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Improved and validated method for the determination of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy-THC in serum, and in human liver microsomal preparations using gas chromatography–mass spectrometry

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Abstract

A validated method for the quantification of Δ^9-tetrahydrocannabinol (THC) and its main metabolites 11-hydroxy-tetrahydrocannabinol (OH-THC) and 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) in serum is presented. The substances were isolated by solid-phase extraction, derivatised by methylation, and analysed by means of GC–MS in the selected ion monitoring mode. Quantitation was achieved by the addition of deuterated analogues as internal standards. The method was linear up to 10 ng/ml for THC and OH-THC, and up to 50 ng/ml for THC-COOH. The limits of quantification were 0.62 ng/ml for THC, 0.68 ng/ml for OH-THC and 3.35 ng/ml for THC-COOH. The limits of detection for the least intensive ions were 0.52 ng/ml for THC, 0.49 ng/ml for OH-THC and 0.65 ng/ml for THC-COOH. The method was validated according to the requirements of the *Journal of Chromatography B.* The method has been routinely used on samples from drivers suspected of ''driving under the influence''. In addition to the forensic application, a cross-validation was carried out by applying the method developed for serum to human liver microsomal preparation samples. © 2002 Published by Elsevier Science B.V.

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1. Introduction

Cannabis is one of the most widespread and most frequently consumed drugs worldwide. Cannabis products like hash, marijuana or hash oil are mostly smoked in pure form or in mixtures with tobacco via cigarette (''joint''), pipe or water pipe (''bong''). Also oral ingestion, e.g., in the form of cakes or tea, is well known. In spite of pro-legalising and of medical use as topics of discussion, at present in most countries around the world the selling, acquiring and possessing of cannabis is sanctioned. From the toxicological point of view, the main psychoactive constituent Δ^9-tetrahydrocannabinol (THC) and the equipotent metabolite 11-hydroxy-tetrahydro-

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cannabinol (OH-THC) as well as the inactive metabolite 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) are of main interest, since recent drug use and type of consumption may be assessed by individual cannabinoid drug levels and ratios of metabolite to parent drug concentration in body fluids. Huestis et al. [1] described that after inhalation, THC can be found rapidly in plasma prior to the end of smoking within the concentration range of 200–300 ng/ml. But due to distribution of the very lipophilic THC especially into fatty tissue, the concentration drops quickly to much lower values [1]. Therefore, in forensic practice, recent cannabis consumption can be assumed even at concentrations of as low as 2 ng/ml. The detection of OH-THC in blood in addition to THC is not required for legal sanctions. However, due to the fact that much more OH-THC could be found after oral ingestion compared to inhalation [2], the ratio of THC and OH-THC may be used for assessment of the type of consumption. However, due to its low concentration level and the short time window of detection, a sensitive analytical method is necessary to determine OH-THC. The pharmacologically inactive THC-COOH is formed by further oxidation. It is detectable in blood much longer than the parent compound and its active hydroxy metabolite [3]. Its concentration level can be used to differentiate between occasional and chronic cannabis consumption, as was described by Daldrup et al. [4].

In response to an increased demand for reliable evidence of cannabis use, several procedures have been developed for analysis of THC and/or its metabolites in blood mostly by means of gas chromatography (GC) in combination with mass spectrometry (MS), both in the electron impact (EI) [5,6] and chemical ionisation (CI) modes [7,8], or in combination with tandem mass spectrometry [9]. Overviews on analytical procedures with appropriate validation data are given in the reviews of Staub [10], and Moeller et al. [11]. In the presented paper, an improved version of a method from Moeller et al. [12] is presented for the simultaneous quantification of THC and its forensically most important metabolites (OH-THC, THC-COOH) in serum after solid-phase extraction (SPE) and methylation. The original method has been applied since 1992 in more than 2000 forensic cases as well as in further research work [13]. The present version has been changed by refining the washing procedure and using deuterated isoforms of each analyte as internal standards. Besides, the method was fully validated according to the requirements of the *Journal of Chromatography B* [14]. The procedure has been routinely applied to drugs and driving cases that were positive for cannabinoids in the immunoassay pre-screening test.

