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Impact of enzymatic and alkaline hydrolysis on CBD concentration in urine

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Abstract

A sensitive and specific analytical method for cannabidiol (CBD) in urine was needed to define urinary CBD pharmacokinetics after controlled CBD administration, and to confirm compliance with CBD medications including Sativex—a cannabis plant extract containing 1:1 Δ^9 -tetrahydrocannabinol (THC) and CBD. Non-psychoactive CBD has a wide range of therapeutic applications and may also influence psychotropic smoked cannabis effects. Few methods exist for the quantification of CBD excretion in urine, and no data are available for phase II metabolism of CBD to CBD-glucuronide or CBD-sulfate. We optimized the hydrolysis of CBD-glucuronide and/or -sulfate, and developed and validated a GC-MS method for urinary CBD quantification. Solid-phase extraction isolated and concentrated analytes prior to GC-MS. Method validation included overnight hydrolysis (16 h) at 37 °C with 2,500 units β -glucuronidase from Red Abalone. Calibration curves were fit by linear least squares regression with $1/x^2$ weighting with linear ranges ($r^2 > 0.990$) of 2.5–100 ng/mL for non-hydrolyzed CBD and 2.5–500 ng/mL for enzyme-hydrolyzed CBD. Bias was 88.7–105.3 %, imprecision 1.4–6.4 % CV and extraction efficiency 82.5–92.7 % (no hydrolysis) and 34.3–47.0 % (enzyme hydrolysis). Enzyme-hydrolyzed urine specimens exhibited more than a 250-fold CBD concentration increase compared to alkaline and

non-hydrolyzed specimens. This method can be applied for urinary CBD quantification and further pharmacokinetics characterization following controlled CBD administration.

Keywords

Cannabidiol; Cannabidiol-glucuronide; Enzymatic hydrolysis; Urine; GC-MS

Introduction

Cannabidiol (CBD) is proposed as oral or oromucosal pharmacotherapy for diverse conditions including dementias, cerebral ischemia, diabetes, inflammatory diseases, nausea, and psychiatric disorders [1–3] over a wide dosage range [4]. CBD concentration in the cannabis plant also may modify psychotic symptoms, as a high CBD/ Δ^9 -tetrahydrocannabinol (THC) ratio could reduce the development of psychotic disorders [5–9]. It has been suggested that CBD can modulate THC's physiological and psychological effects in humans [10], although CBD/THC ratio may be an important factor [11]. In our previous Sativex studies, which evaluated cannabis plant extracts rich in CBD and THC, we did not find any significant interaction with THC's physiological or psychotropic effects [12, 13]. Non-psychoactive CBD has a wide range of therapeutic applications and may also influence psychotropic effects of smoked cannabis [14].

Although growing attention has begun to focus on CBD for clinical and scientific purposes, few methods have quantified urinary CBD and there are no data on phase II metabolism of CBD to CBD-glucuronide or CBD-sulfate. A sensitive and specific CBD in urine assay is thus needed to determine urinary CBD pharmacokinetics, and to confirm compliance with CBD medications including Sativex.

Phase I CBD metabolism primarily occurs through hepatic enzymes [15] CYP2C19 and 3A4 [16]. A high percentage of free-CBD is excreted in the feces [17, 18]. Phase II metabolism by UDP-glucuronosyltransferases yields more hydrophilic metabolites including CBD-6-OH-CBD- and 7-OH-CBD-glucuronides [19–21].

There are few pharmacokinetic data on urinary CBD excretion. Alkaline, enzymatic and/or alkaline-enzymatic tandem hydrolysis may be necessary prior to extraction to release CBD from water-soluble conjugates to facilitate excretion [22–24]. *E. coli* β -glucuronidase is the enzyme of choice for hydrolysis of urinary cannabinoid ether conjugates of THC and the equipotent metabolite 11-hydroxy-THC (11-OH-THC) [23, 24], while alkaline hydrolysis is most effective for increasing 11-nor-9-carboxy-THC (THCCOOH) concentrations. THCCOOH binds to glucuronic acid through an ester linkage. The extent of phase II metabolism of CBD to glucuronide and/or sulfate conjugates remains unknown.

