Application Note

Determination of CBD, 7-hydroxy and 7-carboxy metabolites in human plasma

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- Validated method for CBD in human plasma
- Separation of CBD, 7-hydroxy, and 7-carboxy CBD metabolites
- Accuracy, precision and other validation criteria all met

Introduction

The use of cannabis derived products has been growing in the USA and in other countries as interest in medical uses of cannabis continues. While the main psychoactive component of cannabis, THC, is seeing the greatest potential for use in treatment of medical conditions there is also interest in use of other cannabinoids, particularly cannabidiol (CBD)[1].

CBD has received much attention for use in treating conditions such as Lennox-Gastaut, and Dravet syndrome. A tremendous amount of growth has been observed in the production of hemp, or low THC cannabis, for this purpose. Although only one product has received FDA approval for medical use (Epidiolex, Greenwich Biosciences) there is great interest amongst the public in CBD and related cannabinoids for a host of conditions.

For medicinal use, there are number of studies examining CBD efficacy, toxicity, metabolism, and clearance[2–4] in addition to clinical trials examining possible medical uses[5]. To facilitate these types of studies, methods have been developed and validated[6–7] to provide quantitative data on concentration of CBD in human plasma samples. The method described here, utilizes LC-MS/MS to cover a concentration range of 0.5 to 500 ng/mL using a 20 µL sample volume of plasma. The validation study includes measures of accuracy and precision, stock and working solution stability, matrix effects, recovery, dilution integrity, and effects of hemolysis and lipemia.

BioAgilytix San Diego formerly known as MicroConstants is a bioanalytical CRO that has collaborated with MilliporeSigma in support of identifying solutions to their analytical workflows. The company brings expertise to a variety of bioanalytical services that includes small and large molecule analysis, method development and validation, DMPK assays, biomarker assays and much more.

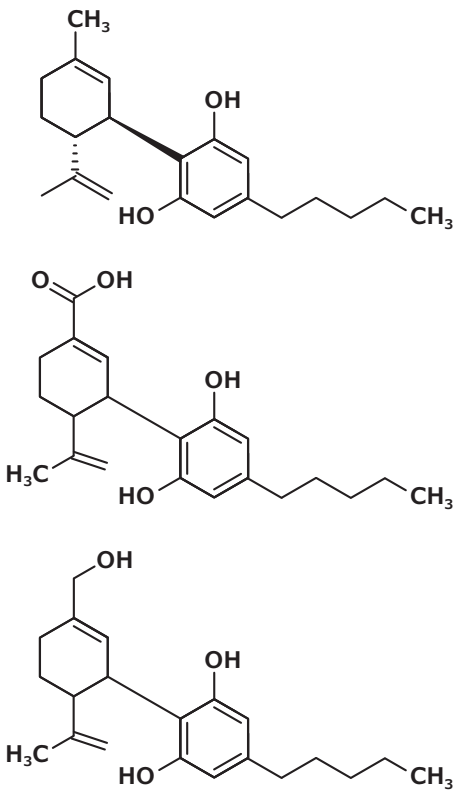

Figure 1. Chemical structures of cannabidiol (top), 7-carboxy cannabidiol (middle), and 7-hydroxy cannabidiol (bottom).

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Validation Experiments

Method validation was carried out as per guidelines provided in the FDA Bioanalytical Method Validation Guidance for Industry (8) and other standard industry practice.

Method and Materials

Instrumentation:

An HTC PAL autosampler (Leap Technologies, NC) was used to inject 20 µL of each sample onto an 1100/1200 Series Agilent HPLC system. Samples were maintained at 10 °C in the autosampler. The mass spectrometer was an API 4000 with Turboionspray source and using Analyst software, v. 1.6.2 (AB Sciex). The ion source was operated in negative ion mode at -2800 V. Mass spectrometer transitions were: CBD, 313.1 > 244.8 and d3-CBD, 316.1 > 247.8 each with a dwell time of 200 ms and collision energy (CE) of -34eV. For 7-COOH CBD, 343.1 > 298.9 (CE -20eV) and 7-OH CBD, 329.1 > 311.2 (CE -24eV). Other parameters were as follows: Declustering potential, -66 V; curtain gas, 25; Gas 1, 40; Gas 2, 35; Source temperature, 600 °C. Both sets of mass resolving quadrupoles were operated at unit resolution.

