RESEARCH PAPER

Development and Validation of a UV Spectrophotometric Method for the Simultaneous Estimation of 5-Fluorouracil and Xanthone with Application to Ultradeformable Liposomes for Cancer Therapy

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ABSTRACT

The objective of this research is to establish a precise, simple and rapid UV spectroscopic technique for the simultaneous estimation of 5-Fluorouracil (5-FU) and Xanthone (XAN) in a combination dosage form. The absorption maxima (λ_{max}) for 5-FU and XAN were recorded at 263 and 258 nm, respectively. Both drugs obey Beers' law within the concentration limits of 2-12µg/ml. A high degree of linearity was attained with correlation coefficient values of 0.9929 for 5-FU and 0.9977 for XAN. The technique was validated according to ICH Q2(R1) guideline exhibiting acceptable accuracy, precision, specificity, LOD of 1.2418 µg/ml and 1.2467µg/ml, while LOQ of 3.765µg/ml and 3.778µg/ml for 5-FU and XAN, respectively. Recovery study (%RSD) showed values ranging from 98 to 102.1% for 5-FU and from 82.3 to 90% for XAN. The results of the developed methods were statistically validated and exhibited consistent performance. The findings verify that this method is reliable and can be effectively implemented for the simultaneous determination of 5-FU and XAN, loaded in an ultradeformable liposome (UDLs) formulation. The established method is essential for quantifying the drugs and supporting formulation characterization parameters such as percent entrapment efficiency and drug release profile..

Keywords: 5-Fluorouracil; Xanthone; UV spectroscopic methods; Simultaneous estimation, Ultradeformable liposomes

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INTRODUCTION

5-Fluorouracil (5-FU) is a widely used antineoplastic agent prescribed for the treatment of several malignancies, such as breast, head, neck, colon, pancreas and stomach cancers[1-3]. 5-Fluoro-2, 4-dihydroxypyridine, commonly known as 5-FU, is a chemotherapeutic agent classified as a fluoropyrimidine antimetabolite. It exerts its anticancer effects primarily by disrupting DNA synthesis, resulting in suppression of tumor cell proliferation[4]. Xanthone (XAN), a natural polyphenol, has a broad spectrum of biochemical and pharmacological properties, including anti-leukemic, anti-inflammatory, anti-cancer, antioxidant, anti-atherosclerotic, anti-hypertensive, antiviral, anti-mutagenic and antidiabetic activity[5-7]. XAN anticancer effects are principally mediated by the induction of apoptosis, cell cycle arrest, suppression of tumor initiation and anti-invasive potential. 5-FU is freely soluble in water and methanol[8,9], whereas XAN is soluble in methanol but has a very low water solubility of 2.03104 mg/L[10,11]. The chemical identity of both drugs, 5-FU and XAN, is presented in **Figure 1** to elucidate a clear understanding of the molecular properties [12,13]. The hypothesis behind the combination of 5-FU with XAN markedly enhances the inhibition of cancer cell proliferation compared to their individual effects, illustrating a synergistic effect that improves therapeutic efficacy while potentially minimizing dose-dependent toxicity [1,14]. The in vitro and in vivo anticancer efficacy of a 5-FU and XAN combination in ultra-deformable liposomes (UDLs) is being investigated in our laboratory to enhance the biodistribution of UDLs compared to conventional liposomes.

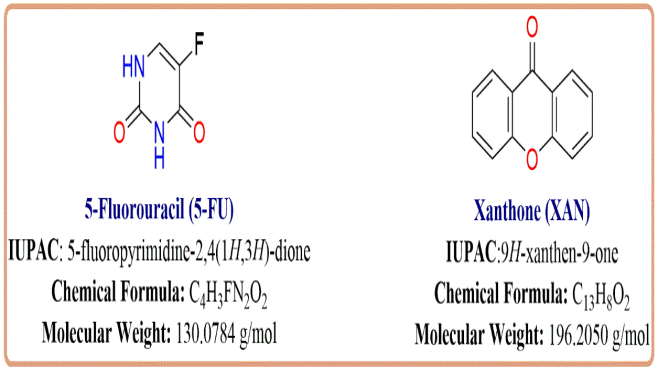

Figure 1. 5-FU and Xan chemical structure, IUPAC, Chemical formula and molecular weight [12,13]

