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Validated method for the simultaneous determination of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy-THC in human plasma using solid phase extraction and gas chromatography–mass spectrometry with positive chemical ionization

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Abstract

A fully validated, highly sensitive and specific method for the extraction and quantification of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in plasma is presented. This method incorporates *Escherichia coli* β-glucuronidase hydrolysis to cleave glucuronic acid moieties to capture total analyte concentrations, and simultaneous solid phase extraction (SPE) of the three analytes in a single eluant with separation and quantification on a bench-top positive chemical ionization (PCI) gas chromatography–mass spectrometry (GC–MS) in the selected ion monitoring (SIM) mode. Quantitation was achieved by the addition of deuterated analogues for each analyte as internal standards (IS). Limits of quantitation (LOQ) were 0.5, 0.5 and 1.0 for THC, 11-OH-THC and THCCOOH, respectively, with linearity ranging up to 50 ng/ml for THC and 11-OH-THC, and 100 ng/ml for THCCOOH. Absolute recoveries ranged from 67.3 to 83.5% for all three analytes. Intra-assay accuracy and precision ranged from 1.2 to 12.2 and 1.4 to 4.7%, respectively. Inter-assay accuracy and precision ranged from 1.4 to 12.2 and 3.1 to 7.3%, respectively. This method was used to analyze plasma samples collected from individuals participating in a controlled oral THC administration study. Statistically significant ($P \leq 0.05$) increases of 40% for 11-OH-THC and 42% for THCCOOH concentrations were found between hydrolyzed and non-hydrolyzed results. This method will be utilized in ongoing controlled cannabinoid administration studies and may be a useful analytical procedure for the fields of forensic toxicology and cannabinoid pharmacology.

Keywords: Δ^9-Tetrahydrocannabinol; 11-Hydroxy-Δ^9-tetrahydrocannabinol; 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol

1. Introduction

The most commonly used illicit drug throughout the world is cannabis. The last decade has seen an expanding global market for food products derived from or containing cannabis material including nutritional supplements. Ingestion of these products may result in exposure to cannabinoid compounds, such as Δ^9-tetrahydrocannabinol (THC), the primary psychoactive compound in cannabis [1,2]. Pharmacological research on the therapeutic potential of cannabinoids [3] has expanded with the elucidation of an endocannabinoid system [4]. Consequently, sensitive and specific analytical methods for the determination of cannabinoids in biological fluids are needed for forensic purposes and for cannabinoid pharmacokinetic and pharmacodynamic studies.

Detection and quantification of urinary cannabinoids remains an important forensic toxicology issue as urine drug

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testing has expanded. However, urine drug concentration data do not provide adequate answers to demanding clinical and forensic questions. These are more readily answered with quantitative plasma data; however, the analysis of plasma is far more complex due to the presence of lipophilic and proteinaceous compounds not usually found in urine, the need for substantially lower sensitivity limits, and the need to measure analytes of strikingly different chemical natures. Compounds of interest elute only in either acidic or basic fractions, making simultaneous extraction and chromatography of all analytes of interest difficult. Therefore, the development of an analytical method with simultaneous extraction of parent and metabolite cannabinoids in human plasma with high sensitivity and specificity would be very useful.

THC is rapidly oxidized to 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), an equipotent psychoactive metabolite, and further to the non-psychoactive 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH). Smaller quantities of other metabolites are produced by minor metabolic pathways [5]. Cytochrome P_{450} enzymes, CYP3A4, CYP2C9 and 11 are responsible for most of the oxidative activity, primarily in the liver, but to a lesser extent in other tissues [6]. The route of drug administration influences metabolite concentrations. A greater amount of 11-OH-THC is found in plasma following oral ingestion compared to inhalation [7]. This effect has been attributed to extensive first pass metabolism [8,9]. These compounds generally undergo further biotransformation to glucuronide conjugates producing water-soluble compounds that facilitate urinary excretion [7,10,11]. With THC, an ether bond forms between the glucuronic acid and the hydroxy-moiety of the phenolic ring. The same reaction occurs with 11-OH-THC, with the possibility of an additional ether bond between the hydroxy-moiety on carbon eleven (C-11). The THCCOOH metabolite may form a C-1 ether bond as well, but predominately forms an ester bond between the glucuronide and the carboxy-moiety at C-11. β-Glucuronidase and alkaline hydrolysis are effective methods for cleaving ester-linked glucuronic acid from THCCOOH. However, alkaline hydrolysis is ineffective in breaking the phenolic ether and the C-11 ether glucuronide bonds [12].

Early research by Wall and Perez-Reyes [7], and Wall and Taylor [13] on the metabolism of THC following inhalation or intravenous administration indicated that conjugated cannabinoids in plasma are low, contributing little to the total cannabinoid concentration. However, low concentrations of conjugated cannabinoids were found in plasma following oral THC administration [7]. Law et al. [14] determined that the ratio of plasma THCCOOH glucuronide to free THCCOOH at peak plasma concentration was approximately 2.8, 4 h after the ingestion of 20 mg THC. Thus, concentrations of conjugated metabolites in plasma following oral THC may be present in significant amounts. In 1995, Kemp et al. [12,15] reported that β-glucuronidase from *Escherichia coli* was most effective in cleaving ether-linked glucuronic acid of cannabinoid metabolites. The glucuronidase enzyme preparations produced significantly greater concentrations of total THC and 11-OH-THC, approximately 40 and 80 % increases respectively, than either the non-treated or base hydrolysis group ($P < 0.05$). Hydrolysis of the ester bond of THCCOOH-glucuronide was affected less by the source of β-glucuronidase. ElSohly and Feng [16] and Feng et al. [17] further validated the usefulness of *E. coli* β-glucuronidase for hydrolysis of cannabinoid-glucuronides in plasma, urine and meconium. The extent of glucuronide conjugate contribution to total cannabinoid concentrations in plasma and urine is still unclear, especially following oral THC administration.