Chromatographic separation was performed on a 5 cm x 2.1-mm ID, 2.7 µm particle, Ascentis Express RP-Amide column and the column oven maintained at 40 °C. The gradient utilized is shown in **Table 1**.

From the time of injection to 1.4 minutes the column effluent was directed to waste before switching to the mass spectrometer source for 1.3 minutes. At 2.7 minutes the effluent was again directed to waste until the end of run at 5.5 minutes.

Table 1. HPLC Gradient

Pump Time (min)	% Mobile Phase A	% Mobile Phase B	Flow Rate (µL/min)
0	35	65	450
2.8	25	75	450
3	5	95	600
4	5	95	600
4.2	35	65	600
4.7	35	65	600
4.8	35	65	450
5.5	35	65	450

A complete list of reagents and standards is found at the end of this application note.

Cannabidiol (CBD), 1mg/mL, and **d3-cannabidiol (d3-CBD)**, 100 ug/mL are certified reference materials provided in sealed glass ampoules.

Extraction solvent: Hexane: 2-butanol, 97.5: 2.5 (v/v).

Extraction buffer: Ammonium phosphate dibasic, 5.3 g is combined with sodium phosphate monobasic dihydrate, 1.6 g in 100 mL of deionized water (pH ~7.5).

Human plasma, K2EDTA: may be purchased from Lampire Biological Laboratories (PA), BioIVT (NY) or BioChemed Services (VA).

Lipemic plasma: purchased from BioChemed Services (VA).

Keeper solution: 4% propylene glycol in isopropanol (v/v)

Reconstitution solution: 62% acetonitrile, 38% water (v/v)

Mobile Phase A: 0.1% propionic acid in water (v/v)

Mobile Phase B: 0.1% propionic acid in acetonitrile (v/v)

CBD and d3-CBD Stock Solutions:

Vortex mix each unopened ampoule and then transfer entire solution to separately labelled 1.8-mL, screw-top, amber, glass injection vials. The solutions may be stored in a freezer set to maintain -20°C. See COA for stock stability.

Working I.S. Solution (100 ng/mL):

Using a pipette, transfer 20.0 µL of d3-CBD Stock Standard Solution (100 µg/mL) into a 20-mL borosilicate amber glass scintillation vial containing 19.98 mL of Working Standard Diluent. Cap and mix well. Store in a freezer set to maintain -20°C.

Working Solutions for Calibrators and QC Samples:

An intermediate solution of 2,500 ng/mL is prepared from the stock CBD solution and then further used to prepare the calibrator working solutions W1-W5 (**Table 2**). These five working standard solutions are prepared in diluent (methanol) at concentrations of 0.5, 2, 10, 50, and 250 ng/mL for preparation of calibrators (**Table 2**). An additional set of working standard solutions (C1-C5) are prepared in a similar fashion for QC sample preparation at concentrations of 10, 30, 3000, 8000, and 100,000 ng/mL. These are then used to prepare plasma QC concentrations of 0.5, 1.5, 150, 400, and 2000 ng/mL by addition of appropriate volumes to bring to 5 mL (2 mL for Dilution QC) total plasma volume as shown in **Table 3**.

Hemolyzed Sample Preparation:

To prepare hemolyzed samples: centrifuge blood and discard plasma. Dilute RBC's 1:1 with deionized water. Freeze and then thaw before adding to plasma at 4% (yields 2% lysed red blood in plasma).

Table 2. Preparation of Calibrator and QC Working Solutions

Working Standard/ Control Spiking ID	Standard Solution Concentration (ng/mL)	Volume of Standard Added (mL)	Volume of Working Standard Added (mL)	Final Volume (mL)	Final Concentration in Plasma (ng/mL)
Intermediate	1,000,000 (Stock)	0.0250	9.975	10.0	2,500
W5	2,500 (Intermediate)	1.00	9.00	10.0	250
W4	2,500 (Intermediate)	0.200	9.80	10.0	50.0
W3	50.0 (W4)	2.00	8.00	10.0	10.0
W2	10 (W3)	2.00	8.00	10.0	2.00
W1	2.00 (W2)	2.50	7.50	10.0	0.500
C5	1,000,000 (Stock)	0.25	2.25	2.50	100,000
C4	100,000 (C5)	0.400	4.60	5.00	8,000
C3	100,000 (C5)	0.150	4.85	5.00	3,000
C2	3,000 (C3)	0.100	9.90	10.0	30.0
C1	30.0 (C2)	2.00	4.00	6.00	10.0

