A SIMPLE COMPREHENSIVE VALIDATED LIQUID CHROMATOGRAPHIC (HPLC) METHODOLOGY FOR SIMULTANEOUS ESTIMATION OF 5-FLUOROURACIL AND CANNABIDIOL

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Abstract
5-Fluorouracil (5-FU) is a conventional anti-cancer agent. Several studies have shown the synergism effect of Cannabidiol (CBD) along with 5-FU for skin cancers, thus making this combination cogitate for researchers for the management of skin cancer.

A novel, economical, sensitive and robust high-pressure liquid chromatography (HPLC) technique was established for the evaluation of 5-Fluorouracil (5-FU) and Cannabidiol (CBD) simultaneously. Chromatographic elution was done using the stationary phase Hypersil™ C18 reverse phase column 250 mm × 4.6 mm (5 µm) and employing mobile phase as Methanol: Water in ratio 90:10 running at a flow rate of 1.0 mL/min and evaluated at 235 nm. The total run time of the proposed technique was 4 min. The retention time was obtained at 1.43 min (5-FU), and 2.27 min (CBD).

The proposed analytical methodology validation was executed in consistent with International Conference on Harmonization (ICH) regulations and the validation parameter taken into consideration were the limit of detection (LOD), robustness, the limit of quantification (LOQ), and system suitability. A linear standard curve was plotted from the 10 to 100 µg/ml concentration range. The LOD of the proposed method was 4.75 ng/ml for 5-FU and 12.12ng/ml for CBD.

All results are under acceptable limits and the method could be suitable for employed in the synchronous determination of sample drugs in quality control and assay.

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1. INTRODUCTION

A famous fluorinated Pyrimidine analogue antimetabolite 5-Fluorouracil (Chemically known as 5-Fluoro-1H,3H-pyrimidine-2,4-dione) (Figure 1) which was accepted as a medicine in 1962 is still widely used conventional anticancer agent after 60 years of its recognition in the medical field (5-Flourouracil, 2022). It is commonly employed in the prescription of different categories of malignancies and skin problems. Evidences of its curative effect in the therapeutics of Breast carcinoma (Raymond , et al., 1997) , GIT (especially colon) (Raymond , et al., 1997) (Arburk, 1989) , ovarian cancer (Raymond , et al., 1997) and skin cancer ((Khan, et al., 2015) , (Iqubal , et al., 2021)) (Diasio & Harris, 1989) is well established.

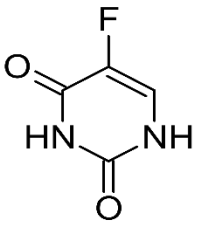


Figure 1: Chemical Structure of 5-Fluorouracil

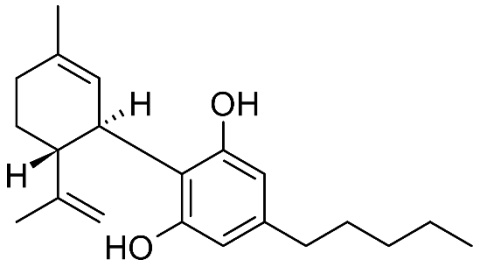


Figure 2: Chemical Structure of Cannabidiol

Cannabidiol (CBD) (chemically named as 2-[(1R, 6R)-6-Isopropenyl-3-methylcyclohex-2-en-1-yl]-5-pentylbenzene-1, 3-diol) (Figure 2) is major non-psychoactive agent derived naturally from plant Cannabis sativa. It has gained fame in the scientific associations due to its pharmacologically activities ranging from antioxidant (Atalay, Jarocka-Karpowicz, & Skrzydlewska, 2020), antipsychotic, antimicrobial (Blaskovich, et al., 2021), anxiolytic (Blessing, Steenkamp , Manzanares, & Marmar, 2015), antiepileptic, antiemetic, anti-inflammatory (Atalay, Jarocka-Karpowicz, & Skrzydlewska, 2020), colorectal cancer (Gustafsson, Lindgren, Jonsson, & Jacobsson, 2009), head and neck squamous cell cancer (Go, Kim, Kim, Chae , & Song, 2020) to skin cancer and lesions ((Scheau , et al., 2020) (Massi, Solinas, Cinquina, & Parolaro, 2012)). (Zhornitsky & Potvin, 2012).

Since both the drug moieties shows well known effect in the area of skin cancer which allows the author to suggest to take this combination ahead for detailed study.

