Chem. Anal. (Warsaw), **51**, 65 (2006)

Determination of Δ^9-Tetrahydrocannabinol (9THC) and 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in Blood and Urine Using Gas Chromatography Negative Ion Chemical Ionization Mass Spectrometry (GC–MS–NCI)

by Maria Kała* and Maciej Kochanowski

Institute of Forensic Research,
ul. Westerplatte 9, 31-033 Cracow, Poland

Keywords: Δ^9-tetrahydrocannabinol; 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol; GC–MS–NCI; Determination

A validated method for the determination of Δ^9-tetrahydrocannabinol (9THC) and its major metabolite 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in blood and urine using gas chromatography–mass spectrometry in negative ion chemical ionization mode has been presented. The analytes were extracted from biological material using liquid-liquid extraction. 9THC was derivatized with trifluoroacetic anhydride in chloroform and THCCOOH with pentafluoropropanol in trifluoroacetic anhydride. Quantification was performed by monitoring the signals of selected target ions: (m/z) 389.3 and 410.3 for 9THC derivative, and 422.3 and 572.3 for THCCOOH derivative. The basis for identification was the presence of two ions (quantitative and qualifier) for each analyte. Extraction yield ranged at 52 and 83% for 9THC and THCCOOH, respectively. The values of validation parameters, expressed in ng mL^{-1}, were the following: limit of detection – 0.25; limit of quantification – 0.5, for both analytes; linearity range – 0.5–100 for 9THC and THCCOOH in blood, and 0.5–200 for THCCOOH in urine. Internal standardization was carried out using deuterated analogues of the analytes. Intra- and inter-day precision did not exceed 25% for 9THC and 7% for THCCOOH. Accuracy was confirmed using Medidrug reference materials. The method was applied to the determination of 9THC and THCCOOH in 15 blood and 2 urine samples collected from drivers suspected of driving under the influence of cannabis.

* Corresponding author. E-mail: mkala@ies.krakow.pl; Fax 012-422-38-50

Przedstawiono zwalidowaną metodę oznaczania Δ^9-tetrahydrokannabinolu (9THC) i jego głównego metabolitu 11-nor-9-karboksy-Δ^9-tetrahydrokannabinolu (THCCOOH) we krwi i w moczu z zastosowaniem techniki chromatografii gazowej ze spektrometrią mas w trybie jonizacji chemicznej jonów ujemnych. Anality wyekstrahowano z materiału biologicznego w układzie ciecz-ciecz. 9THC derywatyzowano z użyciem bezwodnika kwasu trifluorooctowego w chloroformie, a THCCOOH mieszaniną pentafluoropropanolu w bezwodniku kwasu trifluorooctowego. Oznaczenia przeprowadzono rejestrując sygnały od wybranych jonów: (m/z) 389.3 i 410.3 dla pochodnej 9THC oraz (m/z) 422.3 i 572.3 dla pochodnej THCCOOH. Podstawę identyfikacji stanowiła obecność dwóch charakterystycznych (ilościowego i pomocniczego) jonów dla każdego analitu. Oznaczanie kontrolowano za pomocą wzorców wewnętrznych w postaci deuterowanych analogów analitów. Wydajność ekstrakcji wynosiła 52 i 83% odpowiednio dla 9THC i THCCOOH. Wartości parametrów walidacyjnych, wyrażone w ng mL^{-1}, wynosiły: granica wykrywalności – 0,25; granica oznaczalności – 0,5 dla obu związków; zakres liniowości – 0,5–100 dla 9THC i THCCOOH we krwi oraz 0,5–200 dla THCCOOH w moczu. Precyzja metody wyznaczona w seriach wewnątrz- i międzygrupowych nie przekraczała 25% dla 9THC i 7% dla THCCOOH. Dokładność metody kontrolowano za pomocą materiałów referencyjnych Medidrug. Opracowaną metodę zastosowano do oznaczania 9THC i THCCOOH w 15 próbach krwi i 2 próbach moczu pobranych od kierowców podejrzanych o prowadzenie samochodu pod wpływem cannabis.

Drug detection time (the time after drug administration when it is still detectable) is an important factor that has to be considered in the analysis of drug content in biological fluids. Detection time is dependent on pharmacological factors (*e.g.* drug dose, route of administration, and rates of metabolism and excretion) and analytical factors (*e.g.* sensitivity, specificity, and accuracy of the analytical method).

Δ^9-Tetrahydrocannabinol (9THC) is the most active among main components of products, like hashish or marijuana. It is administered either orally or by smoking. Single active dose of 5–10 mg results in euphoria, hallucinations, and often sedation [16, 35]. 9THC is lipophilic and is distributed widely in the body. It is hydroxylated to an active 11-hydroxy-Δ^9-tetrahydocannabinol (11-OH-THC) metabolite that does not achieve high concentration in blood. Its maximum concentration in plasma reaches 7.1 ng mL^{-1} after smoking [11]. 11-OH-THC is further oxidised to the inactive 11-nor--9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH), which is conjugated with glucuronic acid and predominantly excreted with urine. THCCOOH accumulates in organism after a single dose [2, 5, 10]. Concentrations of 9THC in blood decrease below 1 ng mL^{-1} after 4–6 h of exposure to cannabis [11, 31].

