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Box-Behnken Design Assisted Development and Optimization of RP-HPLC-PDA Technique for Determination of Cannabidiol in the Bulk and Nanoformulation

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Abstract

The focus of this research was to implement a new RP-HPLC-PDA method for determining cannabidiol (CBD), which has been established with the help of Quality by Design (QbD). Design expert (version 12) software was used to conduct the factor screening studies. The mobile phase ratio, flow rate, and temperature conditions were used as independent variables, while retention time, peak area, and peak tailing were used as dependent variables in a Box-Behnken design. The best separation was obtained with acetonitrile and water as mobile phase in the ratio of 45:55 (%; v/v), a flow rate of 1.0 mL/min, and an oven temperature of 30 °C with PDA detection at 210 nm. The newly optimized method showed the concentration linearity range in µg/mL (1187.574 - 5997.835) of CBD with an excellent correlation coefficient R^2 = 0.9951. Whereas, the % recovery of the CBD was obtained between 97.84 to 99.34%, and the %RSD was not more than 2%, thus clearly confirming the high level of accuracy in the optimized form. The technique was assessed under the ICH guidelines, which revealed excellent linearity, precision, and robustness. Consequently, the approach was used to determine the retention time in CBD nanoemulsion formulations, which revealed no substantial change in retention time.

Keywords: Cannabidiol, RP-HPLC, ICH guidelines, Box–Behnken design, Quality by Desig.

1. Introduction

Cannabis sativa L. is a long-lived plant that contains over 100 different terpenophenolic compounds [1]. The most-studied compound among the phytocannabinoids is D9-tetrahydrocannabinol (D9–THC), a psychoactive compound, and other valid therapeutic compounds that are cannabidiol (CBD), a non-psychotropic phytocannabinoids [2]. CBD is among the most abundant non-psychoactive phytocannabinoids found in *Cannabis sativa L.* seeds, stalks, and flowers [3, 4]. The IUPAC name of CBD **(Fig. 1)** is "2 -[(1R,6R) -3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl] -5-pentylbenzene -1,3-diol".

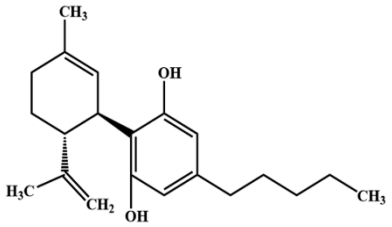

Fig. 1: Chemical structure of Cannabidiol

As per the "Biopharmaceutical Classification System (BCS)" it is categorized as a BCS class II drug candidate due to low solubility (logP 6.33) and high permeability characteristics [5]. CBD is an orally active, potent antipsychotic, antiepileptic, and neuroprotective compound. CBD is a strong antagonist of the GPR55 receptor, an opposite agonist of the GPR3, 6, and 12 receptors, and a slight agonist of the 5-HT1A receptors. Furthermore, CBD also has a low selectivity for CB1 and CB2 receptors from a potential therapeutic perspective. Numerous studies have shown that the active ingredient is an antagonist on both the CB1 and CB2 receptors [6–8]. An open-label study of six Parkinson's disease patients with psychotic symptoms found that CBD administration (150–400 mg for four weeks) was accompanied by significant improvements in psychotic symptoms and large input and has been well considered acceptable [9]. Preclinical studies have revealed that CBD leads to significant autonomous arousal declines, a conditioned manifestation of fear and long-term stress-related anxiety, and a significant improvement of the blockade

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of extinction of fear [10–13]. CBD also exhibits a sedative-hypnotic effect in rodent models after oral administration [14–18]. In animal models and human experiments, fortunately, there has only been limited data for its beneficial effects [19–23]. CBD was expected to lower seizure frequency in patients with DS and other treatment-resistant epilepsies in recent human research [22,24]. CBD is marked as liquid preparation in the brand name of "Epidiolex" (GW Pharmaceuticals, Cambridge, UK) as adjuvant therapy for Dravet syndrome, Lennox-Gastaut syndrome, and extreme myoclonic epilepsy in childhood [25,26].

