

Development and Validation of UV Spectrophotometric Method For Simultaneous Estimation of Biochanin A and 5-Fluorouracil

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Abstract

A validated UV spectroscopic method was established for the simultaneous estimation of Biochanin A and 5-Fluorouracil within a hydrogel system. The method was linear in the range of 0.1 to 1 µg/ml for Biochanin A at 229 nm and 1 to 10 µg/ml for 5-Fluorouracil at 265 nm with regression coefficient near to 1. The UV spectra of Biochanin A and 5-Fluorouracil revealed an isobestic point at 249 nm in phosphate buffer pH 5.5. The accuracy and precision results obtained using simultaneous equation method were observed within acceptable limits. The RSD (%) was found to be less than 2% for intraday and interday precision, demonstrating the acceptable precision of the developed method. Biochanin A showed LOD and LOQ values of 0.064 µg/ml and 0.193 µg/ml at 229 nm respectively while 5-Fluorouracil exhibited corresponding values of 0.314 µg/ml and 0.953 µg/ml at 265 nm. The assay of 5-Fluorouracil and Biochanin A indicated % drug content to be 100.40 % and 103.10 % respectively, indicating accuracy and applicability of developed method.

Key Words: UV spectrophotometric, Biochanin A, 5-Fluorouracil, Isobestic point, Simultaneous estimation.

1. Introduction

The management of complex ailments usually employs combination therapies as they simultaneously target multiple biological pathways and improve therapeutic efficacy. Combination therapies via concurrent use of active pharmaceutical ingredients possessing distinct mechanism of action frequently yield synergistic effects, conferring improved efficacy relative to monotherapy. In addition, combination therapies also help to mitigate resistance development, specifically in conditions like cancer and infectious diseases (Schweim et al., 2014). Furthermore, reduced drug doses of discrete drug molecules can be employed, ameliorating toxicity and deleterious effects. Combined treatment modalities offers comprehensive disease control and attenuate disease progression to a great extent. Collectively, they constitute a mechanistically justified and efficient approach for addressing conditions with complex etiology in modern clinical practice (Abdullah et al., 2024). The existence of multiple APIs in combination therapies complicates their quantitative pharmaceutical analysis. It is often challenging due to distinct physicochemical properties, like solubility, stability, and spectral behavior. These variations typically contribute to overlapping signals and complicate reliable quantification. In this regard, the establishment of simultaneous assay methods becomes essential to resolve the analytical interference for reliable quantification of respective constituent simultaneously in a single analytical run. Such analytical methods decrease analysis time, cost, and solvent usage with superior assay reproducibility. Although, quantification of combination regimens necessitates comprehensive optimization and validation to as certain excellent selectivity, sensitivity, and robustness rendering them essential in contemporary pharmaceutical quality control (Tambe et al., 2021). 5-Fluorouracil (5-FU) is a commonly used chemotherapeutic agent in management of distinct cancers like breast, skin and colon cancer. However, its short half-life and acquired resistance in prolonged therapy due to efflux transporter (e.g., P-glycoprotein) pumps lowering intracellular concentration limits its therapeutic potential (Ahmad et al., 2023; Hasan et al., 2023). Biochanin A (BCA) has demonstrated the ability to overcome resistance by inhibiting efflux transporters (P-gp) and breast cancer resistance protein, enhancing drug accumulation at the target site. It plays a crucial role in modulating the pharmacokinetics of drugs such as taxol, vinca alkaloids and anthracyclines (Dash and Konkimalla, 2017). The available literature reports that combination of Biochanin A with 5-Fluorouracil synergistically enhances apoptosis by sensitizing cancer cells, making it a promising approach for managing multidrug-resistant breast cancer (Mahmoud et al., 2022). Recently, topical formulations have gained more attention for the management of skin cancer. Topical delivery having many benefits involves reduced systemic toxicity, bypassing first-pass metabolism, drug delivery at site of action and improved patient compliance (Nayak et al., 2025). Among various topical carriers, hydrogels are suitable candidates for topical delivery as they are more permeable, biocompatible, flexible, viscoelastic, more hydrating in nature, and enhance residence time between the drug and target site (Gonsalves et al., 2021). Therefore, combination therapy composed of 5-Fluorouracil and