9THC, 11-OH-THC, and THCCOOH are of the main interest in forensic toxicology. Concentration ratios of metabolite to the parent drug and of metabolite to metabolite can be related to the excretion times of individual metabolites [14] and allow one to judge on the time period lasted after the ingestion of marijuana [23]. In addition, several metabolites quantified in body fluids may be used to determine the route of administration. For example, different metabolic routes for oral and inhalant drugs have been

found [13, 32]. Prescription use of synthetic 9THC [26, 33] may be distinguished from the abusive use by determining Δ^9-tetrahydrocannabivarian (THCV) metabolite in urine; this is an analogue of 9THC that exists only in plant material [7].

In response to the increasing demand for reliable evidence of cannabis use, a number of procedures have been developed to determine 9THC and/or its metabolites in body fluids. The procedures involve mainly gas chromatography (GC) coupled with mass spectrometry (MS) and electron impact ionization (EI) [1, 21, 24] and chemical ionization (CI) [4, 7] modes, or in combination with tandem mass spectrometry (MS–MS) [3]. Recently, high performance liquid chromatography (LC) with mass detection and atmospheric pressure chemical ionisation (APCI) has been also applied [9, 22]. Previously reported methods differed in respect of limit of detection (LOD), for instance: in hair [pg mg^{-1}] – 0.3 for THCCOOH [27], 50 for 9THC and THCCOOH, and 500 for 11-OH-THC [34]; in body fluids [ng mL^{-1}] – 0.52 for 9THC, 0.49 for 11-OH-THC and 0.65 for THCCOOH in serum [30], in urine: 0.5–1.5 for 9THC, 8α-hydroxy-9THC, 8β-hydroxy-9THC, 11-OH-THC, 8α,11-dihydroxy-9THC, 8β,11--dihydroxy-9THC and THCCOOH, and 0.6–2.1 for the above seven compounds in plasma [18], 0.5 for 9THC in blood [4], 0.2 for 9THC in saliva, 5 for THCCOOH in urine [28]. Analytical procedures with appropriate validation data are given in the reviews of Moeller *et al.* [25] and ElSohly *et al.* [7]. In all of the above methods derivatization of the target compounds is included.

Numerous immunoassay methods for the analysis of cannabinoids in body fluids are commercially available. Due to the inherent cross-reactivity of many cannabinoids and their metabolites, these methods do not provide specific information on the nature of cannabinoids present in biological samples. These methods are widely used, yet the positive results obtained must be very carefully analysed by comparing them with the results obtained from more specific instrumental methods for medical and legal purposes [17, 33].

In this paper a validated GC–MS–NCI method for the determination of 9THC and THCCOOH followed by the simultaneous extraction of both compounds from 1.0 mL-in-volume blood and urine samples has been reported.

EXPERIMENTAL

Materials and methods

Standards and internal standards (ISs). Δ^9-tetrahydrocannabinol (9THC), 11-nor-9-carboxy--Δ^9-tetrahydrocannabinol (THCCOOH), and ISs (9THC-D_3 and THCCOOH-D_3) were purchased as methanolic solutions from Cerilliant (LGC Promochem, Warszawa, Poland). Concentration of the standards and ISs was 100 μg mL^{-1}. Concentration of 9THC was 1000 μg mL^{-1}.

Chemicals and reagents. Acetonitrile (ACN), acetone, ethyl acetate, n-hexane, n-heptane, water (all HPLC grade) were obtained from Merck (Darmstadt, Germany). Derivatizing reagents, 97% pentafluoropropanol (PFP) and 99% trifluoroacetic anhydride (TFAA) were obtained from Sigma–Aldrich (Warszawa, Poland). Chloroform ($CHCl_3$), sodium hydroxide, and hydrochloric acid (analytical or higher grade) were supplied by POCH (Gliwice, Poland). Potassium phosphate monobasic and potassium phosphate dibasic (POCH) were used for the preparation of 0.5 mol L^{-1} phosphate buffer (pH 6.8). All glass vials and tubes were silanized by immersion in 1% solution of Silon CT in toluene for 1 h, followed by oven drying prior to the use.

Specimens. Drug-free blood (control blood sample) was taken from a local blood bank. Control urine samples were taken from healthy persons. Control liver samples were taken during autopsy from persons without drug history. Drug-free liver samples were certified by an immunoassay screening for cannabinols with negative results. Control specimens were used for the development and validation of the method. To the control blood and urine samples, several (10 and 11) portions of the standards and ISs (20 ng mL^{-1}) were added to construct 10- and 11-point calibration plots, respectively. Control blood samples were spiked with 9THC and THCCOOH to the concentrations of 0, 0.25, 0.5, 1, 2, 5, 10, 20, 50, and 100 ng mL^{-1}. Control urine samples were spiked with THCCOOH to the concentrations of 0, 0.25, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 ng mL^{-1}.

For the accuracy control, the following reference materials were used: Medidrug BTM S-plus serum (Medichem, Stuttgart, Germany) with three assigned 9THC concentrations of 6.13, 5.2 and 3.0 ng mL^{-1}, and Medidrug BTM S serum with two assigned THCCOOH concentrations of 24.0 and 16.4 ng mL^{-1}.

15 real samples of blood and 2 real samples of urine were taken from drivers responsible for road accidents and suspected of being under the influence of drugs.