Kemp et al. [22] developed an assay for THC and metabolites, CBD and cannabinol (CBN) in urine employing liquid-liquid extraction and 11-OH-THC hydrolysis optimization. Grauwiler et al. [25] developed a LC-MSMS method for THC, 11-OH-THC, THCCOOH, CBD and CBN quantification in urine using liquid-liquid extraction and enzyme for THCCOOH-glucuronide hydrolysis. More recently, Scheidweiler et al. [26] developed and validated an LC-MSMS assay for THC, THC-glucuronide, 11-OH-THC, THCCOOH, THCCOOH-glucuronide, CBD, and CBN quantification in urine via supported-liquid extraction. As preparation for an upcoming clinical study involving controlled oral CBD dosing, we developed and validated a quantitative assay for CBD in urine following enzyme hydrolysis. In addition, we optimized hydrolysis conditions to obtain the highest urinary total CBD recovery.

Experimental

Reagents

CBD and its deuterated internal standard cannabidiol-*d*3 (CBD-*d*3) were purchased from Cerilliant (Round Rock, TX, USA). Anhydrous sodium acetate, dibasic, and monobasic potassium phosphate, hydrochloric acid, sodium hydroxide and glacial acetic acid were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). Methanol was from Fischer Scientific (Fair Lawn, NJ, USA). Anhydrous U.S.P. ethanol was acquired from The Warner-Graham Company (Cockeysville, MD, USA) and *n*-hexane, ethyl acetate and acetonitrile from Sigma Aldrich (St. Louis, MO, USA). All solvents and chemicals were at least HPLC and ACS grade, respectively. *E. coli* type IX-A and *H. pomatia* type HP-2 β -glucuronidase were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Red Abalone β -glucuronidase from GOMA Biotec (Santiago, Chile). *N,O*-bis(trimethylsilyl) trifluoroacetamide with 1 % trimethylchlorosilane (BSTFA+1 % TMCS) were from Regis Technologies (Morton Grove, IL, USA). Clean Screen THC solid-phase extraction columns (ZSTHC020) were purchased from United Chemical Technologies (Bristol, PA, USA) for specimen preparation and hydrolysis efficiency evaluation. Blank human urine was provided by drug-free volunteers and evaluated by GC-MS for absence of cannabinoids.

Preparation of standard solutions

Working CBD standard solutions (100, 1000 and 10,000 ng/mL) were prepared by diluting 1.0 mg/mL stock solution with methanol. Working urine calibrators (2.5, 5.0, 10, 25, 50, 75 and 100 ng/mL) were prepared daily by fortifying appropriate amounts of working standard into 2.0 mL blank urine. Working urine calibrators for enzyme hydrolysis (2.5, 5.0, 10, 50, 100, 250, and 500 ng/mL) were prepared daily by spiking into 1.0 mL blank urine.

Quality control (QC) solutions were prepared from different lots of stock solutions than those for preparing standards. Low, medium and high QC samples across the dynamic range of the assay were prepared daily in blank urine at final concentrations of 7.5, 37.5, and 90 ng/mL. The extended linear curve for enzyme hydrolysis analyses (2.5–500 ng/mL) required low, medium, and high QC of 7.5, 90, and 300 ng/mL, respectively.

Deuterated stock internal standard (CBD-*d*3) was diluted with methanol to achieve a working internal standard concentration of 1,000 ng/mL. Twenty microliters working internal standard was added to each sample prior to extraction (or 50 μ L for enzyme hydrolysis analyses), yielding a final internal standard concentration of 10 ng/mL (or 50 ng/mL for enzyme hydrolysis). All standards, quality control and internal standards solutions were stored in amber vials at -20°C .

Hydrolysis and analysis optimization

Authentic urine specimens from this double-blind, placebo controlled and Mount Sinai Institutional Review Board-approved study monitoring urine pharmacokinetics after oral CBD administration (0, 400, and 800 mg) were first analyzed for free CBD with a fully validated assay without hydrolysis. Aliquots of these authentic positive urine specimens (presumed to contain CBD-glucuronide and/or CBD-sulfate) were combined and re-analyzed without hydrolysis, and with alkaline and enzyme hydrolysis to optimize hydrolysis conditions. We evaluated temperature, pH, incubation time, and amount of enzyme to maximize CBD concentrations. Urine specimens were stored at -20°C until analysis.