In addition to the forensic application, the method developed for serum was cross-validated in liver microsomal preparations for further pharmacokinetic studies on the in vitro metabolism of THC since its main initial metabolic pathway in humans is the formation of OH-THC catalysed by cytochrome P 450 isoenzymes [15].

2. Experimental

2.1. Materials, reagents and solvents

The following materials, reagents and solvents were used:

A centrifuge Rotina 46 (Hettich, Tuttlingen, Germany) for centrifugation of whole blood samples to obtain serum, and a Minifuge GL (Hereaus Christ, Germany) for separation of the organic layer after derivatisation and extraction. A Reacti-Therm heating module and a Reacti-Vap evaporation unit (Pierce, Rockford, IL, USA). The C_{18} bonded-phase adsorption columns were Strata C18-E (endcapped) 200 mg, 3 ml, supplied by Phenomenex (Torrance, CA, USA). A Vac-Elute vacuum manifold was used (Model SPS 24) which was purchased from Varian (Darmstadt, Germany).

Methanol, acetone, ethanol, isooctane, iodomethane, magnesium chloride ($MgCl_2$), trifluoroacetic anhydride (TFA) were analytical grade and were provided by Merck (Darmstadt, Germany). Dimethylsulfoxide (DMSO) and tetrabutylammoniumhydroxide (TBAH) were purchased from Fluka (Neu-Ulm, Germany). TBAH was dissolved in DMSO (1:50, v/v, TBAH/DMSO reagent). Nicotinamide adenine dinucleotidephosphate (NADP) was purchased from Biomol (Hamburg, Germany). Isocitric acid and isocitrate-dehydrogenase were obtained from Sigma (Taufkirchen, Germany).

The following were obtained from Radian (Austin,

TX, USA): (−)-Δ^9-THC (catalog No. T-005, 1 mg/ml, in methanol), (−)-Δ^9-THC-d3 (catalog No. T-003, 100 μg/ml, in methanol), (±)-11-hydroxy-Δ^9-THC (catalog No. H-027, 1 mg/ml, in methanol), (±)-11-hydroxy-Δ^9-THC-d3 (catalog No. H-041, 100 μg/ml, in methanol), (−)-11-nor-9-carboxy-Δ^9-THC (catalog No. T-019, 1 mg/ml, in methanol), and (±)-11-nor-9-carboxy-Δ^9-THC-d3 (catalog No. T-008, 1 mg/ml, in methanol).

2.2. Chromatographic system and conditions

GC–MS analysis was performed on a Hewlett-Packard (HP) 6890 GC system (Agilent Technologies, Waldbronn, Germany) connected to an HP MSD 5973 equipped with a polydimethylsiloxane capillary column HP-1 (12 m×0.2 mm I.D., 0.33 μm film thickness). Pulsed splitless injection was used with the HP 6890 autosampler system. The column temperature program was as follows: the initial temperature of 120 °C was held for 1.5 min, followed by an increase in the temperature at a rate of 30 °C/min to 180 °C, the program was then slowed to 10 °C/min until 210 °C was reached, then to the final temperature of 300 °C at 15 °C/min, and held for 12.5 min. The ionisation energy and interface temperature were set at 70 eV and 260 °C, respectively. Helium was used as carrier gas at a constant flow-rate of 0.9 ml/min with an injection pulsed pressure of 16 p.s.i. until 1.60 min (1 p.s.i.=6894.76 Pa).

The chosen ions for each compound for analysis with electron impact ionisation in single ion monitoring (SIM) mode were (values of deuterated ions in parentheses): *m/z* 313 (316), 328 (331), 245 (248) for THC and THC-d3, *m/z* 313 (316), 314 (317), 358 (361) for OH-THC and OH-THC-d3, and *m/z* 313, 357 (360), 372 (375) for THC-COOH and THC-COOH-d3. The quantification of THC and THC-COOH was done using the area ratio of the ions 328/331 and 372/375, respectively; the quantification of OH-THC was done using the signal height ratio of the ions 313/316. The other ions were used as qualifiers; but as evidence of the presence of substances, all ions had to be detected.