Analytical procedure

Sample pretreatment. Specimens were thawed and equilibrated at the room temperature for at least 1 h, next agitated using a vortex mixer before further analysis. 1 mL-in-volume blood, urine, or liver homogenate (1:1 with water, m/v) was placed in 20 mL screw-capped glass tube and 20 µL of 1 ng μL^{-1} methanolic solution of ISs (9THC-D_3 and THCCOOH-D_3) and 1 mL of 0.5 mol L^{-1} phosphate buffer (pH 6.8) were added. The tube was closed and the contents were mixed. Then, the tube was placed in the ultrasonic bath at the room temperature for 10 min, followed by incubation in the water bath at 40°C for 5 min.

Liquid-liquid extraction (LLE). 4 mL of ACN-acetone (9:1, v/v) mixture was added to the sample during vortex mixing in order to precipitate proteins. After that, the sample was centrifuged at 6000 × g for 5 min. Supernatant was transferred into the 20mL-in-volume glass tube and its volume was decreased to approximately 1.5 mL using a TurboVap (Zymark) at 40°C under reduced pressure of nitrogen. pH of the concentrated supernatant was adjusted to 13 by adding 0.5 mL of 2 mol L^{-1} NaOH. Next, 5 mL of hexane–ethyl acetate (7:1, v/v) were added. The mixture was shaken for 30 min at the rate of 60 cycles per minute (cpm). After centrifugation at 6000 × g for 3 min, aqueous phase (bottom layer) containing acidic compounds was transferred into a clean tube in order to determine THCCOOH. Non-acidic 9THC was determined in the organic phase.

Extraction of 9THC. Organic phase was cleaned-up with 5 mL of 0.1 mol L^{-1} HCl. After shaking for 30 min at 60 cpm and centrifugation at 6000 × g for 3 min, 4 mL of the organic phase was transferred to the 1.8 mL-in-volume glass test tube and evaporated to dryness at 40°C under a stream of nitrogen. Organic

phase was transformed very carefully to avoid contamination with the aqueous phase, as even a small amount of water could interfere in the subsequent derivatization reaction.

Extraction of THCCOOH. Aqueous phase was acidified to pH 3 by addition of 1 mL of 1 mol L^{-1} HCl and extracted with 5 mL of hexane–ethyl acetate mixture (7:1, v/v). After shaking for 30 min at 60 cpm, the layers were separated by centrifugation. Afterwards, 4 mL of the organic phase was transferred to the 1.8 mL-in-volume glass tube and evaporated to dryness at 40°C under a stream of nitrogen.

Derivatization. To the dry residue containing 9THC, 100 μL of TFAA and 100 μL of $CHCl_3$ were added. The tube was capped with disposable polypropylene screw cap and then vortex-mixed. THCCOOH was derivatized by adding 50 μL of PFP and 100 μL of TFAA to the dry residue. Derivatization of the analytes was performed for 30 min in the 1.8 mL-in-volume capped tube at 60°C. Reaction mixtures were cooled down to the room temperature and the excess of derivatizing reagents was removed by evaporating to dryness at 40°C under a stream of nitrogen. Dry residues were reconstituted in 50 μL of ethyl acetate and transferred to 2 mL-in-volume glass autosampler vials equipped with a conical glass insert of 100 μL in volume. An aliquot of 2 μL was injected by autosampler into the GC–MS system.

Gas chromatography–mass spectrometry (GC–MS). Analysis of 9THC and THCCOOH was carried out using an Agilent Technologies 6890N gas chromatograph (GC) equipped with a 5973 mass selective detector (MSD) operated in negative chemical ionization (NCI) mode and a 6890 autosampler (Agilent Technologies, Wilmington, USA). Methane at the total flow of 54.3 mL min^{-1} and under pressure of 11.3 psi served as the reagent gas. High-purity helium was used as the carrier gas. GC was equipped with a 30-m DB5 MS (J&W Scientific, Folsom, USA) fused-silica capillary column coated with a 5% phenylmethylpolysiloxane liquid phase of 0.25-mm I.D. and 0.25 μm film thickness. The capillary inlet system was operated in the splitless mode, with temperature zones for the injector port set at 240°C and transfer line at 280°C. The applied temperature programme was: initial temperature 80°C for 1 min, an increase up to 280°C at a rate of 25°C min^{-1}, final temperature 280°C kept for 5 min. The total run time was 14 min. Solvent delay was set at 8.20 min. MS parameters were optimized to find out the most intense ions for the selected ion monitoring (SIM) mode. The 9THC and THCCOOH derivatives, present at the concentration of 50 ng mL^{-1}, were injected individually and analysed in the full-scan mode (m/z range 50–600). Optimized quadrupole temperature was 106°C, and ion source temperature was 150°C. Selection of ions for quantitative (underlined) and qualitative (qualifier) analysis was made on the basis of their abundance and lack of baseline interference. For 9THC and THCCOOH both the quantitative and qualifier ions had to be present and be within 3% of the retention time (RT) of the standards. The following ions (values of deuterated ions in parenthesis) were selected for the determination of each derivative: 9THC (9THC-D_3), m/z 389.3, 410.3 (392.3, 413.3) and THCCOOH (THCCOOH-D_3), m/z 422.3, 572.3 (425.3, 575.3). The obtained data were automatically processed using a G1701CA, Version C.00.00 software with the MSD system, supplied by Agilent Technologies. Each ion of interest was automatically selected, retention times were calculated, and the peak area was determined. All data were checked for interference, peak selection, and baseline determination.