Specimen preparation—no hydrolysis

Blank urine (2 mL) was pipetted into 10-mL conical polypropylene tubes and fortified with appropriate amounts of calibrator or QC solution and 20 μ L working internal standard. One hundred microliters of methanol was added to authentic specimens to account for methanol in standard and QC samples. Cold acetonitrile (2 mL) was added, tubes vortexed and centrifuged at 4 °C for 10 min (1,800 $\times$ g). Supernatants were decanted into clean 10-mL conical polypropylene tubes containing 2 mL 2 M sodium acetate buffer pH 4.0 and vortexed. We applied the buffered supernatant to solid-phase extraction (SPE) columns preconditioned with methanol (2 mL), de-ionized water (2 mL) and 1 mL of 0.1 M potassium phosphate buffer (pH 6.8). After application under gravity flow, columns were washed with deionized water (3 mL) and 3 mL of 0.1 M potassium phosphate buffer (pH 6.8). Samples were then dried under full vacuum for 20 min prior to priming the sorbent bed with 0.2 mL hexane and eluting with 5 mL hexane/ethyl acetate (80:20) into 10 mL conical glass tubes containing 0.5 mL ethanol. Eluants were dried under nitrogen at 40 °C before derivatization for 30 min at 70 °C with BSTFA-1%TMCS (25 μ L). Samples were cooled at room temperature, briefly centrifuged (3 min) at 1800 $\times$ g, and transferred to autosampler vials.

Specimen preparation—alkaline hydrolysis

Alkaline hydrolysis at four different conditions (60 or 70 °C for 20 or 60 min) was evaluated with the positive specimen pool (2 mL). Samples were prepared as described above for non-hydrolyzed analysis, and then 80 μ L 10 M sodium hydroxide (sample pH>10) was added to each tube and samples incubated at 60 or 70 °C for 20 or 60 min. After cooling and neutralization (pH $\approx$ 7.0) with 50 μ L concentrated glacial acetic acid, samples were handled as non-hydrolyzed samples as previously described.

Specimen preparation—enzyme hydrolysis

Similarly, enzyme hydrolysis was evaluated under a variety of conditions, but always at the temperature and pH recommended by the manufacturer [27–29]. At the optimal temperature of 37 °C, and buffer pH values of 6.8 (*E. coli*) and 5.0 (*H. pomatia*) recommended by Sigma-Aldrich and 5.0 (Red Abalone) by GOMA Biotec, multiple amounts (2,500, 5,000, and 10,000 units) of each enzyme were evaluated at two different incubation times (4 and 16 h) to optimize cleavage of glucuronide conjugates.

Briefly, blank urine (1 mL) was pipetted into 16 $\times$ 100-mm glass culture tubes and fortified with appropriate amount of calibrator or QC solution and 50 μ L of working internal standard. One hundred microliters of methanol was added to authentic specimens to account for methanol in standard and QC samples. One milliliter of 0.1 M potassium phosphate buffer pH 5.0 or 6.8 and β -glucuronidase from *E. coli*, *H. pomatia* or Red Abalone were added to each specimen (final concentration 2,500, 5,000, or 10,000 units) and incubated for 4 or 16 h at 37 °C. Following hydrolysis, specimens were cooled to room temperature before adding acetonitrile to precipitate proteins and sodium acetate buffer for SPE as previously described. Sample eluates were evaporated to dryness under nitrogen at 40 °C. For the derivatization of CBD, 25 μ L of BSTFA-1%TMCS and 25 μ L acetonitrile were added and incubated at 70 °C for 30 min.

Samples were cooled at room temperature, centrifuged, and transferred to glass inserts in autosampler vials.

GC-MS

All analyses were performed on an Agilent 6890 GC with Agilent 5973 mass selective detector with electron ionization (EI) mode, equipped with 30 m $\times$ 0.32 mm $\times$ 0.25 μ m

HP-5MS capillary column. The initial oven temperature of 160 °C was increased by 20 °C/min to 250 °C and held for 2 min. Total chromatographic analysis time was 6.5 min. After the run, the temperature was increased to 320 °C at maximum gradient and held for 3 min to clean the column before re-equilibrating to the initial starting temperature. Injection port temperature was 330 °C. A 3 µL splitless injection was utilized for the non-hydrolyzed specimens and a 2 µL 2:1 split injection for the hydrolyzed specimens. Purge flow was 50 mL/min for 1 min. Helium was the carrier gas at a constant flow of 1.5 mL/min. Interface, ion source, and quadrupole temperatures were 280, 230, and 150 °C, respectively. Selective ion monitoring mode was employed with dwell times of 20 ms and +200 eV relative to the daily autotune. Four ions for CBD and three for CBD-*d*3 were monitored (quantification ions are underlined): CBD, *m/z* 390, 458, 443, 301; and CBD-*d*3, *m/z* 393, 461, 340. All ions and ion ratios were utilized.

Validation

We evaluated linearity, sensitivity, selectivity, extraction efficiency, intra- and inter-assay bias and imprecision, dilution integrity, stability, carryover, hydrolysis efficiency and recovery to demonstrate method integrity. Hydrolysis recovery and efficiency were performed after optimal hydrolysis conditions were determined.