Gas chromatography–mass spectrometry (GC–MS) for the separation and quantification of cannabinoids in biological fluids is well established [18–21]. Electron impact (EI) remains the most widely used method of ionization in mass spectrometry [18], although the proportion of quantitative assays using chemical ionization (CI) is increasing as cost declines and the availability of bench-top systems with both EI and CI capabilities increases. Negative chemical ionization (NCI) and positive chemical ionization (PCI) techniques may be employed to improve selectivity and sensitivity [18]. One possible drawback to CI is the smaller number of prominent ion peaks in the mass spectra due to lower fragmentation energy. This concentrates most of the analyte's ion current into a single ion mass, increasing sensitivity, but decreasing the abundance of other characteristic ions.

In order to achieve adequate sensitivity, lengthy and labor-intensive liquid–liquid extraction procedures were used for the quantification of pH neutral (THC and 11-OH-THC) and acidic cannabinoids (THCCOOH) with two separate GC–MS analyses. Recent advances in solid phase extraction (SPE) chemistry have eliminated the labor-intensive manipulations associated with liquid–liquid extractions and new co-polymeric sorbent columns have allowed simultaneous extraction of THC, 11-OH-THC and THCCOOH.

Numerous analytical procedures exist for the quantification of these important analytes in whole blood, serum and plasma. Some methods have utilized NCI detection to achieve the high sensitivity necessary for cannabinoid quantification in plasma. Huang et al. [22], reported plasma limits of quantification (LOQ) of 0.5 and 2.5 ng/ml for THC and THCCOOH with GC–MS–NCI following SPE. Most recently, Steinmeyer et al. [23] described a method for cannabinoids in human serum using SPE followed by a multi-step liquid–liquid extraction and GC–MS–EI with LOQs of 0.62 and 0.68 ng/ml for THC and 11-OH-THC, and a higher LOQ of 3.35 ng/ml for THCCOOH. In 2000, D'Asaro [24] published an automated SPE GC–MS–EI method for cannabinoids in whole blood. The procedure featured a simultaneous extraction and quantification of analytes; however, the analysis was limited to THC and THCCOOH. None of these methods included an alkaline or enzyme hydrolysis step to address the potential presence of

cannabinoid glucuronide conjugates. As Kemp et al. [12] and ElSohly and Feng [16] reported, cannabinoid concentrations in some biological matrices may be underestimated without appropriate hydrolysis of glucuronide metabolites following some routes of administration.

The purpose of this study was to develop and fully validate a highly sensitive and specific procedure for the simultaneous extraction and quantification of THC, 11-OH-THC and THCCOOH in human plasma. This method is necessary for our ongoing controlled cannabinoid administration studies evaluating the pharmacokinetics and pharmacodynamics of cannabis. The proposed analysis, unlike previous methods, incorporates *E. coli* β-glucuronidase hydrolysis to cleave glucuronic acid moieties to capture total analyte concentrations. A simultaneous SPE of the three cannabinoid analytes in a single eluant and separation and quantification on a bench-top positive chemical ionization GC–MS were utilized for increased sensitivity and specificity.

2. Experimental

2.1. Materials, reagents and solvents

THC, 11-OH-THC, THCCOOH, deuterated THC ([$^2H_3$]-THC), deuterated 11-OH-THC ([$^2H_3$]-11-OH-THC), deuterated THCCOOH ([$^2H_3$]-THCCOOH), phenylpropanolamine, ephedrine, pseudoephedrine, phentermine, pentazocine hydrochloride, caffeine, nicotine, clonidine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, methadone, 3,4-methylenedioxyethylamphetamine, fenfluramine, hydromorphone, oxycodone, oxymorphone, hydrocodone, dextromethorphan, phencyclidine, and diphenhydramine hydrochloride were obtained from Cerilliant (Austin, TX). *N*,*O*-bis(trimethyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from Pierce Chemical (Rockford, IL). Cannabinol (CBN), cannabidiol (CBD) and cannabigerol (CBG) were supplied by the National Institute on Drug Abuse (NIDA, Rockville, MD). Data were received with each drug standard indicating that each had a purity equal to or better than 99%. β-glucuronidase (*E. coli*, type IX-A) was purchased from Sigma (St. Louis, MO). Acetonitrile, ethyl acetate, hexane, isopropanol, methanol and methylene chloride were HPLC grade and obtained from Mallinckrodt Baker (Phillipsburg, NJ). Ammonium hydroxide, glacial acetic acid, hydrochloric acid, potassium phosphate dibasic, potassium phosphate monobasic, sodium acetate and sodium hydroxide were ACS reagent grade and purchased from Mallinckrodt Baker (Phillipsburg, NJ). Solid phase extraction columns (Clean Screen ZSDAU020) and vacuum manifolds were obtained from United Chemical Technologies (Bristol, PA). Drug-free expired plasma from a local blood bank was used for the development and validation of the method.

