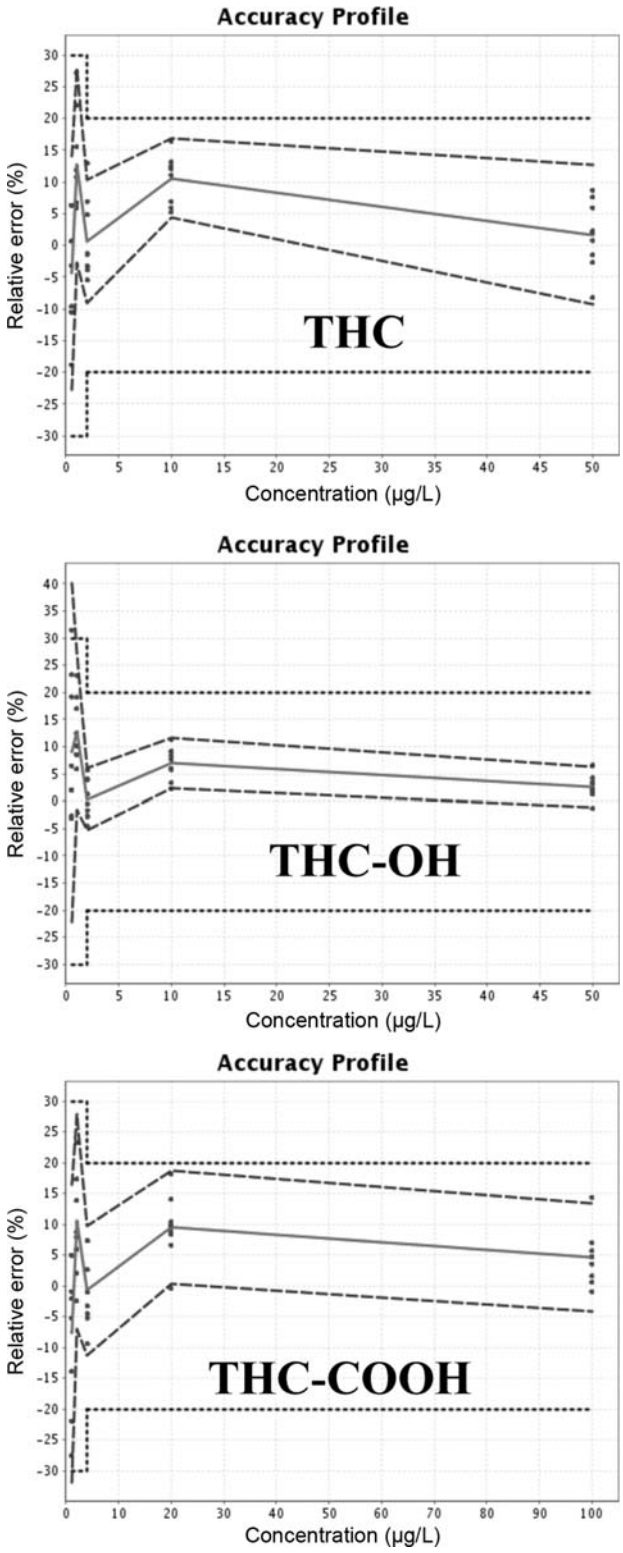

Figure 2. Accuracy profiles obtained for THC, THC-OH, and THC-COOH with e-noval validation software. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits (87.5%), and the dotted curves represent the acceptance limits [30% for concentrations lower than 2 µg/L (4 µg/L for THC-COOH) and 20% for concentrations higher than 2 µg/L (4 µg/L for THC-COOH)]. The dots represent the relative back-calculated concentrations and are plotted with respect to their targeted concentration.

injected; real samples containing other drugs than cannabis were analyzed. No interferences were found.

Matrix effect was analyzed by post-column infusion technique. Analytes were infused post-column via a T-shaped connector at a steady rate of 20 µL/min into the UHPLC eluant stream. At the same time, 10 µL of blank serum extracts were injected. Ten sources of blank samples were evaluated. Ion suppression effect was lower than 10% (24).

Stability of plasma specimens was tested on real samples. Ten samples were stored at a temperature between 2 and 8°C and analyzed after 7 and 14 days; they were still stable after 14 days. Stability of the drug extracts was tested on internal QC and real samples; they were stable at least five days at 10°C (temperature of the sample unit).

Our laboratory took part five times in external QC program BTMF Illicit Drugs in Serum from Arvecon (Waldorf, Germany) and obtained successful results (z-score < 2).

Thirty-six real samples were analyzed in 3 months; 21 were negative and 15 were positive for at least THC-COOH. Concentrations ranged between 0 and 3.6 µg/L for THC, between 0 and 3.6 µg/L for THC-OH, and between 1.7 and 41.4 µg/L for THC-COOH.

Conclusions

UHPLC technique coupled to tandem MS is a very convenient method for rapid, specific, and sensitive quantitative determination of THC, THC-OH, and THC-COOH in plasma. The sample pre-treatment is simple and the three compounds of interest are separated in 3 min. In 2008, Jamey et al. (17) developed a method similar to the proposed method. They used the same apparatus, a column with same physicochemical properties but two times longer, and a mobile phase containing formic acid (pH 2.6) and acetonitrile, whereas we used basic buffer and methanol. They achieved chromatographic separation in 10 min and used a sample volume twice as large compared to our method. Our method is more rapid and needs smaller sample volume, which is appreciable when many DUI analyses have to be performed. Finally, this method was successfully analytically validated using total error approach, which is a really innovative procedure for analytical validation in toxicological laboratories.

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