Method linearity was investigated by calculation of the regression line and expressed by the coefficient of determination (r^2) equal to or higher than 0.990. Seven calibrators were included in each batch and were required to quantify within ± 15 % of target calibrator concentrations [20 % for the limit of quantification (LOQ)] when calculated against the full curve, as well as, Gaussian peak shape, adequate retention time and ion ratios within ± 20 % of averaged calibrator ratios.

Sensitivity was empirically determined by assaying triplicates of low concentrations and determining the limit of detection (LOD) and LOQ. LOD was defined as the lowest analyte concentration with acceptable peak shape and retention time, signal-to-noise ratio ≥ 3 , and qualifier ion ratios within ± 20 % of mean calibrator ratios; LOQ was the lowest concentration within ± 20 % of target, meeting LOD criteria, and exhibiting a signal-to-noise ratio ≥ 10 .

Endogenous and exogenous interferences were evaluated to assess method selectivity. We evaluated ten different urine specimens fortified with internal standard only (negative) and fortified with low QC solution (7.5 ng/mL) to quantify endogenous matrix interferences. Exogenous interferences were evaluated by fortifying 94 illicit and therapeutic drugs and metabolites (Table 1) into low QC samples. The fortified low QC was required to quantify within ± 20 % of target, have acceptable peak shape and retention time and qualifier ion ratios within ± 20 % of mean calibrator ratios.

Extraction efficiency was assessed at each QC concentration by adding standards and internal standards prior to and following SPE ($n=4$). Extraction efficiency was calculated by comparing mean analyte areas before SPE to mean analyte areas following SPE and expressed as a percentage (%).

Bias and imprecision were evaluated at 3 QC concentrations. Intra-assay bias ($n=5$) was calculated by dividing mean CBD QC concentration by target concentration and expressing as % target. Minimum acceptable bias was within 80–120 %. Intra-assay imprecision was calculated by comparing standard deviation of CBD concentrations to mean calculated concentrations to determine the coefficient of variation (%CV). The %CV should not exceed 20 %. Inter-assay bias and imprecision were calculated in 5 replicates over four different

runs ($n=20$). Analysis of variance (ANOVA) was employed to evaluate inter-assay variability and considered significant if two-tailed $p<0.05$.

Dilution integrity was assessed by diluting the high QC sample 1:2 and 1:5 v/v with blank urine. Triplicates at each dilution were evaluated and results were required to be within ± 20 % of target, with acceptable peak shape, retention time and qualifier ion ratios within ± 20 % of averaged calibrator ratios.

Stability was assessed with low and high QC samples in triplicate under five different conditions. Prepared samples were left at room temperature (RT) for 8 and 16 h, refrigerated for 24 and 48 h at 4 °C, and following three freeze/thaw cycles at -20 °C. Derivatized extracts (calibrators, low, medium and high QC) also were re-injected 24 h after initial injections to evaluate autosampler stability. All samples were quantified against the initial calibration curve and were required to be within ± 20 % of initial results.

Carryover was evaluated by injecting a negative specimen (internal standard only) immediately after a specimen fortified at a concentration two times higher than the upper LOQ. The negative specimen was required to quantify $<LOQ$ to document a lack of carryover.

Authentic pooled urine was assayed in triplicate under each hydrolysis condition and compared with non-hydrolyzed CBD concentration to evaluate hydrolysis efficiency. Low QC was analyzed on each hydrolysis condition along with authentic pooled urine and required to meet acceptability criteria as previously described. CBD-glucuronide standard is not commercially available; therefore, an authentic urine pool following controlled CBD administration was employed to optimize hydrolysis conditions.

Data analysis

Specimens were analyzed with Agilent Enhanced ChemStation G1701EA version E. 01.00.237. Compounds were identified based on acceptable retention time, peak shape, signal to noise and qualifier ion ratios. All qualifier ion ratios were required to be within ± 20 % of mean calibrator ratios. Calibration curves were constructed with linear least squares regression with $1/x^2$ weighting factor. ANOVA followed by Bonferroni post-hoc test was performed with SPSS Statistics version 19.0 and considered significant if two-tailed $p<0.05$.

Results and discussion

Several urine cannabinoid methods are available [22, 24–26, 30–33], but most are limited to THC and its metabolites. Additionally, limited information is available on free and conjugated CBD elimination in urine, and no optimization for the hydrolysis of CBD-conjugates has been performed. This method evaluated multiple hydrolysis conditions on a urine pool collected from different participants following controlled oral CBD administration. Also, these are the first data on CBD-conjugated urine concentrations compared to free CBD after oral CBD administration.