Quantification and validation. Calibration plot for 9THC and THCCOOH in blood was constructed using 10 experimental points, within the concentration range of 0.25–100 ng mL^{-1}. The eleven-point plot for THCCOOH in urine covered the range of 0.25–200 ng mL^{-1}. Solutions used for calibration were prepared by adding known amounts each of the IS (9THC-D_3 and THCCOOH-D_3, 20 ng mL^{-1}) and by increasing concentrations of 9THC and THCCOOH in case of blood and IS (THCCOOH-D_3, 20 ng mL^{-1}) and THCCOOH in case of urine. Calibration samples were prepared, extracted, and analysed using

CG–MS–NCI applying the same procedure as in the case of biological materials in forensic studies. Peak area ratios (9THC-to-9THC-D_3; m/z 410.3/413.3, and THCCOOH-to-THCCOOH-D_3; m/z 572.3/575.3) were calculated for each standard and plotted against the known standard-to-IS concentration ratios. Calibration plots were accepted when the corresponding correlation coefficients (r^2) were better than 0.99.

Intra- and inter-day precision of the method was determined by repeating (n = 3, or more) determination 9THC and THCCOOH in blood samples spiked with the analytes to the concentrations of 10 and 50 ng mL^{-1}, and 9THC samples (n = 5) containing the analyte at the concentration of 0.5 ng mL^{-1} (over a period of five days).

Limit of detection (LOD), limit of quantification (LOQ), and limit of linearity (LOL), as well as extraction recovery for 9THC and THCCOOH (both at the concentration of 10 ng mL^{-1}) in spiked blood samples were determined and compared to the results obtained for unextracted drug solutions (derivatised with the matrix of 1 mL drug-free control blood extract).

RESULTS AND DISCUSSION

Chemical structures of 9THC-trifluoroacetyl (9THC-TFA) [29] and THCCOOH--pentafluoropropyl (THCCOOH-PFP/TFA) derivatives are shown in Figure 1.

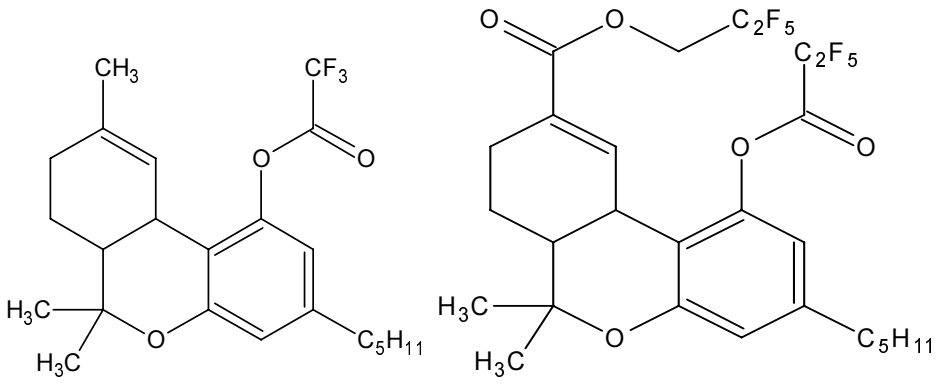

Figure 1. Chemical formulas of Δ^9-tetrahydrocannabinol-trifluoroacetyl (9THC-TFA) and 11-nor-9--carboxy-Δ^9-tetrahydrocannabinol-pentafluoropropyl (THCCOOH-PFP) derivatives

After SIM analysis of 9THC-TFA in blood using the selected ions the obtained chromatograms were free from the interference of endogenous substance (Fig. 2A). Drug-free control blood samples gave no peak in the regions corresponding to 9THC derivative. In drug-free blood (Fig. 2B) and urine samples background peaks were detected close to the retention time of THCCOOH-PFP/TFA. 9THC-TFA and THCCOOH-

PFP/TFA were eluted after 9.20 and 10.05 min, respectively, and deuterated analogues of both analytes one second earlier. Retention times of THCCOOH in liver homogenates and urine samples correlated well with those of measured in blood. The procedure was not validated for the liver matrix.

