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A validated method for the simultaneous quantification of CBD, THC, and their metabolites in human plasma, and application to plasma samples from an oral CBD open label trial

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Abstract

Cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) are the two best known and most extensively studied phytocannabinoids within *Cannabis Sativa.* An increasing number of pre-clinical studies and clinical trials have been conducted with one or both compounds, often probing their therapeutic effects in conditions such as paediatric epilepsy, anxiety disorders or chronic pain. Accurate monitoring of THC and CBD and their metabolites is essential for tracking treatment adherence and pharmacokinetics. However, fully validated methods for the comprehensive analysis of major phase I CBD metabolites are yet to be developed due to a historical lack of commercially available reference material. In the present study, we developed, optimized, and validated a method for the simultaneous quantification of CBD, THC, and their major phase I metabolites 6-hydroxy-cannabidiol (6-OH-CBD), 7-hydroxy-cannabidiol (7-OH-CBD), 7-carboxy-cannabidiol (7-COOH-CBD), 11-hydroxy-tetrahydrocannabinol (11-OH-THC), and 11-carboxy-tetrahydrocannabinol (11-COOH-THC) as per Food and Drug Administration (FDA) guidelines for bioanalytical method validation. The method is accurate, reproducible, sensitive, and can be carried out in high-throughput 96 well formats, ideal for larger-scale clinical trials. Deuterated internal standards for each analyte were crucial to account for variable matrix effects between plasma lots. Application of the method to plasma samples, taken from people who had been administered oral CBD as part of an open label trial of CBD effects in anxiety disorders, demonstrated its immediate utility in ongoing and upcoming clinical trials. The method will prove useful for future studies involving CBD and/or THC and can likely accommodate the inclusion of additional metabolites as analytical reference materials become commercially available.

1. Introduction

Scores of pre-clinical and clinical trials continue to be published involving cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC), with a recent focus on the use of the therapeutic potential of these compounds in treating a range of health problems such as epilepsy, anxiety disorders, neurological conditions and chronic pain.[1-4] Such studies require robust quantification methodologies for CBD, THC, and their metabolites in biological matrices. Methodologies for analysis of human plasma are particularly important, to enable accurate and precise monitoring of therapeutic drug levels and the variations in CBD and THC pharmacokinetics associated with different formulations and routes of administration.

Phase I metabolism of CBD and THC occurs primarily via hydroxylation and subsequent carboxylation accomplished by liver cytochrome P450 isoenzymes.[5,6] This occurs particularly at the 11 position for THC and equivalent 7 position for CBD (Figure 1), before phase II glucuronide conjugation.[7] Accordingly, THC metabolites 11-hydroxy-tetrahydrocannabinol (11-OH-THC) and 11-carboxy-tetrahydrocannabinol (11-COOH-THC) are readily observed in human plasma and urine, often at higher concentrations than THC itself.[8] Similarly, 7-hydroxy-cannabidiol (7-OH-CBD) and 7-carboxy-cannabidiol (7-COOH-CBD) are present at substantial concentrations in human plasma, and 6-hydroxy-cannabidiol (6-OH-CBD) has also been observed as a minor circulating CBD metabolite.[9]

Numerous analytical methods currently exist for the measurement of THC and its primary phase I metabolites in various biological matrices including blood, saliva, and urine.[8,10-14] Equivalent methodologies for measuring phase I CBD metabolites are yet to be developed to a significant extent, reflecting a historical lack of commercially available reference materials and internal standards for 6-OH-CBD, 7-OH-CBD, and 7-COOH-CBD. To our knowledge, existing quantitative methods for these analytes in plasma have only been published as parts of large clinical trials, which often only report partial validation of methods (typically accuracy,

precision, and linearity) and may lack isotopic internal standards.[9,15,16] Some studies have also noted potential analytical issues with 7-OH-CBD material that can prevent quantification of this analyte. Consequently, there is a need for a fully validated, specifically optimized, robust quantitative method for the analysis of CBD metabolites in order to accurately and reliably characterise the pharmacokinetics of CBD beyond that of the parent compound.

We therefore aimed to develop and validate a quantitative assay for these analytes in human plasma, for use in several ongoing and upcoming clinical trials involving CBD and THC administration. A method was optimized specifically for CBD and THC metabolites, using supported-liquid extraction (SLE) and liquid chromatography – tandem mass spectrometry (LC-MS/MS), which was validated for accuracy, precision, linearity, sensitivity, stability, carryover, selectivity, specificity, dilution integrity, extraction efficiency, and matrix effect, according to Food and Drug Administration (FDA) bioanalytical industry guidelines.[17] The method is shown to be sensitive, accurate, reproducible, and to have immediate utility via application of the method to plasma samples taken from participants administered oral CBD over several weeks as part of a recent clinical trial (ACTRN12617000825358).

2. Materials and Method

2.1 Chemicals and reagents

Analytical standards for THC, 11-OH-THC, 11-COOH-THC, CBD, d_3-THC, d_3-11-OH-THC, d_3-11-COOH-CBD, and d_3-CBD were purchased from Cerilliant (TX, USA). 6-OH-CBD, 7-OH-CBD, 7-COOH-CBD, d_9-6-OH-CBD, d_{10}-7-OH-CBD, and d_{10}-7-COOH-CBD were purchased from BDG Synthesis (Lower Hutt, New Zealand). Acetonitrile, methanol, dichloromethane (DCM), ethyl acetate, formic acid, and methyl *tert*-butyl ether (MTBE) were obtained from Thermo Fisher Scientific (NSW, Australia). Ammonium formate was obtained from Sigma-Aldrich (NSW, Australia). CBD capsules for the open label trial each contained 200 mg high-purity (>99.9%) CBD and the excipient Softisan 379 (Trigal Pharma GmbH, Vienna, Austria). All solvents and reagents were at least HPLC or ACS grade, respectively, unless specified otherwise.