Non-hydrolyzed specimens

Calibration and validation—Seven working calibrators were assayed daily. Calculated concentrations of each calibrator were required to be within ± 15 % of target concentration (± 20 % for LOQ) and $r^2 \geq 0.990$. LOD and LOQ were established by decreasing CBD concentrations in fortified blank urine prior to extraction. LOD and LOQ for non-hydrolyzed CBD were 1.0 and 2.5 ng/mL, respectively, with a 2 mL specimen (Table 2). No matrix interferences were observed in 10 different human urine specimens. Low QC samples

prepared in ten urine pools had concentrations within $\pm 20\%$ of target, with acceptable peak shape, retention time, and qualifier ions ratio within $\pm 20\%$ of mean calibrator ion ratios.

Exogenous interferences were evaluated by adding 94 potential interfering illicit drugs and medications (Table 1) to low QC specimens at concentrations of 1,000 ng/mL and 200 ng/mL for cannabinal and cannabigerol. There were no interferences, as all controls quantified within 20 % of target with acceptable chromatography. No CBD conversion to THC occurred in the high QC (90 ng/mL) under non-hydrolyzed specimen preparation.

Extraction efficiency, calculated from four replicates at each QC concentration, ranged from 82.5 to 92.7 %. Efficiencies were improved by adding cold acetonitrile (2 mL) prior to SPE and also by increasing drying time from 10 to 20 min prior to elution.

Bias and imprecision were verified at 3 concentrations (Table 3) across the linear range. Intra- ($n=5$) and inter-assay ($n=20$) bias ranged from 89.0 to 105.3 %. Intra- ($n=5$) and inter-assay imprecision ($n=20$) were less than 6.4 %. ANOVA showed significant differences ($p<0.05$) in inter-assay results; however, differences between days were less than 6.4 % and considered clinically insignificant.

Dilution integrity was performed by diluting high quality control samples (90 ng/mL, $n=3$) with blank urine. A 1:2 (v/v) dilution yielded calculated concentrations between 87.6 and 95.1 % of target. Further dilution (1:5, v/v) produced concentrations from 59.1 to 75.6 % ($n=3$), failing our acceptance criteria of 20 % of target. Previously, we [26] reported almost 70 % of CBD recovered in a fortified sample diluted 1:3 v/v. In light of these results, an authentic non-hydrolyzed specimen with a concentration of 21.2 ng/mL was diluted (1:5, v/v, $n=3$) in blank urine. Calculated concentrations ranged between 94.4–97.5 % of target. These results demonstrate that methanolic CBD spiked in blank urine and diluted with blank urine failed our acceptance criteria; whereas diluted authentic specimens had concentrations 94.4 % of target with 1.7 % CV. The mechanism for CBD losses during dilution integrity studies remains unknown, and reflects differences between fortified and authentic urine specimens that require further investigation.

Assay stability was evaluated in low (7.5 ng/mL) and high (90 ng/mL) QC samples (Table 4) ($n=3$). QC sample concentrations were within 19.8 % of target when refrigerated (4 °C) for up to 48 h, and within 11 % after three freeze/thaw cycles ($-20\text{ }^{\circ}\text{C}$). Stability was more variable at room temperature, yielding mean calculated concentrations from <LOQ (for low QC) to 50.7 % (for high QC) of target concentrations. The reasons for poor stability are currently unknown, as CBD is lipophilic [34, 35], thermolabile [36], and sensitive to oxidation [37, 38], as well as potentially adsorbing to the tube or precipitant material. Further stability testing with authentic urine specimens should be addressed to elucidate possible differences from fortified blank urine [26]. Derivatized extracts re-injected after 24 h were stable according to previously described criteria. A negative urine sample was analyzed following a fortified specimen containing CBD at two times the upper LOQ (200 ng/mL) and no carryover was observed.

Hydrolysis recovery—Due to the lack of a CBD-glucuronide standard and the low prevalence of positive CBD specimens in the non-hydrolyzed assay, specimens collected after controlled CBD administration were pooled to evaluate hydrolysis recoveries. Figure 1 illustrates merged ion chromatograms showing no interferences in blank urine (b), blank urine fortified with CBD at the LOQ (d) and a representative authentic specimen containing 740 ng/mL CBD (f), following 16 h enzyme hydrolysis (Red Abalone 2,500 unit). Figure 2 shows CBD concentrations of pooled positive specimens after each hydrolysis condition. Interestingly, there was no significant difference between alkaline hydrolysis and non-

hydrolyzed CBD concentration in urine (Fig. 2a). Alkaline hydrolysis also was ineffective for THC [23, 24, 39], as ether bonds are not susceptible to cleavage under alkaline conditions. These results suggested no significant increase in CBD concentrations following alkaline hydrolysis; therefore, no further method development of alkaline hydrolysis or tandem enzymatic-alkaline hydrolysis occurred. The data also suggested that there were no CBD-ester conjugates excreted in urine.