Biochanin A loaded hydrogel has been explored. Despite the therapeutic advantages of this combination, no relevant simultaneous determination method of 5-Fluorouracil and Biochanin A is available in pharmacopoeia and published reports till date. This is extremely challenging to estimate both the drugs simultaneously with a valid analytical method during formulation development and quality control. Therefore, it is essential to establish a simple, reliable and reproducible analytical protocol for simultaneous estimation of both the drugs. Thus, this study focused to establish and validate a reliable, accurate and highly sensitive UV-spectroscopy technique for the simultaneous estimation of 5-Fluorouracil and Biochanin A in formulation.

2. Materials and methods

5-Fluorouracil (5-FU) (CAS RN®:51-21-8 > 99.0%) and Biochanin A (BCA) (CAS RN®491-80-5 >98.0%) was purchased from TCI Chemicals, India. All chemicals were used as in their original form without any purification process. Carbopol 934 (CAS No.: 9003-01-4) was purchased from CDH (Daryaganj, New Delhi). The analytical grade solvents and reagents were used.

2.1 Method development

2.1.1 Selection of solvent based on solubility studies

Modified shake flask method was used to determine the solubility of 5-Fluorouracil and Biochanin A respectively. An excess amount of drug dispersed in phosphate buffer pH 5.5 (10 ml) was agitated on shaker for 24 h at room temperature. Subsequently, supernatant collected by centrifuging samples at 10,000 rpm for 10 minutes at 4 °C was quantitatively determined using spectrophotometric analysis.

2.1.2 Absorption maxima estimation

A precisely weighed amount of 5-Fluorouracil was dissolved in phosphate buffer pH 5.5 to obtain a stock solution (1 mg/ml). Suitable dilutions were prepared and screened from 200-800 nm by UV /Visible spectrophotometer (Shimadzu-1900, Japan) to determine the wavelength of maximum absorption (λ_{max}). Similarly, Biochanin A stock solution was prepared in ethanol (1 mg/ml) and dilutions were done with a mixed solvent system composed of ethanol and phosphate buffer pH 5.5 in ratio 3:7 respectively. Suitably diluted samples with phosphate buffer pH 5.5 were screened from 200 - 800 nm utilizing UV/Visible spectrophotometer to determine the λ_{max} respectively. Biochanin A (1 mg/ml) and 5-Fluorouracil (1 mg/ml) solution were mixed to yield a mixture having a strength of 0.5 mg/ml for each drug. Suitably diluted samples using phosphate buffer pH 5.5 were screened from 200 - 800 nm utilizing UV/Visible spectrophotometer to determine the λ_{max} respectively.

2.1.3 Preparation of calibration curve

Different dilutions of 5-Fluorouracil from stock solution of 5-Fluorouracil (1mg/ml) were prepared and their absorbance was recorded at 265 nm. Biochanin A stock solution (1 mg/ml) in ethanol was diluted with mixed solvent system composed of ethanol and phosphate buffer pH 5.5 in 3:7 ratio. Further, dilutions were prepared using phosphate buffer pH 5.5 respectively and absorbance was recorded at 229 nm. A calibration graph was obtained by plotting absorbance versus concentration ($\mu\text{g/ml}$) of different dilutions prepared.

2.2 Analytical Method Development for Simultaneous drug Quantification

2.2.1 UV overlay spectral study

Suitably diluted sample of Biochanin A (1 $\mu\text{g/ml}$) and 5-Fluorouracil (10 $\mu\text{g/ml}$) was screened at 229 nm and 265 nm respectively. The overlay was prepared to detect the isobestic point. The absorbance of both drugs in mixture (1:10 ratio) was estimated at their highest absorption wavelength utilizing simultaneous equation approach. The absorption coefficient of Biochanin A and 5-Fluorouracil was determined at their respective absorption wavelength. The concentration of drug in each combination was ascertained by followed equation

$$C_n = \frac{X_2 \cdot a_{m1} - X_1 \cdot a_{m2}}{a_{n2} \cdot a_{m1} - a_{n1} \cdot a_{m2}} \quad (i)$$

$$C_m = \frac{X_1 \cdot a_{n2} - X_2 \cdot a_{n1}}{a_{m2} \cdot a_{n1} - a_{m1} \cdot a_{n2}} \quad (ii)$$

