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Development of a simple and sensitive HPLC–UV method for the simultaneous determination of cannabidiol and Δ^9-tetrahydrocannabinol in rat plasma

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ABSTRACT

There has been increased interest in the medical use of cannabinoids in recent years, particularly in the predominant natural cannabinoids, cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC). The aim of the current study was to develop a sensitive and reliable method for the quantification of CBD and THC in rat plasma.

A combination of protein precipitation using cold acetonitrile and liquid–liquid extraction using n-hexane was utilised to extract CBD and THC from rat plasma. Samples were then evaporated and reconstituted in acetonitrile and 30 μL was injected into an HPLC system. Separation was achieved using an ACE C18-PFP 150 mm × 4.6 mm, 3 μm column at 55 °C with isocratic elution using a mobile phase consisting of acetonitrile–water (62:38, v/v) at 1 mL/min for 20 min. Both cannabinoids, as well as the internal standard (4,4-dichlorodiphenyltrichloroethane, DDT) were detected at 220 nm.

Our new method showed linearity in the range of 10–10,000 ng/mL and a lower limit of quantification (LLOQ) of 10 ng/mL for both cannabinoids, which is comparable to previously reported LC–MS/MS methods. Inter- and intra-day precision and accuracy were below 15% RSD and RE, respectively. To demonstrate the suitability of the method for *in vivo* studies in rats, the assay was applied to a preliminary pharmacokinetic study following IV bolus administration of 5 mg/kg CBD or THC.

In conclusion, a simple, sensitive, and cost-efficient HPLC–UV method for the simultaneous determination of CBD and THC has been successfully developed, validated and applied to a pharmacokinetic study in rats.

1. Introduction

The first recorded use of *Cannabis sativa*, commonly called hemp, was by the ancient Chinese as early as 4000 BC. It was cultivated as a fibre source for the manufacturing of textiles, ropes, and paper. Medical, psychological, and recreational uses of cannabis were well documented by Indians, Chinese, and Assyrians centuries before the Christian era [1–4]. Cannabis is a unique source of around 60 chemical compounds collectively known as cannabinoids. Although used for centuries, the chemical constituents of cannabis extracts were not identified until the end of the 19th century [5]. Two main cannabinoids, the non-psychoactive cannabidiol (CBD; Fig. 1A) and the psychoactive Δ^9-tetrahydrocannabinol (THC; Fig. 1B), were isolated from the oil extract of hemp in 1940 and 1942, respectively [5]. Two decades later, Raphael Mechoulam and colleagues successfully achieved the chemical synthesis of CBD and THC [5,6]. The discovery of endogenously occurring cannabinoids (endocannabinoids) and their cognate cannabinoid receptors 1 and 2 (CB_1 and CB_2) have renewed the interest in cannabinoids as pharmacologically active compounds [7,8] and there has been an escalating interest in the therapeutic benefits of cannabinoids over recent decades. The therapeutic benefits of cannabinoids are supported by evidence-based studies and testimony from patients who benefited from the use of medical cannabis [9].

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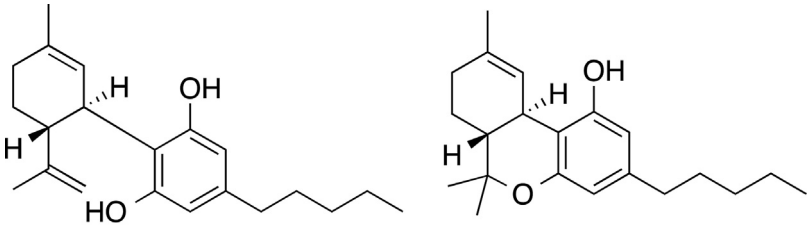

Fig. 1. Chemical structures of cannabidiol (A) and Δ^9-tetrahydrocannabinol (B).

Subsequently, increasing public and political pressures supported the legalisation of cannabis for medical use. At present, cannabis is legalised for medical use in 23 states of the US, as well as in Canada, the Netherlands, and Israel. In addition, there are other states and countries which are currently considering the legalisation of medical cannabis, such as Australia and New Zealand [10].