Several Liquid Chromatography (HPLC) methods has been documented for the determination of 5-Fluorouracil (5-FU) individually plus in amalgamation with several different drug moieties in Biological matrices (Muhammad, et al., 2018), (Semail, et al., 2020), along with ion pairing technique (Rustum & Hoffman, 1988), along with Fluorescence derivatisation (Iwamoto, Yoshida, & Hirose, 1984) and in Bulk drug and marketed preparation (Larson, Khazaeli, & Dillon, 2003), (Sinha, Kumar, & Bhinge, 2009) , (Haq, et al., 2013), (Muhammad, et al., 2018), (Tomar, Sharma, Kumar, Jain, & Ahirrao, 2021).

Similarly, several HPLC methods are also developed for Cannabidiol (CBD) in Biological matrices (Zgair , et al., 2015) and in Raw Material, Bulk drug and marketed preparation (Mandrioli , Tura , Scotti, & Toschi, 2019), (Nahar, Onder, & Sarker, 2019), (Vlad, et al., 2019), (Ahmed, Rizwanulla, Usman, Mir, & Amin, 2022), (Piani, Ferfuia, Bortolomeazzi,, Verardo, & Baldini , 2022), (Analakkattillam, Langsi, Hanrahan , & Moore, 2022).

Many HPLC methods are reported for 5-FU and CBD individually in literature, but to the contrary no method has been reported in the literature for the estimation of 5-Fluorouracil (5-FU) and Cannabidiol (CBD) simultaneously. Thus, instigating the authors to develop an innovative synchronous estimation method for 5-Fluorouracil and Cannabidiol which can be intended to be used in assay studies, and routine analysis due to its cost-effectiveness (Tiwari et al 2021).

The intent of the ongoing investigation was to simply develop a rapid, economical and modernistic Reverse Phase HPLC analytical technique for the simultaneous quantification of 5-Fluorouracil (5-FU) and Cannabidiol (CBD) and validate it according to the guidelines of ICH (ICH, 2005).

As per literature, 5-FU has been quantified at various wavelengths, i.e. 205nm, 210nm, 254nm, 275nm, 266nm and 269nm, by HPLC method (Semail, et al., 2020). Furthermore, CBD has been quantified at 221nm by HPLC method (Nahar, Onder, & Sarker, 2019). While developing the method for simultaneous estimation, 5-FU was observed at 267nm and CBD at 221 nm individually. Though, the Isobestic wavelength was found to be 235nm which provides an efficient way for data analysis in the simultaneous quantification of two different drug moieties. Thus, using the optimised wavelength mentioned earlier, an understandable and methodical analytical way was

refined for estimation of 5-FU and CBD simultaneously which was able to beat the issues of the former individual methods. Validation of the current method was in consonance as per ICH guidelines.

2. MATERIAL AND METHODOLOGY

2.1. Material Used

5-Fluorouracil (5-FU) was bought from Sigma-Aldrich and Cannabidiol (CBD) was acquired as a kind from Netherlands, Europe. For mobile phase, Water, Methanol and Acetonitrile (ACN), all were of HPLC grade and were procured from Merck India. HPLC Column used for analysis was Hypersil C-18 which was procured from Thermo fisher India. The syringe filters used were from Merck Millipore. All other reagents used were of analytical (AR) or pharmaceutical grade.

2.2. Identification of Standard Drug:

Bulk drugs' (5-FU & CBD) identification were carried out by determining melting point, Infrared spectroscopy and solubility.

2.3. HPLC Instrument and Chromatographic Conditions

Analytical equipment used for this method development was Waters e-2695 Separation Module, along with Waters e-2998 PDA detector equipped with Software EMPOWER 3 for data acquisition and analysis. Stationary Phase used was Hypersil Reverse Phase column C-18 (250mm* 4.6mm, 5µm). Mobile phase used for the analysis was Methanol: Water (90:10) using isocratic elution mode at temperature 25ºC. Detection was done at 235nm using PDA detector. The flow rate was 1ml/min with ambient column temperature. The volume of injection was 10µl with the total run time of 4 minutes.