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A review of the literature suggests that UV spectrophotometry has been effectively utilized to estimate both 5-FU and XAN individually. There are many methods reported for determining 5-FU when combined with other drugs [9,15–19]. There is currently no analytical method reported for the simultaneous estimation of 5-FU and XAN in mixed or combined dosage forms. This study aims to provide a cost-effective UV spectrophotometric technique for the simultaneous quantification of two drugs when dispensed in combination. The objective is to develop an ultra-deformable liposome (UDLs) formulation that integrates 5-FU and XAN as pharmacologically active compounds, advancing from conventional therapy to more advanced treatment regimens for skin cancer treatment. A combination of drugs is difficult to estimate and quantify the active ingredients in the formulation. Using UV spectrophotometry, simultaneous estimation can be performed using the derivative method in UV multicomponent analysis, specifically in cases involving mixtures with spectral overlap issues. UV spectrophotometry remains a widely used method due to its simplicity, specificity, rapid analysis and low operational cost [17, 20].

This research aimed to develop a rapid, economical, accurate and robust procedure for the simultaneous determination of 5-FU and XAN in topical UDL formulations. The in vitro and in vivo anticancer efficacy of a 5-FU and XAN combination in ultra-deformable liposomes (UDLs) is being investigated in our laboratory to enhance the biodistribution of both drugs compared to conventional liposomes. In comparison to sophisticated chromatographic techniques like HPLC and HPTLC, the proposed method offers a simpler and cost-effective alternative for the quantification of drugs [21,22]. The procedure was validated as per the ICH Q2(R1) guidelines [23,24]. To enhance quality assurance, an Ishikawa (fishbone) diagram was developed to evaluate possible causes and contributing factors of a problem or effect known as critical management practices (CMPs) (**Figure 2**) [25,26]. The successful application of developed and validated methods to UDLs highlights its importance in solving critical pharmaceutical challenges, providing a robust strategy for quality control and assurance in the simultaneous assessment of combination medications [27].

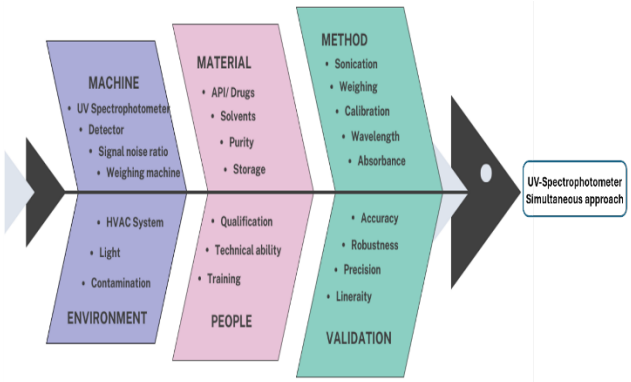

Figure 2. Ishikawa cause and effect diagram showing the method performance characteristics of the UV simultaneous estimation method development

2. MATERIALS AND METHODS

2.1 Materials

Pure 5-FU and XAN were purchased from Sigma Aldrich. Soya phospholipid was a kind gift from Lipoid GmbH, Germany. Tween 80, Ethanol (99%), and methanol were purchased from local suppliers. The other chemicals and reagents were of analytical grade and obtained from a local vendor.

2.2 Instruments

A digital balance machine (Contech CAI Series) was used to weigh samples accurately. Instrument used for method validation (Shimadzu UV-1900i Model with UV Probe software version 2.70), using two sets of quartz cuvettes (1 cm path length). The formulation characterization processes utilized a rotary evaporator (D Lab, Made in China), a magnetic stirrer (REMI), a centrifuge machine (NEYA 16R, REMI, Mumbai, India) and an ultrasonic bath sonicator from Borosil Scientific (UCB model).

2.3 Determination of λ_{max}

To produce a stock solution of 100 µg/ml, 10 mg of 5-FU and XAN were dissolved in separate 100 ml volumetric flasks and the volume was make up with methanol. 1 ml from the stock solution flask was added to a 10 ml volumetric flask to achieve a concentration of 10 µg/ml. Blank samples and previously prepared samples with 10 µg/ml concentration were scanned between 200 and 400 nm to determine the λ_{max} of both active constituents [17,28].

2.4 Preparation of working solution

Stock solutions of 5-FU and XAN were prepared in methanol individually by dissolving the required amount of each drug in a volumetric flask to get a final concentration of 100µg/ml [29].

2.5 Simultaneous equation method

The absorptivity values for 5-FU and XAN were determined in methanol within the concentration range of 2-12 µg/ml. Using a UV spectrophotometer, the maximum absorbance (λ_{max}) of 5-FU was observed at approximately 263 nm, while that of XAN was found at 258 nm. The concentration of the two drugs in combination can be estimated using the simultaneous equation method, as represented below [30,31].