Data were automatically processed by software (G1701BA, Version 1.0) supplied by Hewlett-Packard with the MSD system. Each ion of interest was automatically selected, retention times were calculated, and the peak area respectively the peak height was determined. All data were checked for interferences, peak selection, and baseline determination.

2.3. Serum

Approx. 250 ml of blood was obtained from non-abusing volunteers. For ascertainment of the selectivity and extraction efficiency, five blood samples were taken from different non-abusers. All blood samples were centrifuged for 8 min at 2500 rpm at room temperature, and the serum was isolated and stored at −20 °C until assayed.

The authentic blood samples were from drivers suspected of ''driving under the influence'' according to the orders of the police, prosecution or administrative authorities. The drivers were selected by specially trained police officers if in traffic control situations indications of the use of drugs were obvious.

2.4. Human liver microsomes and preparation of incubation samples

Pooled human liver microsomes (protein content 20 mg/ml in 250 m*M* sucrose) were purchased from Gentest (Woburn, MA, USA). Assay results with enzyme activities and donor information were provided by the manufacturer. The microsomes were stored at −80 °C until needed.

Reaction mixtures containing 6 μl human liver microsomes, 358 μl of aqueous phosphate buffer (0.1 *M*, pH 7.4), 50 μl of NADP (10 mg/ml in phosphate buffer), 50 μl of isocitric acid/$MgCl_2$ (5 m*M* each in phosphate buffer) and 1 μl of isocitrate-dehydrogenase were placed in a water bath and warmed up to a temperature of 37 °C. After addition of 30 μl TFA solution (1:3, v/v in water) and 5 μl of a methanolic solution of THC and OH-THC at different concentrations, the mixtures were incubated for 10 min. The TFA solution was added before the addition of THC, in order to avoid microsomal metabolism during the incubation process. Then the vials were transferred immediately to an ice bath and subsequently stored at −20 °C. In total, 500 μl of each sample was obtained, and aliquots of 25 μl were used for SPE.

2.5. Preparation of stock and working solutions

A standard stock solution containing THC, OH-THC and THC-COOH was prepared in ethanol at a concentration of 2 μg/ml of both THC and OH-THC, and of 10 μg/ml of THC-COOH. The solution was kept frozen at −20 °C until needed. With this stock, both a serum working solution and a microsomal incubation working solution was prepared, containing 10 ng both of THC and OH-THC, and 50 ng THC-COOH in 1.0 ml of blank serum, or 25 μl of blank incubation mixture. The appropriate working solutions were prepared prior to each assay in order to independently prepare the calibration standards and quality controls.

2.6. Sample preparation and derivatisation procedure

Strata C_{18} endcapped cartridges were activated by washing with 2×3 ml methanol and 3 ml distilled water, successively. To 1.0 ml serum in a Pyrex tube (either spiked serum or authentic serum to be analysed) 50 μl internal standard solution (containing 5 ng THC-d3, 5 ng OH-THC-d3, 25 ng THC-COOH-d3) was added as well as 200 μl ethanol and 2.0 ml phosphate buffer solution (0.1 *M*, pH 9). To 25 μl of microsomal incubation sample, 50 μl internal standard solution was added as well as 200 μl ethanol and 2.675 ml phosphate buffer solution. Additionally, 300 μl of blank serum was added to the microsomal incubation sample to improve recovery and sensitivity. The samples were vortexed and cleaned-up by SPE. The cartridges were subsequently washed with 3.0 ml ethanol (8%, v/v, in water), 3.0 ml acetic acid (0.25 *M*), 1.5 ml water, 3.0 ml $NaHCO_3$ (5%, in water), 3.0 ml ethanol (8%, v/v, in water), 80 μl acetone and then allowed to dry for 20 min under maximum vacuum. The analytes were eluted from the columns with 2.0 ml acetone–methanol (1:1, v/v) under gravity. The eluates were evaporated to dryness at 60 °C under a stream of nitrogen, the residues were dissolved in 250 μl of TBAH/DMSO reagent, and after 2 min at room temperature, 50 μl of iodomethane was added as derivatisation reagent. After 10 min at room temperature, 300 μl of 0.1 *M* aqueous hydrochloric acid was added, and the reaction mixtures were extracted with 2.0 ml isooctane by shaking (1 min). After centrifugation, the organic phase was transferred to a clean tube and evaporated to dryness at 60 °C under a stream of nitrogen. The derivatised residues were reconstituted in 40 μl of isooctane, and a 1.0 μl aliquot was injected into the GC–MS system.