The most popular method used for quantitative assay of CBD in compounds is RP-HPLC/PDA technique. RP-HPLC/PDA technique is a precise, effective, and sustainable method widely used for drug discovery. RP-HPLC/PDA method validation according to ICH guidelines is not very reliable in terms of reducing method variability beyond conventional sturdy testing. Therefore, consistency by Quality by Design (QbD) standards for the production of analytical methods is now commonly used to obtain optimal effectiveness and improved process efficiency. A QbD facilitates methodical and risk-based understanding of the source of variability accompanied by critical manufacturing parameters identification risk analysis, and evaluation of experiments to identify high-risk variables with significantly important relative impacts, followed by customization using a performance-enhancing method based on appropriate experimental designs [27]. According to different scientific reports, numerous analytical techniques for optimizing chromatographic conditions in RP-HPLC/PDA methods with using Box–Behnken design have indeed been revealed [28–33].

Therefore, this study aimed to develop and optimize a new, simple, robust, and reproducible RP-HPLC technique by the Box-Behnken design for the quantification of CBD in pure form and its application to CBD nanoemulsion.

2. Materials and Methods

CBD was procured from Sigma-Aldrich, USA. HPLC-grade acetonitrile, water, methanol, and polysorbate 80 (Tween 80) were obtained from S.D Fine Chemicals, Ltd. (Mumbai, Maharashtra, India). Transcutol P and capryol 90 were obtained as a gift sample from Gattefosse India Pvt Ltd (Mumbai, Maharashtra, India). All other chemicals and reagents were used in the study were of analytical reagent (AR) grade.

2.1. Instrumentation

The LC-20 AD binary pump, rehodyne-injector with 20 µl capability per injection, CTO-10AS VP oven column compartment, and a photodiode array (SPD-M20A) sensor were the complement of RP-HPLC-PDA (Shimadzu Corporation, Kyoto, Japan). The LC solution program version 1.25 managed the HPLC system. With the assistance of the analytical balance SHIMADZU AUX-120, all weighing operations for the required study were performed. CBD was separated using a Princeton SPHERE Ultima (250 mm × 4.6 mm × 5 µm) C-18 column. The temperature of the column was held at 30 °C. Acetonitrile: water in 45:55 ratio was used as the binary mobile phase. Retained the solvent system flow rate at 1 mL/min. The average time of testing was hardly 5 minutes with a wavelength of 210 nm photodiode array detector (PDA).

2.2. Statistical engine

Design Expert® software, version-12, Stat-Ease Inc., Minneapolis, USA, was used to statistically optimize the RP-HPLC-PDA technique. Microsoft excel 2013 was used to conduct the continuing calculations for the analysis (Microsoft, USA).

2.3. Chromatographic Conditions

The chromatographic condition used during our study is summarized in **Table 1.**

Table 1. Chromatographic conditions for the development of the RP-HPLC-PDA technique for CBD.

HPLC system	RP-HPLC-PDA/LC-20 AD (Shimadzu Corporation, JA)
Detector	(SPD-M20A) a photodiode array
Column	Princeton SPHERE Ultima C18 column
Dimensions	(250 mm × 4.6 mm × 5 µm)
Mobile phase	acetonitrile 45% (v/v), water 55% (v/v)
Mode	Isocratic
Flow rate	1.0 ml min^{-1}
Temperature	Ambient temperature
Detection wavelength	210 nm
Injection volume	20 µl

2.4. Preparation of CBD standard stock solution

The CBD standard stock solution of 100 µg/mL was formulated. Various dilutions of the standard solutions were prepared using sequential concentrations of the stock solution diluted with mobile phase combination to achieve the required concentrations. The dilutions were filtered via membrane filter of 0.22-µm size, for obtaining the concentrations of 1187.574, 1839.212, 2868.117, 4079.157, 5028.104, and 5997.835 µg/mL for the standard curve of CBD. The calibration curve was prepared by plotting the ratio of the peak area of the drug to internal Standard (I.S) and concentration. To measure drug concentrations, the peak area ratio was extrapolated to the calibration data via inclines and the intercepts.