C_{5FU} or $C_x = A_1\, ay_2 - A_2\, ay_1 / ax_1\, ay_2 - ax_2\, ay_1$

C_{XAN} or $C_y = A_1\, ax_2 - A_2\, ax_1 / ay_1\, ax_2 - ay_2\, ax_1$

Where 5-FU and XAN concentrations are expressed as C_x and C_y, respectively, a mixture of 5-FU and XAN (concentration from 2-12 µg/ml) [18]. The absorbance of two solutions at 263 and 258 nm is shown by A_1 and A_2, respectively. Where ax_1 and ax_2 represent the absorptivity of 5-FU at λ_1 and λ_2, respectively, and ay_1 and ay_2 represent the absorptivity of XAN at λ_1 and λ_2, respectively. At both wavelengths, the drug absorbance maxima were measured. The methodology of simultaneous equations was used to determine the concentration. All experiments were performed in triplicate (n = 3).

2.6. Validation

2.6.1 Linearity (calibration curve)

Calibration curves for 5-FU and XAN were established within a concentration range of 2 and 12 µg/ml. An aliquot standard solution of 2, 4, 6, 8, 10 and 12 µg/ml was

accurately prepared in 10 ml volumetric flasks. The absorbance of 5-FU and XAN was measured at their respective λ_{max} values of 263 and 258 nm using pure methanol as a blank. The calibration curve was created by plotting the absorbance versus concentration and the regression equations, along with the correlation coefficient (R^2), were calculated for both 5-FU and XAN [23,30].

2.6.2 Precision

For intra-day precision, 5-FU and XAN at different concentrations of 6, 8 and 10 µg/ml were analyzed three times on the same day at three-hour intervals [21]. Inter-day precision was determined by analyzing the same concentration (06, 08, and 10 µg/ml) across three distinct days. The accuracy of both intra- and inter-day studies was represented as the percentage relative standard deviation (%RSD) [23,32].

2.6.3 Detection Limit (LoD)

The LoD has been calculated using a group of six calibration curves that were analyzed to evaluate the method's linearity [33].

The limit of detection (LoD) was computed using the formula provided below:

LoD = 3.3*σ/S

Here, σ = relative std. deviation of the response, S = slope of the calibration curve [23].

2.6.4 Quantitation Limit (LoQ)

The limit of quantification (LoQ) was calculated using a group of six calibration curves that were analyzed to evaluate the method's linearity.

The LoQ was computed using the following formula:

LoQ = 10*σ/S

Here, σ = relative std. deviation of the response, S = slope of the calibration curve [23].

2.6.5 Accuracy

Absorbance values were recorded for various concentrations of 5-FU and XAN (06, 08, 10 µg/ml). The drug concentrations were subsequently determined using the linear regression equation derived from the calibration curve. produced. The accuracy percentage was evaluated using the following equation [33].

$$\% \text{ Accuracy} = \frac{\text{Experimental value}}{\text{True value}} \times 100$$

A total of three different runs were successfully completed.

2.6.6 Robustness

Three separate 5-FU and XAN (6, 8, 10 µg/ml) concentrations have been prepared and analyzed using various wavelengths. 5-FU was analyzed at 262 nm, 263 nm, and 264 nm, whereas the drug solution of XAN was examined at 257 nm, 258 nm, and 259 nm. The absorbance at various wavelengths was measured, and the % RSD was estimated [23].

2.6.7 IR data of standard drug

The Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectra were recorded using a Bruker Alpha II spectrophotometer equipped with an ECO ATR accessory. For analysis, each drug sample (5-FU and XAN) was triturated into a fine powder and scanned in % transmission mode over the spectrum range of 4000-400 cm^{-1} with an optimum resolution. The observed FTIR spectra were compared to the reference spectra of 5-FU and XAN to confirm the identity of the samples.

The ATR-FTIR spectra of 5-FU displayed characteristic peaks associated with its functional group, including a broad band between 3000-3500 cm^{-1} indicative of N-H stretching, a sharp peak at 1721 cm^{-1} corresponding to C=O called carbonyl stretching, a peak at 1645 cm^{-1} representing C=C stretching in the pyrimidine ring and a peak at 1233 cm^{-1} indicating C-N stretching within the cyclic structure. Additional peaks comprise C-H bending vibrations at 1416 cm^{-1} and less intense bands for C-H stretching at 1168 cm^{-1} as shown in **Figure 3** [12,34]. The FTIR spectra of pure XAN represent characteristic peaks at 1649 cm-1 (C=O ketone stretching), 1597 cm-1 (C=C aromatic ring stretching), and 1445 and 1327 cm-1, which correspond to C-O and C–O–C ether stretching vibrations. Peaks at 873 and 615 cm^{-1} due to ring deformation vibrations in the fingerprint region, which were consistent with reported IR data for XAN (**Figure 04**) [13,35].