In 1985 and 1992, dronabinol (Marinol®), the first oral preparation of synthetic THC, was approved for the treatment of nausea and vomiting associated with cancer chemotherapy and for the decrease in appetite associated with weight loss in AIDS patients. Another commercially available product containing cannabinoids is nabiximols (Sativex®), an oromucosal spray approved for multiple sclerosis-associated spasticity. Sativex® was the first natural cannabinoid extract to be approved as a prescription drug. It provides CBD and THC in a ratio of 1:1 [11]. More recently, Epidiolex®, which is a lipid-based oral solution of CBD, was approved by the FDA as an investigational drug in the treatment of Dravet syndrome in children [12].

In addition to the above-mentioned therapeutic applications already in clinical use, efficacy studies have shown that both CBD and THC have a broad spectrum of other therapeutic activities [13–16]. A significant number of these studies have been conducted in animal models of the diseases in question, such as multiple sclerosis [17], glaucoma [18], acute colitis [19], epilepsy [20–24], oedema and hyperalgesia [25], anxiety [26,27], and psychosis [28]. In the light of this interest in studying CBD and THC in a variety of rat models, it is essential to develop and validate analytical methods capable of monitoring the levels of CBD and THC in rat plasma to support pharmacokinetic studies. Early assays used the unspecific radioactivity-based methods [29–32]. Subsequently, liquid chromatography–mass spectrometry (LC–MS)-based assays were developed and validated for the detection of THC [33] and tandem mass spectrometry assays (LC–MS/MS) for CBD [34] in rat plasma. The lower limit of quantification (LLOQ) in these assays was 5 and 10 ng/mL, respectively. In general, mass spectrometers offer more sensitive and selective analysis than ultra-violet (UV) detectors. However, equipment and maintenance, as well as sample running costs are a considerable drawback of these methods [35,36]. For the detection of CBD and/or THC in human plasma, bioanalytical methods had been developed using radioactivity assays [37], high-performance liquid chromatography (HPLC)-UV detection [38], HPLC-electrochemical detection [39], gas chromatography–mass spectrometry (GC–MS) [40–43], and LC–MS [43,44]. However, issues of unspecific detection and/or high blood sampling volumes needed for the analysis preclude the use of these methods to conduct pharmacokinetic studies in rats.

Therefore, the aim of the present study was to develop and validate a simple, sensitive, and cost-efficient method for simultaneous detection of CBD and THC in rat plasma using HPLC coupled with UV detection.

2. Material and methods

2.1. Chemicals and reagents

CBD (CAS: 13956-29-1) and THC (CAS: 1972-08-3) were donated by GW Pharmaceuticals (Cambridge, UK). 4,4-Dichlorodiphenyltrichloroethane (DDT, CAS: 50-29-3) was purchased from Sigma Aldrich (Dorset, UK) and used as the internal analytical standard (IS). HPLC grade n-hexane, acetonitrile and water were purchased from Fisher Scientific (Leicestershire, UK). Male Sprague Dawley rat plasma was purchased from BD Biosciences (Oxford, UK). All other reagents used were of HPLC grade or higher and used without further purification.

2.2. Method development

2.2.1. Preparation of standard solutions and quality control samples

Stock standard solutions of CBD, THC and DDT were prepared in acetonitrile at concentrations of 1.92, 1.32 and 0.5 mg/mL, respectively, and stored at −20 °C. Working standard solutions of 200, 200 and 50 μg/mL, respectively, were prepared by dilution of stocks in acetonitrile and were also stored at −20 °C.

The working solutions of CBD and THC were diluted in acetonitrile immediately before preparation of calibration curves to concentrations of 0.15, 0.2, 0.3, 0.4, 1, 2, 4, 10, 20, 100 and 200 μg/mL. Plasma calibration curves were prepared at concentrations of 7.5, 10, 15, 20, 50, 100, 200, 500, 1000, 5000, and 10,000 ng/mL by spiking 7.5 μL of the appropriate working solution of CBD and THC into a 135 μL of rat plasma to make up to a total sample volume of 150 μL.

Independently prepared THC and CBD stock solutions of 0.2, 0.4, 2 and 20 μg/mL were used for the preparation of LLOQ, low quality control (LQC), medium quality control (MQC), and high quality control (HQC) samples, respectively. These samples were then processed as described below.