2.5. Optimization of RP-HPLC-PDA method by Box-Behnken design

Firstly, the design expert helps in the optimization of the RP-HPLC-PDA method by selecting the accurate value from each of the factors. In addition, the trial-and-error technique was used to learn more knowledge about the performance of the RP-HPLC-PDA method and to check the impacts of the independent variables on the dependent variables.

Table 3. ANOVA results for the entire Box-Benkhen responses

Responses	±SD	Mean	%C.V.	Press	R^2	Adjusted R^2	Predicted R^2	Adequate precision	P-value
Retention time	0.0057	2.06	0.2756	0.0025	0.9993	0.9981	0.9892	74.5241	0.0430
Peak area	305.56	65539.60	0.4662	7.462E+06	0.9985	0.9957	0.9757	51.6972	0.0330
Tailing factor	0.0021	1.24	0.1772	0.0003	0.9999	0.9998	0.9991	243.1330	0.2893

SD: Standard deviation; C.V. : Coefficient of variation; R^2: Coefficient of correlation; Press: Predicted residual sum of squares

The foremost significance of the RP-HPLC-PDA method was mainly to check the purity of CBD compound, perform the RP-HPLC-PDA method, and recommend that the resolution was greater than 2.0. In addition, the Box Behnken configuration with a surface response was used to improve the method's experimental conditions. In this research, experiments according to Box–Behnken design were designed and carried out. As per **Table 2,** three stages of independent variables were used in this investigation, resulting in 15 experimental cycles. The stated value for each of the three variables: acetonitrile composition in the mobile phase (A) flow rate of sample (B), oven temperature of the HPLC © were 45%, 1 mL/min, and 30°C, respectively. In order to refine the compositional considerations and to determine the quadratic special effects of acetonitrile volume in the mobile phase, oven temperature, and flow rate, a statistical screening design of Box–Behnken was used.

ANOVA produced a linear polynomial equation, which is shown below.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2$$

β_0 is the symbol of the polynomial equation intercept which represents the average arithmetic mean, $\beta_1 - \beta_{33}$ are the regression coefficients computed from the observed experimental values of Y (response), and A, B, and C are the coded level of the independent variables. Where 'Y' is the mean measured response (dependent variable). While A represents the acetonitrile composition in the mobile phase, B denotes flow rate and C is the oven temperature respectively. The considered responses were retention time (Y_1), peak area (Y_2), and tailing factor (Y_3). In this situation, acetonitrile composition in mobile phase volume was selected as 40-50 %, the flow rate was also raised from 0.8 to 1.2 mL/min, while the
oven temperature was appropriately set between 25 and 35°C.

2.6. Process validation method

The proposed procedure was validated for a number of the parameters proposed by the International Conference on Harmonization (ICH) 2005, such as device suitability, precision, limits of detection and quantification, robustness, and specificity.

2.6.1. System suitability

The standard solution (20 µg/mL) was injected into the column to analyze system appropriateness. System suitability was observed with respect to low retention time (Y_1) and tailing factor (Y_2), and high peak area (Y_3) were analyzed.

2.6.2. Linearity study

As per ICH guidelines, the linearity analysis of the proposed method was assessed. Linearity range within 10–60 µg/mL concentration was observed for the CBD samples and the calibration curve was obtained by plotting the peak area vs. concentration.

2.6.3. Accuracy

The proposed method's accuracy was evaluated using the standard addition method, which included some of the amount of the standard added in three different levels 80, 100, and 120 % to the standard concentration of CBD and checking the results for all three sets. The % recovery and the % relative standard deviation (%RSD) techniques for CBD were carried out by spiking three different levels of (80, 100, 120) to the standard stock of the CBD. The % recovery and %RSD were estimated for each level.

2.6.4. Precision

The data analysis for the precision was measured by the intra-day and inter-day sample analysis. Low, medium, and high dilutions, i.e., 10, 20, and 30 µg/mL of CBD were tested in triplicate for repeatability. The experiment was analyzed for three consecutive days for inter-day precision. The percent value of the RSD was computed for all the concentrations prepared within a day and for three days.