X1 = Binary mixture's absorbance at 229 nm

X2 = Binary mixture's absorbance at 265 nm

a_{n1} = Value of absorptivity of Biochanin A (λ_{max} 229)

a_{n2} = Value of absorptivity of Biochanin A (λ_{max} 265 nm)

a_{m1} = Value of absorptivity of 5-Fluorouracil (λ_{max} 229 nm)

a_{m2} = Value of absorptivity of 5-Fluorouracil (λ_{max} 265 nm)

C_n = Biochanin A concentration in $\mu\text{g/ml}$

C_m = 5-Fluorouracil concentration in $\mu\text{g/ml}$

2.3 Method validation using UV spectrophotometry Validation of the UV spectroscopic method for sample analysis was executed to examine linearity, range, precision, accuracy, LOD and LOQ.

2.3.1 Linearity

The calibration graph of absorbance versus concentration of Biochanin A (0.1-1 µg/ml) and 5-Fluorouracil (1-10 µg/ml) was prepared respectively at 229 nm and 265 nm. The linearity of relation of absorbance vs concentration was established via linear regression analysis.

2.3.2 Precision

The method's precision was evaluated by analyzing Biochanin A (0.1, 0.4, and 1 µg/mL) and 5-Fluorouracil (1, 4 and 10 µg/mL) at three concentration levels (Majithia et al., 2020).

2.3.3 Intraday precision (Repeatability)

Intraday precision was evaluated via analyzing different concentrations of Biochanin A (0.1, 0.4 and 1 µg/ml) and 5-Fluorouracil (1, 4 and 10 µg/ml) in triplicate within the same day. Variation of intraday was determined by calculating the % RSD of the concentrations.

2.3.4 Interday precision

Method's interday precision was analyzed by measuring distinct concentrations of Biochanin A (0.1, 0.4 and 1 µg/ml) and 5-Fluorouracil (1, 4 and 10 µg/ml) in triplicate for three days. Interday variation was determined by calculating the % RSD of the concentrations.

2.3.5 Accuracy assessment

Recovery studies were executed to evaluate the accuracy of method. Previously analyzed samples were fortified with additional amount of Biochanin A and 5-Fluorouracil at 80%, 100%, 120% and the mixture was then evaluated by the developed method in triplicate. This analysis was conducted to measure drug recovery across different concentrations (Mahmood et al., 2020).

2.3.6 LOD and LOQ determination

Detection limit (LOD) of method refers to lowest amount of substance that can be assessed in a sample. The quantitation limit (LOQ) is the smallest amount of substance that can be quantitatively measured. Detection limit and quantitation limit of Biochanin A and 5-Fluorouracil were determined using given equations (Sharma et al., 2023).

$$\text{Detection Limit} = \frac{\alpha}{SL} \times 3.3 \quad (\text{iii})$$

$$\text{Quantitation limit} = \frac{\alpha}{SL} \times 10 \quad (\text{iv})$$

Where α represents response's standard deviation, and SL denotes regression line's slop.

2.4 Hydrogel formulation

Briefly, pure drug (5-Fluorouracil and Biochanin A) combinations were uniformly dispersed in aqueous solution of Carbopol-934 (1%w/v) under continuous stirring for 1h. Subsequently, triethanolamine (0.05 w/w) was added to obtain hydrogel with optimum viscosity (Prashanthini et al., 2023).