2.2. Calibrators and controls

For the calibrator samples, three working solutions were prepared in methanol at the following concentrations: 0.01, 0.1 and 0.5 ng THC and 11-OH-THC/μl, and 0.02, 0.2 and 1.0 ng THCCOOH/μl. Additional methanolic solutions were prepared for quality control (QC) samples at concentrations of 1.0 ng/μl for THC, 11-OH-THC and 20 ng/μl for THCCOOH. Calibrator and QC working solutions were made from different source lots. All working solutions were stored at −20 °C when not in use. Daily calibration samples were prepared by fortifying 1.0 ml of blank plasma with known amounts of THC, 11-OH-THC at concentrations ranging from 0.5 to 50 and 1.0 to 100 ng/ml for THCCOOH. Low, medium and high QC specimens were also prepared daily at concentrations of 2.0, 20, 40 ng/ml for THC and 11-OH-THC, and 4.0, 40, 80 ng/ml for THCCOOH.

For the deuterated internal standard (IS), a working solution of 0.2 ng [$^2H_3$]-THC/[$^2H_3$]-11-OH-THC and 0.4 ng [$^2H_3$]-THCCOOH/μl in methanol was prepared and stored at −20 °C when not in use. Twenty five microliters of this working solution was added to each sample prior to extraction, giving a final deuterated IS concentration of 5.0 and 10 ng/ml for [$^2H_3$]-THC/[$^2H_3$]-11-OH-THC and [$^2H_3$]-THCCOOH, respectively.

2.3. Sample preparation and derivatization procedure

Calibrators, and QC samples were prepared in separate 16 mm × 100 mm culture tubes by the addition of 1.0 ml blank plasma, appropriate amount of calibrator or QC working solution and IS while gently vortexing. 1.0 ml of plasma from study participant samples was pipetted into tubes and IS added while gently vortexing. Tubes were capped and allowed to equilibrate for 1 h at room temperature. To each tube was added 1.0 ml of 0.1 M potassium phosphate buffer (pH 6.8) followed by 250 μl of a 20,000 units/ml solution of β-glucuronidase in the same buffer (a total of 5000 units). The tube was capped and vortexed gently, placed in a 37 °C waterbath and incubated for 16 h. After allowing the sample to cool to room temperature, 1 ml of cold acetonitrile was gradually added while gently vortexing the tube. Tubes were centrifuged for 15 min at 1500 × *g* and the supernatant was decanted into clean 16 mm × 100 mm tubes. After addition of 2.0 ml of 2N sodium acetate buffer (pH 4.0), the tubes were vortexed and centrifuged for 15 min at 1500 × *g*. SPE columns were conditioned with sequential addition and elution of 1.0 ml freshly prepared primary elution solvent (methylene chloride: 2-propanol: concentrated ammonium hydroxide, 80:20:2 by volume), 3 ml methanol, 3 ml double deionized water and 2 ml 2N sodium acetate buffer (pH 4.0). Supernatants were decanted onto conditioned SPE columns on a vacuum manifold. Vacuum was applied at a rate of 1.0 ml/min to the columns to elute solvents and samples. Columns were washed by the sequential addition and elution of 2 ml double deionized water and 1.25 ml 0.2N

hydrochloric acid and then dried under vacuum for 5 min. Analytes were eluted into 10 ml conical centrifuge tubes by the addition of 2.5 ml primary elution solvent and 2.5 ml of a secondary elution solvent of hexane: ethyl acetate (80:20 by volume) at a rate of 1.0 ml/min. The combined eluates were dried under a stream of nitrogen at 40 °C using a Zymark Turbovap® LV Evaporator. Extracted residues were reconstituted with 20 μl acetonitrile and vortexed, to increase recovery from tube walls, then centrifuged for 5 min at 1500 × *g*. The acetonitrile layer was transferred from each tube to a 250 μl polypropylene injection vial with a glass insert, 20 μl of BSTFA containing 1% TMCS was added, the vial was capped and heated at 80 °C for 45 min. The trimethylsilyl derivatives (2 μl) were injected on the GC–MS system.

2.4. Chromatographic and detection system conditions

GC–MS analysis was performed on an Agilent 6890 gas chromatograph interfaced with an Agilent 5973 mass-selective detector. Splitless injection was used with the Agilent 7683 autosampler system. Separation of analytes was achieved with a HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) with helium as carrier gas at a flow rate of 1.0 ml/min. The initial column temperature of 120 °C was held for 0.5 min, followed by an increase to 200 °C at 15 °C/min with a 0.5 min hold. The oven temperature was increased to 250 °C and again to 300 °C at 15 °C/min and held for 2 min at each temperature. The MS was operated in the positive ionization mode, methane (Grade 4.0, 99.99% pure) was used as reactant gas at an apparent pressure of 1.0×10^{-4} Torr in the ionization source. MS interface, source and quadrupole temperatures were 295 °C, 250 °C and 150 °C, respectively. Selective ion monitoring was used with a dwell time of 100 ms per ion. One ion for each analyte was monitored: [$^2H_3$]-THC, *m/z* 390; THC, *m/z* 387; [$^2H_3$]-11-OH-THC, *m/z* 462; 11-OH-THC, *m/z* 459; [$^2H_3$]-THCCOOH, *m/z* 492; and THCCOOH, *m/z* 489.