2.2 Calibrators, quality controls, and internal standards

Cannabinoid-free blank plasma was obtained from the Australian Red Cross Blood Service (NSW, Australia) and from Lee Biosolutions (MO, USA). Absence of cannabinoids was verified via UHLPC-UV and by LC-MS/MS as part of the method validation. Stock solutions of individual cannabinoids were prepared or obtained at 100 or 1000 µg/mL in methanol. Stock solutions were combined and spiked into blank plasma to generate calibrators at seven ascending levels to generate standard curves. We selected calibrator concentrations from 0.5 to 100 ng/mL for all analytes, since pilot experiments showed that extending the range higher (to 1000 ng/mL, for example) compromised accuracy and precision at very low concentrations (e.g. 0.5 ng/mL), and also because this is the typical range in which THC, CBD, and their hydroxylated metabolites have been observed in human plasma.[8,18-21] Repeated or high doses of CBD or THC can result in carboxylated metabolites reaching

plasma concentrations above this range, so dilution integrity at 10X and 100X dilution factors was validated to extend the quantitative range as necessary (see Section 2.5.5).

Quality control (QC) samples were prepared similarly, using stocks generated from different supplier lot numbers where possible, to generate QCs at four levels: lower limit of quantification (LLOQ), low (QCL), medium (QCM), and high (QCH; Table 1). LLOQ was determined experimentally (Section 2.5.2), QCL was set at 3 ng/mL (near the bottom of the calibration range and approximately 3-6X that of the LLOQ) for all analytes except 6-OH-CBD, which was set as 10 ng/mL because early testing revealed a higher LLOQ for this analyte relative to the other analytes . QCM was set at 50 ng/mL (the mid-point of the calibration range), while QCH was set at 90 ng/mL (within but near the top of the calibration range).

Deuterated forms of each analyte were used as internal standards (ISs). Internal standard stock solutions were obtained or prepared at 100 µg/mL in methanol, and were combined and spiked into plasma to yield a concentration of 50 ng/mL for each IS. For preparation of calibrator, QC, and IS stocks and samples, we avoided pipetting less than 5 µL volumes via use of serial dilutions.

All peaks were identified and integrated with Shimadzu LCMS Solutions software. For a positive identification, quantifier to qualifier transition ion ratios were required to fall within ±15 % of pre-defined nominal values, and peak retention time within 5 % of that of the reference material.

2.3 Development and optimization of method

2.3.1 Chromatographic optimization

Chromatographic separation was performed using a Shimadzu (Kyoto, Japan) LC-30AD UHPLC system equipped with an Agilent (CA, USA) Zorbax XDB-C18 analytical column

(50 x 2.1 mm, 3.5 µm particle size) and Agilent XDB-C8 guard column (12.5 x 2.1 mm, 3.5 µm particle size).

Acetonitrile and methanol were assessed as mobile phase solvents (mobile phase B), coupled with 0.1% formic acid in water (mobile phase A). Chromatography was then optimized to produce sufficient separation of isomeric compounds (i.e, separation of 7-COOH-CBD and 11-COOH-THC, of 6-OH-CBD, 7-OH-CBD, and 11-OH-THC, and of CBD and THC), while maintaining a total run time of under 6 min. Injection volumes of 10 and 20 µL were compared in terms of peak size, shape, and matrix effect. The analytical column, oven temperature (50 °C), and mobile phase flow rate (0.6 mL/min) were selected based on previous success with 11-OH-THC and 11-COOH-THC analyses.[8]

2.3.2 Mass spectrometric optimization

Mass spectrometric data was collected using a Shimadzu LCMS-8040 tandem-mass spectrometer, equipped with an electrospray ionisation (ESI) source. MS1 and MS2 data for each analyte and IS were collected in positive and negative ESI mode, with collision energies set at 10, 20, 30, or 40 eV. Optimal mass transitions and collision energies were then further optimized and selected for multiple reaction monitoring (MRM), ensuring at least one confirmatory transition in addition to the quantifier transition for each analyte. Qualifier transitions were required to exceed 20% of the peak area of the quantifier transition and be free from nearby matrix interference. Other parameters such as nebulising and drying gas flows, heat block temperature, and desolvation line temperature were selected based on previous success with 11-OH-THC and 11-COOH-THC analyses.[8]

2.3.3 Optimization of sample preparation and extraction

We adapted our existing methodologies for analysis of phytocannabinoids in plasma, testing key parameters as necessary. A sample volume of 100 µL was selected to minimise the amount of plasma required while maintaining our target sensitivity (≤ 5 ng/mL LLOQ for all analytes). Supported liquid extraction (SLE) was performed semi-autonomously using a Biotage (Uppsala, Sweden) Extrahera extraction automation system.[22] SLE was assessed with and without a preceding protein precipitation step. When it became apparent that a protein precipitation step was advantageous, several reconstitution solvents for the subsequent SLE were trialled (0.1% formic acid in water, 0.1% ammonium formate in water, with and without 90 µL acetonitrile). A variety of extraction solvents – DCM, ethyl-acetate, and MTBE, and combinations thereof – were evaluated for best recovery and minimal matrix effect. Other extraction parameters were selected based on manufacturer recommendations (Biotage) and previous phytocannabinoid analysis methodologies.[8,10,18,23]

2.4 Optimized method

2.4.1 Sample preparation and protein precipitation

Frozen plasma samples were thawed at room temperature and placed on ice as they finished thawing. 100 µL aliquots were transferred to 1.5 mL capacity polypropylene centrifuge tubes. Calibrator and QC samples were prepared by spiking blank plasma with appropriate amounts of analytes dissolved in methanol (spike volume not exceeding 20 µL; see Section 2.2), then treated identically to analysis samples. All samples were spiked with 5 µL of an internal standard solution containing d_3-CBD, d_9-6-OH-CBD, d_{10}-7-OH-CBD, d_{10}-7-COOH-CBD, d_3-THC, d_3-11-OH-THC, and d_3-11-COOH-THC, to bring the spiked concentration of each IS to 50 ng/mL. 300 µL of ice-cold acetonitrile was added to all samples, briefly vortexed, and then centrifuged at 6000 x *g* for 10 min at 4 °C. The resultant supernatants were decanted into a

glass-lined 2 mL 96-well plate. The samples were dried under a steady flow of nitrogen, then reconstituted in 90 µL acetonitrile, followed by addition of 300 µL 0.1% ammonium formate in water.

