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Original Article

Development and validation of reverse phase high performance liquid chromatography method for the determination of delta-9-tetrahydrocannabinol and cannabidiol in oromucosal spray from cannabis extract

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ABSTRACT

A simple, fast, precise, accurate and responsive high performance liquid chromatography method was developed and validated carefully for determining an amount of delta-9-tetrahydrocannabinol and cannabidiol. A reverse phase Zorbax C-18 column 4.6 mm × 100 mm, 3.5 μm was eluted by using a mixture (85:15) of methanol and water as the mobile phase in an isocratic system with a flow rate of 1.0 ml/min and the injection volume was 10 μl, at a wavelength of 220 nm. The method was developed and validated through linearity, accuracy, precision and detection and quantitation limits studies. A good linear relationship (R^2) of delta-9-tetrahydrocannabinol and cannabidiol were 0.9998 and 0.9995, respectively. The obtained % recoveries were found to be 98.3% and 96.5%. The relative standard deviations values of peak areas were found to range from 0.20% to 2.55% and 0.30% to 3.28% for delta-9-tetrahydrocannabinol and cannabidiol, respectively. Delta-9-tetrahydrocannabinol and cannabidiol presented limits of detection of 0.12, 0.23 μg/ml and limits of quantitation of 0.40, 0.76 μg/ml. The developed method could be employed for quantitative analysis of cannabis extract and oromucosal spray formulation.

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Introduction

Cannabis (*Cannabis sativa* L.) or marijuana belongs to the family of the Cannabaceae. In Thailand, cannabis is an illegal plant but has limited laws allowing the use of hemp (*C. sativa* L. subsp. *sativa*) containing high cannabidiol (CBD) and low delta-9-tetrahydrocannabinol (THC). It contains wide variety chemicals, approximately 500 compounds have been identified. The major chemical cannabinoids constituents are including delta-9-tetrahydrocannabinol (**1**, THC), cannabidiol (**2**, CBD) and cannabinol (**3**, CBN). The structure formula of THC and CBD were bi- and tri-cyclic compounds, contained twenty-one atoms of carbon, thirty of hydrogen and two of oxygen. The psychotropic effects of cannabis are mediated by THC. However, CBD and CBN (the degradation product of THC) have gained attention as they have pharmacological properties in addition the former might antagonize some of the effects produced by THC (Pertwee, 2008). Cannabinoids was concentrated in a resinous secretion produced by the trichomes of the female pollen. Cannabis is the medicinally use for chronic neuropathic pain (Ben, 2006), glaucoma (Hepler and Frank, 1971), intestinal dysfunction (Manara et al., 2002), schizophrenia (Giuffrida et al., 2004) and rheumatoid arthritis (Lynch and Campbell, 2011). Cannabis formulation commonly use in a capsule such as Cannador (THC 2.5 g and CBD 1.2 mg), Dronabinol (THC 2.5, 5 and 10 mg) and Nabilone (THC 0.25, 0.5 and 1 mg). Nabiximal, a cannabis extract oromucosal spray containing THC 27 mg/ml and CBD 25 mg/ml. Nabiximal is a solution that has to be administered by spray onto the buccal surface to bypass the "first pass" metabolism of the cannabinoids associated with intestinal absorption. Therefore, THC and CBD were absorbed fairly rapidly.

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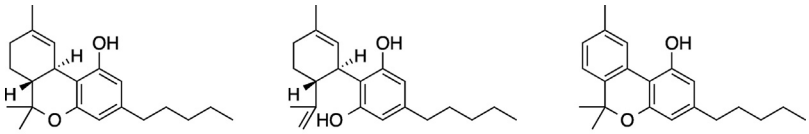

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The high performance liquid chromatography (HPLC) method for the quantitative detection of THC and CBD in cannabis formulation has been developed, completely validated and applied to analysis cannabis spray formulation. The gradient elution reverse phase HPLC with diode array detector (DAD) methods for the measurement of cannabinoids in cannabis plant material were published previously (Patel et al., 2017) but it does not permit the determination of isomer forms during analysis. The isocratic elusion normal phase HPLC with diode array detector (DAD), the simplest method for the determination (Backer et al., 2009) with long run time of 36 min makes them unsuitable for a routine determine of THC and CBD with a large number of samples.