2.7. Selectivity

To assess peak-purity and selectivity, five different blank serum samples were analysed and checked for peaks interfering with the detection of the analytes or the internal standards.

2.8. Calibration curves and limits

Calibration curves were prepared on 3 separate working days by the same analyst. The serum working solution was diluted with blank serum to obtain calibration concentrations of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 ng/ml both of THC and of OH-THC, and 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 ng/ml of THC-COOH. The characteristics of the calibration graphs established with serum were calculated with ProControl program, version 3.1 (Isomehr, Saarbruecken, Germany). The limits of the method were calculated according DIN 32645 (German Industry Standard) at low concentrations close to the limit of detection (LOD), which is determined by using the ions with least intensity (m/z 245 for THC, m/z 358 for OH-THC, m/z 357 for THC-COOH). The calculations of the limits of quantification (LOQs) were done by using those ions with which the quantification was done (m/z 328 for THC, m/z 313 for OH-THC, m/z 372 for THC-COOH).

2.9. Accuracy and precision

Independent serum control samples ($n=5$) were prepared from the stock solution at a low concentration level of 1.5 ng/ml THC and OH-THC, and 7.5 ng/ml THC-COOH, and at a high concentration level of 8 ng/ml THC and OH-THC, and 40 ng/ml THC-COOH. The samples were analysed on 3 different working days together with an independent calibration curve, respectively. The accuracy was assessed by comparing the mean concentrations of THC, OH-THC and THC-COOH in

the samples with the target concentration, and expressed as percentages. An estimate of the precision was obtained using one-way analysis of variance (ANOVA) with day as grouping variable: intra-assay precision was determined by calculating the percent ratio between the standard deviation within groups and the mean value of all samples, and inter-assay precision was determined by calculating the percent ratio between the standard deviation between groups and the mean value of all samples [16]. In addition, the method has also been applied in national and international proficiency testing programs.

2.10. Extraction efficiency

To determine the extraction efficiency of THC and its two metabolites, two batches of five independent blank serum samples spiked at two different concentrations for each compound were prepared (THC and OH-THC, 1.5 and 8 ng/ml, respectively; THC-COOH, 7.5 and 40 ng/ml). The first batch was extracted as described. For the second batch, the internal standard had been added to the eluate after SPE. The extraction efficiencies of THC and THC-COOH were calculated by comparing the peak area ratios of analyte to internal standard obtained for each compound in the first batch with the appropriate peak area ratios obtained in the second batch. For OH-THC, the calculation was made by comparing the appropriate peak height ratios instead of the peak areas.

2.11. Determination of stability

Numerous studies were carried out in order to determine the stability of the analytes during storage and analysis [17–20]. In our study, the stability of serum samples stored at −20 °C was investigated since in normal routine work serum samples will not be analysed immediately following collection but days or even weeks later. The freezer stability was assessed as follows: five serum samples, each containing 5 ng/ml of both THC and OH-THC, and 25 ng/ml THC-COOH, were prepared in 10-ml tubes and divided into aliquots; the first part was analysed immediately, and the second part was frozen for a time span of 1 month and then analysed after thawing.

2.12. Application to blood samples from suspected drivers

In the year 2000, 764 blood specimens of drivers involved in traffic offences were investigated in our institute for the presence of illicit drugs. In 512 cases, GC–MS analysis was performed for the determination of cannabinoids in serum by using the presented method.

2.13. Cross-validation in human liver microsomal preparation as matrix

The analytical method was applied to human liver microsomal preparation samples, and a cross-validation was performed by analysing replicate samples ($n=5$) on 3 different working days spiked at two concentrations for each compound (both THC and OH-THC 1.5 and 8 ng/50 μl) in order to determine the accuracy and the precision using the serum calibration curves, estimated by ANOVA (cf. Section 2.9). The microsomes to be used were inactivated before incubation by addition of TFA solution to avoid biotransformation of THC to OH-THC. THC-COOH was not included in the cross-validation, because its formation is not catalysed by microsomal enzymes [21].