Table 3. Preparation of Quality Control Samples

QC Sample	Standard Solution Concentration (ng/mL)	Volume of Standard Added (mL)	Volume of Plasma Added (mL)	Final Volume (mL)	Final Concentration in Plasma (ng/mL)
Dilution	100,000 (C5)	0.04	1.96	2	2,000
High	8,000 (C4)	0.25	4.75	5	400
Intermediate	3,000 (C3)	0.25	4.75	5	150
Low	30.0 (C2)	0.25	4.75	5	1.5
LLOQ*	10.0 (C1)	0.25	4.75	5	0.5

* This QC sample for validation purposes only

Sample Preparation Procedure

1. Four hundred µL of phosphate extraction buffer is added to a set of labeled 16 x 100-mm borosilicate glass test tubes followed by addition of 20.0 µL of each sample or QC sample or blank matrix to be used for the calibration curve, control zero and matrix blank samples.
2. Mix tubes on a multi-tube vortex mixer for 30 seconds.
3. The calibration curve is prepared as described in **Table 4** including duplicate ULOQ and LLOQ standards, a reagent blank (Extraction Buffer only), a matrix blank (20.0 µL of blank plasma) and a control zero (20.0 µL of blank plasma fortified with I.S).
4. Next add 20.0 µL of internal standard working solution (100 ng/mL) to each standard, sample, and QC sample, resulting in 100 ng/mL I.S. in plasma.
5. Using a repeating pipette, bring total volumes to 60.0 µL with diluent, based on volumes previously combined. [For example, where 20 µL of standard solution is added to 20 µL of plasma, as shown in **Table 4**, an additional 20 µL of diluent is added.]
6. Again, mix all tubes on a multi-tube vortex mixer for two minutes.
7. Let samples sit undisturbed for approximately two minutes.
8. Using a bottle top dispenser, add 4 mL of hexane: 2-butanol extraction solvent to each tube. Extract samples by vortex mixing all tubes for 5 minutes.
9. Next centrifuge tubes at 2,500 rpm (~575x g) for 5 minutes before carefully removing from the centrifuge so as not to disturb the aqueous/solvent interface. Cover the tubes with foil and place in a -70 °C freezer for approximately 20 minutes. The upper organic layer is then poured into labeled 16 x 100-mm borosilicate glass test tubes, into which 20.0 µL of Keeper Solution has been previously added. Evaporate the contents of the tubes to dryness under nitrogen at 30 °C.
10. Reconstitute the extracts with 200 µL of Reconstitution Solution and vortex mix all tubes for two minutes. Transfer the extracts into appropriately labeled 500-µL polypropylene injection vials before capping.
11. Store extracts in a refrigerator or autosampler set to maintain 10 ±5 °C.

Table 4. Scheme for preparation of calibration standards

Working Standard	Working Standard Concentration (ng/mL)	Volume of Working Standard Added (µL)	Volume of Plasma Added (µL)	Final Concentration in Plasma (ng/mL)
W1	0.5	20	20	0.5
W1	0.5	40	20	1.0
W2	2	20	20	2.0
W2	2	40	20	4.0
W3	10	20	20	10
W3	10	40	20	20
W4	50	20	20	50
W4	50	40	20	100
W5	250	20	20	250
W5	250	40	20	500