2.5. Data analysis

Each specified ion was automatically selected, retention times were calculated, and peak abundances determined. All data were checked for interferences, peak shape and selection, and baseline determination. Calibration, using internal standardization, was done by linear regression analysis over a maximum concentration range from 0.5 to 50 ng/ml for THC and 11-OH-THC and 1.0 to 100 ng/ml for THCCOOH. For each standard curve, a minimum of five different concentrations was used. Peak abundance ratios of analytes to their respective deuterated IS were calculated for each concentration. Data were fit to linear least-squares regression curves with a weighting factor of $1/x$ to account for unequal variances (heteroscedasticity) over the calibration range.

2.6. Selectivity

To evaluate peak-purity and selectivity, blank plasma samples (no analyte or IS added) were analyzed with each batch to check for peaks that might interfere with detection of analytes or internal standards. Also, unextracted methanolic IS samples were analyzed by full scan mode to verify no appreciable amounts (<1%) of non-deuterated analyte ions and negative samples (blank plasma + IS) were analyzed to verify the absence of native analyte in the IS solution. To assess possible interferences, low quality control samples were spiked individually to contain 1000 ng/ml of cannabinol, cannabidiol, cannabigerol and 10,000 ng/ml of phenylpropanolamine, ephedrine, pseudoephedrine, phentermine, pentazocine hydrochloride, caffeine, nicotine, clonidine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyamphetamine, methadone, 3,4-methylenedioxyethylamphetamine, fenfluramine, hydromorphone, oxycodone, oxymorphone, hydrocodone, dextromethorphan, phencyclidine, and diphenhydramine hydrochloride.

2.7. Linearity, carry-over and limits of quantitation and detection

Calibration curves were prepared daily by spiking blank plasma with corresponding analytical working solutions to obtain calibration concentrations of 0.5,1.0, 2.5, 10, 50 ng/ml THC and 11-OH-THC, and 1.0, 2.0, 5.0, 20, 100 ng/ml of THCCOOH. Validation samples were prepared in triplicate at the following concentrations: 0.1, 0.25, 75, 100, 150, 500 ng/ml THC and 11-OH-THC, and 0.25, 0.5, 150, 200, 250, 300, 1000 ng/ml THCCOOH to assess the method's accuracy above and below the calibration curve. Negative quality control samples were analyzed after each linearity sample to evaluate potential carry-over.

The limit of detection (LOD) of the method was determined by analyzing validation samples ($n = 5$) to determine if acceptance criteria were met for each analyte. The LOD was defined as the lowest concentration at which the analyte ion signal-to-noise ratio (determined by peak height) was ≥3/1, and chromatography (peak shape and resolution) and relative retention time (±2% of target RT) were acceptable. The LOQ was defined as the lowest concentration that met LOD criteria and had analyte quantification within ±20% of target value.

2.8. Accuracy and precision

Inter- and intra-assay accuracy and precision data for THC and its two metabolites were determined with the low, medium and high QC samples. Intra-assay data were assessed by comparing data from within one run ($n = 10$) and inter-assay data were determined between five separate runs ($n = 34$). Data were evaluated using one-way analysis of variance (ANOVA) with day as the grouping variable. Accuracy, expressed as a percentage, was calculated by taking

the difference between mean calculated concentrations and target concentrations, dividing by the calculated mean and multiplying by 100. Precision, expressed as percent relative standard deviation (%R.S.D.), was determined by calculating the percent ratio of the standard deviation divided by the calculated mean concentration times 100.

2.9. Extraction efficiency

The recovery or extraction efficiency (%) for each analyte was determined at low, medium and high concentrations ($n = 5$). Relative recovery was assessed by adding IS working solution to one set of spiked plasma samples before extraction and to the second set after extraction but prior to evaporation. Samples were derivatized and analyzed. A third set of samples was prepared for the determination of absolute recovery. Analyte and IS working solutions were added to clean tubes followed by evaporation, derivatization and analysis. The relative extraction efficiency was calculated by comparing the peak area ratios of analyte to internal standard for each compound in the first set with the appropriate peak area ratios in the second, and the absolute extraction efficiency was determined by comparing peak area ratios between the first and third set.

2.10. Determination of stability

Several studies were conducted to assess the stability of analytes. Spiked unextracted plasma samples were analyzed after varying storage conditions and times. A set of low, medium and high validation samples ($n = 5$) were subjected to three cycles of freezing at $-20\,^{\circ}C$ for 24 h, thawing to ambient temperature for approximately 4 h and refreezing. A second set of samples was stored at ambient temperature for 24 h, while the third and fourth sets of unextracted spiked plasma samples were stored at $4\,^{\circ}C$ for 72 h and $-20\,^{\circ}C$ for 30 days prior to extraction and analysis, respectively. Concentrations of cannabinoids in the stability study samples were calculated and compared with freshly prepared and freshly analyzed quality control samples.

Stability of analytes after derivatization also was examined. GC injector vials containing derivatized low, medium and high validation samples ($n = 5$) were stored at ambient temperature for 72 h following initial analysis. Concentrations of analytes in stored vials were compared to cannabinoid concentrations of freshly prepared quality control samples.

2.11. Oral THC administration study plasma specimens

Plasma specimens for the verification of method validity were collected from participants in an Intramural Research Program, NIDA cannabis administration protocol evaluating the pharmacokinetic and pharmacodynamic effects of oral THC. The NIDA Institutional Review Board approved the study and all participants provided written informed consent. The participants were under continuous medical supervision and were financially compensated for their participation.