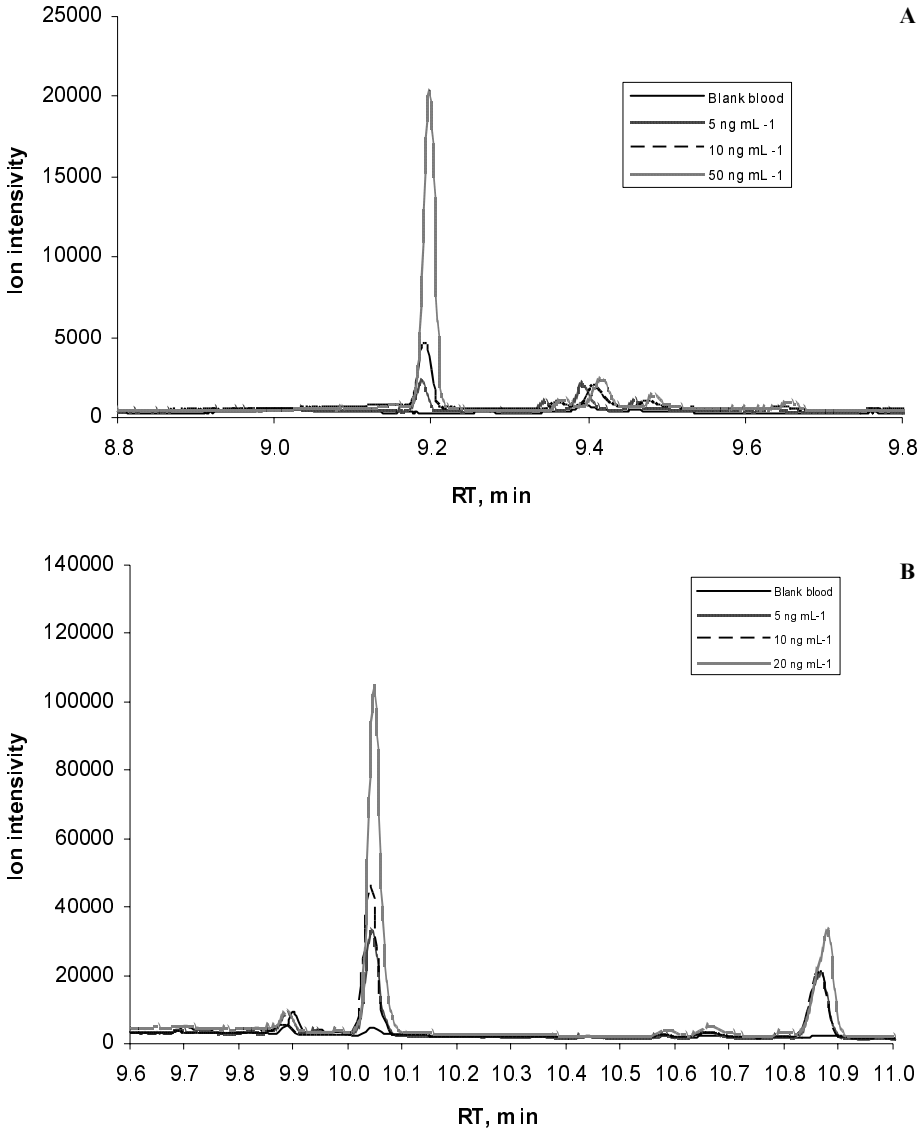

Figure 2. GC–MS–NCI SIM chromatograms of the extracts of control blood samples spiked with 9THC (A) and THCCOOH (B) to the concentrations of 0, 5, 10, and 50 or 20 ng mL^{-1}. Target ions (m/z): 410.3 for 9THC, and 572.3 for THCCOOH

Validation data of the method including recovery, linearity, intra- and inter-day precision, LOD, and LOQ are given in Table 1.

Table 1. Validation of the GC–NCI–MS determination procedure for Δ^9-tetrahydrocannabinol (9THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in blood and THCCOOH in urine

Parameter	Blood		Urine
	9THC	THCCOOH	THCCOOH
Retention time [min]	9.20	10.05	10.05
Recovery[a] [%]; C[b] = 10	52 ± 4 (8%); n = 3	83 ± 2 (3%); n=3	–
Linear equation	y = 0.736x+0.003	y=1.200x+0.114	y = 0.552x+0.171
r^2	0.996	0.991	0.995
Intra-day precision[a]; C[b] = 50	37.3 ± 9.3 (25); n = 3	39.7 ± 0.33 (0.8); n=3	–
Intra-day precision[a]; C[b] = 10	8.16 ± 1.2 (15); n = 3	9.66 ± 0.33 (4); n=3	–
Inter-day precision[a]; C[b] = 50	40.7 ± 9.6 (23); n = 7	42.1 ± 2.7 (6); n=7	–
Inter-day precision[a]; C[b] = 10	8.64 ± 1.6 (18); n = 6	10.04 ± 0.6 (6); n=6	–
Inter-day precision[a]; C[b] = 0.5	0.52 ± 0.04 (7); n = 5	–	–
LOD[b]	0.25		
LOQ[b]	0.5		
LOL[b]	0.5–100		0.5–200

[a] Mean ± SD (RSD, %);
[b] [ng mL^{-1}].
„–" – not determined.
$x = C_{St}/C_{IS}$.

LOD was defined as the minimum concentration equivalent to or higher than three times the background noise that could be still detected for each of the two ions (389.3, 410.3 for 9THC, and 422.3, 572.3 for THCCOOH). LOD was estimated as 0.25 ng mL^{-1} in blood and urine samples. In nine blood and urine control samples spiked with 9THC and THCCOOH to the concentration of 0.25 ng mL^{-1} the measured concentration was 0.22 ng mL^{-1}. Inter-day precision of the assay was as 80%, which was not satisfactory. Therefore, the concentration of 0.25 ng mL^{-1} was taken as LOD. This value is equal to or even better than the published values for GC–MS–NCI. Thus, the method is sufficiently sensitive to allow detection of the recent cannabis use [12, 15, 20]. LOQ was defined as the equivalent of the lowest calibration level that can be quantified with precision of at least ±20%. The calculated LOQ value was 0.5 ng mL^{-1}. LODs and

LOQs did not differ for blood and urine samples. For 9THC, the obtained LOQ of 0.5 ng mL^{-1} allowed one to conclude that the method is sufficiently sensitive for the routine determination of this compound for forensic purposes. This LOQ value was lower than a minimum requirement performance limit (MRPL; 2 ng mL^{-1}) approved by the Ministry of Health Decree on 11th of June 2003 (Dz. U. 2003, Nr 116, poz. 1104) for the determination of 9THC in blood samples taken from drivers.