2.4. Formulation of Standard Solutions and Working Standards

5-Fluorouracil and Cannabidiol standard solution measuring 1000µg/ml concentration were formulated and were marked as SS1 & SS2 respectively. Stock Solutions of concentration 100µg/ml were formulated from the standard solution (S1& S2) and were marked as A1 and A2 respectively. Working stock solution of different concentration (10, 20, 40, 60, 100 µg/ml) were formulated from the standard solution (A1& A2) for both 5-Fluorouracil and Cannabidiol and the diluent used for working stock solution was Methanol. Furthermore, the samples of FU-CBD combination were formulated in methanol at 10, 20, 40, 80, and 100 µg/ml concentration for both the sample moieties. All the sample solutions were stored in amber-coloured bottles in the freezer at 4ºC before the set analysis. All the samples were filtered via a syringe filter of 0.22µm pore size. (Wrightson, Myers, & Galandiuk, 1995)

3. VALIDATION

Subsequently, the developed optimized method was validated for the synchronous determination of 5-Fluorouracil and Cannabidiol. The validation was attained in accordance with the protocol of ICH guidelines Q2 (R1) (ICH, 2005). The developed HPLC method should be validated on various domains to ensure that the strategy's comprehensive efficacy attributes meet the requirements of its intended purpose only (Zothanpuii & Selvakumar, 2020). The parameter taken into consideration are system suitability testing, linearity, specificity, ruggedness, limit of detection (LOD) and limit of quantification (LOQ).

3.1. System Suitability Test

This system suitability test (SST) is of key importance in liquid chromatography as it aids in the validation of the given testing procedure and ensures reproducibility. The suitability testing was done in six replicates with the sample size of 20µg/ml for both 5-Fluorouracil and Cannabidiol by scrutinizing the R_T and AUC at UV detection value of 235nm. According to the guidelines laid by US-FDA, Relative Standard Deviation (%RSD) should be NMT 2%.

3.2. Specificity

Specificity is described as the capacity of the analytical technique to isolate the sample of analytical nature from the combination (excipients, degraded products, impurities), which is the main distinctive feature of HPLC. Chromatograms of drug samples were compared to blank solution and their amalgamation sample to evaluate specificity. The blank solution did not contain 5-FU or CBD; the rest of the constituents and preparation process were identical to the drug sample.

3.3. Linearity

Linearity of the analytical procedure can be determined by preparing sample mixture of 10, 20, 40, 80, and 100 µg/ml concentration from standard solution. The area under the curve is calculated for each concentration of the sample (individual or mixture). Thus, plotting the calibration graph with concentration (x-axis) and area under the curve (y-axis) helps in derivatisation of regression equation. All standard curves of 5-Flourouracil, Cannabidiol and their combination were devised for linear equation.

3.4. Ruggedness

Ruggedness means having the capacity to generate an outcome under different conditions, like
Employing varied investigator or device of unsimilar make. We have utilised three different samples of 10, 20, and 30 µg/ml concentration, that were examined by various investigator on the same Liquid chromatographic equipment located in the similar and different laboratory. Ruggedness score was calculated by evaluating the Recovery (%) of 5-Fluoruracil and Cannabidiol in both the situations.

3.5. Sensitivity

Sensitivity of the HPLC analytical method can be determined by calculating the minimum amount of drug which can be isolated and quantified i.e limit of detection (LOD) and limit of quantification (LOQ).

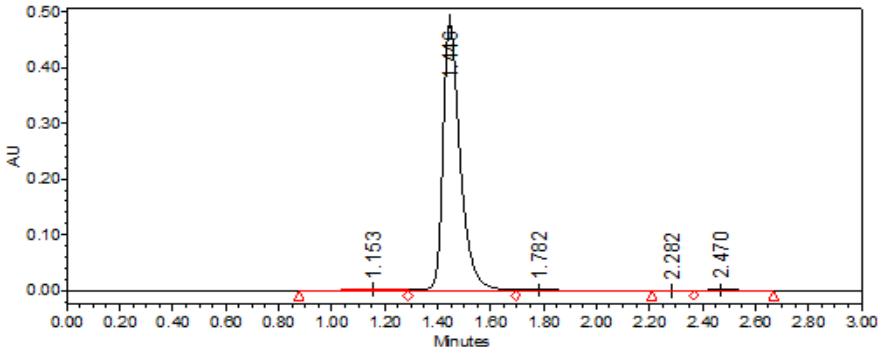
	LOD	**LOQ**
Formula **S= S.D.** **s= Slope**	3.3* S/s	10*S/s
Signal to noise ratio	3:1	10:1