2.4.2 Supported Liquid Extraction (SLE)

The samples were extracted using a Biotage 400 µL SLE 96 well plate (Biotage ISOLUTE® SLE+). The reconstituted samples were transferred to the extraction plate, and pushed into the extraction media with nitrogen at 5 bar for 5 seconds and left to equilibrate for 5 min. The analytes were eluted into a fresh glass-lined 96 well plate in two steps: (1) 700 µL of DCM, allowed to flow under gravity for 5 min followed by a nitrogen push of 0.5 bar for 30 s, (2) 900 µL of MTBE, allowed to flow under gravity for 5 min followed by a nitrogen push of 0.5 bar for 120 s, then a final push of 5 bar for 120 s. The eluate was evaporated under a gentle stream of nitrogen. The samples were reconstituted in 60 µL of methanol and 40 µL of 0.1% formic acid in water, added sequentially and mixed thoroughly via shaking at 1200 rpm for 2 min. The sample plate was sealed and placed in the LC autosampler held at 8 °C for immediate analysis.

2.4.3 LC-MS/MS analysis

Analytes were eluted in gradient mode at a flow rate of 0.6 mL/min. Initially, the mobile phase was comprised of 60% B, where it was held for 0.5 min, before increasing to 65% B at 0.75 min. Concentration was further increased to 69% B at 1.9 min, before ramping again to 85% B at 2 min and held until 3 min, before a final increase to 100% B at 3.1 min. This composition was held until 4 min, and then re-equilibrating at 60% B for a total run-time of 5 min. The column oven was held at 50 °C.

The LCMS-8040 mass spectrometer was operated in positive ESI mode with nitrogen nebulising and drying gas flows at 3 and 13 L/min, respectively, and the heat block and

desolvation line held at 400 and 250 °C, respectively. Analytes were identified and quantified using multiple-reaction monitoring (Table 2).

2.5 Method validation

Accuracy, precision, linearity, sensitivity, specificity, extraction efficiency, matrix effect, stability, dilution integrity, and carryover were systematically evaluated per FDA bioanalytical method validation guidelines.[17]

2.5.1 Accuracy and precision

Accuracy and precision were assessed during three separate days consisting of quality control samples (LLOQ, low, medium, and high; Table 1), with six replicates per level, measured against freshly prepared standard curves. Low, medium, and high QCs were required to have intra- and inter-day accuracies within ±15 % of nominal values and ≤15 % RSD, while LLOQ QCs required accuracies of ± 20% of nominal values and ≤20 % RSD.

2.5.2 Linearity and sensitivity

Linearity was measured using the calibrator prepared throughout the validation (n = 4 standard curves, each prepared in duplicate), which were prepared fresh each day at 7 levels in duplicate. Standard curves were computed via unweighted linear regression. The lowest R^2 value observed throughout the validation was used to validate linearity, which was required to exceed 0.99 for each analyte.

ULOQ was set at the highest calibrator that maintained an accuracy of ± 15 % nominal value and a precision of ≤ 15% RSD. LLOQ was selected based on the lowest calibrator sample that retained a ≥ 500 % response compared to zero calibrators, maintained an accuracy of ± 20%, and precision ≤ 20% RSD, and LOD set at signal-to-noise ≤ 3.

2.5.3 Carryover, selectivity, and specificity

Blank samples were injected after samples prepared at analyte concentrations of 10,000 ng/mL to check for carryover (6 blanks total). Selectivity was measured by comparing blanks, extracted from 6 different plasma sources, and zero calibrators to LLOQ QC samples, checking for interfering peaks at analyte retention times (6 blanks and 8 zero calibrators total). Specificity was assessed by spiking blank extracted pooled plasma with other phytocannabinoids likely to be in cannabis-related study samples (CBDV, CBDVA, CBC, CBG, CBGA, CBN, THCA, THCV, and THCVA; 50 ng/mL each) and checking for interferences at analyte retention times (6 blanks total).

2.5.4 Extraction efficiency and matrix effect

Extraction efficiency and matrix effect were determined by comparison of plasma spiked with analytes pre- and -post-extraction, in conjunction with neat mobile phase (40:60 0.1% formic acid in water:methanol) spiked with analytes.[24,25] For each condition (pre-extraction, post-extraction, and mobile phase), 6 replicates were prepared at QCL, QCM, and QCH concentrations.

Extraction efficiency was calculated as:

$$\text{Extraction efficiency (\%)} = \frac{\text{Peak Area (Post-extraction spike)}}{\text{Peak Area (Pre-extraction spike)}} * 100$$

There were no minimum criteria for extraction efficiency, but analyte and IS extraction efficiency were required to be similar (±15%) and consistent (RSD ≤ 15%).

Matrix effect was calculated as:

$$\text{Matrix Effect (\%)} = \left(1 - \frac{\text{Peak Area (Post-extraction spike)}}{\text{Peak Area (Mobile-phase)}}\right) * 100$$

Matrix effect was observed to be variable between different plasma lots, so IS-normalised matrix effect was further computed as:

$$\text{IS-Normalised Matrix Effect (\%)} = \left(1 - \frac{\text{Response (Pre-extraction spike)}}{\text{Response (Mobile-phase)}}\right) * 100, \text{ where:}$$

$$\text{Response} = \frac{\text{Peak Area (Analyte)}}{\text{Peak Area (IS)}}$$

Average IS-normalised matrix effect was required to fall below 20%.

2.5.5 Stability and dilution integrity

Short-term benchtop stability was assessed by leaving thawed samples at room temperature for 5 h before extraction. Freeze-thaw stability was measured in spiked plasma, from 6 different sources, prepared at QCL and QCH concentrations, across four freeze-thaw cycles, frozen at -30 °C. Autosampler stability was assessed by leaving samples in the LC autosampler held at 8 °C for 24 h prior to injection. Re-injection stability was measured by re-injecting a full plate of samples and assessing calibrator and QC samples for accuracy and precision on the second injection. Dilution integrity was tested using plasma spiked to create samples with concentrations 10X or 100X that of the low and high concentration QC samples. These highly concentrated samples were then diluted back into the calibration range (i.e. 10X or 100X) with blank plasma and extracted and analyzed as usual. For all stability and dilution integrity experiments, the accuracy and precision of QCL and QCH samples (6 replicates per level) were required to fall within 15% of nominal values and <15% RSD, respectively.