2.6.5. Limit of Detection (LOD) and limit of quantification (LOQ)

The standard deviation method was used for the determination of LOD and LOQ. Equations (1) and (2) were used to determine the LOD and LOQ. With the assistance of the slope (σ) that presents the linearity plot and the standard deviation (s) of the blank sample, LOD and LOQ were calculated.

$$LOD=3.3\times\sigma/S \quad \text{.....(1)}$$

$$LOQ=10\times\sigma/S \quad \text{.....(2)}$$

Chromatogram of nanoemulsion formulation was compared with the blank to reveal obstruction of formulation excipients.

2.6.7. Robustness

The slight intentional changes in the acetonitrile volume (±2 %v/v), the flow rate of the sample (±1%), and the temperature of the column's oven (±2 °C) were analyzed to study the effect on the responses. In the experiment, a control standard of CBD that contained 10 µg/mL was used and the average percent recovery, as well as percent RSD, were calculated.

2.7. Application of the analytical method in pharmaceutical formulation

The marketed formulation of the CBD is not available in the Indian market. Therefore, the nanoemulsion formulation was prepared. The developed analytical procedure for CBD determination was applied to estimate the CBD in nanoemulsion formulation. An optimized nanoemulsion formulation was prepared by employing Transcutol P, Capryol-90, and Polysorbate 80 (Tween 80) in the quantified amount for dissolving 3 mg of CBD. For estimating CBD, its nanoemulsion (~1 mg of CBD) was dissolved in 1 mL of methanol with the help of sonication. The compound was extracted in methanol to achieve a concentration of 100 µg/mL after sufficient dilution. The solution was filtered and injected into the column for the quantification of CBD.

3. Results and discussion

3.1. Development and optimization of RP-HPLC-PDA technique

Fifteen experimental compositions were generated by the Box–Behnken design to gain more knowledge about the responses like retention time, peak areas, and tailing factors **(Table2).** The computer-generated plots were used to generate statistical models of dependent and independent variables. The significant terms of the selected model on the responses were identified by performing ANOVA analysis **(Table 3).** Based on the value of the press, a quadratic model was chosen. The model terms were considered statistically significant when the p-value was <0.05. The low coefficient of variation (CV) and coefficient of correlation (R^2) was indicative of a strong relationship between the investigational data and that of the equipped model.

Adequate precision between the adjusted R^2 value and the predicted R^2 value revealed the appropriateness of the method. The polynomial equations generated by the design expert software for the actual components and factors that were used for predicting how each factor responded to certain changes are shown below:

Retention time (Y_1) = $+2.09267 - 0.001250A - 0.168000B - 0.002750C - 0.001000AB - 0.00050AC + 0.005000BC - 0.001583A^2 - 0.046583B^2 - 0.006583C^2$ .....(3)

Peak area (Y_2) = $+64993.66667 + 64.50000A - 6165.50000B - 18.75000C - 210.25000AB - 37.75000AC + 148.25000BC + 249.54167A^2 + 687.04167B^2 + 87.04167C^2$ .....(4)

Tailing factor (Y_3) = $+1.23433 - 0.000875A + 0.209500B + 0.000125C - 0.003000AB + 0.000250AC - 0.000500BC + 0.000458A^2 + 0.009208B^2 - 0.000042C^2$ .....(5)

Generally, a positive value is used to denote an effect that favors optimization; however, in some cases, a negative effect could occur as a result of inverse relationships between the factors and responses. The p-level significance of factors and their interactions is shown in **Table 3,** indicating that the p-shading value was less than 0.05, indicating adequate and potential outcomes from interactions of method variables. A 3D response surface plot demonstrated the effect of acetonitrile volume in a mobile phase, flow rate, and oven temperature of the HPLC system on responses: retention time, peak area, and tailing factor. **Fig. 2** to **4** illustrates the 2D contour plots and 3D response surface plots of each of the independent variables (factors) and represents the impact of the factors on each response. **Fig. 2** and polynomial equation (3) represents the influences of the independent variables on the retention time (Y_1) and it was revealed that the independent variables like acetonitrile composition (A) in the mobile phase, flow rate (B), and oven temperature (C) revealed a decrease in the retention time value. The optimum retention time value was recorded at lower acetonitrile composition in the mobile phase, flow rate, and oven temperature.