Enzyme hydrolyzed specimens had more than a 250-fold increase in CBD concentrations compared to alkaline and non-hydrolyzed specimens. Hydrolysis efficiency was significantly affected by enzyme source, amount and incubation time. With the addition of 2,500 units, *E. coli* yielded higher CBD concentrations than Red Abalone ($p<0.001$) and *H. pomatia* ($p<0.001$) after 4 h at 37 °C; while CBD concentrations were higher after a 4 h Red Abalone hydrolysis (2,500 units) than after hydrolysis with *H. pomatia* ($p<0.001$). Higher CBD concentrations were achieved with *E. coli* and Red Abalone vs *H. pomatia* ($p<0.001$) when using 5,000 or 10,000 units at 4 h incubation times at 37 °C. CBD concentrations significantly increased with units of Red Abalone enzyme and *H. pomatia* enzyme (Fig. 2b), while *E. coli* showed no difference among all 3 concentrations during 4 h incubation time at 37 °C.

Overnight enzyme incubation (16 h) at 37 °C produced similar CBD concentrations with all enzyme sources and amounts, except for *H. pomatia* 2,500 units that was significantly lower (Fig. 2). Optimal hydrolysis conditions included overnight hydrolysis (16 h) at 37 °C after adding 5,000 units of *E. coli* to each specimen. For method validation and specimen analyses, we selected β -glucuronidase from Red Abalone (2,500 units) over *E. coli*, as there was a considerable difference in cost that outweighed the slight (although non-significant) increase in efficiency.

The manufacturers of Red Abalone and *H. pomatia* also reported secondary sulfatase activity at 8,000 units, with the same optimum temperature and pH as for β -glucuronidase. With a 16-h incubation and 5,000 or 10,000 units, we did not observe any significant CBD concentration differences following Red Abalone, *H. pomatia* or *E. coli* hydrolysis, which may indicate that insignificant CBD sulfate is formed. Over 50 CBD free and glucuronide metabolites were previously identified in human urine [20, 40, 41] with no direct evidence of sulfate conjugates.

Enzyme-hydrolyzed specimens

Calibration and validation

Elevated CBD concentrations after enzyme hydrolysis required modifications to our existing assay. In order to extend linearity and avoid detector saturation, we reduced sample volume from 2 to 1 mL, and injection volume from 3 to 2 μ L, and changed mass spectrometry injection technique from splitless to split injection. This allowed us to maximize our linearity without compromising the LOQ. This enzyme-hydrolysis method was validated for linearity, sensitivity, extraction efficiency, intra- and inter-bias and imprecision, and carryover. Seven working calibrators were assayed daily. Calculated concentrations of each calibrator were within ± 15 % of target calibrator concentrations (± 20 % for LOQ) and r^2 0.990. LOD and LOQ for enzyme hydrolysis were 2.5 ng/mL (Table 2).

Extraction efficiencies after enzyme-hydrolysis (34.3 to 47.0 %) were lower than non-hydrolyzed extraction efficiencies (92.7 %). Decreased extraction efficiency after enzyme hydrolysis also was reported for other cannabinoids in urine using ZSTHC020 SPE column [24]. Kemp et al. [22] reported 50.1–88.8 % recovery for CBD from urine by liquid-liquid

extraction following hydrolysis. Although the extraction efficiency in enzyme hydrolyzed specimens was below 50 % in our method, we achieved desired analyte sensitivity.

Intra- ($n=5$) and inter-assay ($n=20$) bias for enzyme-hydrolyzed samples (Table 3) ranged from 88.7 to 104.1 %. Intra-assay ($n=5$) and inter-assay imprecision ($n=20$) were less than 3.4 %. ANOVA indicated a significant difference ($p<0.05$) in inter-assay results in the enzyme-hydrolyzed condition; however, differences between days were less than 3.4 % and were considered clinically insignificant. There was no evidence of carryover; and no CBD conversion to THC up to 2,000 ng/mL ($n=3$). CBD can convert to THC under acidic conditions as previously reported [26, 42–44]. Abraham et al. [24] showed an average of 1.7 % conversion to THC at 500 ng/mL CBD. Although we utilized the same SPE column as previously reported [24], replacing 0.1 N acetic acid and 0.1 N hydrochloric acid/acetonitrile (70:30, v/v) during column conditioning and washing, respectively, with 0.1 M phosphate buffer pH 7.0 eliminated cannabidiol isomerization to THC. This change resulted in interferences in all monitored THC ions (m/z 371, 386, and 303), with poor chromatography at the expected THC retention time. However, ion ratios always failed ± 20 % limit of the average THC calibrator ion ratios. Therefore, no false-positive THC results occurred, although false negative results were possible.