2.6 Application of method to trial samples

To demonstrate the utility of the validated method, it was applied to plasma samples that were recently collected as part of larger open label clinical trial examining the effects of escalating doses of CBD (fixed flexible dosing of 200-800 mg CBD days across 12 weeks)

on 31 young persons (aged 12-25) with treatment-resistant anxiety. CBD was administered once (200mg) or twice (400mg – 800mg) daily in the form of oral capsules.

Plasma was analyzed from a subset of 12 participants from this clinical trial who were administered the highest dose sequence: CBD 200 mg/day CBD for the first week (week 1), escalating to 400 mg/day for the next three weeks (weeks 2-4), then 600 mg/day for the next four weeks (weeks 5-8), and 800 mg/day to the completion of the 12 week study (weeks 9-12). For each participant, plasma was sampled on week 0 (baseline, before dosing), week 4 (after 1 week of 200 mg/kg followed by 3 weeks of 400 mg/kg CBD), week 8 (after a further 4 weeks of 600 mg/kg CBD), and week 12 (after a further 4 weeks of 800 mg/kg CBD). All samples were analyzed in triplicate.

The trial was sponsored by Orygen and reviewed and approved by an independent human research ethics committee (Bellberry Ltd.; HREC2017-02-107). The study was approved under the Clinical Trial Notification Scheme by the Therapeutic Goods Administration (TGA).

3. Results

3.1 Development and optimization of method

3.1.1 Chromatographic optimization

Larger peak heights and areas were obtained with methanol compared to acetonitrile, so methanol was selected as mobile phase B. Separation of 6-OH-CBD and 7-OH-CBD required precise control of the mobile phase gradient; these compounds are isomeric and produce very similar fragmentation patterns, and are consequentially very difficult to distinguish via mass spectrometry alone. Other isomeric groups (e.g. 7-COOH-CBD and 11-COOH-THC, or CBD and THC) were easily separated (Table 2 and Figure 2H-N). Both 10 µL and 20 µL injection volumes produced sharp, symmetrical analyte peaks with broadly equivalent matrix effect and baseline signal. 20 µL was therefore selected as absolute peak magnitude was larger, thereby maximising sensitivity.

3.1.2 Mass spectrometric optimization

Details of selected mass transitions and collision energies for all analytes are presented in Table 2. Comparison of positive and negative ESI modes revealed superior ionisation in positive mode for all analytes. Although carboxylic acids often ionize best in negative ESI mode,[26] we nevertheless observed greater ion counts in positive ESI mode for 7-COOH-CBD and 11-COOH-THC.

3.1.3 Optimization of sample preparation and extraction

Both 100 and 200 µL initial plasma volumes provided sufficient sensitivity (< 5 ng/mL LLOQs for all analytes), so the smaller 100 µL sample volume was selected. Trials without protein precipitation with acetonitrile yielded similar sensitivity to samples prepared with the

precipitation step, but substantially decreased QC precision beyond acceptable limits, so a protein precipitation step preceding SLE extraction was included in the final method.

SLE solvent trials showed that ethyl acetate, DCM/ethyl acetate, and DCM/MTBE in two separate steps yielded the best overall analyte recovery and similar matrix effect, but the DCM/MTBE combination produced the least variable recovery and was therefore selected for the final method (Figure 3). We observed a steady loss of signal for some analytes and ISs when kept in regular polypropylene 96 well plates while waiting for sample injection into the LC-MS/MS system, compromising sensitivity over time. Switching to glass-lined 96 well plates resolved this issue while maintaining the 96 well format for greater sample throughput.

3.2 Method validation

3.2.1 Accuracy and precision

Analyte quantification was accurate and reproducible for all analytes (Table 3). For low, medium, and high QCs, intra- and inter-day accuracy ranged from 94.9 – 110.7% and 98.1–106.8%, respectively, and intra- and inter-day precision ranged from 2.6 – 13.0% and 6.2 – 14.7% RSD, respectively. For LLOQs, intra- and inter-day accuracy ranged from 92.2 – 109.6% and 91.4 – 105.1%, respectively, and intra- and inter-day precision ranged from 3.6 – 13.6% and 6.7 – 19.6% RSD, respectively.

3.2.2 Linearity and sensitivity

Across the validation, all calibration curves for all analytes were linear ($R^2 > .99$) across the ranges specified in Table 1. LLOQs ranged between 0.5 – 1 ng/mL for all analytes except 6-OH-CBD, which ionized poorly compared to the other analytes, yielding a slightly higher LLOQ of 3 ng/mL (Table 1 and Figure 2H-N). LODs typically ranged between 0.1 – 0.5 ng/mL for all analytes.

3.2.3 Carryover, selectivity, and specificity

The method was selective and specific; several spiked phytocannabinoids (CBDV, CBDVA, CBC, CBG, CBGA, CBN, THCA, THCV, and THCVA) are expected not to interfere with quantification of any analytes or internal standards. No major interfering peaks were observed in extracted blank plasma (Figure 2A-G). No carryover analyte peaks were detected in blank samples injected directly after samples spiked to 10 µg/mL with each analyte..

3.2.4 Extraction efficiency and matrix effect

Extraction efficiency exceeded 75% for all metabolites and exceeded 60% for the parent compounds, and was similar for each analyte-IS pair. Matrix effects were generally low, although sometimes exceeded 20% without IS-normalisation, and were somewhat variable (RSD > 25%) across different plasma lots. When normalised against the IS, matrix effect reliably fell within ±10% for all analytes regardless of concentration (Figure 3).

3.2.5 Stability and dilution integrity

Autosampler and re-injection stability fell within acceptable limits (100 ± 15 %) for all analytes (Figure 4). All analytes were stable at room temperature for 5 h pre-extraction, except for 6-OH-CBD which degraded at low concentrations. All analytes were stable up to two freeze-thaw cycles, but high concentrations of 11-OH-THC degraded on cycles three and four, while low concentrations of CBD and 7-COOH-CBD degraded by cycle four. Therefore, we recommend minimisation of freeze-thaw cycles and swift extraction post-thaw for accurate and sensitive quantification of these analytes. A 10X or 100X dilution in blank plasma did not adversely impact the accuracy or precision of low or high QC samples for all analytes (Table 4).