In order to determine the percentage of free and glucuronidated cannabinoids in plasma after oral THC, a set of ten plasma samples, five each from two individuals participating in the same THC administration protocol, were divided into two, with one group of aliquots assayed as described above and the other group analyzed without the enzyme hydrolysis step.

Plasma samples ($n = 75$) from one individual who received five different oral THC doses while participating in a THC administration protocol were analyzed with the validated method.

3. Results

3.1. Selectivity

Blank plasma samples were analyzed with each validation run ($n = 6$). All six samples were free of co-eluting peaks at the retention times of THC, 11-OH-THC, THCCOOH and their respective deuterated IS. Representative selected ion monitoring chromatograms for a blank plasma sample and a 10 ng/ml THC and 11-OH-THC, 20.0 ng/ml THCCOOH calibrator sample are shown in Fig. 1. Analysis of negative plasma samples in each assay also demonstrated that the IS did not contain relevant amounts of native cannabinoids. Of the 20 interference compounds added to a low validation sample (2.0 ng/ml THC and 11-OH-THC; 4.0 ng/ml THCCOOH) at a concentration of 1000 ng/ml for CBD, CBN and CBG and 10,000 ng/ml for 17 other drugs, none yielded analyte concentrations outside the ±20% limits of expected concentration.

3.2. Linearity, carry-over and limits of quantitation and detection

An overview of characteristic calibration data over a dynamic range from the LOD/LOQs to 50 ng/ml for THC and 11-OH-THC and 100 ng/ml for THCCOOH is presented in Table 1. A linear relationship between concentration and peak area was demonstrated. This calibration range encompasses the expected concentrations to be found in actual plasma samples following oral THC exposure.

Additional quality control samples ($n = 3$) were analyzed to evaluate the upper limit of linearity and potential carryover. The 75 ng/ml THC and 11-OH-THC and 150 ng/ml THCCOOH samples quantified within the acceptable criteria of ±20% of target concentration. Negative samples were analyzed between samples of increasing analyte concentration. No detectable carryover occurred following the 150 ng/ml THC and 11-OH-THC and 300 ng/ml THCCOOH sample; however, quantifiable amounts of cannabinoids (2.9, 1.6 and 3.2 ng/ml, respectively) were measured

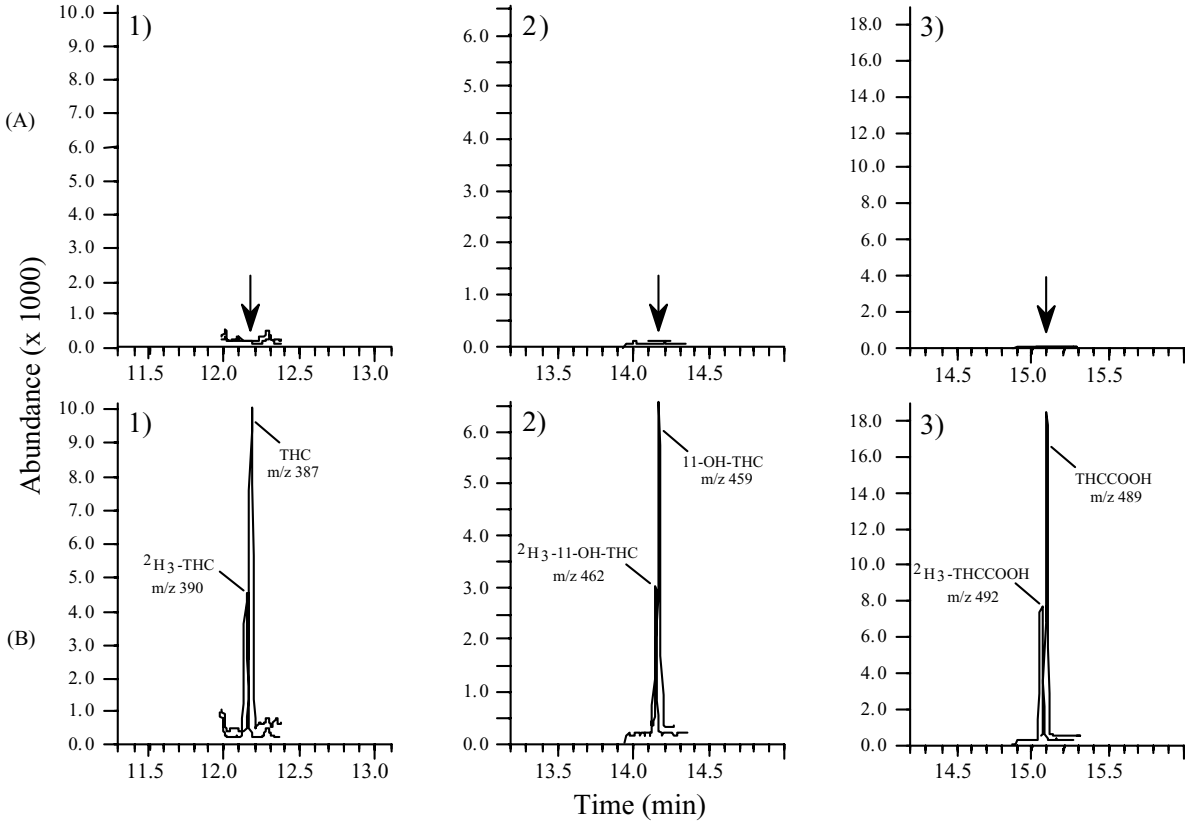

Fig. 1. Single ion chromatograms of Δ^9-tetrahydrocannabinol (THC) (1), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) (2) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) (3) from extracted plasma samples by positive chemical ionization gas chromatography–mass spectrometry. (A) Blank plasma sample with no internal standard. (B) Quality control sample fortified with 10, 10 and 20 ng/ml THC, 11-OH-THC and THCCOOH, respectively, and 5.0, 5.0 and 10 ng/ml of respective deuterated internal standards.

in the negative sample following the 500 ng/ml THC and 11-OH-THC and 1000 ng/ml THCCOOH sample.