Average recoveries for 9THC and THCCOOH in blood (both at the concentration of 10 ng mL^{-1}) were 52% (n = 3) and 83% (n = 3), respectively (Tab. 1). The method used allowed one to determine two target analytes in one blood sample applying two successive extraction steps at different pH.

In our methods, the accuracy control was based on: (1) quantification of 9THC and THCCOOH in reference Medidrug materials (Tab. 2), and (2) comparison of 9THC determination results obtained by the proposed method and previously validated method [22] (Tab. 3). Using the reference method, 9THC was extracted from acidified (pH 3) blood samples and determined by LC–MS–APCI. Intra- and inter-day precision (expressed as RSD) studies were conducted using control blood samples containing 9THC

Table 2. Certified and determined (inter- and intra- day tests) concentrations of Δ^9-tetrahydrocannabinol (9THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in Medidrug reference materials

No.	Concentration, ng mL^{-1}				Bias %
	assigned	confidence range	intra-day[a]	inter-day[a]	
	9THC				
1	6.13	3.61–8.66	4.45 ± 0.30 (7)	5.20 ± 1.06 (20)	in range
			5.95 ± 0.36 (6)		
2	3.0	1.8–4.2	4.03 ± 0.01 (0.4)	4.24 ± 0.30 (7)	in range
			4.46 ± 0.13 (2)		
3	5.2	3.3–7.1	8.16 ± 0.54 (7)	–	15
THCCOOH					
4	16.4	11.5–21.3	23.3 ± 0.56 (2)	23.6 ± 2.86 (12)	11
			25.8 ± 0.14 (0.6)		
			20.1 ± 0.79 (3)		

[a] Mean ± SD (RSD %).

„–" – not determined.

Table 3. Comparison of Δ^9-tetrahydrocannabinol (9THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) concentrations [mean ± SD (RSD (%), ng mL^{-1}] determined by LC–MS–APCI and GC–MS–NCI methods in blood and urine samples collected from drivers suspected of driving under the influence of cannabis

Case No.	Concentrations [mean ± SD (RSD %), ng mL^{-1}] determined by methods			
	LC–MS–APCI	GC–MS–NCI	Bias [%]	GC–MS–NCI
	9THC			THCCOOH
1	4.65 ± 0.35 (7)	3.16	–32	45.6 ± 0.49 (1)
2	4.10 ± 1.10 (27)	3.73 ± 1.36 (36)	–9	26.9 ± 1.77 (7)
3	4.30	5.77 ± 1.10 (19)	+ 34	23.1 ± 0.21 (1)
4	1.90[a]	2.77 ± 1.73 (62)	+ 46	16.5 ± 0.42 (3)
5	> 2[a]	0.37 ± 0.07 (19)	–	27.8 ± 0.28 (1)
6	3.23 ± 0.11 (3)	2.86 ± 0.43 (30)	- 11	61.8 ± 3.04 (5)
7	3.60	4.00 ± 0.06 (15)	+ 11	123.7 ± 8.56 (7)
8	3.47 ± 0.02 (0.6)	3.84 ± 0.28 (7)	+ 11	40.2 ± 3.68 (9)
9	–	22.50	–	79.1
10	–	6.59	–	5.94
11	> 2[a]	3.01	–	45.30
12	> 2[a]	> 0.5[b]	–	3.81 ± 0.5 (13)
13	> 2[a]	> 0.5[b]	–	17.71 ± 1.36 (8)
14	> 2[a]	> 0.5[b]	–	2.84 ± 0.06 (2)
15	> 2[a]	> 0.5[b]	–	4.92 ± 0.49 (10)
16*	–	–	–	109.44
17*	–	–	–	45.63

[a] LOQ for LC–MS–APCI method.
[b] LOQ for GC–MS–NCI method.
„–" – not determined.
* – urine specimen.

and THCCOOH at the concentrations of 10 and 50 ng mL^{-1}, respectively. The found values of intra- and inter-day precision did not exceed 25 and 6%, respectively. Precision for the reference materials was 20% for 9THC and 12% for THCCOOH. The method was successfully included into the international proficiency testing programmes and the obtained results were accepted in several legal cases.

The developed GC–MS–NCI method was applied to the determination of 9THC and THCCOOH in blood and urine samples taken from 17 randomly chosen drivers. Concentrations of both analytes in blood were in the range 0.4–5.77 ng mL^{-1} for 9THC (n = 15) and 3–124 ng mL^{-1} for THCCOOH. Concentration of THCCOOH in urine (n = 2) fell in the range 46–109 ng mL^{-1}. When 9THC concentration in blood was found higher than 2 ng mL^{-1}, it was concluded that these drivers have consumed cannabis recently and were still under the influence of this psychoactive compound. THCCOOH has been chosen to indicate the past use of cannabis. THCCOOH concentration in blood lower than 100 ng mL^{-1} can be attributed to either single or occasional consumption [11]. In contrast, THCCOOH concentrations in blood ranging from 100 to 200 ng mL^{-1} indicate addiction [30].