Application of method

The study design was a double-blind randomized placebo-controlled cross-over study to evaluate CBD as a treatment intervention for opiate relapse. During this three group, two-session, double-blind design; six participants were administered placebo, six received oral CBD 400 mg, and six participants received oral CBD 800 mg. Sessions were separated by at least a 1-week interval to ensure a sufficient drug washout period. Urine specimens were collected at baseline and at 45 min, 2 h, 4 h, 6 h, 7 h, and 8 h post-dose for a total of seven specimens. These urine samples will help to provide information on the urinary excretion of CBD. Data from a participant receiving both 400 mg CBD doses are presented in Table 5, and representative chromatography in Fig. 1. CBD concentrations after non-hydrolyzed analysis of these specimens were all <LOQ. However, after hydrolysis, only the initial baseline specimen was <LOQ. Concentrations ranged from 3.5–7,824.8 ng/mL after the first 400 mg dose, and from 6.9–5,213.8 ng/mL after the second dose; demonstrating the necessity for hydrolysis.

Conclusions

This is the first method to evaluate CBD hydrolysis and quantification in urine by GC-MS. The method is robust, sensitive and specific for quantification of total CBD in human urine, with a fast and simple sample preparation. This method will be useful to determine CBD pharmacokinetics and free/glucuronide ratios after oral CBD or cannabis-based medicinal extract administration. Determining the urinary excretion of CBD following controlled oral CBD, oral mucosal Sativex, or smoked cannabis administration with this new validated method may improve interpretation of urine cannabinoid results.

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Fig. 1. Merged ion chromatograms for cannabidiol-*d3* (*CBD-d3*) [393, 340, 461] and cannabidiol (*CBD*) [m/z 390, 301, 458, 443] following 16 h hydrolysis at 37 °C by 2,500 units Red Abalone β -glucuronidase; (**a** and **b**) extracted blank urine; (**c** and **d**) extracted blank urine fortified at limit of quantification (2.5 ng/mL); and (**e** and **f**) authentic specimen at 740 ng/mL *CBD* concentration in urine. Quantification ions are *underlined*

Fig. 2.

Hydrolysis efficiency of CBD concentrations in urine after: **a** authentic non-hydrolyzed and alkaline hydrolyzed pool; **b** enzyme hydrolysis with 4 h incubation time and three different sources of β -glucuronidase (*E. coli*, Red Abalone, and *H. pomatia*) at three different enzyme amounts (2,500, 5,000, and 10,000 units); **c** enzyme hydrolysis with 16 h incubation time and three different sources of β -glucuronidase (*E. coli*, Red Abalone, and *H. pomatia*) at three different enzyme amounts (2,500, 5,000, and 10,000 units). Bars represent mean concentrations ($n=3$) with standard deviation; * represents significant difference from *E. coli* β -glucuronidase; + represents significant difference from Red Abalone β -glucuronidase

Table 1

Exogenous interferences investigated by fortification into a low cannabidiol quality control sample.
Interference was ruled out if cannabidiol concentration quantified within ± 20 % of target