2.4.1 Analysis of 5-Fluorouracil and Biochanin A in hydrogel formulation

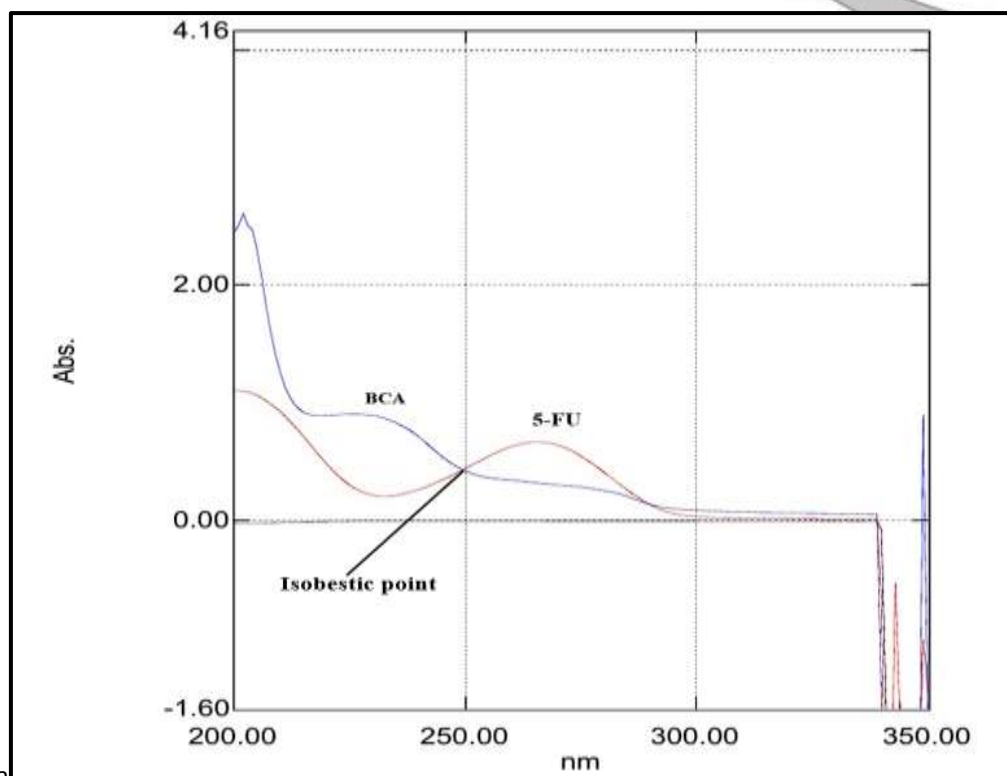
A definite amount of pure drug combination loaded hydrogel (10 mg each drug) were dispersed in solvent system composed of phosphate buffer (pH 5.5) and ethanol in 7:3 ratios with continuous stirring at 750 rpm for 2.5 h. Later, clear supernatant collected by centrifuging sample at 14,000 rpm for 15 min was analyzed spectrophotometrically to quantitate Biochanin A and 5-Fluorouracil content in optimized formulation respectively.

3. Results and discussion

3.1 Method development

The choice of an appropriate solvent in analytical method development is largely depending on drug's solubility. The solubility of Biochanin A in water and phosphate buffer pH 5.5 was observed to be 5 µg/ml and 5.41 µg/ml respectively. The solubility of 5-Fluorouracil in water and phosphate buffer pH 5.5 was found to be 11.51 mg/ml and 17.25 mg/ml respectively. The absorption maximum of 5-Fluorouracil and BCA was obtained at 265 and 229 nm using U.V./Visible spectrophotometer respectively. A wavelength at which both drugs showed identical absorptivity is referred as isobestic point. The isobestic wavelength was found to be at 249 nm, which is essential for the simultaneous estimation analysis of Biochanin A and 5-Fluorouracil in binary mixture.

Figure 1 represented the U.V./Visible spectra of Biochanin A and 5-Fluorouracil, indicating their respective λ_{max} and the isobestic point. The method of simultaneous equations was chosen for the quantitative analysis of both drugs in binary system.



a
Figure 1: Overlay UV-visible Spectrum of Biochanin A (BCA) and 5-Fluorouracil (5-FU), revealing an iso-absorptive point at 249 nm

3.2 Validation of method

The calibration curve of Biochanin A was developed in phosphate buffer pH 5.5 in a suitable concentration range respectively as shown in Figure 2 by analyzing samples spectrophotometrically. The calibration curve of 5-Fluorouracil was developed in phosphate buffer pH 5.5 in a suitable concentration range between 1-10 µg/ml as depicted in Figure 3 by analyzing samples spectrophotometrically. Method validation was executed considering range, linearity, accuracy, precision, detection limit and quantitation limit.