3. Results

3.1. Selectivity

All five different blank serum samples tested were free from substances interfering with THC and THC-COOH. Near the retention time of OH-THC, background peaks were detected for m/z 316, 317. As a consequence, the appropriate concentrations of OH-THC were calculated by determining the peak height instead of the peak area in order to avoid evaluation problems. The identities of the background peaks could not be elucidated. However, in spite of the presence of these peaks, validation data of the method was acceptable. Representative mass fragmentograms are shown in Fig. 1.

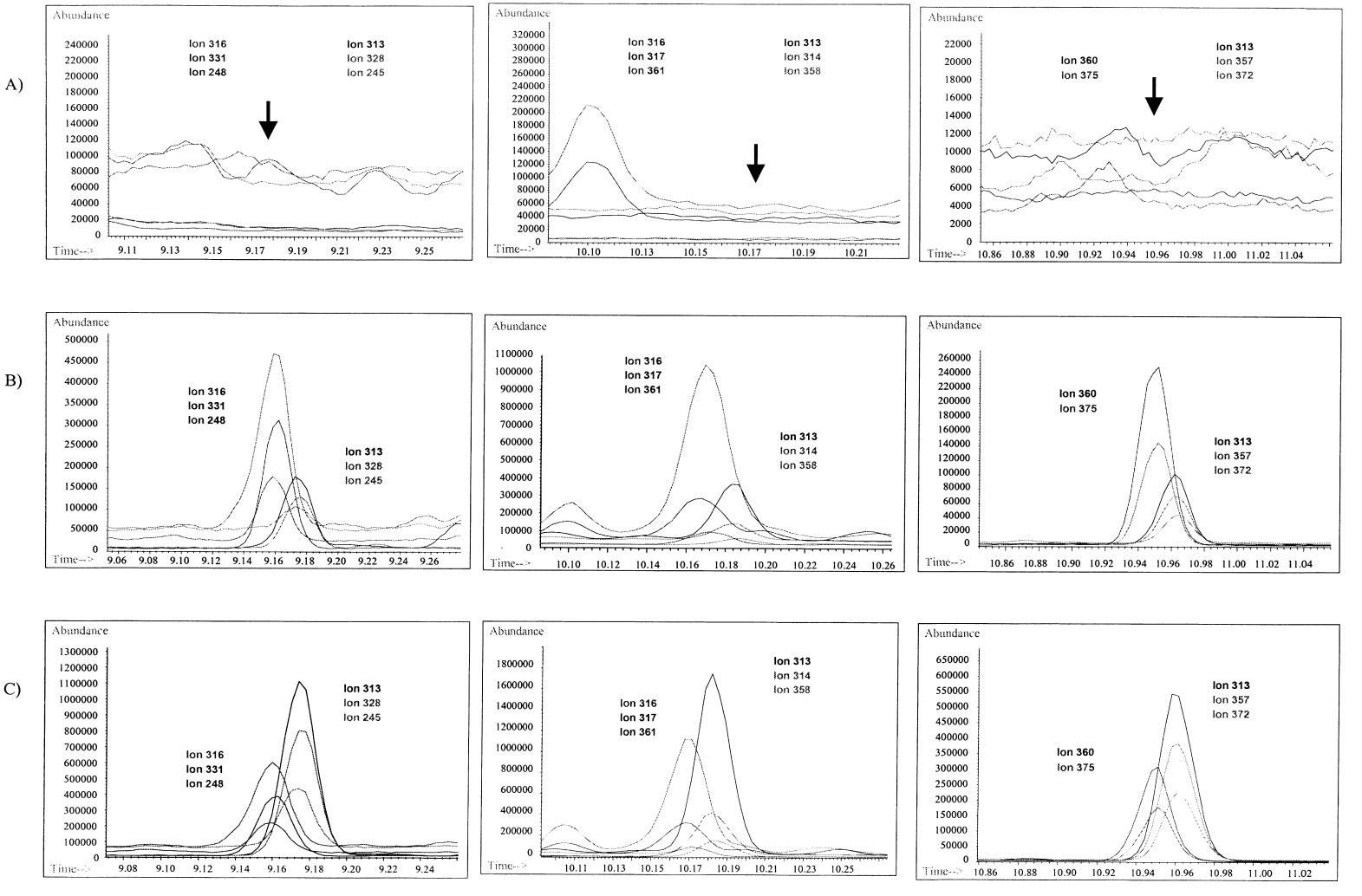

Fig. 1. Merged mass fragmentograms of extracted serum samples at the corresponding retention times of THC (left side), OH-THC (middle) and THC-COOH (right side). (A) Blank serum sample, (B) blank serum sample spiked with 1.5, 1.5 and 7.5 ng/ml THC, OH-THC and THC-COOH, respectively, and (C) blank serum sample spiked with 8.0, 8.0 and 40 ng/ml THC, OH-THC and THC-COOH, respectively. B and C additionally contained 5.0, 5.0 and 25 ng/ml THC-d3, OH-THC-d3 and THC-COOH-d3, respectively.

Table 1
Characteristics of the calibration graphs

Compound	Regression equation of standards	Correlation coefficient (r)	Limit of detection (ng/ml)	Limit of quantification (ng/ml)
THC	$y=0.26x+0.07$	0.99973	0.52 m/z 245	0.62 m/z 328
OH-THC	$y=0.21x+0.05$	0.9992	0.49 m/z 358	0.68 m/z 313
THC-COOH	$y=0.03x+0.01$	0.99996	0.65 m/z 357	3.35 m/z 372

3.2. Calibration curves and limits

An overview on the characteristic data of the method is given in Table 1. The results demonstrated a linear relation of concentration to peak area for THC up to 10 ng/ml, and for THC-COOH up to 50 ng/ml. Also the relation of concentration to peak height for OH-THC was linear up to 10 ng/ml. The LOQs were 0.62 ng/ml for THC, 0.68 ng/ml for OH-THC and 3.35 ng/ml for THC-COOH. The LODs for the least intensive ions were 0.52 ng/ml for THC, 0.49 ng/ml for OH-THC and 0.65 ng/ml for THC-COOH.