3.3 Application of method to trial samples

Mean plasma concentrations of CBD, 6-OH-CBD, 7-OH-CBD, and 7-COOH-CBD across 11 of the 12 selected participants are shown in Figure 5A. CBD metabolites tracked the escalating dosing regimen but varied substantially in absolute concentration, with 7-COOH-CBD being far more concentrated than 7-OH-CBD, which was again more concentrated than 6-OH-CBD. One of the twelve participants had detectible THC, 11-OH-THC, or 11-COOH-THC in all plasma samples across the study, indicating unsanctioned use of cannabis or THC (Figure 5B), and were therefore excluded from Figure 5A.

4. Discussion

We developed and validated a quantitative LC-MS/MS method for the simultaneous analysis of CBD and THC metabolites in human plasma. Analyte quantification was accurate, reproducible, and sensitive, such that all analytes except 6-OH-CBD were quantifiable down to 1 ng/mL or lower. The analysis was resilient to a 10X or 100X dilution, effectively extending ULOQs to 10,000 ng/mL for all analytes. Extraction efficiency was optimised for the metabolites, yielding analytical recoveries exceeding 75%. Slightly lower extraction efficiencies for CBD and THC were obtained, which is typical of similar methods.[10] Ninety-six well formats can be used for most of the extraction, improving sample throughput for larger-scale analyses. Use of deuterated internal standards for each analyte was crucial to compensate for variability in matrix effect between samples. To our knowledge, this is the first fully validated (per FDA requirements) method for the analysis of primary CBD phase I metabolites that included deuterated internal standards for each analyte.

Application of the method to plasma samples, taken from a clinical trial involving the use of escalating doses of oral CBD capsules to treat pathological anxiety, demonstrated the utility of the method. Plasma concentrations of CBD, 6-OH-CBD, 7-OH-CBD, and 7-COOH-CBD followed the ascending dosing regimen and the measured concentrations were in-line with existing reports.[9,15,16] Very high 7-COOH-CBD concentrations relative to CBD and 7-OH-CBD may position this analyte as a particularly useful proxy for monitoring CBD, even using less sensitive instruments or assays. Conversely, in our samples and in previous reports, 6-OH-CBD only appears at low levels (< 10 ng/mL), unless very high doses of CBD are used (e.g. 20 mg/kg/day), and may consequently be less suitable for monitoring purposes.[9] However, intake of high-fat food with CBD can dramatically increase circulating CBD concentrations,[27,28] possibly leading to corresponding increases in metabolite concentrations. Unfortunately, CBD dosing was not controlled relative to food intake in our clinical trial.

Variations in CBD metabolite concentrations as a function of food intake may be an interesting avenue for future research.

Simultaneous quantification of THC, 11-OH-THC, and 11-COOH-THC enabled the detection and exclusion of one participant with THC exposure that may have otherwise skewed results via pharmacodynamic or pharmacokinetic interactions.[29,30] More broadly, simultaneous measurement of CBD and THC metabolites enables deeper study of pharmacokinetic interactions between these two compounds, which are often administered together (e.g. in the widely used cannabis-based medicine nabiximols (Sativex)™ or in cannabis that is enriched in both cannabinoids.[18,23]

A previous report noted an unspecified analytical issue with 7-OH-CBD material that prevented accurate quantification of that analyte.[9] We observed no problems with 7-OH-CBD quantification during our method development, aside from co-elution with 6-OH-CBD unless chromatographic conditions were precisely controlled. Indeed 6-OH-CBD proved to be the most challenging analyte; it ionized less efficiently than 7-OH-CBD and we observed degradation if held at room temperature for 5 h at low concentrations in plasma. Indeed, one batch of powdered 6-OH-CBD, but not 7-OH-CBD or 7-COOH-CBD, degraded to less than 90% purity (determined via UHPLC-UV) during an extended stay at room temperature after being temporarily seized during shipment by the Australian Border Force (data not published). We would therefore recommend transport and storage of 6-OH-CBD at low temperatures and carrying out sampling and extraction procedures on ice where possible.

This method, while robust and sensitive, does carry some limitations. The protein precipitation step takes some time to carry out, albeit with minimal analyst attention. It may be possible to omit this step if expected analyte concentrations are relatively high (> 50 ng/mL) or if precision requirements are less stringent. The remainder of the method requires several steps, although these can be carried out using semi-automated systems and multi-channel

pipettes, substantially improving throughout. In our experience, a single analyst can prepare two 96-well plates in a day. The glass-lined 96-well plates are more expensive than standard polypropylene plates, but greatly improve recovery. Although we screened for potential interferences arising from several phytocannabinoids, we did not check for interferences arising from common concomitant medications that may be present in clinical trial cohorts (antidepressants, anxiolytics, etc). Finally, to maximise sensitivity, the method uses a relatively large injection volume (20 µL). Because similar matrix effects were observed at 10 and 20 µL injection volumes, we would encourage analysts to adjust the injection volume as necessary for their instrumentation and sensitivity requirements, and to conduct small validation experiments to confirm sufficient accuracy, precision, and other key metrics at that volume.