Simultaneous extraction of 9THC and THCCOOH from a single 1.0 mL-in-volume sample is especially required in forensic applications, where sample volumes are often limited and small. Determination of other drugs present in the sample requires separate sampling and thus a portion of the blood sample should be set aside for the independent re-test.

Our validation data were comparable to the published ones and obtained by other methods [4, 6]. In these methods, nobody utilised the same derivatising reagents for THCCOOH as in the presented method. Recovery was comparable to the value obtained for blood or plasma by other methods [4, 8, 18, 30]. However, direct comparison should be done very carefully as different methods were used for recovery calculations.

Since extraction of the target analytes from blood is difficult, in the most of LLE methods plasma has been used instead as a matrix. However, plasma samples are not always available, particularly in forensic cases. In the majority of the published assays it has been reported that in the analysed clinical samples blood, especially postmortem blood, caused additional difficulties such as variable quality and putrefaction thus possibly affecting reliable extraction and recovery [4, 24]. Moreover, quantitative results concerning determination of tetrahydrocannabinols in blood should be interpreted very carefully since terms: blood and plasma are sometimes used as synonyms. Therefore, many authors use a term: whole blood to emphasise the type of specimen.

Optimization of the described method led to the significant improvements in the assay performance. Our initial experiments as well as the study by Lechowicz [23] have shown that short heating of the sample (5 min at 40°C) after dilution of the phosphate buffer resulted in higher and more reproducible recovery of the analytes. Foltz *et al.* [8] have found that addition of a buffer prior to extraction is advantageous since it favours precipitation of proteins in blood and consequently improves reproducibility. D'Asaro [6] has concluded that heating of the blood sample prior to precipitation and addition of 10% acetone to ACN limits a degree of precipitate „clumping", that commonly occurs at the room temperature in case of whole blood samples and 100% ACN

used for precipitation. In consequence, blood sample and the precipitating solvent solution were more efficiently mixed during vortex mixing, which might partly explain the increased recovery of 9THC. In the method described in this paper sonication of blood was applied with satisfactory results. Chi Chu *et al.* [4] have performed sonication of blood to assess whether the recovery increases due to the decreased size of solid particles. However, sonication had no effect on recovery compared to the samples that were gently mixed. Noticeably, the quality of chromatography was lowered after sonication due to the increased baseline noise.

9THC and THCCOOH are extensively metabolized and were found as glucuronide conjugates in blood and urine. For the analysis of the free compounds in biosamples it is necessary to hydrolyse the glucuronide bond. Ether bonds in 9THC and 11-OH--THC molecules were resistant to the cleavage under basic conditions and, therefore, had to be hydrolyzed enzymatically. Glucuronide ester-linked to THCCOOH was relatively resistive to β-glucuroidase while easily hydrolyzed using base without heating and incubation time [19]. The work of Kemp *et al.* [18, 19] is consistent with our findings that for the cleavage of THCCOOH–glucuronide conjugates alkalization of the medium before extraction is sufficient. Noteworthy, the first step in this extraction procedure was addition of 2 mol L^{-1} NaOH, followed by extraction of 9THC with hexane–ethyl acetate mixture (7:1, v/v).

Several liquid-liquid and solid-phase techniques for extraction of tetrahydrocannabinols from blood and urine have been published. Many solvent combinations were used for extraction of 9THC and THCCOOH from biological matrices [4, 18]. The mixture of hexane–ethyl acetate (7:1, v/v) is used most often; thus it has been chosen also by us.

In the proposed method derivatization of hydroxy groups plays a double role: it makes both tetrahydrocannabinols less polar, thereby improving their gas chromatographic characteristics, as well as makes electron affinities of 9THC and THCCOOH increase by introduction of trifluoroacetyl and pentafluoropropyl groups, respectively. Trifluoroacetate derivatives are easily hydrolyzed. Therefore, it was essential not to transfer any amount of the aqueous phase with the organic phase after cleaning-up 9THC extract. Both 9THC and THCCOOH derivatives were stable for 1 day. No more attempts have been made to study the stability of derivatives. According to the previously published results [8] heptane solutions of the derivatized extracts were also stable for at least 1 day at the room temperature. If derivatized sample had to be stored for a longer period, it was necessary to repeat the treatment with trifluoroacetic anhydride just before GC–MS analysis.

Our experiments on fragmentation of 9THC and THCCOOH derivatives have shown that under more gentle fragmentation conditions during chemical ionization only one ion for each analyte was produced and reflected in the mass spectrum. The signals of target analytes must be free from the influence of the interfering peaks and the moni-

tored ions must be sufficiently specific to the drug being analysed to provide its undoubtful identification. Under the conditions applied in our procedure, two ions were generated for both analytes.

CONCLUSIONS

Concentration of 9THC in blood may rapidly decrease after smoking to the low ng mL^{-1} level. For this reason, sensitive and reliable MS method for detection and quantification of 9THC and THCCOOH in whole blood and urine samples for clinical and forensic toxicology purposes has been developed. For 1 mL-in-volume blood or urine sample, LOQ and LOD equalled 0.5 ng mL^{-1} and 0.25 ng mL^{-1} for both compounds.