The objective of the present study is to develop and validate a simple RP-HPLC/DAD method, allowing a good separation and short run time followed by a qualitative and quantitative determination of THC and CBD in oromucosal spray from cannabis extract which is important for understanding the stability of cannabis products.

Materials and methods

Chemicals and reagents

The 40 kg of dried cannabis samples (*Cannabis sativa* L., Cannabaceae) were supplied from an illegal narcotic drugs in Thailand arrested by Narcotics Suppression Bureau and legally authorized representative in research by Office of the Narcotics Control Board, Food and Drug Administration, Thailand. THC and CBD reference standards were isolated by The Herbal Medicinal Products Research and Development Center (Cooperation between Rangsit University and Harbin Institute of Technology and Heilongjiang University of Chinese Medicine) and Department of Pharmacognosy, Rangsit University, Thailand. The standard references were prepared by column chromatography, semi-preparative HPLC and identified by GC-MS and NMR, the purity more than 95%. Ultrapure water was obtained from Puris-Expe UP water system (Korea; resistivity 18.2 MΩ cm). HPLC grade methanol and AR grade ethanol were purchased from Burdick and Jackson®, SK Chemicals (Korea). All other chemicals used in the spray formulation were of pharmaceutical grade.

Instrumental and analytical conditions

Quantitative analysis of THC and CBD were analyzed using a high performance liquid chromatography (HPLC) method, coupled to a UV detector set to 220 nm. UV spectra scanning from 190 to 400 nm were recorded online for peak identification. The HPLC system consisted of a quaternary pump system (Agilent, 1260 VL), autosampler (Agilent, 1260 TCC) and UV/VIS with diode array detector (Agilent, 1260 DAD VL). A reverse-phase Zorbax C-18 column 4.6 mm × 100 mm, 3.5 μm was eluted by using a mixture (85:15) of methanol and ultrapure water as the mobile phase with a flow rate was set to 1.0 ml/min and the injection volume was 10 μl as presented in Box 1. All experiments were carried out at 25 ± 0.5 °C. The identification peak was performed by comparing the retention times and UV absorption spectra of the samples with those of the standard solutions.

Box 1: Chromatographic condition of HPLC analysis.

Column	A reverse-phase Zorbax C-18 column 4.6 mm × 100 mm, 3.5 μm
Mobile phase	Methanol:water (85:15, v/v)
Flow rate	1 ml/min
Detector	UV detector 220 nm
Injection volume	10 μl

Method validation

The method was developed and validated through linearity, accuracy, precision and detection and quantitation limits studies.

Linearity

THC was tested a six point calibration curve with the following concentrations was used: 2.5, 5, 10, 20, 40 and 80 μg/ml in methanol and CBD with the following concentrations was used: 3.125, 6.25, 12.5, 25, 50 and 100 μg/ml in methanol. Calibration curves for the different concentrations versus peak area were plotted and subjected to regression analysis by least squares method.

Accuracy

To ensure the accuracy and reliability of the method, recovery studies were carried out at three concentration levels of test concentration. Method of accuracy was tested through spiking experiments. THC with the following concentrations was used: 5, 20 and 40 μg/ml in methanol and CBD with the following concentrations was used: 6.25, 25 and 50 μg/ml in methanol. At each level, samples were prepared in triplicate and the recovery percentage was determined.

Precision

The precision of method was validated through intra-day and inter-day testing. The intra-day precision of the assay method was evaluated by carrying out six independent assays of THC and CBD test samples against qualified reference standard on same day and these studies were also repeated on six consecutive days to determine inter-day precision. The percentage of the relative standard deviations (%RSD) of six assay values was calculated.