Fig. 3 and polynomial equation (4) represents the influences of the independent variables on the peak area (Y_2) and it was revealed that the acetonitrile composition (A) in the mobile phase represents a positive influence on the peak area, while the flow rate (B) and oven temperature (C) represents negative influences on the peak area. The maximal peak area was observed with a lower flow rate and oven temperature and higher acetonitrile composition in the mobile phase.

Fig. 4 and polynomial equation (5) represents the influences of the independent variables on the tailing

factor (Y_3) and it was revealed that the independent variables like acetonitrile composition (A) in the mobile phase represents a negative impact on the tailing factor whereas, the flow rate (B) and oven temperature (C) are revealing a positive influence on the tailing factor.

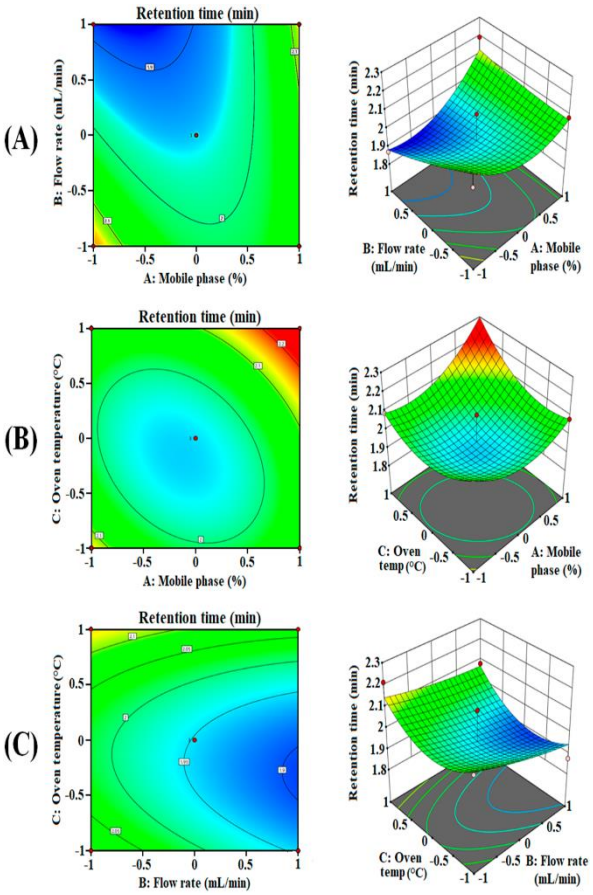

Fig. 2: Effect of variables on the retention time of the drug depicted through 2D contour and 3D RSM plots.

The optimum tailing factor value occurred at a lower acetonitrile composition in the mobile phase, higher flow rate and oven temperature. It was noticeably demonstrated that the empirical value of the chromatographic requirement was almost close to the expected value. As a result, the highlighted chromatographic conditions, acetonitrile volume (45%), flow rate (1 mL/min), and oven temperature (30 °C) were the optimized variables for the CBD standard chromatogram.

3.2. Optimized chromatographic solution

The optimal solution for chromatography was found through numerical optimization by customizing various independent variables to meet their desired goals, i.e. maximizing the peak area and minimizing the retention time and tailing factor to obtain the desirability that will be close to 1.