11-OH-THC	Clonidine	Norbenzoylecgonine
2-Ethyl-5-methyl-3,3-diphenylpyrrolidine	Cocaethylene	Norbuprenorphine
2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	Cocaine	Norcocaethylene
3,4-(Methylenedioxyphenyl)-2-butanamine	Codeine	Norcocaine
3,4-Dihydroxymethamphetamine	Cotinine	Norcodeine
3,4-Methylenedioxyamphetamine	Diazepam	Norcotinine
3,4-Methylenedioxyethamphetamine	Diphenhydramine	Nordiazepam
4-Bromo-2,5-dimethoxyphenethylamine	Ecgonine	Norfluoxetine
4-Hydroxy-3-methoxyamphetamine	Ecgonine ethyl ester	Normorphine
4-Hydroxy-3-methoxymethamphetamine	Ecgonine methyl ester	Noroxycodone
6-Acetylcodeine	Ephedrine	Noroxymorphone
6-Acetylmorphine	Ethylamphetamine	Oxazepam
7-Aminoclonazepam	Fenfluramine	Oxycodone
7-Aminoflunitrazepam	Flunitrazepam	Oxymorphone
7-Aminonitrazepam	Fluoxetine	Paroxetine
Acetaminophen	Flurazepam	Pentazocine
Acetylsalicylic acid	Hydrocodone	Phencyclidine
Alprazolam	Hydromorphone	Phentermine
Amphetamine	Ibuprofen	Phenylpropanolamine
Anhydroecgonine methyl ester	Imipramine	<i>p</i> -Hydroxybenzoylecgonine
Benzoylecgonine	Lorazepam	<i>p</i> -Hydroxycocaine
Bromazepam	Methadone	<i>p</i> -Hydroxymethamphetamine
Brompheniramine	Methamphetamine	<i>p</i> -Methoxyamphetamine
Buprenorphine	<i>m</i> -Hydroxybenzoylecgonine	<i>p</i> -Methoxymethamphetamine
Caffeine	<i>m</i> -Hydroxycocaine	Propoxyphene
Cannabigerol ^a	Morphine	Pseudoephedrine
Cannabinol ^a	Morphine-3-glucuronide	Temazepam
Cathinone	Morphine-6-glucuronide	THC
Chlorpheniramine	<i>n</i> -methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine	THC-glucuronide
Clomipramine	Nicotine	THCCOOH
Clonazepam	Nitrazepam	THCCOOH-glucuronide <i>trans</i> -3'-Hydroxycotinine

THC Δ^9 -tetrahydrocannabinol, 11-OH-THC 11-hydroxy- Δ^9 -tetrahydrocannabinol, THCCOOH 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol

* Compounds fortified at 1,000 ng/mL

^a Compounds fortified at 200 ng/mL

Table 2Validation parameters for cannabidiol (CBD) in urine by GC-MS (*n*=5)

Analyte	Internal standard	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)	Slope (mean $\pm$ SD)	Intercept (mean $\pm$ SD)	r^2 (mean $\pm$ SD)
No hydrolysis							
CBD	CBD- <i>d</i> 8	1.0	2.5	2.5–100	0.0954 $\pm$ 0.0072	0.0328 $\pm$ 0.0178	0.9959 $\pm$ 0.0023
Red Abalone β -glucuronidase hydrolysis							
CBD	CBD- <i>d</i> 8	2.5	2.5	2.5–500	0.0212 $\pm$ 0.0002	0.0090 $\pm$ 0.0011	0.9920 $\pm$ 0.0014

SD standard deviation, *LOD* limit of detection, *LOQ* limit of quantification

Table 3

Intra- and inter-assay bias and imprecision for cannabidiol (CBD) in urine by GC-MS

Analyte	Target (ng/mL)	Intra-assay bias (n=5; % CV)	Inter-assay bias (n=20; % CV)	Intra-assay imprecision (n=5; % target)	Inter-assay imprecision (n=20; % target)
No hydrolysis					
CBD	7.5	96.4	95.8	5.6	3.9
	37.5	104.6	105.3	2.3	3.6
	90	89.0	96.4	1.5	6.4
Red Abalone β -glucuronidase hydrolysis					
CBD	7.5	97.7	97.2	1.4	3.4
	90	101.9	104.1	3.2	2.2
	300	88.7	90.3	1.5	2.0

CV coefficient of variation

Table 4

Cannabidiol (CBD) urine stability data by GC-MS ($n=3$) based on fortified blank urine

Analyte	Target (ng/mL)	Stability			
		RT 8 h	RT 16 h	4 °C 24 h	4 °C 48 h
CBD	7.5	-49.5 %	N/A ^a	-17.7 %	-19.8 %
	90	-18.9 %	-50.7 %	-10.5 %	-13.7 %
RT room temperature					

^aSpecimens quantified below limit of quantification (2.5 ng/mL)

Table 5

Cannabidiol (CBD) concentrations in urine collected from a volunteer after oral CBD administration

Participant	Dose	Collection times	Non-hydrolyzed CBD (ng/mL)	Hydrolyzed CBD (ng/mL)
T	Dose 1 (400 mg)	Baseline	<LOQ	<LOQ
		+45 min	<LOQ	3.5
		+2 h	<LOQ	7824.8
		+4 h	<LOQ	2963.0
		+6 h	<LOQ	3737.8
		+7 h	<LOQ	2590.6
		+8 h	<LOQ	739.9
T	Dose 2 (400 mg)	Baseline	<LOQ	6.9
		+45 min	<LOQ	300.9
		+2 h	<LOQ	3652.0
		+4 h	<LOQ	876.4
		+5 h	<LOQ	5213.8
		+7 h	<LOQ	2402.6
		+8 h	<LOQ	839.2

LOQ limit of quantification (2.5 ng/mL)