4. RESULT AND DISCUSSIONS

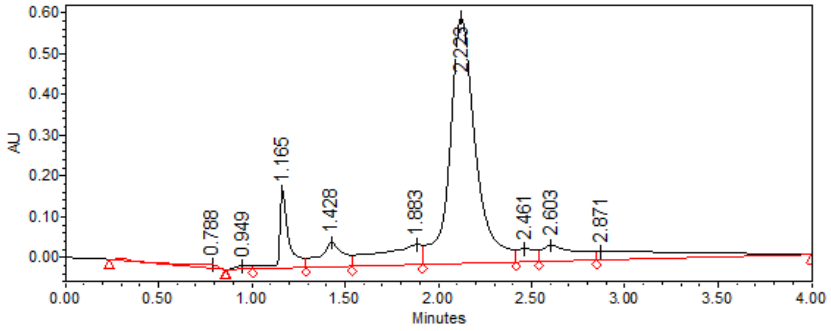
4.1 Development and Optimisation of the Technique

In order to make the prepared approach more financially viable and credible, an isobestic wavelength was screened for the analysis of the both the drug moieties, which is postulated on the basis of the absorption maxima of both drug samples at a particular wavelength. The methanolic solutions of both drug samples (5-FU and CBD) of concentration 10 µg/ml each were analysed by UV spectroscopy in the 400-200 nm spectrum. 5-FU and CBD had the highest absorbance at 267 nm and 221 nm respectively. A single wavelength of 235 nm was chosen as per the Spectral data of both drugs and validated by a PDA detector.
Mobile phase selection for method development was done among ACN/Methanol and Water in varied concentrations in isocratic and gradient flow. The best separation of 5-Florouracil and Cannabidiol was marked in the mobile phase Water: Methanol in a 90:10 ratio and the flow rate of the mobile phase was 1ml/min along with the total run time of 4 mins. The retention time of 1.43 minutes for 5-FU and 2.27 minutes for CBD was obtained. 5-Florouracil and Cannabidiol both analytes were tested independently at λmax 267 and 221 nm respectively (Figure. 1a and 1b). The combination of 5-FU and CBD was also estimated, through which the peaks got separated at 1.4 and 2.2 min respectively for 5-FU and CBD suggesting no change in the retention time of the individual and that combination (Fig. 1c).
The developed method for analysing 5-FU, CBD, and its combination determination indicates supremacy in comparison to the different established techniques because of its accelerated efficacy, and ability to detect both analytes within only 4 minutes of run time.

a) Chromatogram of 5-Fluorouracil at λmax 267nm,



b) Chromatogram of Cannabidiol at λmax 221 nm



c) Chromatogram of 5-Fluorouracil and Cannabidiol at isobestic λmax 235 nm

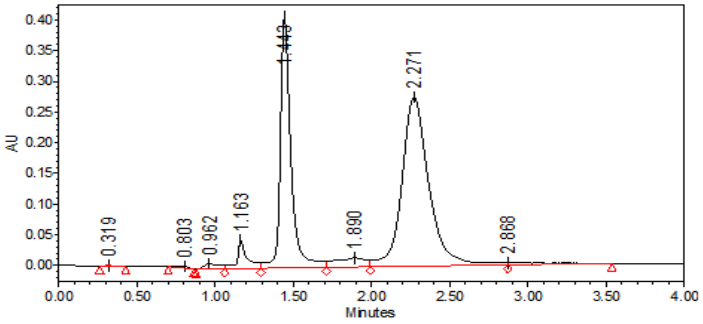
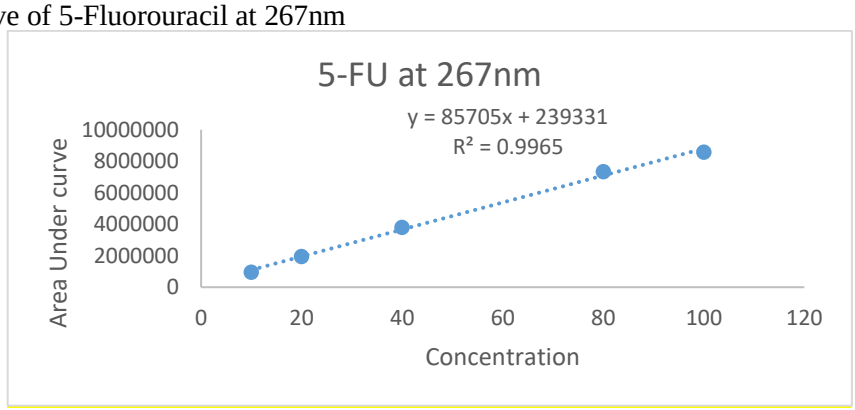