Detection and quantitation limits

Limit of detection (LOD) (signal-to-noise ratio of 3) and limit of quantification (LOQ) (signal-to-noise ratio of 10) were followed equation: $LOD = 3(\sigma/S)$ and $LOQ = 10(\sigma/S)$. Determination of the signal-to-noise ratio was performed by comparing measured signal samples with known low concentration of analysis with those of blank samples establishing the minimum concentration at which the analysis can be reliably detected.

Application to oromucosal spray

Prepared cannabis spray stock solution in 10 ml volumetric flask by weighed accurately 17 mg of spray, dissolved in methanol and

adjusted volume to 10 ml. To determine the specificity of the analytical method, standard stock solution of THC and CBD, placebo solution, cannabis extract solution and cannabis spray solution dissolve in the same solution. Prepared other solutions by weighed each sample accurately 17 mg of placebo and 3.7 mg of extract, dissolved in methanol and adjusted volume to 10 ml. All solutions were sonicated for 15 min and filtered through 0.45 μm Nylon filter. Equal volumes (10 μl) of each sample were injected into the chromatograph by autosampler and peak areas were measured.

Results and discussion

There are number of analytical methods reported in recent pharmaceutics literature for the quantitative measurement of cannabinoids in cannabis. These include high performance liquid chromatography (HPLC). The chromatographic parameters were evaluated using a reverse-phase Zorbax C-18 (4.6 mm × 100 mm, 3.5 μm) column and a mobile phase composed of methanol and water (85:15, v/v) isocratic. The isocratic elution reverse-phase HPLC is the simplest method for the determination. The mobile phase has been investigated percentage of methanol (80, 85 and 90%, v/v), approximately pH 7.15. It was found that methanol 85% (v/v) provide a good separation and presented a sharp, symmetry and no tailing peak. Several HPLC methods have been described in the literature for pH of mobile phase. Although a good separation was achieved at approximately pH 5 (Backer et al., 2009; Patel et al., 2017), pH 7.15 was observed in baseline, retention time and peak characteristic (symmetry and tailing). This mobile phase was developed and proved to be a good linear relationship; R^2 more than 0.999 of CBD and THC. Therefore, pH was not influenced on HPLC chromatographic profile with this condition.

The conditions were performed to obtain a good peak of CBD and THC (Fig. 1A). The retention time of CBD and THC were shown the peak area at 3.033 min and 6.380 min, respectively and a short run time (10 min). UV spectrum in the range of 190–400 nm was evaluated for CBD and THC. The UV λ_{max} of CBD and THC were 210–220 nm, the wavelength of 220 nm was selected for detection due to the suitable molar absorptivity of CBD and THC in this part and the higher selectivity of this wavelength respecting possible interfering ingredients in the sample. This wavelength was kept the baseline noise minimum and achieved optimum system suitability parameters.

A column is the major component in HPLC. It contains the chromatographic packing material (stationary phase) effected the separation. The preliminary screening of a column studies were carried out in various columns including Zorbax C-18 column (4.6 mm × 100 mm, 3.5 μm), Poroshell C-18 column (3.0 mm × 150 mm, 2.7 μm) and ACE C-18 column (4.6 mm × 250 mm, 5 μm) column. The column studies were reversed phase, different in pore sizes, column lengths and particle sizes, which may present a variation in separation efficacy. It was found that Zorbax C-18 column (4.6 mm × 100 mm, 3.5 μm) provide a best separation and a short run time. The structure formula of THC and CBD were bi- and tri-cyclic compounds, contained twenty-one atoms of carbon, thirty of hydrogen and two of oxygen. THC and CBD have poor solubility in water, but good solubility in most organic solvents, particularly in lipids and alcohols. Both THC and CBD are present in cannabis in a mixture of acidic forms. The mobile phase mixture methanol and water as 85:15 was improved peak shape, run time and resolution.