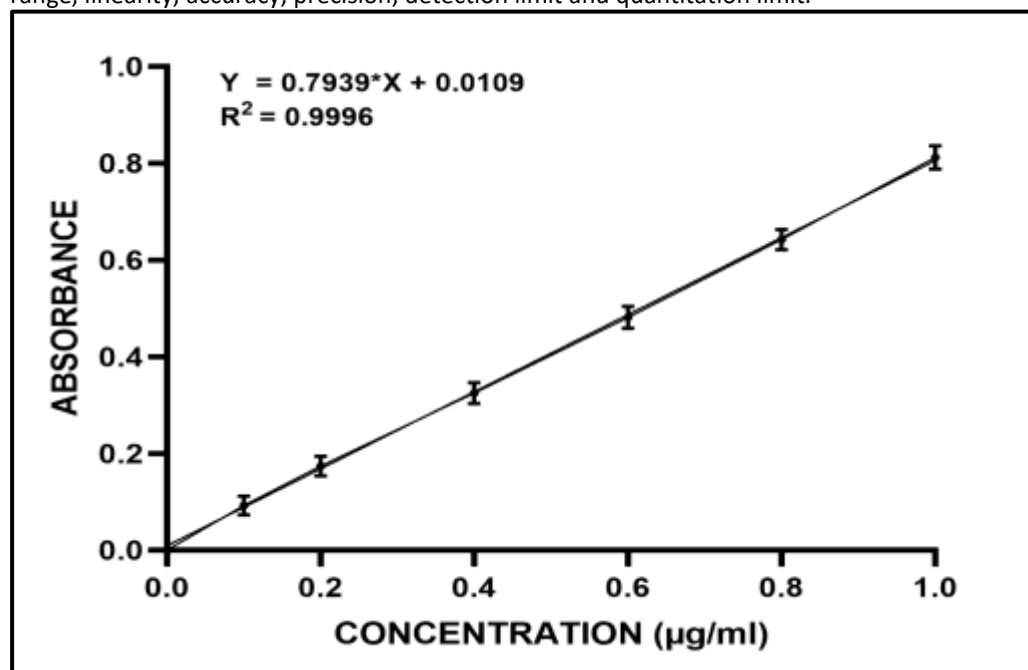


Figure 2: Calibration curve of Biochanin A at 229 nm

The calibration curve was linear for Biochanin A (0.1-1 µg/ml) at 229 nm (Figure 2) and for 5-Fluorouracil (1-10 µg/ml) at 265 nm (Figure 3) both complying with Beer-Lambert's law. The R^2 values close to 1 for Biochanin A as

well as 5-Fluorouracil obtained validate the linearity over the concentration range used for both drugs. The absorbance values were recorded at the chosen wavelengths and absorptivity for both drugs was determined at each wavelength (Table1) (Figure 4 and 5). The findings of the regression analysis are presented in Table 2.