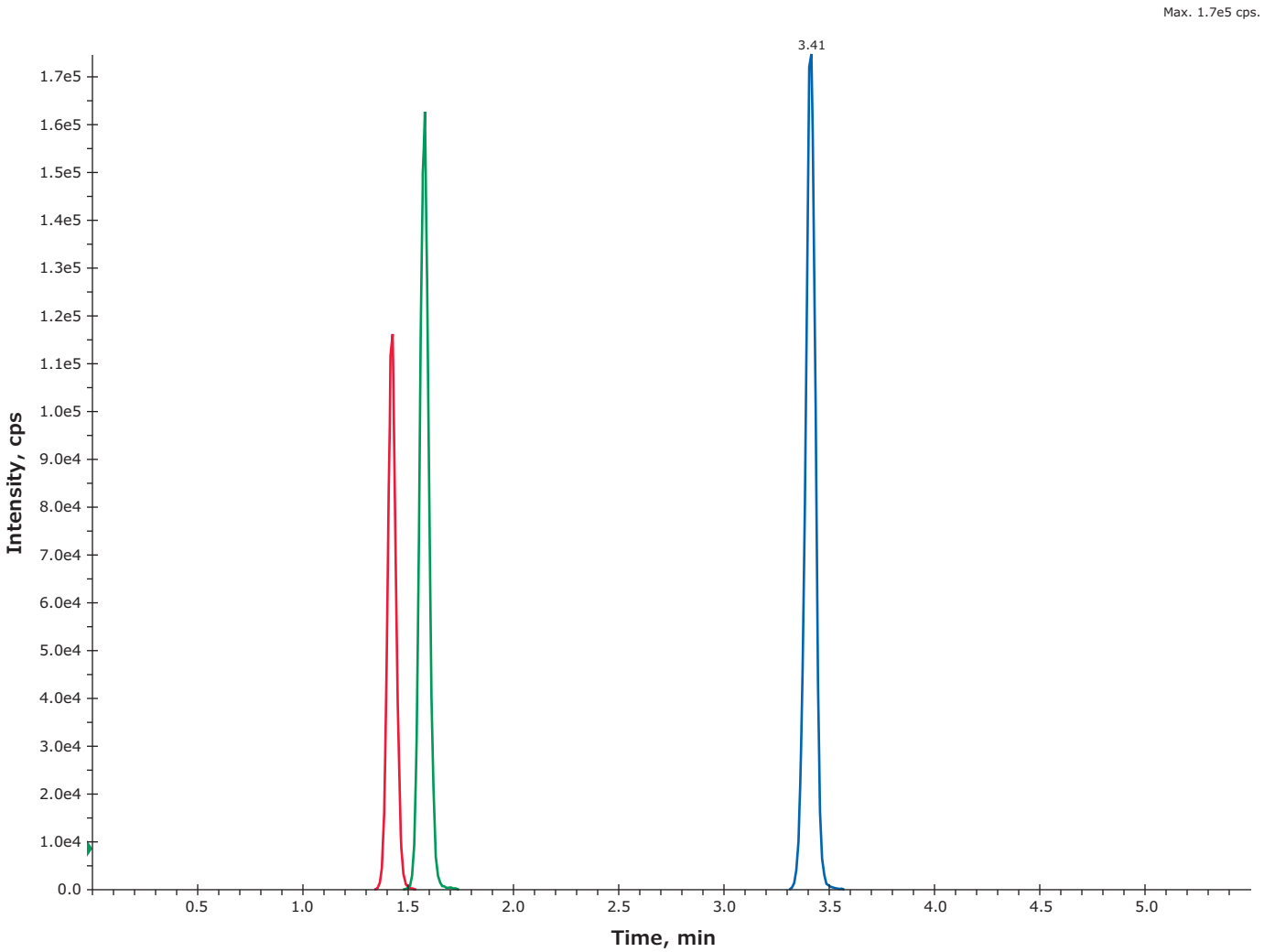

Figure 2. Chromatogram showing elution of 7-OH CBD at 1.4 minutes, 7-COOH CBD at 1.6 minutes and CBD at 3.4 minutes.

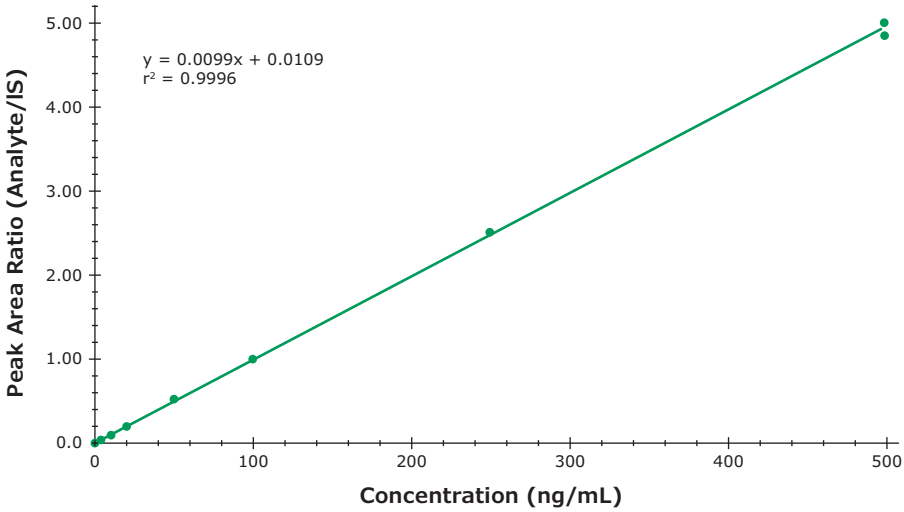

Figure 3. Example calibration curve showing range, fit, and correlation.