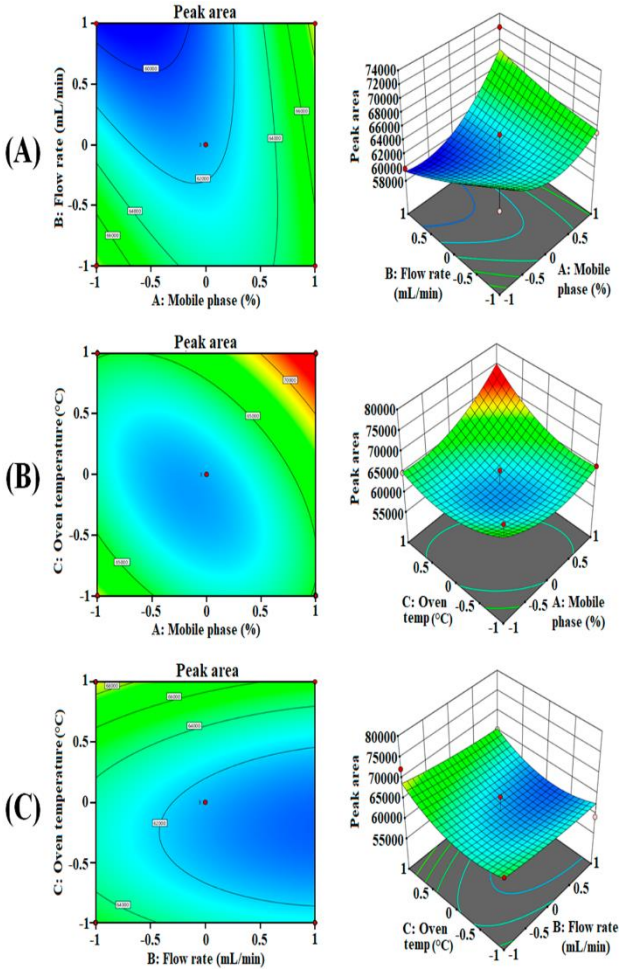

Fig. 3: Effect of variables on the peak area of the drug depicted through 2D contour and 3D RSM plots.

In addition, the optimized solution of the RP-HPLC showed that the acetonitrile composition in the mobile phase in the range of 45-55% mixture of the acetonitrile and water, the flow rate of 1 mL/min, and oven temperature of 30 ℃led to desirability that is close to 1 associated with all the independent variables in the desired ranges.

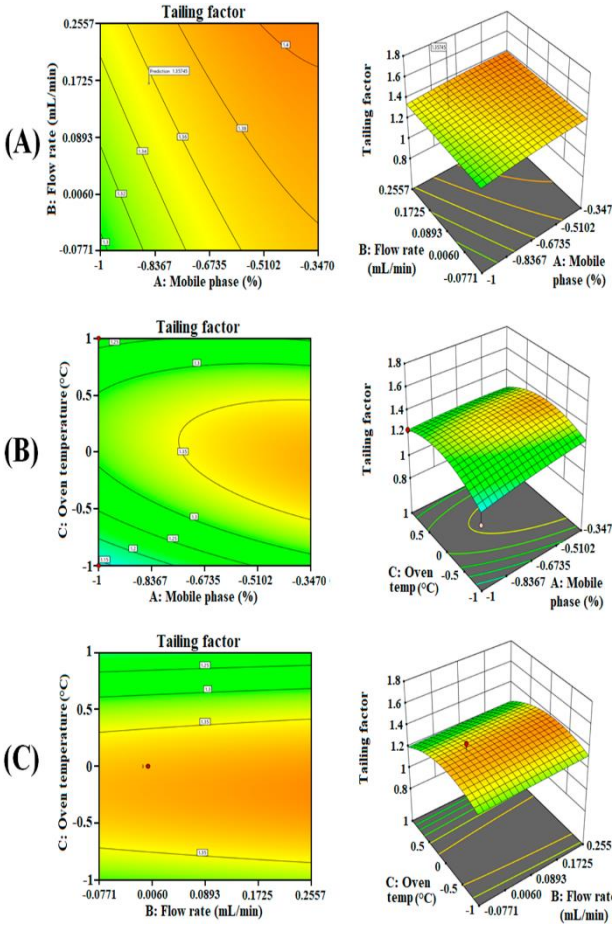

Fig. 4: Effect of variables on the tailing factor of the drug depicted through 2D contour and 3D RSM plots.

3.3. Validation of the proposed method

The proposed method was validated as per the ICH guidelines to study the integrity of the system and its suitability to assess linearity, precision, accuracy, and robustness.

