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VALIDATION AND COMPARISON OF METHOD USING DIFFERENT WAVELENGTH DETECTION AT 210 AND 228 NM FOR QUANTITATIVE ANALYSIS OF CBD IN SUBLINGUAL CANNABIS OIL PRODUCTS

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Abstract: Cannabidiol or CBD oil market is expected to grow 31.50% annually till 2030. Therefore, the method to accurately determine the quantity of CBD in oil products must be developed and validated. Various HPLC methods have been commonly developed with different ultraviolet (UV) wavelengths. In this research, we would like to compare new developed HPLC-UV method with isocratic elution using the wavelength of 210 nm, which is the maximal UV absorbance wavelength and 228 nm, which is recommended wavelength by previous work and Thai Pharmacopoeia to quantify CBD content based on ICH guidelines. Specificity, accuracy, precision, linearity, range, limit of detection (LOD) and limit of quantitation (LOQ) were evaluated. It was found that both methods exhibited a good linear relationship of CBD with R^2 equal to 0.9997. Per cent of RSD from both methods were less than 2%, and the mean %recovery was in the range of 99-100%. LOD and LOQ for the 210 nm method were 5.93 and 17.96 µg/ml, respectively and for the 228 nm, LOD and LOQ were 5.77 and 17.49 µg/ml. It can be concluded that both methods were suitable for the analysis of CBD content in oil formulations, and no significant difference ($p>0.05$) between both methods was found. Additionally, these methods were used to analyse CBD content from sublingual oil formulations which contain 50 and 100 mg/g of CBD. Percent labelled amount of CBD in CBD sublingual oil has complied with the criteria of Thai Pharmacopoeia. Our developed method was verified to be accurate and simple without the tedious procedure of sample preparation.

Keywords: CBD, HPLC, validation

INTRODUCTION

Cannabidiol or CBD exhibits many pharmacological activities such as pain relief (Hammell et al., 2016), anti-anxiety (Blessing et al., 2015), anti-inflammatory (Burstein, 2015; Sangiovanni et al., 2019), anti-epilepsy (Silvestro et al., 2019), anti-acne (Oláh et al., 2014) without psychoactive effect. Therefore, it is gaining much attention in pharmaceutical, wellness and cosmeceutical markets (Jhawar et al., 2019). Many CBD products are now

available and legal in some countries. Additionally, an oral solution of CBD "Epidiolex®" manufactured by GW Pharmaceuticals has been approved by the U.S. FDA for epilepsy treatment. CBD oil market are expected to growth 31.50% annually till 2031 (*CBD Oil Market*, 2021). Therefore, the method to accurately determine the quantity of CBD in products must be developed and validated. Many analysis methods to quantify CBD have been published, including the liquid and gas chromatography (GC) methods (Pourseyed Lazarjani et al., 2020). The high-pressure liquid chromatography (HPLC) method does not require heat; therefore, the conversion of compounds by heat can be avoided. Conversion of acid from delta-9 tetrahydrocannabinolic acid A to tetrahydrocannabinol in the GC injector was found (Dussy et al., 2005). Various HPLC methods have been commonly developed with gradient methods, and various ultraviolet (UV) wavelength detection has been utilized (Micalizzi et al., 2021). From the monograph of Thai Pharmacopoeia for cannabis sublingual drops, which are "the cannabis extract in a suitable vegetable oil such as sesame oil, olive oil or coconut oil", a gradient method with multiple steps of sample preparation was required. Briefly, samples will be dissolved in a mixture of methanol and chloroform, then sonicated for 30 min and frozen for 90 min. After freezing, the sample has to be filtered through a solid-phase extraction kit. Obtaining clear solution will be further evaporated until dried, redissolved, and filtered with 0.45 um porous membrane before analyzing by the HPLC gradient method (TP supplement, 2020). Therefore, we preferred to develop the HPLC analysis with a simple method for sample preparation and using the isocratic elution method because the isocratic method ensures that the system's simple and constant polarity can be maintained.