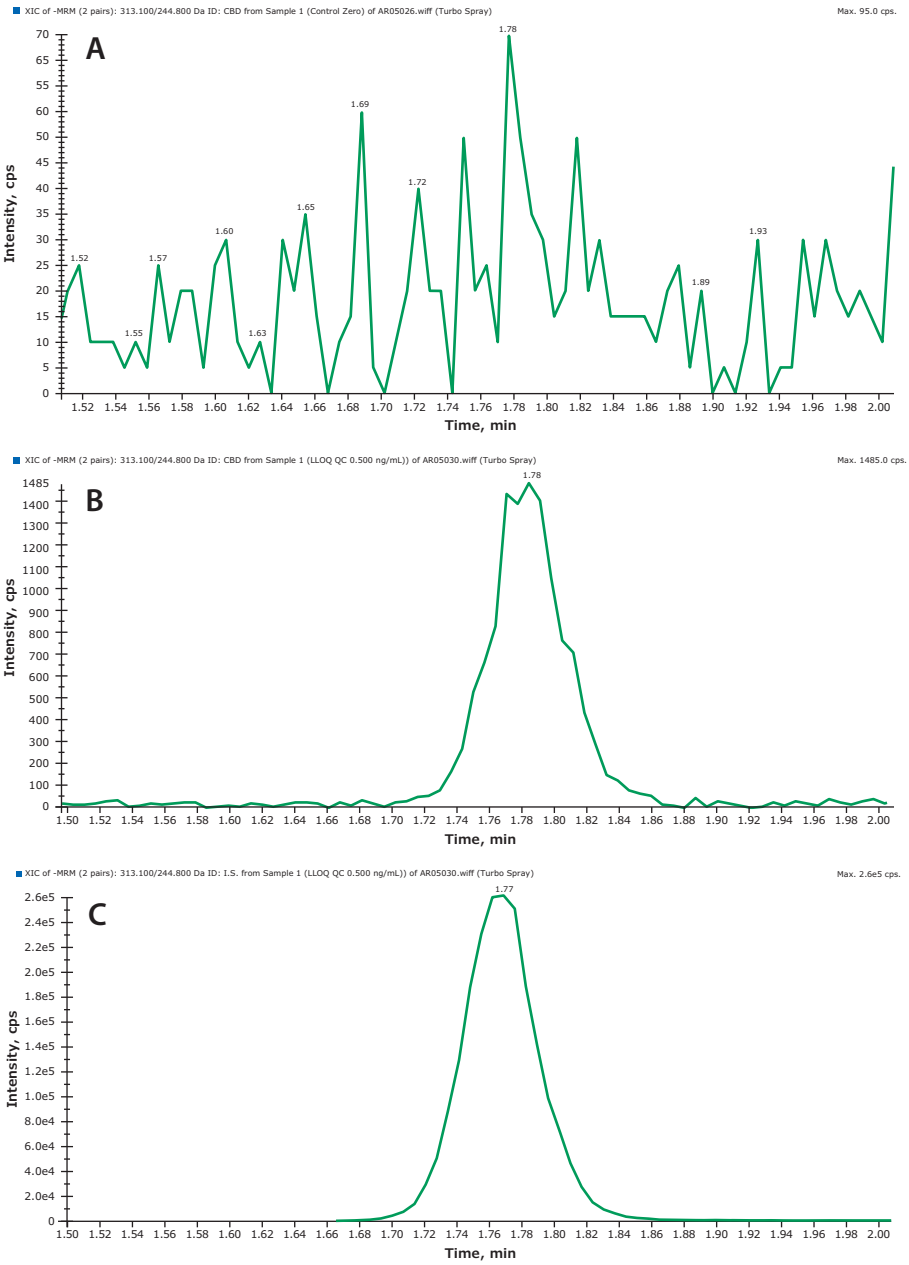

Figure 4. Chromatograms showing CBD transition in blank (A), at lower limit of quantitation (B) and representative peak for internal standard transition (C).

Results

A method was developed and validated for determination of CBD in human plasma using LC-MS/MS across the range of 0.5 to 500 ng/mL. The method uses 20 µL of plasma which is diluted with buffer and then extracted with organic solvent. After evaporating off the solvent the sample is reconstituted and submitted for analysis.