2.2.2. Sample preparation

Samples were prepared for HPLC–UV analysis by a combination of protein precipitation and liquid–liquid extraction steps. Fifteen microliters of the IS (50 μg/mL, DDT) was added to 150 μL of rat plasma sample in a 16 mm × 150 mm glass tube. Plasma proteins were precipitated by the addition of 600 μL of cold acetonitrile (stored for 5 min at −20 °C), and the sample was then vortex-mixed for 1 min. Water (600 μL) was added and the sample was vortex-mixed again for 1 min. n-Hexane (3 mL) was added to each tube and vortex-mixed for 5 min. The tubes were centrifuged at 1160 *g* for 15 min at 10 °C (Harrier 18/80R, UK) and the upper organic layer was then carefully decanted by glass pipette to a new glass tube. The organic layer was evaporated to dryness under a stream of nitrogen gas at 35 °C (Techne DRI-Block type DB-3D, Cambridge, UK),

reconstituted in 150 μL of acetonitrile, and 30 μL was injected into the HPLC system.

2.2.3. Chromatographic conditions

A Waters Alliance 2695 separations module equipped with Waters 996 photodiode array detector was used for the analysis. Samples and column temperatures were controlled by the fitted chiller and heater at 4 and 55 °C, respectively. Separation was achieved using an ACE C18-PFP 150 mm × 4.6 mm, 3 μm particle size column (Hichrom Ltd., Reading, UK), protected by an ACE C18-PFP 3 μm guard cartridge. The mobile phase was a mixture of acetonitrile and water in a ratio of 62:38 (v/v). The flow rate was set at 1 mL/min for 20 min. The absorbance of all three compounds of interest (THC, CBD and DDT) was monitored at 220 nm. Data processing was carried out using Empower™ 2 software.

2.3. Method validation

A full validation of the current method was performed in accordance with the FDA Guidance for Bioanalytical Method Validation [45].

2.3.1. Selectivity

The selectivity of the newly developed method was examined by comparing chromatograms of extracted blank rat plasma from six different batches with samples spiked with CBD and THC at the LLOQ, as well as with samples obtained following intravenous (IV) bolus administration of CBD and THC to rats [45].

2.3.2. Intra- and inter-day precision and accuracy

Precision and accuracy (also called trueness) were expressed as relative standard deviation (RSD) and relative error (RE), respectively. The intra-day precision and accuracy were determined by analysing six replicates of rat plasma samples containing both CBD and THC at concentrations of 20 (LQC), 100 (MQC), and 1000 ng/mL (HQC). The inter-day precision and accuracy were determined at six separate days at the same concentrations. A method is considered to be precise and accurate if the values of RSD and RE are within the acceptable limits of the FDA guidelines (RSD ≤ 15% and RE within ±15%) for both intra- and inter-day runs [45].

2.3.3. Sensitivity

The validated LLOQ for CBD and THC was the lowest tested concentration of spiked plasma sample that had sufficient precision and accuracy (RSD ≤ 20% and RE within ±20%) for intra-day analyses [45,46].

2.3.4. Recovery

To assess the extraction efficiency of the assay, the recovery of CBD and THC was determined by comparing the peak areas from extracted samples at three concentrations (LQC, MQC, and HQC) with non-extracted acetonitrile solutions of equivalent concentrations ($n = 5$ for each level) [45]. The recovery of the IS was determined in the same way (at one concentration level).

2.3.5. Stability

Storage stability of CBD and THC in rat plasma was evaluated under different conditions: freeze-thaw stability (3 cycles from −80 °C to room temperature), room temperature stability (6 h at room temperature), short-term stability (24 h at −80 °C), and long term stabilities (30 and 60 days at −80 °C) were performed at LQC, MQC and HQC ($n = 6$ for each level). In addition, autosampler stability of the processed samples (16 h at 4 °C) was performed at LQC, MQC and HQC ($n = 6$ for each level). Samples were considered stable if precision and accuracy values were within the acceptable limits (RSD ≤ 15% and RE within ±15%, respectively).

2.4. Pharmacokinetic experiment

To demonstrate the application of the new bioanalytical method to *in vivo* studies, a preliminary pharmacokinetic experiment was performed. The protocol for this study was approved by The University of Nottingham Ethical Review Committee in accordance with the Animals [Scientific Procedures] Act 1986. Four male Sprague Dawley rats (Charles River Laboratories, UK) weighing 330–350 g were used in this study. The rats were housed in the University of Nottingham Bio Support Unit, and kept in a temperature controlled, 12 h light–dark cycle environment with free access to water and food.