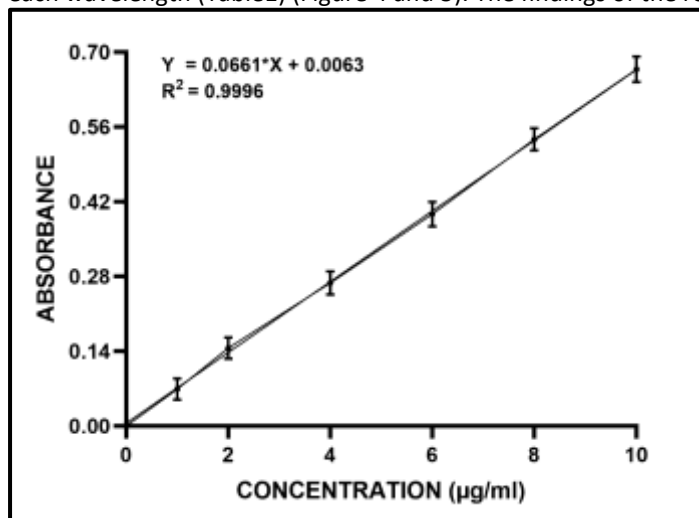


Figure 3: Calibration graph of 5-Fluorouracil at 265 nm

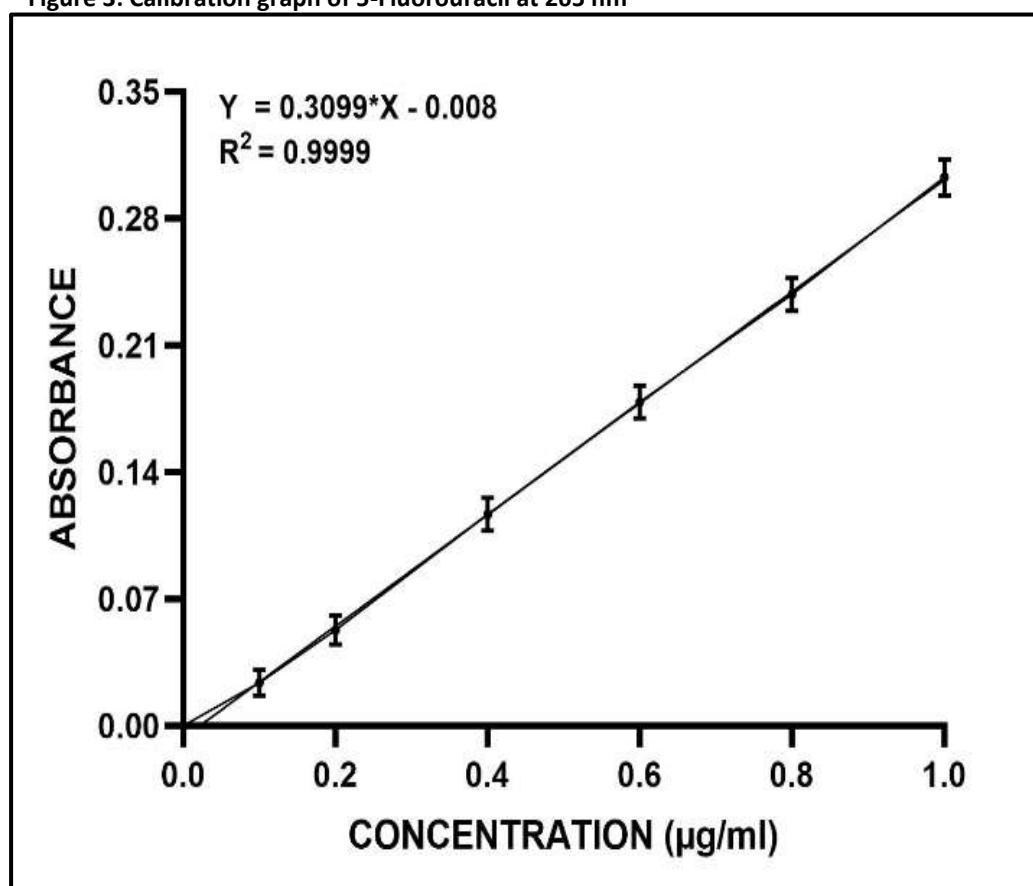


Figure 4: Calibration graph of Biochanin A at 265 nm

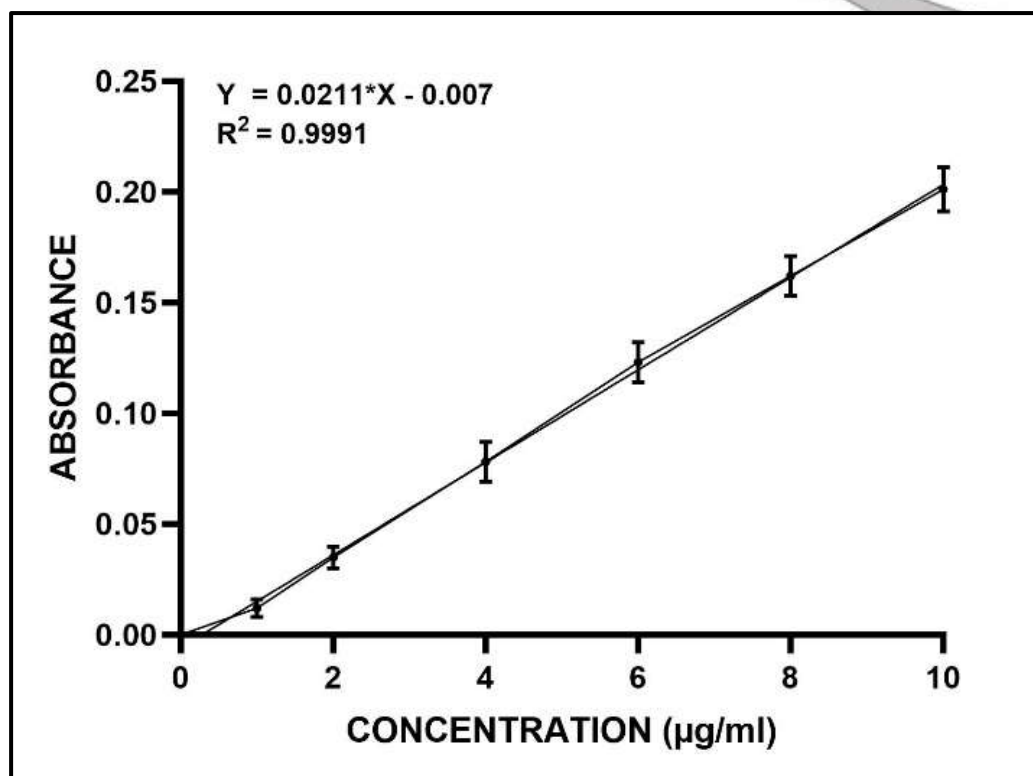


Figure 5: Calibration Curve of 5-Fluorouracil at 229 nm

The outcomes of inter- and intra- day precision studies were assessed utilizing % RSD. The calculated % RSD values were found to be less than 2 demonstrating the acceptable precision of the developed method according to ICH recommended acceptable limit (Table3). These results ascertain strong repeatability and minimal intra- and inter-day variability revealing good precision and accuracy of method. The adequacy of proposed method was validated by recovery experiments yielding 99.49-100.21 % for Biochanin A and 99.92-100.79 % for 5-Fluorouracil after spiking known amounts into previously analyzed samples. Table 4 illustrated the findings of accuracy assessment. The recovery percentage for