Figure 3: Chromatogram of 5-Flurouracil, Cannabidiol and Combination at their respective wavelength

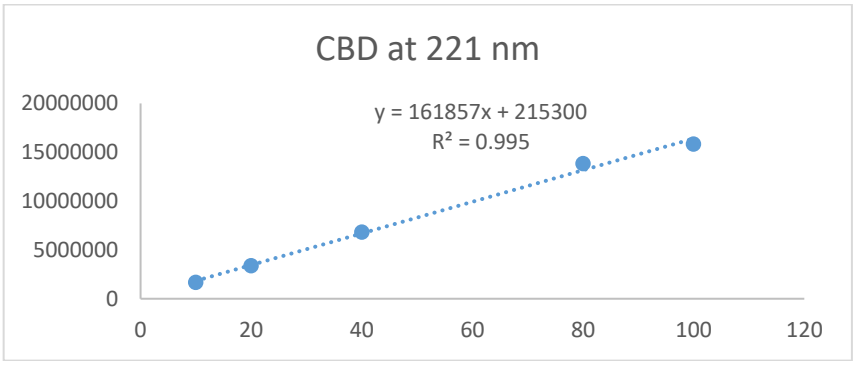
4.2. Method Validation

4.2.1. Linearity

a) Calibration curve of 5-Fluorouracil at 267nm



b) Calibration curve of Cannabidiol at 221nm



c) Calibration curve of 5-Flourouracil & Cannabidiol at Isobestic λmax 235nm

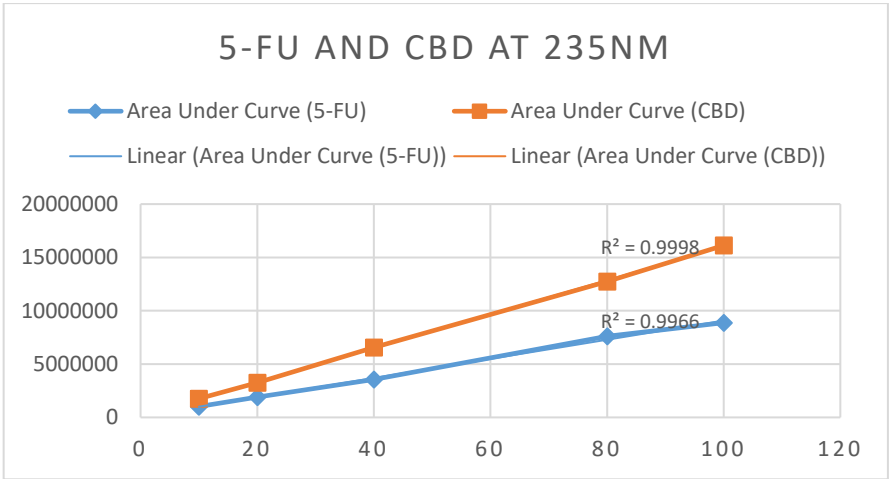


Figure 4: Calibration Curve of 5-Fluorouracil, Cannabidiol and Combination at respective wavelength

Five different samples of 5-FU, CBD and 5FU-CBD of concentration 10, 20, 40, 80 and 100 µg/ml were formulated and analysed independently. To determine linearity of the current HPLC method, 5-Flourouracil, Cannabidiol and their combination were monitored at 267nm, 221nm and 235 nm respectively. A regression graph was devised between the concentration on X-Axis and Area under the curve (AUC) on Y-Axis. The Correlation Coefficient value (R2) for 5-FU at λmax 267nm was 0.9965, for CBD at λmax 221nm was 0.995 and for 5-FU-CBD at λmax 235nm was 0.9966 for 5-FU and 0.9998 for CBD. (Fig 4). The above values showed the current HPLC method's linearity in the stated range of concentration.