5. Conclusions

The described method provides a fully validated, robust, sensitive, high-throughput LC-MS/MS approach to the simultaneous quantification of CBD, THC, and metabolites 6-OH-CBD, 7-OH-CBD, and 7-COOH-CBD, 11-OH-THC, and 11-COOH-THC. The method is specifically optimised for sensitive analysis of trace concentrations of CBD metabolites, but the quantitative range can be extended with appropriate sample dilutions. It is readily applicable to a variety of experimental work involving the administration of CBD and/or THC to human participants. Given the chemical similarity of other hydroxylated, carboxylated, or decarbonylated CBD and THC metabolites,[7,31] we anticipate that this method could be expanded to include other relevant metabolites with minor modification to chromatographic parameters as reference material becomes more widely available.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Tables

Table 1. Quantitative validation parameters for the analysis of CBD, THC, and their metabolites

Analyte	**LLOQ, low, medium, and high QC concentrations (ng/mL)**	**Linear R^2 (minimum over 4 standard curves)**	**LOD (ng/mL)**	**Quantification range (LLOQ – ULOQ; ng/mL)**	**Effective analytical range (100X dilution; ng/mL)**
CBD	0.5, 3, 50, 90	0.992	0.25	0.5 - 100	0.5 – 10,000
6-OH-CBD	3, 10, 50, 90	0.990	0.5	3 - 100	3 – 10,000
7-OH-CBD	1, 3, 50, 90	0.990	0.5	1 - 100	1 – 10,000
7-COOH-CBD	0.5, 3, 50, 90	0.994	0.25	0.5 - 100	0.5 – 10,000
THC	0.5, 3, 50, 90	0.996	0.1	0.5 - 100	0.5 – 10,000
11-OH-THC	1, 3, 50, 90	0.995	0.5	1 - 100	1 – 10,000
11-COOH-THC	0.5, 3, 50, 90	0.996	0.25	0.5 - 100	0.5 – 10,000

Table 2. Mass spectrometric parameters for the analysis of CBD, THC, and their metabolites.

	Retention time (min)	**Quantifier transition (*m/z*; collision energy in parentheses)**	**Qualifier transitions (*m/z*; collision energy in parentheses, eV)**
Analytes			
6-OH-CBD	1.65	331.2 → 193.1 (21)	331.2 → 123.1 (32)
7-COOH-CBD	1.88	345.2 → 299.2 (18)	345.2 → 119.2 (27) 345.2 → 193.0 (26)
7-OH-CBD	1.95	331.2 → 193.1 (22)	331.2 → 105.1 (37) 331.2 → 123.1 (35)
11-OH-THC	2.73	331.2 → 313.2 (15)	331.2 → 193.0 (24)
11-COOH-THC	2.79	345.2 → 299.1 (22)	345.2 → 193.0 (34)
CBD	2.83	315.1 → 193.1 (23)	315.1 → 135.1 (21) 315.1 → 259.1 (20)
THC	3.34	315.1 → 193.1 (21)	315.1 → 259.1 (20)
Internal standards			
d_9-6-OH-CBD	1.65	340.2 → 322.2 (13)	340.2 → 280.2 (18)
d_{10}-7-COOH-CBD	1.88	355.2 → 337.2 (15)	355.2 → 309.2 (19) 355.2 → 203.1 (25)
d_{10}-7-OH-CBD	1.95	341.2 → 323.2 (11)	341.2 → 203.2 (25) 341.2 → 105.2 (35)
d_3-11-OH-THC	2.73	334.3 → 316.3 (15)	334.3 → 196.0 (27)
d_3-11-COOH-THC	2.79	348.3 → 330.3 (16)	348.3 → 302.3 (22)
d_3-CBD	2.83	318.1 → 196.1 (20)	318.1 → 123.1 (32) 318.1 → 93.1 (23)
d_3-THC	3.34	318.1 → 196.1 (24)	318.1 → 123.1 (32)

Note. Each deuterated compound was used as the internal standard for the corresponding non-deuterated analyte (e.g. d_3-CBD for CBD, and so on).

Table 3. Accuracy and precision of cannabinoid quantification in human plasma via supported-liquid extraction and liquid chromatography – tandem mass spectrometry.

Analyte	**Concentration (ng/mL)**	**Accuracy (% nominal)**		**Precision (% RSD)**	
		Intra-day (n = 6)	**Inter-day (n = 3 days)**	**Intra-day (n = 6)**	**Inter-day (n = 3 days)**
CBD	0.5	104.7	96.1	12.9	15.4
	3	103.5	102.6	9.0	12.3
	50	94.9	102.5	10.1	13.1
	90	103.6	98.6	4.9	12.1
6-OH-CBD	3	100.2	102.0	3.6	6.7
	10	99.7	102.2	6.0	7.3
	50	105.3	102.6	11.9	11.5
	90	109.7	103.3	9.9	10.9
7-OH-CBD	1	96.0	97.9	13.6	14.1
	3	96.0	103.0	6.0	11.5
	50	97.7	99.1	6.5	9.6
	90	106.9	98.1	7.1	9.3
7-COOH-CBD	0.5	93.1	99.3	12.1	19.6
	3	110.7	105.2	7.5	14.7
	50	97.7	100.4	7.9	9.4
	90	105.1	101.1	7.1	8.3
THC	0.5	92.2	91.4	7.2	18.2
	3	104.3	99.6	13.0	13.4
	50	95.7	98.7	7.0	7.4
	90	104.1	99.1	2.6	6.2
11-OH-THC	1	104.7	100.7	7.8	15.0
	3	109.8	100.6	8.6	10.5
	50	106.3	101.5	8.7	12.4
	90	105.1	99.7	6.9	9.2
11-COOH-THC	0.5	109.6	105.1	9.7	18.0
	3	107.2	106.8	9.6	13.6
	50	106.5	103.2	7.9	11.8
	90	104.1	100.1	7.9	11.8

Table 4. 10 and 100X dilutions did not adversely impact analyte accuracy or precision at low or high final concentrations.

Analyte	Dilution Factor	Accuracy (%)		Precision (% RSD)	
		Low QC	High QC	Low QC	High QC
CBD	10X	108.8	90.7	13.3	9.0
	100X	100.6	100.8	7.5	10.8
6-OH-CBD	10X	104.1	103.1	10.9	12.8
	100X	98.1	101.7	11.8	7.9
7-OH-CBD	10X	103.2	99.5	6.1	12.3
	100X	98.1	98.3	7.8	7.4
7-COOH-CBD	10X	98.7	94.3	10.9	9.5
	100X	100.4	103.4	8.5	9.7
THC	10X	90.9	96.6	9.8	4.6
	100X	94.1	96.2	6.6	11.3
11-OH-THC	10X	100.5	98.6	10.7	6.7
	100X	97.3	97.9	9.8	9.7
11-COOH-THC	10X	100.3	101.5	9.4	5.7
	100X	98.9	104.9	13.3	7.6

Figure Legends

Figure 1. Major phase I metabolites of CBD and THC. By convention CBD and THC follow different numbering schemes but are primarily hydroxylated in the same position (7-OH-CBD and 11-OH-THC, respectively) and further oxidised to the corresponding carboxylic acids. 6-OH-CBD also appears as a minor circulating metabolite.