After 5 days of acclimatisation, the right external jugular vein was cannulated with a two-part catheter consisting of polyethylene tubing (PE-50) connected to silastic tubing. The next morning, two animals were administered an IV bolus of 5 mg/kg CBD (10 mg/mL solution in propylene glycol–ethanol–sterile water (80:10:10, v/v/v)), and two other animals were administered an IV bolus of 5 mg/kg THC in the same vehicle. Blood (0.35 mL) was sampled from the cannula before dosing, and at 5, 15, 30, 120, and 420 min following the administration of either compound. Plasma was separated by centrifugation (3000 g, 10 min, at 15 °C) and stored at −80 °C until analysis. Phoenix WinNonlin 6.3 (Pharsight, Mountain View, CA, USA) software was used for pharmacokinetic analysis of the data using a non-compartmental approach.

3. Results and discussion

3.1. Method development

Initially, a liquid–liquid extraction method (tetrahydrofuran–hexane) was attempted for sample preparation, which was based on a previously published study on other lipophilic cannabinoids [47]. However, the chromatography resulting from this method showed significant interference between background plasma peaks and the peaks of CBD and THC (data not shown), and tetrahydrofuran in particular was not efficient in precipitating proteins. Therefore, a different protein precipitation method using acetonitrile was attempted, as it had previously been shown to be an effective agent for plasma protein precipitation [48], and also for the extraction of lipophilic compounds [49–51]. The use of cold (compared with room temperature) acetonitrile appreciably decreased the size of background plasma peaks, but interfering peaks were still present at the same retention times as CBD and THC peaks (data not shown). Subsequently, addition of a liquid-liquid extraction stage using n-hexane significantly improved separation of analytes from the background plasma peaks. A cleaner baseline was obtained by dilution of the plasma samples with water. This could possibly be explained by increased specificity of the extraction to more lipophilic compounds, by improved retention of hydrophilic contaminants in the water-miscible phase.

A number of different HPLC–UV columns were tested for their ability to separate CBD and THC from background peaks. The columns that were tested included a Phenomenex Luna C18 100 mm × 2.1 mm, 2 μm column, an ACE Super C18 100 mm × 4.6 mm, 5 μm column, and finally the ACE C18-PFP 150 mm × 4.6 mm, 3 μm column, which provided the best separation efficiency.

Compounds that were tested as an IS in this method included dexamethasone, bifonazole, testosterone, benz[a]pyrene, cholecalciferol (vitamin D), α-tocopherol (vitamin E), and probucol. It was