3.3. Accuracy and precision

Precision and accuracy of the method were evaluated at three concentrations over the linear dynamic range (low, medium and high). One-way ANOVA analysis indicated no statistically significant difference ($p \leq 0.05$) between inter-assay data sets. Data for both intra-assay ($n = 10$) and inter-assay ($n = 34$) are presented in Table 2. Intra-assay accuracy (percent difference between mean and target concentrations) and precision (%R.S.D.) ranged from 1.2 to 12.2 and 1.4 to 4.7%, respectively. Inter-assay accuracy and precision ranged from 1.4 to 12.2 and 3.1 to 7.3%, respectively.

3.4. Extraction efficiency

The extraction efficiencies of the method for the three analytes in quality control samples ($n = 5$) are presented in Table 3 as percent recovery. The method provided good relative and absolute recoveries of 88.2 to 96.4 and 67.3 to 83.5%, respectively, for all three cannabinoids across the linear dynamic range.

Table 1
Characteristics of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) calibration curves[a]

Analyte	Range (ng/ml)	Regression equation of calibrators[b]	Correlation coefficient (r)[b]	LOD[c] and LOQ[d] (ng/ml)
THC	0.5–50.0	$y = 0.07(0.13)x + 0.19(0.02)$	0.999 (0.001)	0.5
11-OH-THC	0.5–50.0	$y = 0.18(0.08)x + 0.18(0.02)$	0.996 (0.002)	0.5
THCCOOH	1.0–100.0	$y = 0.07(0.05)x + 0.09(0.01)$	0.997 (0.001)	1.0

[a] $N = 5$.
[b] Mean value and standard error.
[c] Limit of detection.
[d] Limit of quantitation.

Table 2
Accuracy and precision for the simultaneous determination of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in human plasma by gas chromatography–mass spectrometry with positive chemical ionization after β-glucuronidase hydrolysis

Analyte (ng/ml)	Intra-assay ($n = 10$)			Inter-assay ($n = 34$)		
	Mean (ng/ml)	Accuracy[a] (%)	Precision[b] (%)	Mean (ng/ml)	Accuracy (%)	Precision (%)
THC						
2.0	2.2	10.1	4.3	2.2	9.0	5.2
20	22.1	9.4	1.6	21.8	8.0	3.9
40	42.2	5.1	2.7	41.6	3.7	3.9
11-OH-THC						
2.0	2.2	8.6	4.5	2.2	8.0	7.1
20	22.8	12.2	2.0	22.8	12.2	3.1
40	40.5	1.2	2.3	40.6	1.4	3.3
THCCOOH						
4.0	4.3	7.6	4.1	4.3	6.1	7.3
40	42.8	6.6	1.4	44.2	9.6	6.5
80	73.7	−8.5	4.7	78.0	−2.6	6.9

[a] Percent difference between mean and target concentration.
[b] Percent relative standard deviation.

3.5. Determination of stability

Analyte concentrations in short and long-term stability experiments were within ±20% of the target concentration. The results, expressed as mean calculated concentrations, are presented in Table 4. Analyte concentrations were stable in plasma after three freeze–thaw cycles, at 4 °C for 72 h, at room temperature for 8 h and at −20 °C for 30 days. Stability of derivatized analytes in capped GC vials at room temperature was assessed after 72 h and analyte concentrations were within acceptable criteria.

Table 3
Percent extraction efficiencies of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) from human plasma samples ($n = 5$) of research subjects

Analyte (ng/ml)	Relative recovery[a] ± S.D. (%)	Absolute recovery[b] ± S.D. (%)
THC		
2.0	91.6 ± 2.7	70.9 ± 6.7
20	93.3 ± 3.3	67.3 ± 5.2
40	91.4 ± 5.7	69.1 ± 2.0
11-OH-THC		
2.0	90.4 ± 4.6	79.2 ± 3.6
20	91.9 ± 5.4	80.9 ± 4.2
40	92.9 ± 4.7	83.5 ± 1.8
THCCOOH		
2.0	88.2 ± 6.4	77.6 ± 4.4
20	92.7 ± 5.7	78.1 ± 2.8
40	96.4 ± 1.7	83.4 ± 1.6

[a] Relative recovery was assessed by comparing results when IS was added to samples before and after SPE.
[b] Absolute recovery was assessed by comparing results from extracted samples to results from unextracted methanolic samples.