To determine the specificity of the analytical method, standard stock solution of THC and CBD, placebo solution, cannabis extract solution and cannabis spray solution dissolve in the same solution. All solutions were filtered through 0.45 μm Nylon filter. Equal volumes (10 μl) of each sample were injected into the chromatograph by autosampler and peak areas were measured. The chromatogram of placebo, cannabis extract and cannabis spray was compared with the chromatogram of CBD and THC. As a result, the chromatogram of placebo was not shown the peak area at 3.033 min and 6.380 min, respectively (Fig. 1C). Cannabis extract solution and cannabis spray solution were presented peak at 5.180 min (Fig. 1B, D). These chromatogram was cannabinol (CBN), produced by oxidative degradation reaction of THC. CBN was commonly found degradation product in cannabis (Backer et al., 2009).

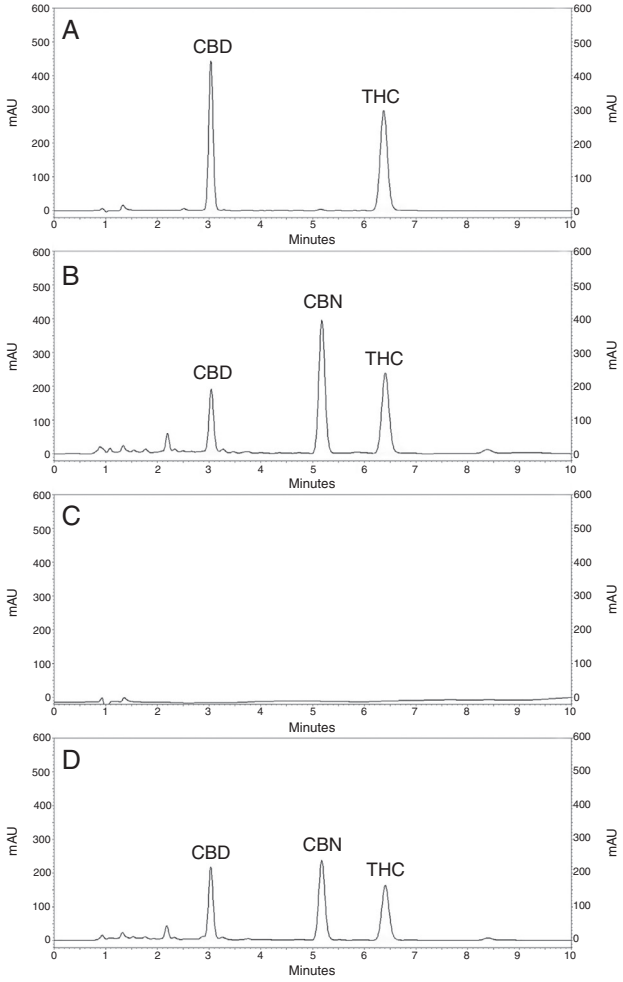

Fig. 1. The chromatogram of CBD and THC. (A) Standard solution; (B) cannabis extract solution; (C) placebo solution; and (D) cannabis spray solution.

Table 1
Calibration curve data for THC and CBD.

Regression parameters	THC	CBD
Regression coefficient (R^2)	0.9998	0.9995
Linear equation	$y = 81{,}316x + 10{,}573$	$y = 56{,}689x + 33{,}076$
Concentration range (μg/ml)	2.5–80	3.125–100
Number of points	6	6

Validation method of HPLC analysis

Linearity

The linearity was studied by preparing standard solutions at six different concentration levels. When the concentrations of THC, CBD and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship; $R^2 = 0.9998$ for THC and $R^2 = 0.9995$ for CBD was observed between

Table 2
Validation results of the analytical method.