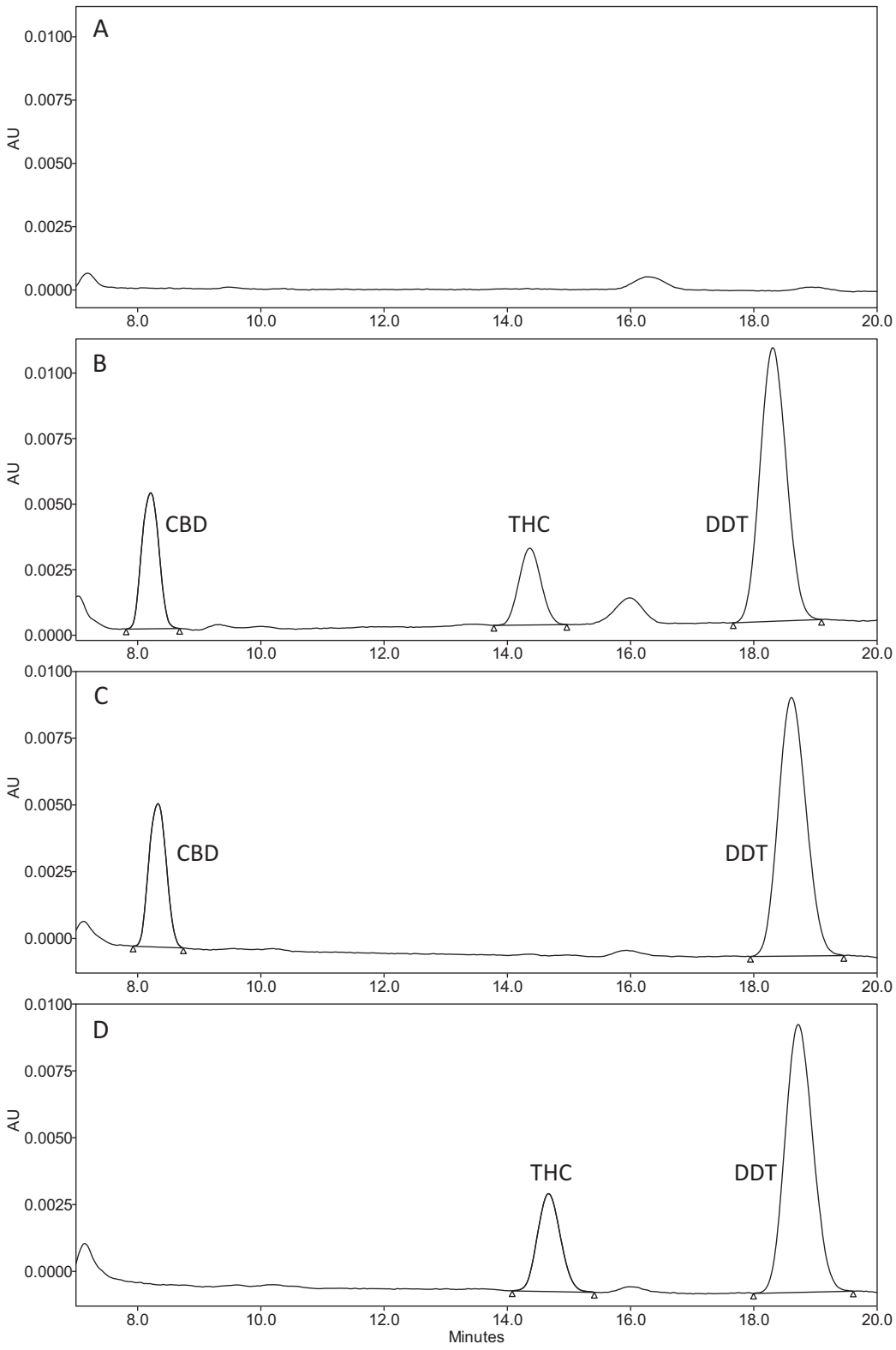

Fig. 2. Representative chromatograms of blank rat plasma (A), plasma spiked with cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) at concentrations of 1000 ng/mL in addition to DDT (B), rat plasma following IV injection of CBD (C), and rat plasma following IV injection of THC (D); all detected at λ = 220 nm.

found that the elution times of these compounds under the instrumentation conditions used were either too short (dexamethasone, bifonazole, testosterone, benz[a]pyrene), or too long (probucol, vitamin D, vitamin E). Finally, DDT was chosen as the most appropriate IS, based on its elution being a short time after THC.

A simple buffer-free acetonitrile–water based mobile phase was used in this method, which is also compatible with LC–MS analysis, if necessary. Column temperature, flow rate and mobile phase composition were all optimised to give the final instrumentation conditions described in Section 2.2.3.

Table 1
Intra- and Inter-day precision and accuracy data for the detection of CBD and THC in rat plasma.

Level	Intra-day ($n=6$)		Inter-day ($n=6$)	
	RSD%	RE%	RSD%	RE%
CBD				
LLOQ (10 ng/mL)	8.10	6.55	–	–
LQC (20 ng/mL)	8.02	13.6	9.8	0.84
MQC (100 ng/mL)	3.23	−3.00	4.11	−0.82
HQC (1000 ng/mL)	2.60	0.73	5.47	−0.96
THC				
LLOQ (10 ng/mL)	16.00	−15.87	–	–
LQC (20 ng/mL)	14.33	5.17	8.25	6.48
MQC (100 ng/mL)	4.97	3.13	4.62	3.23
HQC (1000 ng/mL)	3.54	1.81	3.25	−0.91

CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol; RSD, relative standard deviation; RE, relative error; LLOQ, lower limit of quantification; LQC, MQC, and HQC, lower, medium, and high quality controls, respectively.

3.2. Method validation

3.2.1. Selectivity

The method showed good selectivity since matrix related peaks from blank plasma did not interfere with either CBD or THC (Fig. 2A and B) at the detection wavelength (220 nm). Likewise, no endogenous peaks were observed at the elution times of either cannabinoid in samples obtained from rats before IV bolus administration. Typical chromatograms of rat plasma after IV bolus administration of CBD and THC are shown in Fig. 2C and D, respectively. No endogenous and/or drug metabolite peaks were observed at the elution times of CBD and THC in chromatograms after IV administration of the other cannabinoid (Fig. 2C and D). Therefore, this method can be used for simultaneous determination of CBD and THC in rat plasma.