3.3. Accuracy and precision

The data on precision and accuracy are presented in Table 2. For THC and THC-COOH, good accuracy and precision were obtained, shown by the low percent values clearly below 10% at the two concentration levels. For OH-THC, the accuracy and precision values were higher but below 20% for the low concentration. In addition, application of the method in national and international proficiency testing programs was successful.

3.4. Extraction efficiency

In Table 3, the mean percentage extraction efficiencies of THC, OH-THC and THC-COOH at different concentrations are listed. The method provided good extraction efficiencies (73–88%) for all three analytes from serum at both concentration levels.

3.5. Determination of stability

The serum samples did not show any considerable change in concentration over a 1-month period. The results, expressed as the mean calculated concentrations, are presented in Table 4.

3.6. Analysis of blood samples from suspected drivers

THC (always in addition to THC-COOH) was detectable in 71.1% (364) of the 512 specimens

Table 2
Summary of assay precision and accuracy data for the determination of THC, OH-THC and THC-COOH in human serum samples ($n=15$)

Compound	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	Within-assay precision (%)	Between-assay precision (%)
THC	1.50	1.55	3.34	2.20	5.60
	8.00	8.28	3.50	1.93	3.21
OH-THC	1.50	1.47	−2.00	6.63	15.05
	8.00	8.11	1.38	3.46	10.52
THC-COOH	7.50	7.44	−0.80	2.01	3.70
	40.0	40.30	0.75	1.47	1.81

Table 3
Extraction efficiencies for THC and its two metabolites from serum ($n=5$)