Samples	Added standard (μg/ml)	Linearity (R^2)	Accuracy (%recovery)	Precision (%RSD)		LOD (μg/ml)	LOQ (μg/ml)
				Intra-day	Inter-day		
THC	5 20 40	0.9998	98.21 99.33 97.32	0.20	2.55	0.12	0.40
CBD	6.25 25 50	0.9995	95.56 97.06 96.89	0.30	3.28	0.23	0.76

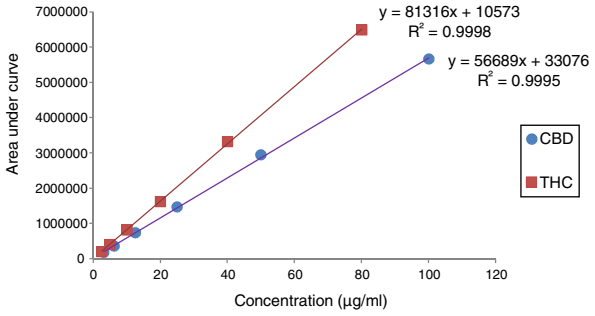

Fig. 2. Linearity of THC and CBD determined at 220 nm.

the concentrations of THC, CBD and the respective peak areas in the range 2.5–80 μg/ml and 3.125–100 μg/ml, respectively as presented in Fig. 2. The regression equation was found to be $y = 81{,}316x + 10{,}573$ and $y = 56{,}689x + 33{,}076$ (Table 1), where y is the peak area and x is the concentration of THC and CBD.

Accuracy

To ensure the accuracy and reliability of the method, recovery studies were carried out in the range 90–110% of test concentration. The accuracy of the quantitative analysis method was investigated by means of addition THC and CBD. The obtained %recoveries were found to be 98.3% and 96.5% for THC and CBD, respectively. The results obtained are presented in Table 2.

Precision

The intra-day precision of the assay method was evaluated by carrying out six independent assays of THC and CBD test samples against qualified reference standard on same day and these studies were also repeated on six consecutive days to determine inter-day precision. The percentage of relative standard deviation (RSD) of six assay values was calculated and found to be below 2% and 5% for intra-day and inter-day, respectively. The RSD values of peak areas were found to range from 0.20–2.55% and 0.30–3.28% for THC and CBD, respectively. The results obtained are presented in Table 2. Thus, it concluded that assure the precise of the HPLC method.

Detection and quantitation limits

Limit of detection (LOD) and limit of quantification (LOQ) were performed by comparing measured signal samples with known low concentration of analysis with those of blank samples establishing the minimum concentration at which the analysis can be reliably detected. According to the determined signal-to-noise ratio, THC and CBD presented limits of detection of 0.12, 0.23 μg/ml and limits of quantitation of 0.40, 0.76 μg/ml, respectively. The results obtained are presented in Table 2 and indicated that sensitivity of this HPLC method was suitable for the quantitative determination of THC and CBD in cannabis extract and oromucosal spray formulation.

Application to oromucosal spray

The chromatogram of cannabis spray solution was compared with chromatogram of standard, cannabis extract and placebo. As a result, the chromatogram of placebo was not shown the peak area at 3.033 min and 6.380 min, respectively as shown in Fig. 1C. Oromucosal spray from cannabis extract formulation was composed of THC and CBD as 1:1, the chromatogram was shown peak THC and CBD at 3.033 min and 6.407 min, respectively. The contents of THC and CBD from spray solution were 1:1 and related to amount of extract which added into the formulation.

Conclusions

The analytical RP-HPLC method was developed and validated carefully for determination an amount of THC and CBD. This method was proved to be linearity, accuracy, precision and detection and quantitation limits. The developed method was simple, fast, precise, accurate and responsive which could be employed for quantity analysis of formulation and stability product.

Author's contributions

WS was contributed in parts of oromucosal spray preparation and analysis HPLC. AS was contributed in parts of validate and evaluate. Both author were approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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