For the detection of CBD, a wavelength of 228 nm was recommended for cannabis sublingual drops analysis (TP supplement, 2020) and routine analysis of CBD and impurities (Micalizzi et al., 2021). However, the maximal UV absorbance of CBD is 209.09 (Ryu et al., 2021). Typically, the maximal UV absorbance should be selected for an assay. However, CBD has the maximal UV absorbance close to the UV cut-off of mobile phases such as methanol (205 nm) or acetonitrile (190 nm). Therefore, CBD's optimal wavelength can differ from the maximal wavelength to avoid interference from the mobile phase (Ryu et al., 2021). Therefore, the question of this study is whether the detection of CBD at the wavelength of 228, which is the recommended wavelength and 210 nm, which is the maximal wavelength, is significantly different. However, there is no research to compare analysis with these two wavelengths of detection. Additionally, in this research, we would like to validate our new HPLC-UV method using isocratic elution with a simple sample preparation based on ICH guidelines Q2R1 (2005) to quantify CBD content in oil formulations which can also be used for stability tests. Furthermore, CBD content was analysed with validated methods to evaluate the quality of CBD sublingual oil.

MATERIALS AND METHODS

Standard cannabidiol (99.8%) 1 mg/ml in 1 ml of methanol was purchased from Sigma Aldrich. Methanol and water HPLC grade were purchased from RCI LabScan Limited.

Standard and sample preparation

Standard CBD solutions were diluted with methanol to the desired concentration (approximately 100 μg/ml). For sample preparation, CBD sublingual oils were weighed with a four-digit balance, then dissolved and adjusted the volume by methanol to 5 ml before analyzing with the validated HPLC method.

HPLC condition

HPLC system equipped with a quaternary pump, temperature control for column and UV DAD (Agilent 1260 Infinity II) was used for analysis. A chromatographic column was the C-18, 5 um particle size, 4.6 mm ID x150 mm length column (Shimadzu shim-pack GIST). The mobile phase was methanol and water in the ratio of 82.5:17.5. Flow rate was 1 ml/min. The sample injection volume was 10 µl. The temperature of the column was controlled at 30 °C. The detection wavelengths were 210 and 228 nm. The run time was 15 min.

Specificity

HPLC chromatograms of standard CBD in methanol solution, CBD in sublingual oil, and oil base were compared between the 2 methods. Additionally, the UV spectrum from 190-400 nm of CBD peak from CBD in methanol solution and CBD in sublingual oil were compared.

Linearity

Various concentrations of standard CBD solution were prepared by diluting CBD standard (1 mg/ml) with methanol to obtain the final concentration of 31.25, 62.5, 95, 125, and 250 µg/ml. Calibration curves were generated by plotting the area under the curve (AUC) of CBD peak against CBD concentration. The correlation coefficient (R^2) and linear regression equation were obtained from the calibration curve. Quantification of CBD content was calculated from a linear regression equation.

Accuracy

The amount of CBD was spiked to the oil base composing of all excipients as CBD sublingual oil formulations but without CBD to produce CBD oil at the concentration of 50, 100 and 150 µg/ml. Then, the exact amount of CBD oil was weighed with a 4-digit analytical balance, diluted, and the volume was adjusted to 5 ml with methanol by volumetric flasks. The sample was then filtered with a 0.45 µm nylon filter before HPLC analysis. Each concentration was independently prepared in triplicate. % Recovery were calculated by this equation:

$$\% \text{ Recovery} = [\text{Calculated amount}/\text{Actual amount}]x100$$

Precision

Three concentrations of standard CBD solution (62.5, 125 and 250 µg/ml) was prepared in triplicate. For intra-day and inter-day precision, the analysis were performed on the same day and on 3 different days, respectively. % RSD were calculate by this equation:

$$\% \text{ RDS} = [\text{SD}/\text{Mean}]x100$$

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated from calibration curve data by these equations:

$$\text{LOD} = 3.3\text{SD}/\text{slope}$$

$$\text{LOQ} = 10\text{SD}/\text{slope}$$

SD is standard deviation and slope is the slope of the calibration curve.