REFERENCES

1. Baptista M.J., Monsanto P.V., Marques E.G.P., Bermejo A., Ávila S., Castanheira A.M., Margalho C., Barroso M. and Vieira D.N., *Forensic Sci. Int.*, **128**, 66 (2002).
2. Baselt R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 6th edn, Biomedical Publications, Foster City, California, 2002, p. 1004.
3. Chiarotti M. and Costamagna L., *Forensic Sci. Int*., **114**, 1 (2000).
4. Chi Chu M.H. and Drummer O., *J. Anal. Toxicol*., **26**, 575 (2002).
5. *Clarke's Analysis of Drugs and Poisons*, 3rd edn, [Moffat A.C., Osselton M.D., Widdop B., Eds], Pharmaceutical Press, London 2004, p. 740.
6. D'Asaro J.A., *J. Anal. Toxicol*., **24**, 289 (2000).
7. ElSohly M.A. and Salem M., in: *Handbook of Analytical Separations*, vol. 2 [Bogusz M., Ed.] Elsevier Science B.V., New York 2000, p. 163.
8. Foltz R.L., McGinnis K.M. and Chinn D.M., *Biomed. Mass Spectrom*., **10**, 316 (1983).
9. Giroud Ch., Ménétrey A., Augsburger M., Buclin T., Sanchez-Mazas P. and Mangin P., *Forensic Sci. Int*., **123**, 159 (2001).
10. Harvey D.J., in: *Analytical methods in human toxicology*, 1st edn, vol. 1, [Curry A.S., Ed.], VCH, Weinheim 1985, p. 258.
11. Huestis M.A., Henningfeld J.E. and Cone E.J., *J. Anal. Toxicol*., **16**, 276 (1992).
12. Huestis M.A., Henningfeld J.E. and Cone E.J., *J. Anal. Toxicol*., **16**, 283 (1992).
13. Huestis M.A., in: *Current approaches in forensic toxicology*, [Forensic Toxicologist Certification Board, Inc., Ed.] Tampa 1994, Chapter 6, p. 1.
14. Huestis M.A., Mitchell J.M. and Cone E.J., *J. Anal. Toxicol*., **20**, 441 (1996).
15. Huestis M.A. and Cone E.J., in: *Drug Abuse Handbook*, [Karch S.B., Ed.], CRC Press, New York, 1998, p. 799.
16. Huestis M.A., *Forensic Sci. Review*, **14**, 17 (2002).
17. Kała M., *Toxicological Analysis of Drugs of Abuse*, Wydawnictwo Instytutu Ekspertyz Sądowych, Kraków 2001, p. 168 (in Polish).

18. Kemp P.M., Abukhalaf I.K., Manno J.E., Manno B.R., Alford D.D. and Abusada G.A., *J. Anal. Toxicol*., **19**, 285 (1995).
19. Kemp P.M., Abukhalaf I.K., Manno J.E., Manno B.R., Alford D.D., McWilliams M.E., Nixon F.E., Fitzgerald M.J., Reeves R.R. and Wood M.J., *J. Anal. Toxicol*., **19**, 292 (1995).
20. Kintz P. and Samyn N., *Therapeutic Drug Monitoring*, **24**, 239 (2002).
21. Kudo K., Nagata T., Kimura K., Imamura T. and Jitsufuchi N., *J. Anal. Toxicol*., **19**, 87 (1995).
22. Lechowicz W., *Methods of the Hallucinogens Determination in Biological Fluids for Forensic Toxicology Purposes*, Doctor Thesis, Jagiellonian University, Kraków 2003.
23. Manno J.E., Manno B.R., Kemp P.M., Dempsey A.D., Abukhalaf I.K., McWilliams M.E., Hagaman F.N. and Fitzgerald M.J., *J. Anal. Toxicol*., **25**, 538 (2001).
24. Moeller M.R., Doerr G. and Warth S., *J. Forensic Sci*., **37**, 969 (1992).
25. Moeller M.R., Steinmeier S. and Kraemer T., *J. Chromatogr. B*, **91**, 713 (1998).
26. Moffat A.C., *Science & Justice*, **42**, 55 (2002).
27. Moore Ch., Guzaldo F. and Donahue T., *J. Anal. Toxicol*., **25**, 555 (2001).
28. Niedbala S.R., Kardos W.K., Fritch F.D., Kardos S., Fries T., Waga J., Robb J. and Cone J.E., *J. Anal. Toxicol*., **25**, 289 (2001).
29. Pfleger K., Maurer H.H. and Weber A., *Mass Spectral and GC Data of Drugs*, Poisons, Pesticides, Pollutants and their Metabolites, 2nd edn, VCH, Weinheim 1992, p. 2911.
30. Steinmeyer S., Bregel D., Warth S., Kraemer T. and Moeller R., *J. Chromatogr. B*, **772**, 239 (2002).
31. Sunshine I., *Monograph: Marijuana (THC)*, Marion Laboratories Inc., New York 1985, p. 153.
32. Wall M.E. and Perez-Reyes M., *J. Clin. Pharmacol*., **21**, 178 (1981).
33. Widdop B., *Drugs of Abuse, Immunoassay Handbook*, 2nd edn, The Pharmaceutical Press, London 2000, p. 781.
34. Wilkins D., Haughey H., Cone E., Huestis M., Foltz R. and Rollins D., *J. Anal. Toxicol*., **19**, 483 (1995).
35. Wills S., *Drugs of Abuse*, 1st edn, The Pharmaceutical Press, London 1997, p. 37.

Received August 2005
Accepted November 2005