3.3.1. System suitability

The results of system suitability are represented in **Table 4.** System suitability experiments show an insignificant change in the dependent variables like peak area, retention time, and tailing factor. A study of six replicates of 20 µg/mL of the CBD standard was adequately made to understand the effect on retention time, peak area, and tailing factor. Percent RSD was found to be less than 2% to show the adequacy of strategic development.

Table 4. . Results of system suitability analysis for the developed HPLC-PDA method.

Runs	System suitability parameters		
	Retention time (min)	Peak area	Tailing factor
1	2.091	69485	1.233
2	2.092	64988	1.237
3	2.093	64986	1.234
4	2.092	64984	1.235
5	2.095	64986	1.233
6	2.091	64987	1.232
Mean	2.0923	64986	1.234
SD	0.0007	1.2909	0.0016
%RSD	0.0343	0.0019	0.1323

3.3.2. Linearity

Linearity study demonstrates that the developed method can analyze the drug solution in a specific concentration range. The standard plot of CBD **(Fig. 5)** between peak area vs drug concentration was found to be linear. The linear regression data for the standard plot revealed a linear association with peak area. The linear regression equation was found to be y = 34252x + 48913 with a regression coefficient (R^2) of 0.999.

3.3.3. Accuracy

The accuracy of the developed RP-HPLC-PDA technique is the closeness of the test results obtained by that method to the actual value for the drug sample. Accuracy is expressed as %recovery and in this study, the recovery of CBD was observed between 97.84% to 99.34%, and the %RSD was not more than 2%.

Therefore, the obtained result confirms the high level of accuracy of the developed RP-HPLC technique. The results of this study are summarized in **Table 5.**

3.3.4. Precision

To determine the precision of the proposed method, three distinct CBD concentrations were prepared at distinct times on day one and then on three consecutive days (intra-day and inter-day precision) in accordance with ICH guidelines. The results of inter-day and intra-day precision are represented in **Table 6.**

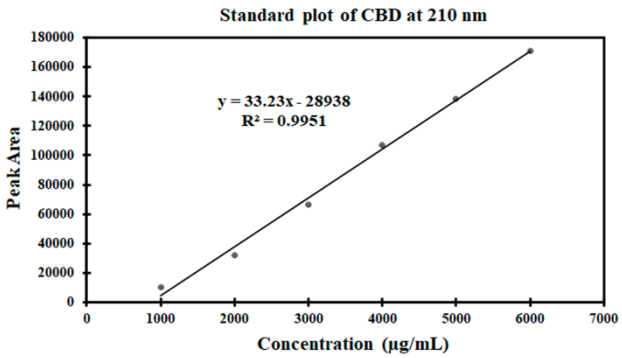

Fig. 5: Standard plot of Cannabidiol at 210nm

Table 5. Accuracy data for the developed RP-HPLC method of CBD

Standard concentration	Level (%)	Concentration (µg/ml)	Amount recovered (µg/ml +SD)	Recovery (%)	RSD (%)
10 µg/mL	LQC 80	16	15.67 ± 0.25	99.34	1.12
	MQC 100	18	17.18 ± 0.16	97.84	0.57
	HQC 120	20	19.27 ± 0.21	98.68	0.69

Table 6. Intra and Inter day precision data for the developed RP-HPLC of CBD

Standard Concentration (µg/ml)	Amount recovered (µg/ml)			Recovery (%)			Mean %Recovery	SD	%RSD
	Intra-day precision								
	1	2	3	1	2	3			
10	9.76	9.77	9.75	97.60	97.70	97.50	97.6	0.1	0.102
20	20.14	20.04	19.98	100.72	100.24	99.91	100.291	0.409	0.408
30	30.04	30.15	30.08	100.15	100.52	100.28	100.316	0.187	0.187
	Inter-day precision								
	1	2	3	1	2	3			
10	10.149	9.981	10.016	101.49	99.81	100.16	100.486	0.886	0.882
20	19.982	20.048	20.017	99.91	100.33	100.08	100.108	0.210	0.210
30	29.875	30.024	30.172	99.58	100.08	100.57	100.077	0.496	0.496

The suggested approach showed a percent RSD of 0.24 to 0.54 and 0.19 to 0.47% (i.e. <2%) respectively for intraday and inter-day precision indicating the repeatability of the developed technique.