CBD sublingual oil analysis

CBD sublingual oil formulations composed of CBD, medium chain triglyceride (MCT) oil, vitamin E acetate, menthol terpene aroma and benzyl alcohol were prepared. The base oil was prepared the same as CBD sublingual oil, except no CBD was added. Three sample preparations of two CBD sublingual oil formulations with the concentration of CBD at 50 and 100 mg/g were performed independently. The amount of CBD was calculated by

linear regression equation in triplicate. Percent labelled amount of formulations were calculated by this equation:

[%Labelled amount = Amount of CBDx100/Labelled amount]

Statistical analysis

Data were collected from triplicate independent measurements. T-test at a significance level of 0.05 was performed to statically analyze data.

RESULTS AND DISCUSSION

Currently, many methods of HPLC analysis of CBD have been developed with gradient and isocratic elution methods. An isocratic method is simpler to perform, and the constant polarity can be maintained. Therefore, in this research, the objective is aimed to develop the method of HPLC analysis with isocratic elution conditions. The condition of HPLC analysis was modified from the previous study (Saingam and Sakunpak, 2018) by slightly changing the mobile phase ratio and the detection wavelength. In a previous study, the wavelength at 220 nm was selected for CBD and THC analysis. In this research, we would like to compare the detection of CBD at 210 nm providing the maximal UV absorbance to 280 nm, which is the wavelength that Thai Pharmacopoeia recommend for cannabis sublingual drops.

Specificity

HPLC chromatograms of standard CBD in methanol, oil formulations and oil base were compared for their homogeneity and to confirm no interference peak from the oil base (Figure 1). The peak of standard CBD and CBD in sublingual oil formulation had a similar retention time of about 6.68 min, while the oil base did not have a peak that could interfere CBD peak. Additionally, UV spectra of CBD peak from standard CBD in methanol and sublingual oil formulation were similar, which confirms that the peak found in sample chromatograms was CBD (Figure 1A-B).

Linearity

The linear regression equation obtained by plotting AUC against the concentration of CBD standard was $y = 73.158x+243.04$ ($R^2 = 0.9997$) for 210 nm method and $21.919x+58.233$ for 228 nm method ($R^2 = 0.9997$). Both methods exhibited R^2 over 0.999, showing an excellent correlation between measured AUC and CBD concentration (Figure 2).