Biochanin A and 5-Fluorouracil also revealed that excipients of hydrogel exhibited no interference to quantitation of both drugs. Additionally, analytical sensitivity is reflected by low LOD and LOQ for both drugs respectively (Table 5).

Table 1: Absorbance and absorptivity of Biochanin A and 5-Fluorouracil at each other's wavelengths (n=3)

Name of drug	Concentration (µg/ml)	Mean Absorbance (nm ± SD)		Absorptivity	
		229	265	229	265
Biochanin A	0.1	0.092±0.012	0.023±0.064	0.927	0.230
	0.2	0.174±0.0022	0.052±0.073	0.870	0.260
	0.4	0.324±0.0101	0.118±0.063	0.811	0.295
	0.6	0.481±0.004	0.179±0.081	0.802	0.298

	0.8	0.642±0.0033	0.238±0.033	0.802	0.297
	1	0.812±0.0005	0.303±0.054	0.812	0.303
				an1:0.837	an2: 0.281
5-Fluorouracil	1	0.012±0.002	0.069± 0.043	0.012	0.069
	2	0.035±0.003	0.146±0.022	0.017	0.073
	4	0.078±0.011	0.268±0.052	0.019	0.067
	6	0.123±0.004	0.397±0.073	0.020	0.066
	8	0.162±0.002	0.537±0.044	0.020	0.067
	10	0.201±0.021	0.668±0.069	0.020	0.066
				am1:0.0183	am2:0.0681