Compound	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Recovery (%)
THC	2.0	1.48±0.06	73.8
	8.0	6.36±0.10	79.5
OH-THC	2.0	1.68±0.08	83.8
	8.0	6.96±1.24	87.0
THC-COOH	10.0	8.73±0.21	87.3
	40.0	35.36±0.95	88.4

measured with the presented method. OH-THC could also be detected in 82.4% of the 364 positive cases. Determination of OH-THC was not included in the previous method [12] due to the non-availability of a deuterated internal standard. THC-COOH alone was found in 22.6% of all cases. The serum samples were found negative for cannabis in 6.3% of all samples. The high prevalence of positive results can be explained by the fact that the police officers had a special training in drug recognition. In 52.7% of the 364 THC-positive cases, THC was found at concentrations higher than 2 ng/ml. It was concluded, that these drivers seemed to have had recent cannabis consumption.

60.2% of the THC-COOH positive cases showed concentrations lower than 100 ng/ml. This can be attributed to single or occasional consumption [1]. 18.0% concentrations were in the range from 100 to 200 ng/ml, indicating transition to chronic consumption. For 14.8% of the cases, THC-COOH concentrations were higher than 200 ng/ml, corresponding to chronic consumption of cannabis, on the scale of several times per week or even daily. The ''high-score'' cases of each compound were: 72.0 ng/ml for THC (furthermore, 28.0 ng/ml OH-THC, 561 ng/ml THC-COOH), 34.8 ng/ml for OH-THC (furthermore, 67.4 ng/ml THC, 952 ng/ml THC-COOH), and 1070 ng/ml for THC-COOH (furthermore, 16.7 ng/ml THC, 6.8 ng/ml OH-THC). The median of the forensically most relevant analyte THC was 4.1 ng/ml. In Fig. 2, the mass fragmentograms of an extracted serum sample of an authentic case for THC, OH-THC and THC-COOH are presented. It has to be mentioned that serum samples with concentrations of THC, OH-THC or THC-COOH measured above the appropriate working range were diluted with buffer, and the determination was repeated.

The fully validated method has also been successfully applied to national and international proficiency testing programs and its results have been accepted in numerous cases in court. It has been shown to be superior to the previous method [12].

3.7. Application to human liver microsomal preparation as matrix

The appropriate data are shown in Table 5. The found accuracy and precision values for both levels of THC, as well as for both levels of OH-THC, are excellent. All data are clearly below 20% for the low concentrations. The good results can be explained by the fact that there are far less matrix effects in the

Table 4
Stability of THC and its two metabolites in human serum ($n=5$) for a period of 1 month

Compound	Nominal concentration (ng/ml)	Initial concentration (ng/ml)	Final concentration (ng/ml)
THC	5.0	5.51±0.05	5.20±0.07
OH-THC	5.0	4.91±0.35	4.80±0.31
THC-COOH	25.0	24.83±0.31	24.41±0.23