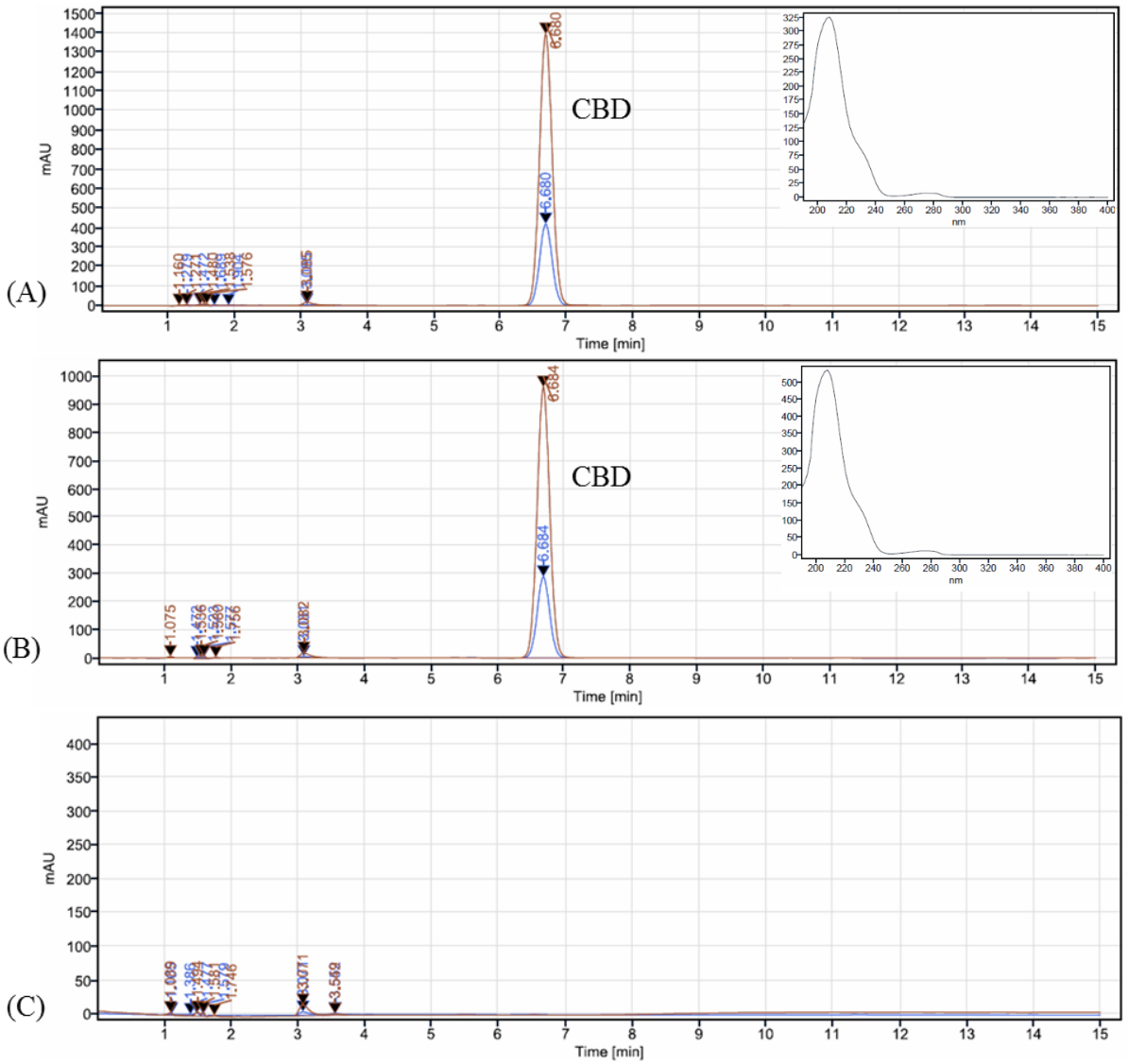
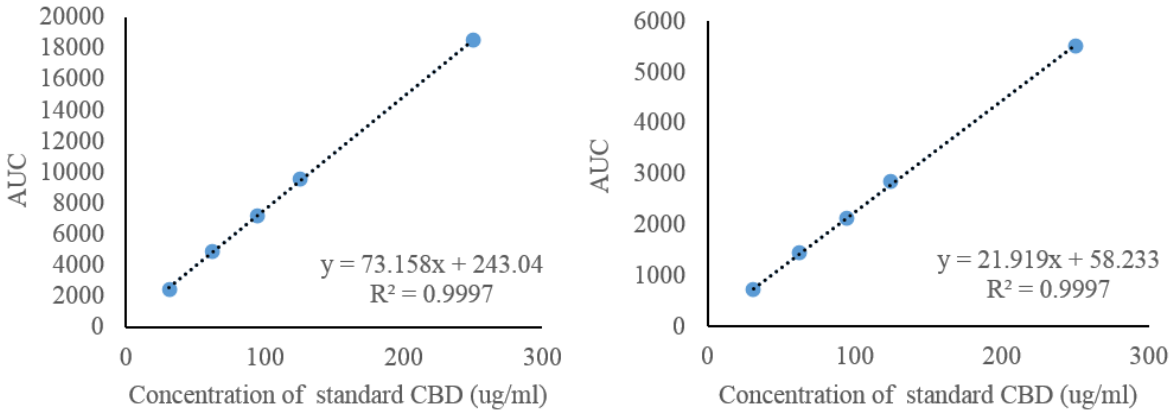


Figure 1. HPLC chromatograms and spectrum of CBD peak of standard CBD in methanol (A), CBD oil formulation (B) and oil base (C) at the wavelength of 210 nm (red) and 228 nm (blue).