3.6. Application to authentic plasma samples

This simultaneous THC, 11-OH-THC and THCCOOH analytical method was applied to human plasma samples obtained from two participants in an oral THC administration study [25]. The effect of *E. coli* β-glucuronidase hydrolysis on the concentration of THC, 11-OH-THC and THCCOOH is presented in Table 5. Enzyme hydrolysis increased THC concentrations the least with a mean ±S.D. percent increase of 18 ± 17%, for samples with quantifiable THC concentrations. 11-OH-THC concentrations increased by a mean of 40 ± 25%, and THCCOOH concentrations had the largest mean increase of 42 ± 12%. Statistical analysis of data indicated significant differences ($P \leq 0.05$) between the hydrolyzed and non-hydrolyzed results for 11-OH-THC and THCCOOH, but not for THC.

The reported method also was used to analyze 75 samples from a third participant who completed the 10-week oral THC administration study. Plasma concentration data for the first 24 h following administration of two 2.5 mg dronabinol doses (Marinol®, synthetic THC in sesame oil) at 4.5 and 10.5 h, are presented in Fig. 2. Maximum concentrations were 0.7, 1.3 and 28.1 ng/ml for THC, 11-OH-THC and THCCOOH, respectively.

4. Discussion

This manuscript describes an analytical procedure for the simultaneous quantification of THC, 11-OH-THC and THCCOOH in human plasma by GC–MS with PCI following *E. coli* β-glucuronidase hydrolysis and SPE. This validated method provides specific and accurate results over an analyte concentration range that is consistent with expected plasma concentrations following oral THC. This method may be a

Table 4
Stability of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) in human plasma ($n = 5$)

Analyte (ng/ml)	Freeze–thaw ×3, mean ± S.D. (ng/ml)	72 h at 4 °C, mean ± S.D. (ng/ml)	8 h at room temperature, mean ± S.D. (ng/ml)	30 day at −20 °C, mean ± S.D. (ng/ml)
THC				
2.0	2.2 ± 0.05	2.4 ± 0.02	2.4 ± 0.05	2.2 ± 0.06
20	22.7 ± 0.19	21.6 ± 0.21	21.7 ± 1.11	21.8 ± 0.48
40	43.1 ± 0.26	40.5 ± 0.77	41.2 ± 0.60	40.8 ± 0.40
11-OH-THC				
2.0	2.3 ± 0.02	2.2 ± 0.07	2.2 ± 0.01	2.2 ± 0.05
20	23.7 ± 0.18	21.3 ± 0.18	21.3 ± 0.46	21.3 ± 0.18
40	41.0 ± 0.15	36.1 ± 0.84	36.8 ± 0.01	38.8 ± 0.24
THCCOOH				
4.0	4.7 ± 0.17	4.5 ± 0.10	4.6 ± 0.15	4.3 ± 0.06
40	47.1 ± 0.17	43.1 ± 0.71	43.2 ± 0.19	42.4 ± 0.41
80	83.3 ± 0.20	79.8 ± 1.97	75.0 ± 0.42	82.0 ± 0.52

Table 5
Effect of *E. coli* β-glucuronidase hydrolysis on the concentration of Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) in human plasma samples (ng/ml)

Specimen	THC buffer[a]	THC enzyme[b]	11-OH-THC buffer	11-OH-THC enzyme	THCCOOH buffer	THCCOOH enzyme
A	0.0	0.0	0.0	0.0	0.0	0.0
B	0.0	0.0	0.0	0.0	3.9	5.2
C	1.8	2.3	0.0	2.6	6.2	9.8
D	1.6	1.6	1.7	2.6	9.1	13.2
E	0.0	0.0	0.5	1.4	4.7	11.6
F	0.9	1.0	1.2	1.6	5.3	7.7
G	0.0	0.0	0.0	0.0	4.0	10.0
H	0.0	0.0	1.5	1.8	6.0	11.4
I	1.3	2.2	2.1	2.4	15.2	26.4
J	0.0	0.0	0.6	1.0	11.9	21.5

[a] Potassium phosphate buffer (pH 6.8) added to samples and incubated for 16 h.
[b] Potassium phosphate buffer (pH 6.8) and 5000 IU of *E. coli* β-glucuronidase added to 1.0 ml plasma samples and incubated for 16 h.

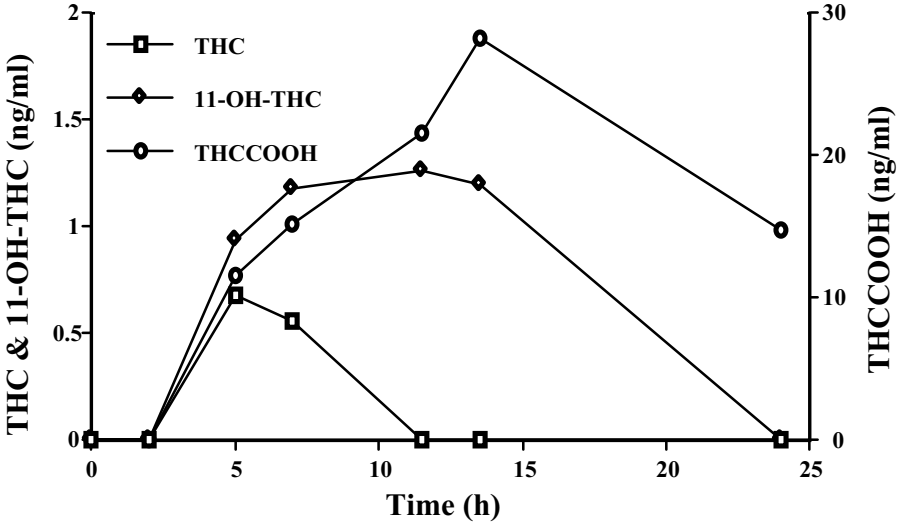

Fig. 2. Plasma concentrations ($n = 1$) over 24 h for Δ^9-tetrahydrocannabinol (THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCCOOH) following administration of two 2.5 mg dronabinol (synthetic THC), doses at 4.5 and 10.5 h.

useful analytical procedure for the fields of forensic toxicology and cannabinoid pharmacology.