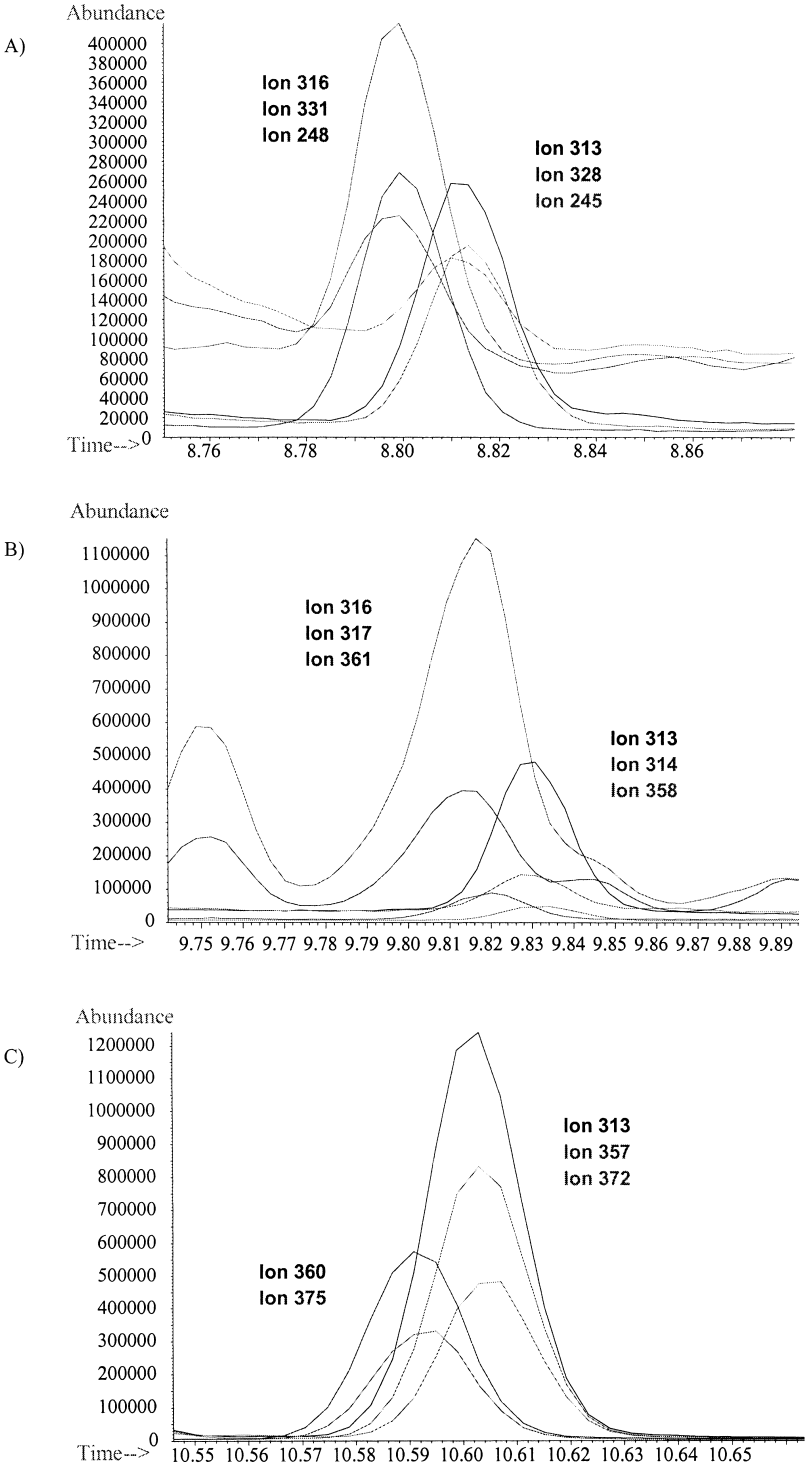

Fig. 2. Merged mass fragmentograms of an extracted serum sample of an accident involved driver at the corresponding retention times of THC, OH-THC and THC-COOH. (A) THC, 2.9 ng/ml; (B) OH-THC, 2.1 ng/ml and (C) THC-COOH, 47.3 ng/ml.

Table 5
Summary of assay precision and accuracy data for the determination of THC and OH-THC in human liver microsomal preparation ($n=15$)

Compound	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	Within-assay precision (%)	Between-assay precision (%)
THC	2.00	2.03	1.50	1.32	4.07
	8.00	8.12	1.50	1.39	0.96
OH-THC	2.00	1.88	−6.00	6.35	3.79
	8.00	8.24	3.00	3.15	12.04

microsomal preparation samples than in the serum samples. This method was applied to kinetic studies of THC metabolism in microsomal preparations. The results will be published elsewhere [22].

4. Conclusions

This report describes a simple and sensitive GC–MS procedure for the quantification of THC and its forensically most important metabolites. The method has been routinely used on samples from drivers suspected of impaired driving for administrative offences regarding the German Road Traffic Law (§ 24a StVG, Strassenverkehrsgesetz) and for criminal offences regarding the German Penal Code (§§ 316, 315c StGB, Strafgesetzbuch), respectively. It has been successfully applied to national and international proficiency testing programs and its results have been accepted in numerous cases in court. The method has also been used for analyses of haemolytic post-mortem samples, but this application is very rare.

In addition to the determination of cannabinoids in serum, the method was also suitable for determination of THC and OH-THC in human liver microsomal preparations, allowing application to in vitro studies on the metabolism and pharmacokinetics of THC.

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