(A) (B)

Figure 2. Calibration curves of standard CBD detected with 210 nm (A) and 228 nm (B)

Precision

The precision results of both methods were shown in Table 1. The value of %RSD of intra-day precision was found in the range of 0.8-1.3 % for the 210 nm method and 0.7-1.4% for the 228 nm method. For inter-day precision, the %RSD value was 0.6-1.9 % for the 210 nm and 228 nm method. % RSD values of both methods were not more than 2% limit, indicating good precision. The statistical calculation of %RSD value of two methods by the independent samples *t* test showed no significant difference between the % RSD of both methods ($p>0.05$).

Table 1. Precision of HPLC methods with 210 and 228 nm detection

Wavelength of detection (nm)	Concentration (µg/ml)	AUC (mAU)			Mean	SD	% RSD
		1	2	3			
210	Intra-day Precision						
	250	18903	18463	18785	18717	227.71	1.22
	125	9711	9567	9609	9629	74.03	0.77
	62.5	4984	4911	4856	4917	64.37	1.31
	Inter-day Precision						
	250	18903	19482	18824	19070	359.06	1.88
	125	9711	9765	9821	9766	55.12	0.56
	62.5	4984	4815	4902	4900	84.81	1.73
228	Intra-day Precision						
	250	5669	5521	5630	5607	76.78	1.37
	125	2886	2851	2856	2864	19.04	0.66
	62.5	1483	1456	1441	1460	21.12	1.45
	Inter-day Precision						
	250	5669	5830	5627	5709	107.05	1.88
	125	2886	2909	2922	2906	18.26	0.63
	62.5	1483	1432	1455	1457	25.66	1.76

Accuracy

Accuracy of both methods were performed by spiking CBD into a blank oil base. The mean per cent recovery was in the range of 98-102%, with % RSD less than 2% from both methods (Table 2), indicating that these methods are acceptable as the results comply with the AOAC guideline as AOAC, 2019, guideline recommend the acceptable range of mean recovery of 0.01% analyte is 90-107%.

Table 2. Accuracy of HPLC methods with 210 and 228 nm detection

Wavelength of detection (nm)	Concentration (µg/ml)	% Recovery			Mean	SD	% RSD
		1	2	3			
210	50	98.42	98.47	100.96	99.28	1.45	1.46
	100	100.65	98.71	99.12	99.50	1.02	1.03
	150	100.44	98.75	100.05	99.74	0.89	0.89
228	50	98.43	99.27	101.48	99.73	1.58	1.58
	100	100.25	98.25	98.47	98.99	1.10	1.11
	150	100.86	98.83	100.25	99.98	1.04	1.04

LOD and LOQ

LOD and LOQ for the 210 nm method were 5.93 and 17.96 µg/ml, respectively and for the 228 nm, LOD and LOQ were 5.77 and 17.49 µg/ml.

Results from all experiments can be concluded that both methods were suitable for CBD content determination from oil formulations. Although the retention time of CBD from our new method was longer, the %RSD of precision and %recovery of CBD were improved when compared with the previous method (Saingam and Sakunpak, 2018).

CBD sublingual formulations Analysis

CBD sublingual formulations containing the same excipients as in oil bases with CBD at 50 and 100 mg/g were determined for their % labelled amount of CBD (Table 3). It was found that both formulations complied with the criteria of Thai Pharmacopoeia (TP), as cannabis sublingual drops must contain not less than 90.0 and not more than 110% of the labelled amount of CBD (TP supplement, 2020).

Table 3. Percent labelled amount of CBD from CBD sublingual formulations

Wavelength of detection (nm)	Concentration (mg/ml)	% Labelled amount			Mean	SD	% RSD
		1	2	3			
210	50	98.45	96.33	97.79	97.53	1.09	1.11
	100	100.58	101.92	98.58	100.36	1.02	1.02
228	50	98.66	96.58	97.97	97.74	1.58	1.61
	100	100.65	102.14	98.57	100.46	1.10	1.10

CONCLUSION

The new HPLC method to determine CBD content in oil formulations with the 210 and the 228 nm detection were developed and validated. Both methods were accurate analysis methods and suitable for analysis of CBD content in oil formulations. The percent labelled amount of CBD in CBD sublingual formulation has complied with the criteria of Thai Pharmacopoeia.

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