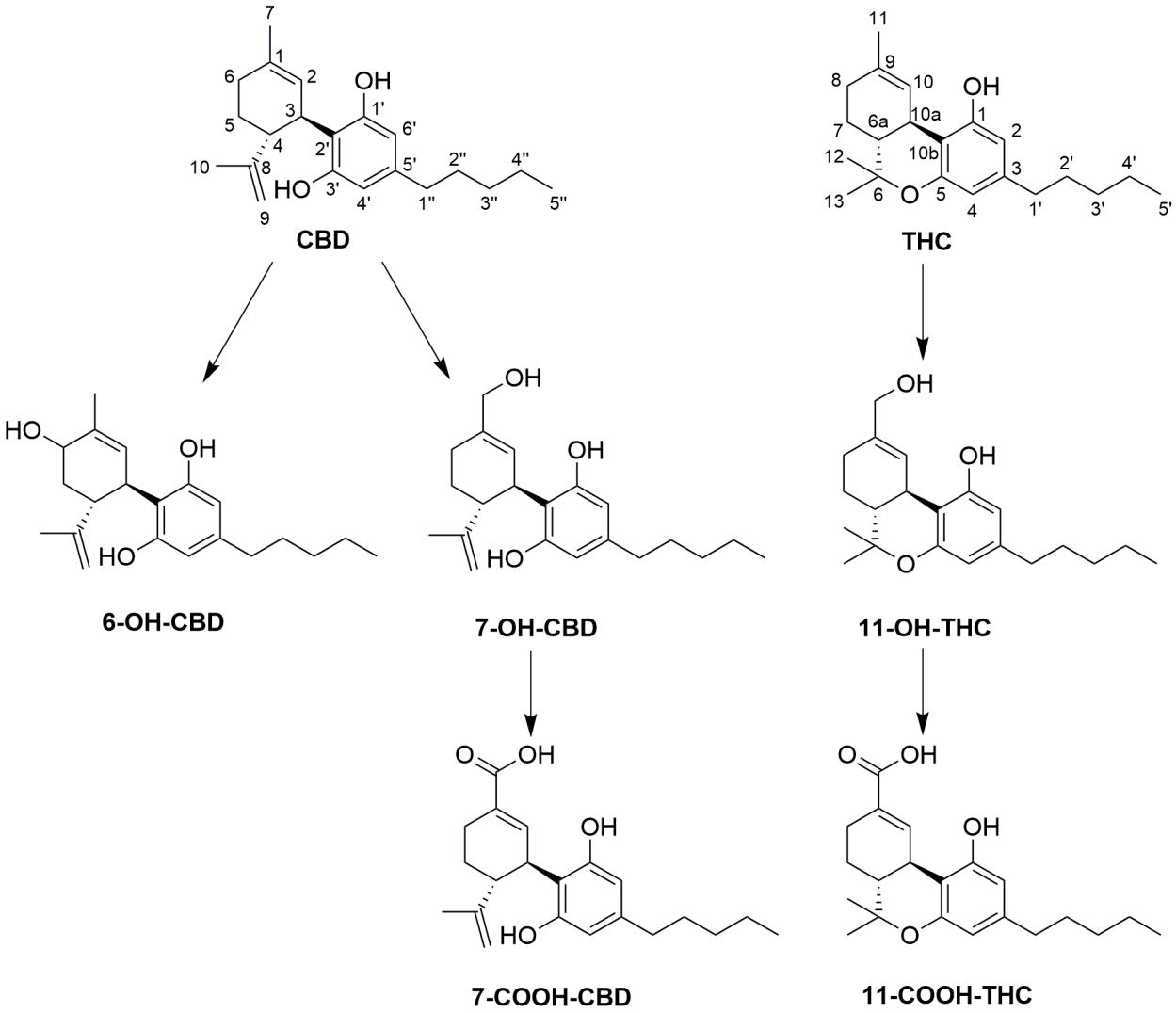
Figure 2. (A-G) Representative extracted MRM chromatograms from blank extracted plasma samples (H-N) representative extracted MRM chromatograms for LLOQ-spiked extracted plasma.

Figure 3. (A) Extraction efficiencies and (B) matrix effect using dichloromethane (DCM), ethyl acetate (EtOAc), methyl *tert*-butyl ether (MTBE), and combinations thereof, for CBD, THC, and metabolites (n = 4 per analyte per solvent system). (C) Extraction efficiency and (D) internal standard-normalized matrix effect for low, medium, and high concentrations of CBD, THC, and metabolites using DCM/MTBE. n = 6 plasma lots per concentration; error bars represent standard deviation.

Figure 4. Stability of CBD, THC, and metabolites during four freeze-thaw cycles at (A) high and (B) low concentrations, (C) after 24 h in the autosampler at 8 °C, (D) after being held at room temperature prior to extraction for 5 h, and (E) after being re-injected into the LC-MS/MS system after completion of a full 96-well batch. n = 6 per data point or bar; error bars represent standard deviation.

Figure 5. (A) CBD, 6-OH-CBD, 7-OH-CBD, and 7-COOH-CBD plasma concentrations from 11 participants, administered CBD orally over 12 weeks. CBD was dosed at 200 mg/day

for the first week, 400 mg/day for the next three weeks, 600 mg/day for the next four weeks, and finally 800 mg/day for the final four weeks. Plasma was sampled before dosing on week 0 and before dose escalation thereafter. Error bars represent standard error. (B) THC, 11-OH-THC, and 11-COOH-THC observed in one participants' plasma, indicating unsanctioned cannabis or THC use. The remaining participants had no detectible THC, 11-OH-THC, or 11-COOH-THC.