The column, mobile phases and gradient developed provide separation of the 7-hydroxy and 7-carboxy CBD metabolites from CBD itself, as shown in **Figure 2**. This ensures that CBD quantitation in patient samples will not suffer from interferences of these metabolites.

Validation was performed over the course of five analytical runs to generate accuracy and precision statistics. Calibration standards were prepared according to the scheme shown in **Table 4** and a linear $1/x^2$ weighted regression used. Correlation coefficients over five validation batches were all 0.996 or higher.

QC samples were prepared at four different concentrations of 0.5, 1.5, 150, and 400 ng/mL and evaluated for precision and accuracy across each of the five analytical runs in replicates of six per run.

The mean, percent deviation from theoretical, along with % coefficient of variation, are shown in the following table.

Table 5. Overall summary of validation statistics from 6 replicates in each of 5 analytical runs (n=30)

	LLOQ 0.5	QC 1.5	QC 150	QC 400
Mean	0.483	1.51	154	397
% CV	11.5	6.23	5.49	5.99
% DEV	–3.4	0.667	2.67	–0.75

In addition, other studies were performed to evaluate reinjection stability, storage stability of stock and working solutions, freeze/thaw, benchtop, and cold storage stability of samples, as well as the effects of lipemia and hemolysis on CBD quantitation. The results of these tests passed bioanalytical acceptance criteria with reinjection stability shown valid for up to 78 hours at 10 °C storage; stock and working solutions were stable for up to 30 days at –20 °C and up to six hours at room temperature; freeze/thaw stability was shown adequate through 4 cycles; QC samples can be stored for up to 24 hours at RT, and up to 29 days at –20 °C or colder.

Both hemolysis and lipemic conditions were evaluated for potential influence on quantitation and in both cases shown to create a slightly negative bias but within 15% of expected concentrations.

An examination of six different lots of human plasma indicated that significant interferences do not occur at the retention time and transition of CBD, or d3-CBD, and therefore would not influence CBD concentrations at the LLOQ.

The matrix effect experiment conducted with the same set of 6 plasma lots indicated an average of 12% suppression. Carryover studies indicated that in each of six replicates peak area was less than 20% of the lowest low calibration standard and less than 5% of the lowest internal standard peak area. These determinations were made after injection of the high calibration standard.

The extraction recovery was determined to be 75% or higher across the three QC sample concentrations.

The dilution QC samples, prepared at both 20,000 and 200,000 ng/mL, were prepared with 10X dilution, and a 100X serial dilution (2 x 10X), respectively. In both cases, concentrations were found within 6% of theoretical and with CV of less than 5%.

Conclusion

A method has been validated for the determination of cannabidiol (CBD) in human plasma. The method passed all acceptance criteria including accuracy and precision, linearity, lipemic and hemolyzed matrix effects, both benchtop and autosampler stability studies, as well as storage stability, recovery and carryover. The method has a relatively short run time and two major metabolites of CBD have been shown not to interfere with CBD quantitation due to earlier elution.

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Ordering Information

Product Description	Cat. No.
HPLC Column	
Ascentis™ Express RP-Amide, 2.1 mm x 50 mm, 2.7 µm particles	**53911-U**
Reagents	
Cannabidiol solution, 1 mg/mL	**C-045-1ML**
D3-cannabidiol solution, 100 ug/mL	**C-084-1ML**
7-OH Cannabidiol solution, 1 mg/mL	**C-180-1ML**
7-COOH Cannabidiol solution, 1 mg/mL	**C-181-1ML**
Methanol, for HPLC, >99.9%	**34860-4L-R**
Acetonitrile, LiChrosolv® Hypergrade for LC-MS	**1000294000**
Water, LiChrosolv® for LC-MS	**1153334000**
Propionic acid, ACS Reagent grade	**402907-100ML**
Hexanes, HPLC, 98.5%	**HX0290**
2-butanol	**B85919-1L**
Ammonium phosphate dibasic	**79762**
2-propanol, HPLC, >99.9%	**34863-4L**
Propylene glycol (1,2-propanediol), >99.5%	**398039-25ML**
Formic acid	**533002**
Consumables	
Pyrex culture tubes	**CLS982016**
Polypropylene vial kit w screw caps	**29389-U**
Labware	
Brand bottle top dispenser, 1–10 mL	**Z627569**

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