4.2.2. System Suitability Test

a) SST of 5-Fluorouracil at 235nm		
SST Criteria	**T_R**	**AUC**
1	1.46	1962575
2	1.45	1996652
3	1.39	1914854
4	1.41	1985485
5	1.44	1952548
6	1.42	1995241
Mean	1.428333333	1967892.5
Standard Deviation	0.026394444	31500.34069
RSD	1.847919057	1.600714505
b) SST of Cannabidiol at 235nm		
SST criteria	**T_R**	**AUC**
1	2.19	3308678
2	2.24	3348182
3	2.17	3395527
4	2.27	3336826
5	2.23	3382535
6	2.18	3324173
Mean	2.213333333	3349320.167
Standard Deviation	0.039327683	35074.94097
RSD	1.776853157	1.047225682

Table 1: a) Retention time & Area under peak data for system suitability test of 5-Flurouracil at 235 nm
b) Retention time & Area under peak data for system suitability test of Cannabidiol at 235 nm

Six samples runs of 5-FU and CBD were conducted to calculate the Relative standard deviation (RSD) of retention time and Area under the curve. The average retention time for 5-FU was 1.4284± 0.026min with RSD of 1.84% and the average retention time for CBD was 2.21± 0.039min with RSD of 1.77%. The average area under the curve of 5-Fluorouracil was observed as 1967892.5± 31500.34 with RSD of 1.60% and the average curve area of CBD was found to be 3349320.167± 35074.94 with RSD of 1.04%. (Table 1). All the obtained Relative Standard deviation (RSD) values for both retention time and area under the curve for 5-Fluorouracil and Cannabidiol are below 2%, thus complying with the parameters for system suitability and reproducibility for the current liquid chromatographic method.

4.2.3. Specificity

By correlating the chromatograms of 5-Fluoruracil, Cannabidiol, and Fluorouracil Cannabidiol mixture's standard solution along with the blank solution, the assessment of the specificity of the current HPLC procedure was established.

The standard sample which was injected was of 10 µl volume each and all were analysed separately. When the retention time of both drug moieties (5-FU & CBD) were examined individually, they were found to be 1.44 and 2.2 minutes, respectively. 5-Fluorouracil and Cannabidiol had a retention time of 1.44 and 2.2 minutes, respectively, in the 5-Fluorouracil-Cannabidiol mixture (Fig 1). Thus, implies that the retention time is unaltered irrespective the samples are analysed individually or simultaneously, therefore indicating the specific nature of the current analytical method.

4.2.4. Sensitivity

Drug Sample	**Slope of the curve**	**Standard deviation (SD)**	**LOD**	**LOQ**
5-Flourouracil at λmax 267nm and Cannabidiol at λmax 221nm				
5-FU	85705	53979	2.07841666	6.298232309
CBD	161857	373689	7.61890867	23.08760202
5-Fluorouracil and Cannabidiol at Isobestic λmax 235 nm				
5-FU	89770	129341	4.75465412	14.40804278
CBD	159246	585240	12.1277269	36.75068762

Table 2: Sensitivity Table

The sensitivity of the HPLC analytical method is done by evaluating the calibration curves of the samples and combination to analyse the lowest detection amount of the drug (LOD) and lowest quantified drug value (LOQ). Thus, LOD for 5-Fluorouracil was 2.08 ng/ml at λmax 267nm and LOD for Cannabidiol was found to be 7.6 ng/ml at λmax 221nm. LOD obtained at the common isobestic wavelength 235nm was 4.75ng/ml for 5-Fluorouracil and 12.12ng/ml for Cannabidiol (Table 2). The minimal amount of the analyte that can be measured is known as LOQ, which was calculated to be 6.29 ng/ml for 5-FU at λmax 267nm and LOQ for CBD was estimated to be 23.08 ng/ml at λmax 221nm. The LOQ for the current designed HPLC method at λmax 235nm were calculated to be 14.04 ng/ml for5-Fluorouracil and 36.75 ng/ml for Cannabidiol (Table 2).

Hence, the statistics of LOD and LOQ shows promising evaluation scope for the current HPLC analytical method in assay procedures, and drug development.