There are many analytical methods for the quantification of THC and its major metabolites in plasma and other biological fluids [19,20]. Increasing demands in forensic and pharmacological science are placing greater expectations on analytic methods for the quantification of cannabinoids. High sensitivity and small sample volume are continuous goals. Higher sensitivity increases windows of detection and permits a more accurate determination of pharmacokinetic parameters. The potent psychoactivity of 11-OH-THC justifies the inclusion of this analyte in quantitative cannabinoid methods. Previous methods for the quantification of THC, 11-OH-THC and THCCOOH in plasma have met most of these requirements, but to-date, few if any have encompassed all requirements. Our method provides good sensitivity with LOQs of 0.5 ng/ml for THC and 11-OH-THC and 1.0 ng/ml for THCCOOH from a 1.0 ml plasma sample. This method employs SPE, eliminating the laborious liquid–liquid extraction and allowing for potential automation. Previous methods have depended on large expensive GC–MS systems, generally using NCI or tandem MS detection, to obtain the desired low LOQs. Most laboratories now can afford bench-top GC–MS systems with EI, PCI and NCI capabilities, such as the one used in this analysis. We have developed a dependable, robust analytical procedure with acceptable linearity, precision and accuracy.

Also, few methods include a hydrolysis step to release the analyte from the glucuronide moiety for quantification of total cannabinoids [15,16]. Kelly and Jones [26], reported that after frequent and infrequent marijuana users were administered 5 mg THC intravenously, the ratio of free THCCOOH to its glucuronide form was greater than 2 at 30 min postinfusion. Free THCCOOH/ THCCOOH glucuronide plasma ratios of 0.47 ± 0.27 were reported following the ingestion of 20 mg THC in cannabis resin [14]. These two studies emphasize the need to include a hydrolysis step in the analysis of plasma THCCOOH to obtain more accurate total concentrations. Kemp et al. [12] evaluated different hydrolysis methods, including two forms of β-glucuronidase, in the quantification of THC and its two major metabolites in urine. Hydrolysis at 37 °C for 16 h with *E. coli* glucuronidase produced significant ($P \leq 0.05$) increases, approximately 5 fold, in the concentration of free THC and 11-OH-THC in urine collected following marijuana smoking. The authors found no significant differences in THCCOOH concentration with basic and enzymatic hydrolysis. ElSohly and Feng [16] also reported considerable increases in cannabinoid concentrations following enzymatic hydrolysis of meconium at 37 °C for 16 h. Data from the present oral THC administration study demonstrate similar significant ($P \leq 0.05$) increases in 11-OH-THC and THCCOOH concentrations following hydrolysis with *E. coli* β-glucuronidase. THC did not increase significantly. These preliminary data demonstrate that *E. coli* β-glucuronidase hydrolysis may be necessary in cannabinoid plasma analyses following oral THC.

An important aspect of any method, especially if used in forensic analysis, is interference by non-targeted drugs. Cannabis contains at least 61 cannabinoid compounds [27], including CBD, CBN and CBG. CBD and CBN generally represent 1–2% by weight of cannabis preparations, while CBG is present in much smaller amounts [28]. Maximum plasma concentrations of approximately 100 ng/ml have been reported for CBD and CBN following cannabis smoking [29]. Our interference study documented that concentrations of these other natural cannabinoids well above expected levels did not interfere with the accurate quantification of THC, 11-OH-THC and THCCOOH. In addition, 17 other commonly used licit and illicit drugs did not interfere with accurate cannabinoid quantitation at concentrations of 10,000 ng/ml.

There is active pharmacological research in the area of cannabinoid therapeutic agents. In 1999, the United States National Academy of Sciences, Institute of Medicine called for clinical trials to test the effectiveness of cannabinoids in pain relief, control of nausea and vomiting, appetite stimulation, other indications, and to improve delivery systems [30]. Interest in the pharmacological actions of cannabinoids has been enhanced in recent years by the elucidation of a mammalian endocannabinoid system. Cannabinoid receptors (CB_1 and CB_2) and endogenous ligands (anandamide and 2-arachidonylglycerol) have been identified [31,32]. Studies on the administration of THC and other cannabinoids via oral, inhalation and sublingual routes are being conducted. To assess drug effectiveness and bioavailability of routes of delivery, accurate plasma concentrations are essential.

In conclusion, this report describes a sensitive and specific GC–MS–PCI procedure for the simultaneous quantification of THC, 11-OH-THC and THCCOOH in human plasma with *E. coli* β-glucuronidase hydrolysis to achieve more accurate total cannabinoid concentrations. The method has suitable linearity, accuracy and precision with high analyte recoveries. Application of the method to plasma samples collected from individuals participating in a controlled oral THC administration study provided preliminary data suggesting that *E. coli* β-glucuronidase hydrolysis is a useful means to more accurately measure total cannabinoid concentrations following oral THC administration.

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