Table2: Spectral and regression parameters of Biochanin A and 5-Fluorouracil

Parameters	Biochanin A		5- Fluorouracil	
Wavelength(nm)	229	265	229	265
Beer's law range	0.1-1 µg/ml	0.1-1 µg/ml	1-10 µg/ml	1-10 µg/ml
Regression equation	y= 0.7939x + 0.0109	y=0.3099x+-0.008	y=0.0211x-0.007	y=0.0661x + 0.0063
Slope	0.7939	0.3099	0.0211	0.0661
Intercept	0.0109	-0.008	-0.007	0.0063
Regression Coefficient	0.999	0.999	0.999	0.999

Table 3: Intraday and intermediate precision results of Biochanin A and 5-Fluorouracil (Mean $\pm$ SD, n = 3)

Drug	Standard concentration ($\mu\text{g/ml}$)	Mean concentration ($\mu\text{g/ml}$)				% RSD			
		Intraday	Interday (Days)			Intraday	Interday (Days)		
			1	2	3		1	2	3
Biochanin A	0.1	0.101 $\pm$ 0.091	0.100 $\pm$ 0.001	0.101 $\pm$ 0.001	0.100 $\pm$ 0.002	0.935	1.295	0.9897	1.295
	0.4	0.397 $\pm$ 0.007	0.398 $\pm$ 0.001	0.399 $\pm$ 0.002	0.398 $\pm$ 0.0037	1.012	0.2668	0.624	0.952
	1	1.009 $\pm$ 0.013	0.999 $\pm$ 0.013	1.019 $\pm$ 0.012	1.017 $\pm$ 0.008	1.331	1.331	1.221	0.788
5-Fluorouracil	1	0.998 $\pm$ 0.030	0.986 $\pm$ 0.011	0.983 $\pm$ 0.014	1.002 $\pm$ 0.012	1.102	1.171	1.511	1.231
	4	3.974 $\pm$ 0.067	3.948 $\pm$ 0.031	3.941 $\pm$ 0.076	3.932 $\pm$ 0.007	1.161	0.791	0.191	0.191
	10	9.962 $\pm$ 0.077	9.903 $\pm$ 0.032	9.911 $\pm$ 0.044	9.921 $\pm$ 0.038	0.512	0.312	0.441	0.380

Table 4: Analyte recovery studies using the developed assay method.

Drug	Amount Added %	Recovery % $\pm$ SD	% RSD
Biochanin A	0	100.11 $\pm$ 0.001	0.071
	80	99.99 $\pm$ 0.013	0.912
	100	100.3 $\pm$ 0.021	0.321
	120	100.21 $\pm$ 0.013	0.730
5-Fluorouracil	0	99.92 $\pm$ 0.164	0.920
	80	100.27 $\pm$ 0.089	0.491
	100	100.55 $\pm$ 0.145	0.711
	120	100.79 $\pm$ 0.171	0.772

Table 5: Limit of detection (LOD) and limit of quantification (LOQ) of Biochanin A and 5-Fluorouracil utilizing proposed method.

Drug	Wavelength (nm)	LOD (µg/ml)	LOQ (µg/ml)
Biochanin A	229	0.064	0.193
5-Fluorouracil	265	0.314	0.953

3.3 Analysis of 5-Fluorouracil and Biochanin A in hydrogel formulation

This method was utilized to analyze 5-Fluorouracil and Biochanin A in combination in hydrogel system. The assay method confirmed 100.40 % and 103.10 % content of 5-Fluorouracil and Biochanin A in hydrogel system respectively (Table 6).

Table 6. Analysis of 5-Fluorouracil and Biochanin A in hydrogel formulation

Drug	Actual drug loaded in hydrogel (mg)	Estimated amount of drug in hydrogel (mg)	Drug in hydrogel (%)
5-Fluorouracil	10	10.04	100.42
Biochanin A	10	10.31	103.10

4. Conclusion

The established spectrophotometric method was observed to be precise, specific, linear and accurate to estimate 5-Fluorouracil and Biochanin A in optimized hydrogel system. Accuracy assessment findings confirmed the method's ability to precisely detect minor changes in drug concentration. Furthermore, the sensitivity of method was validated by low LOD and LOQ value of both drugs. Thus, developed method enabled simultaneous quantification of both drugs without interference.

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