3.2.2. Intra- and inter-day precision and accuracy

RSD and RE values are shown in Table 1. The intra- and inter-day precision and accuracy for CBD and THC were within the acceptable limits for all QC samples as indicated by the values of RSD (<15%) and RE (within ±15%), respectively [45]. These results indicate that the method is precise and accurate for the determination of CBD and THC in rat plasma.

3.2.3. Sensitivity and linearity

RSD and RE values for CBD and THC were within the acceptable limits at the LLOQ (Table 1), which was found to be 10 ng/mL for both cannabinoids. The linearity of the method was confirmed over the concentration range of 10–10,000 ng/mL based on 10 concentration levels with correlation coefficient (r^2) values ≥0.9996 in all calibration curves. Compared to previously reported assays in rat plasma, the LLOQ of CBD obtained in our study is similar to the LLOQ (10 ng/mL) reported by Deiana et al. [34] in a study for the determination of CBD in rat plasma using LC–MS/MS. Valiveti and Stinchcomb [52], on the other hand, were able to develop a more sensitive method (LLOQ 5 ng/mL) for the detection of THC in rat plasma using LC–MS. However, the run time in their study was relatively long (30 min) which could increase the cost and overall time needed for sample analysis. Taken together, the developed HPLC–UV method for the determination of CBD and THC in rat plasma provides a technique with a similar sensitivity to reported LC–MS/MS methods, but in a more cost-efficient and simple way.

3.2.4. Recovery

Mean values of absolute recoveries (mean ± SEM, $n=5$) of CBD from rat plasma at the LQC, MQC, and HQC concentrations were 90.5 ± 3.1%, 86.2 ± 1.1%, and 91.0 ± 1.3%, respectively. Likewise, THC recoveries from rat plasma at the LQC, MQC, and HQC concentrations were 94.6 ± 1.9%, 86.7 ± 1.0%, and 93.1 ± 1.3%, respectively. The recovery of the IS, was 74.6 ± 0.7%. The good sensitivity achieved by this method reflects the high efficiency of the extraction method for CBD and THC.

3.2.5. Stability

The stability of CBD and THC at LQC, MQC, and HQC under different conditions is presented in Table 2. RSD and RE values indicate that both CBD and THC were stable in rat plasma after three cycles of freeze-thawing (−80 °C to room temperature), bench-top storage (6 h at room temperature), short term storage (24 h at −80 °C), and long term storage (30 and 60 days at −80 °C). In addition, both cannabinoids were stable in processed samples after 16 h at 4 °C (autosampler stability). These results confirm that the developed method is suitable for routine analysis. To the best of our knowledge, this study reports for the first time detailed stability data for CBD and THC in rat plasma. Some studies have evaluated the stability of CBD and THC in human plasma [42,43] and the results were consistent with those obtained in our study for rat plasma.

3.3. Pharmacokinetic study

To demonstrate the suitability of the developed method for pharmacokinetic studies, the method was applied to a preliminary pharmacokinetic study in rats. The mean plasma concentration–time profiles observed following a single IV bolus administration of CBD or THC at a dose of 5 mg/kg are presented in Fig. 3. The pharmacokinetic parameters from these concentration–time profiles calculated by non-compartmental

Table 2
Stability results ($n=6$) of CBD and THC under various conditions.

Level	Freeze-thaw (3 cycles)		Room temperature (25 °C, 6 h)		Short term (−80 °C, 24 h)		Long term (−80 °C, 30 days)		Long term (−80 °C, 60 days)		Autosampler (4 °C, 16 h)	
	RSD%	RE%	RSD%	RE%	RSD%	RE%	RSD%	RE%	RSD%	RE%	RSD%	RE%
CBD												
LQC (20 ng/mL)	11.7	12.4	9.1	6.3	11.2	−2.4	12.8	10.5	6.4	−15	7.3	14.4
MQC (100 ng/mL)	6.1	−12.4	6	−9.1	2.8	−9.2	7.3	−12.5	3.5	−5.8	3.8	5.6
HQC (1000 ng/mL)	4.9	−14.1	2.9	−4.9	3.9	−7.3	5.9	−9.5	2.2	−6.8	2.1	−0.8
THC												
LQC (20 ng/mL)	12.1	14.5	7.2	−14.5	14.9	−12.7	9.4	9.6	11	−6	9.9	−12.6
MQC (100 ng/mL)	6.1	−11.1	6.2	−12.6	6.8	−8.9	6.1	−12.2	9.3	11.6	7.7	−1.8
HQC (1000 ng/mL)	3.9	−3.9	2.9	−5.9	2.1	−0.6	2.4	−12.3	3.3	−13	1.5	1

CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol; RSD, relative standard deviation; RE, relative error; LLOQ, lower limit of quantification; LQC, MQC, and HQC, lower, medium, and high quality controls, respectively.