3.3.5. Sensitivity

The sensitivity of the anticipated method found using the proper equation for the detection limit and quantification limit (LOD and LOQ) revealed values of 0.483622 and 1.465521 µg/mL respectively for CBD. It seems to be a sensitive method for the quantification of CBD.

3.3.6. Robustness

The Box-Behnken design was used to assess the robustness of the proposed method. The flow rate of the sample, mobile phase ratio of the two liquid phases that were used and oven temperature were all considered. When these requirements were varied as stated in the investigational section, there was no substantial alteration in the retention time and peak area of CBD. The low RSD value as represented in **Tables 5** and **6,** indicated that the procedure was reliable. The tailing factor is another variable that should be monitored with help of the ICH guidelines, and it was found to be in the range set by these guidelines. These system-suitability experiments improved the validation of the process, ensuring that the RP-HPLC system and proper procedure can provide results of sufficient quality. Therefore, even though the flow rate of the samples and mobile phase ratio differed somewhat, the technique generated satisfactory results.

3.4. Method application in CBD nanoemulsion formulation

The chromatograms of CBD in internal standard and nanoemulsion formulation are shown in **Fig. 6.** Both chromatograms showed no noticeable difference in the drug retention time, as well as the absence of any excipient peak. This demonstrated the selectivity of the validated RP-HPLC method for determining CBD in pharmaceutical formulations.

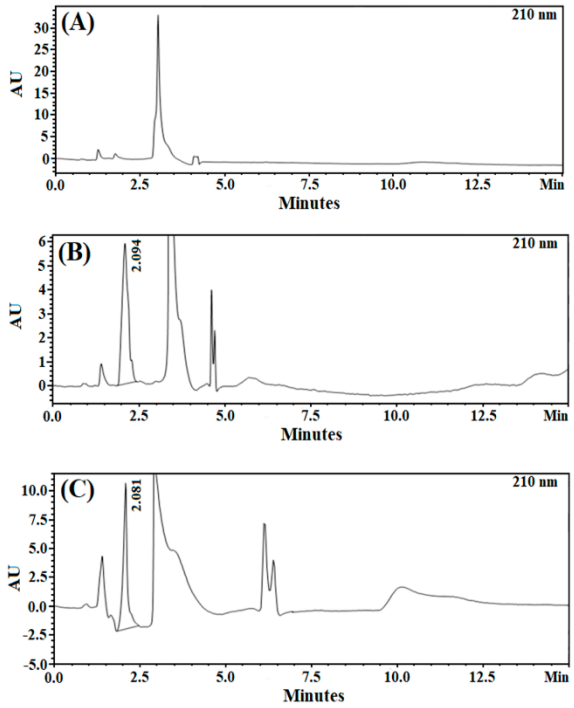

Fig. 6: HPLC chromatograms obtained through developed method (A) Blank chromatogram, (B) CBD through validated method, (C) CBD nanoemulsion formulation.

4. Conclusion

For estimating CBD in bulk and nanoemulsion formulation, an accurate and complete method based on reversed-phase HPLC technique at an effective cost was developed and optimized based on Box–Behnken design. By adopting the Design of Experiments (DOE), a more reliable and reproducible method was developed to meet the analytical target profile for the drug estimation. Box–Behnken architecture helped to evaluate the independent elements effectively. In the proposed method, the acetonitrile composition was considered the most inflexible element for the drug retention time. The flow rate and oven temperature were the more influential factor for responses: tailing factor and the peak area. It was observed that using this validated method; overall test runs needed to refine and improve the RP-HPLC process were decreased. The excellent percentage recovery of CBD from its nanoemulsion showed that CBD determination could not be hampered by the excipients found in the nanoemulsion formulation. Thus, it has been concluded that the established method could be successfully used for the routine testing of CBD and its bulk pharmaceutical formulations.

Conflicts of interest

There are no conflicts to declare.

Formatting of funding sources

Not applicable

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