4.2.5. Ruggedness

(A)Ruggedness of 5-Fluorouracil and Cannabidiol on varied HPLC unit by same investigator					
Drug Sample	**Sample Concentration (µg/ml)**	**Amount Recovered concentration (in µg/ml)**		**Recovery (In percentage)**	
		HPLC-I	**HPLC-II**	**HPLC-I**	**HPLC-II**
5-FU	10	9.48 ± 0.22	9.61 ± 0.43	94.89	96.14
	20	19.24 ± 0.49	19.08 ± 0.89	96.24	95.42
	30	29.86 ± 0.2	29.54 ± 0.75	99.53	98.49
CBD	10	9.78 ± 0.23	9.50 ± 0.93	97.89	95.02
	20	19.36 ± 0.56	19.73 ± 0.58	96.82	98.66
	30	29.05 ± 0.69	29.03 ± 0.74	96.83	96.78
(B) Ruggedness of 5-Fluorouracil and Cannabidiol by varied investigators on the same HPLC unit.					
Drug Sample	**Sample Concentration (µg/ml)**	**Amount Recovered concentration (in µg/ml)**		**Recovery (In percentage)**	
		Investigator-I	**Investigator-II**	**Investigator-I**	**Investigator-II**
5-FU	10	9.97 ± 0.90	9.64 ± 0.72	99.72	96.48
	20	19.08 ± 0.45	19.68 ± 1.03	95.44	98.43
	30	28.80 ± 0.46	29.79 ± 0.96	96	99.31
CBD	10	9.76 ± 0.41	9.71 ± 0.44	97.67	97.19
	20	19.62 ± 0.89	19.42 ± 0.66	98.11	97.11
	30	28.81 ± 0.59	28.99 ± 1.00	96.04	96.64

Table 3: Ruggedness Score of 5-Fluorouracil and Cannabidiol at λmax 235nm

The robustness test was used to assess the reproducibility of outcome values procured on varied HPLCs with the similar investigator and on single liquid chromatographic setup with various investigators. Then, the test was performed by the same investigator on different HPLC. The amount recovered of 5-FU for sample concentrations 10, 20 and 30 µg/ml at 235nm came out to be 9.48 ±0.22, 19.24 ±0.49 and 29.86 ±0.2 µg/ml along with percentage recovery of 94.89, 96.24 and 99.53 % subsequently on HPLC-1 and the recovered concentration was 9.61 ± 0.43, 19.08 ± 0.89 and 29.54 ± 0.75 µg/ml with percentage recovery 96.14, 95.42 and 98.49 % respectively on HPLC -2. Similarly, the recovered concentration of CBD by the same investigator for sample concentration 10, 20 and 30 µg/ml at 235nm came out to be 9.78 ± 0.23, 19.36 ± 0.56 and 29.05 ± 0.69µg/ml along with percentage recovery of 97.89, 96.82 and 96.83% respectively on HPLC-1 and the recovered concentration was 9.50 ± 0.93, 19.73 ± 0.58and 29.03 ± 0.74µg/ml with percentage recovery 95.02, 98.66 and 96.78% respectively on HPLC -2. (Table 3(A)).

Then, the ruggedness data was collected by different analysts on the same HPLC (HPLC-1). The recovered concentration of 5-FU for sample concentrations 10, 20 and 30 µg/ml at 235nm came out to be 9.97 ± 0.90, 19.08 ± 0.45 and 28.80 ± 0.46µg/ml along with percentage recovery of 99.72, 95.44 and 96% respectively by analyst-1 and the recovered concentration was 9.64 ± 0.72, 19.68 ± 1.03 and 29.79 ± 0.96µg/ml with percentage recovery 96.48, 98.43 and 99.31% respectively by analyst-2. Similarly, the recovered concentration of CBD for sample concentrations 10, 20 and 30 µg/ml at 235nm came out to be 9.76 ± 0.41, 19.62 ± 0.89 and 28.81 ± 0.59µg/ml along with percentage recovery of 97.67, 98.11and 96.04% respectively by analyst-1 and the recovered concentration was 9.71 ± 0.44, 19.42 ± 0.66 and 28.99 ± 1.00µg/ml with percentage recovery 97.19, 97.11and 96.64% respectively by analyst-2. The study findings show an excellent level of

ruggedness implying that the current analytical method is capable of producing concurrent results on various HPLC analytical setups and by various researchers. The validation data produced tends to be consistent with the reported work. (Sharma, Goyal, & Chauhan, 2018), (Swartz & Krull, 1997).

CONCLUSION

The current study presents a novel approach for simultaneous determination of 5-FU and C B D in assay procedures and quality control. The developed method is simplified, fast, highly selective, sensitive and meticulous, and the proposed technique was validated in consistent with ICH guidelines.

The run time of the method is less than 4 minutes i.e. faster elution with great resolution, thus saving analyst time and solvents, making it to be cheap for routine analysis. This method could employed as rapid and economical way to quantify both the analytical samples (5-FU and CBD) simultaneously in formulation development and quality control checks.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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