Table 3
Pharmacokinetic parameters (means ± SEM) derived from plasma concentration–time profiles following single IV bolus administration of CBD or THC to rats at a dose of 5 mg/kg ($n = 2$).

	AUC_{inf} (h.ng/mL)	AUC_{0-t} (h.ng/mL)	C_0 (ng/mL)	V_d (mL/kg)	CL (mL/h/kg)	$t_{1/2}$ (h)
CBD	2581 ± 58	2546 ± 71	12,917 ± 2325	1614 ± 274	1939 ± 44	1.42 ± 0.15
THC	4736 ± 53	4679 ± 47	20,620 ± 85	979 ± 74	1056 ± 12	1.32 ± 0.01

CBD, cannabidiol; THC, Δ^9-tetrahydrocannabinol.

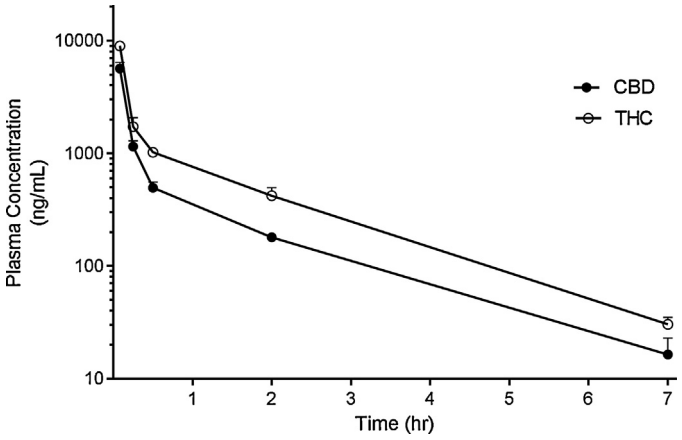

Fig. 3. Plasma concentration–time profiles (means ± SEM) following single IV bolus administration (5 mg/kg) of cannabidiol (CBD) or Δ^9-tetrahydrocannabinol (THC) to rats ($n = 2$ for each cannabinoid).

analysis are summarised in Table 3. The pharmacokinetic parameters derived from the IV bolus administration of CBD and THC in rats showed a number of differences between the two cannabinoids. CBD showed lower area under the curve, and higher apparent volume of distribution and clearance values than THC.

Few pharmacokinetic studies have been reported for CBD and THC in rats. Different bioanalytical methods were used in these previous studies, including unspecific radioactivity assays in early reports [30,31]. The variability of pharmacokinetic parameters obtained in previous works is very high. Siemens et al. [30] reported $t_{1/2}$ and V_d parameters following single IV bolus administration of radiolabeled CBD (4 mg/kg). The parameters stated in that study were significantly higher than those reported in subsequent works, as well as in our study. Indeed, the $t_{1/2}$ obtained in our study is comparable to the $t_{1/2}$ values more recently reported by Deiana et al. [34] who used LC–MS/MS method for the detection of CBD. Similarly, the pharmacokinetic parameters obtained for THC in the current study are more comparable with the recent pharmacokinetic study by Valiveti et al. [53], who used LC–MS technique to detect THC. Further studies will be required to accurately assess the pharmacokinetic parameters for CBD and THC after intravenous as well as oral administrations.

4. Conclusion

A simple, sensitive, and cost-efficient HPLC–UV method was developed and fully validated for the determination of CBD and THC in rat plasma. The method was shown to be precise and accurate, and has a LLOQ of 10 ng/mL for both cannabinoids. This LLOQ is similar to previously reported values but achieved with a simpler and more cost-efficient methodology. The stabilities of CBD and THC in rat plasma were investigated for the first time, and were found to be satisfactory under all tested conditions. The method was applied to a pharmacokinetic study after IV administration of CBD or THC to rats which demonstrated that this assay can be used for simultaneous detection of CBD and THC.

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