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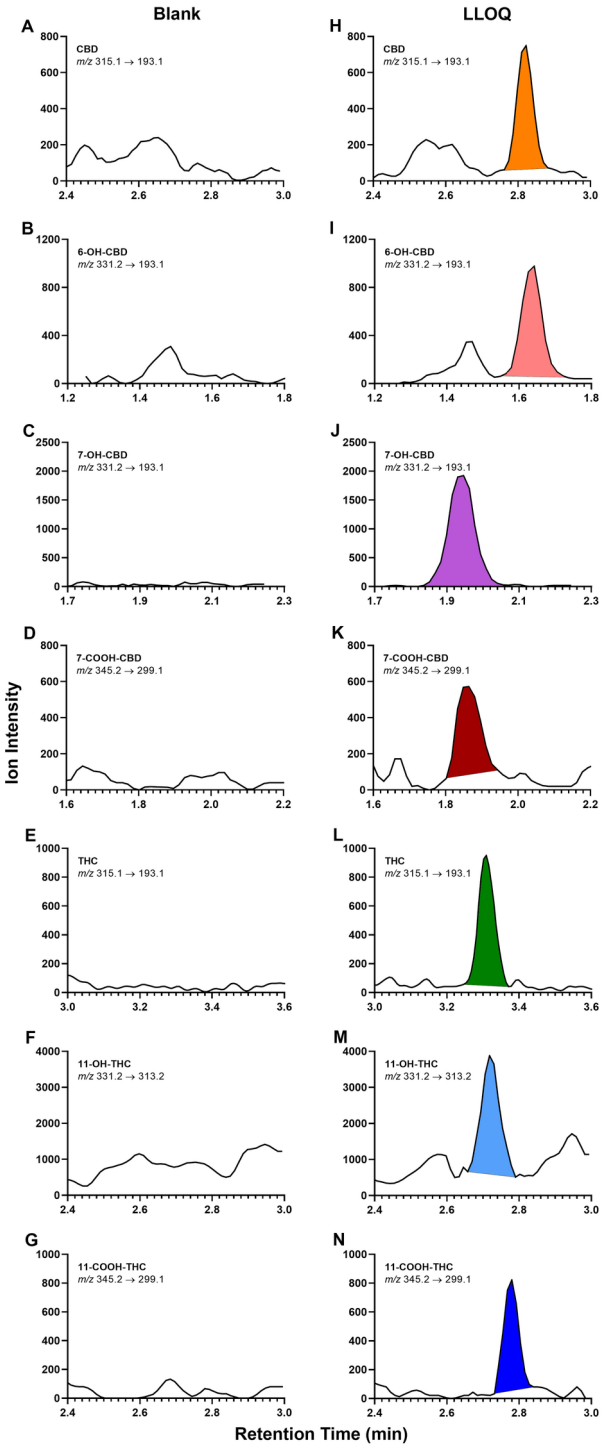

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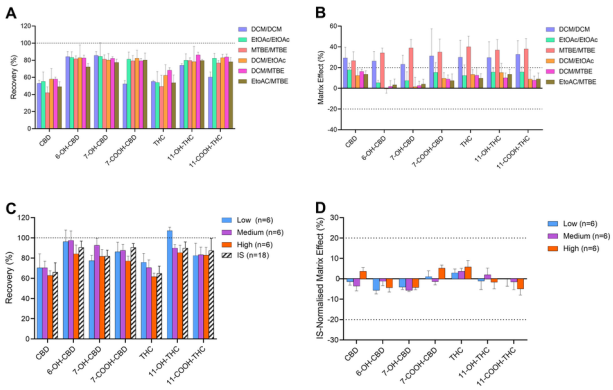

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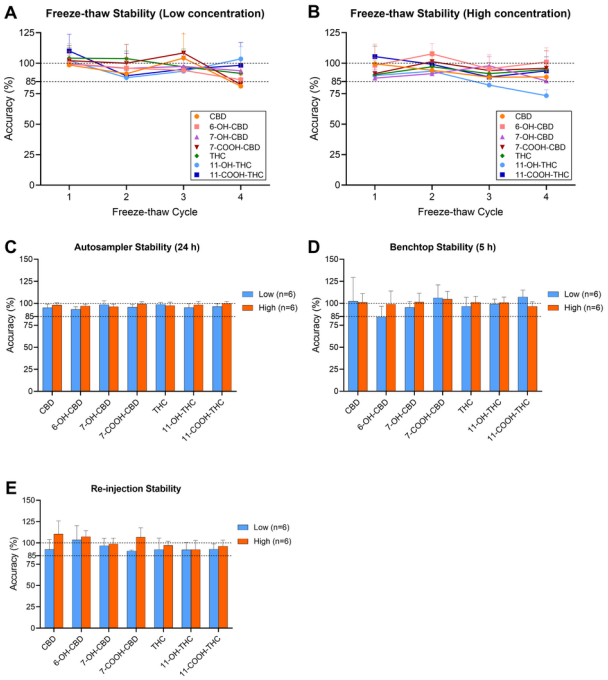

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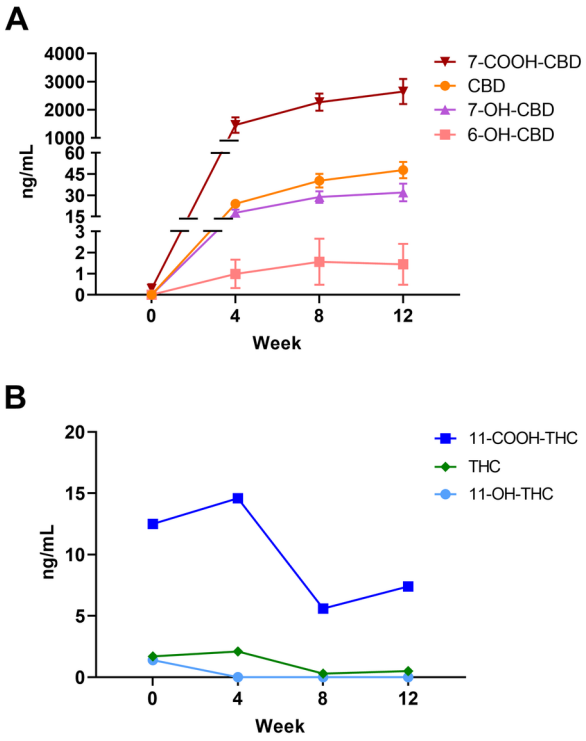

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