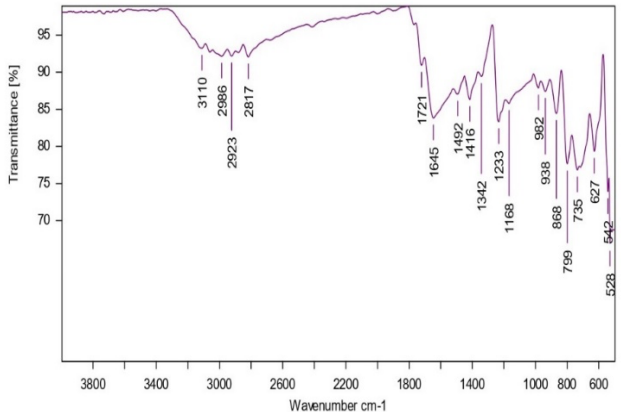

Figure 3. ATR FTIR spectra of 5-FU [12]

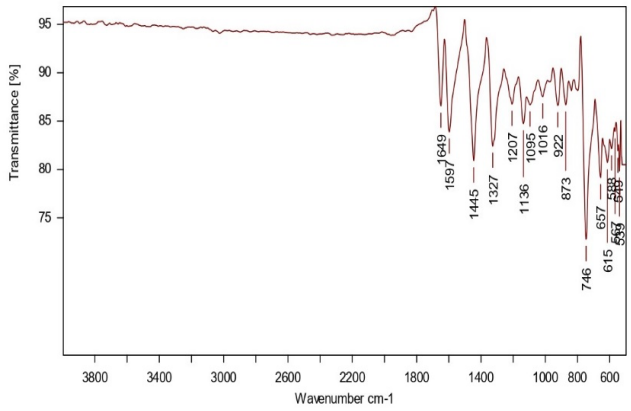

Figure 4. ATR FTIR spectra of XAN [13]

2.6.8 Application of the developed and validated method

2.6.8.1 5-FU and XAN entrapment efficiency by UDLs

The indirect method was employed for estimating the amount of 5-FU and XAN entrapped within the UDLs. The UDL formulation was centrifuged using a cooling centrifuge (NEYA 16R, REMI, Mumbai, India), maintained at a temperature of 4 °C and spun at 14,000 rpm for 30 minutes to separate the unentrapped drug. 1 ml of supernatant was examined for drug concentration using a UV spectrophotometer (Shimadzu UV-1900i) at 263 nm

and 258 nm for 5-FU and XAN, respectively [36]. The quantification was performed in triplicate using a previously validated simultaneous UV spectrophotometric method. The % EE have been determined using the following formula [15].

$$\% \text{ EE} = \frac{\text{Amount of total drug taken} - \text{Unentrapped drug}}{\text{Amount of total drug taken}} \times 100$$

Additionally, as characterization parameters of the prepared UDLs, particle diameter and polydispersity index (PDI) were assessed using a Litesizer 500 (Anton Paar, Australia) equipped with dynamic light scattering (DLS) technology. Particle size measurements were done using diluted dispersions at 25°C and with an angle of detection of 173°. Each sample was analyzed in triplicate (n = 3) and the results are presented as the mean ± standard deviation (SD). Additionally, the Litesizer was used to calculate the zeta potential, which reflects the particle electrophoretic mobility. The Litesizer evaluated zeta potential, which assesses the electrophoretic mobility of particles. TEM studies were performed to establish the surface morphology of the prepared UDLs. [37,38].

3. Result

A UV spectroscopic technique was developed and validated for the simultaneous estimation of 5-FU and XAN when used in combination therapy. The approach was simple, sensitive, accurate, robust, and precise in nature. The λ_{max} of 5-FU and XAN was determined using 10 µg/ml solution scanned across the wavelength range of 200-400 nm. The maximum absorbance peak was observed at 263 nm for 5-FU (**Figure 5)** and at 258 nm for XAN (Figure 6); comparative spectra of both drugs are shown in **Figure 7**.

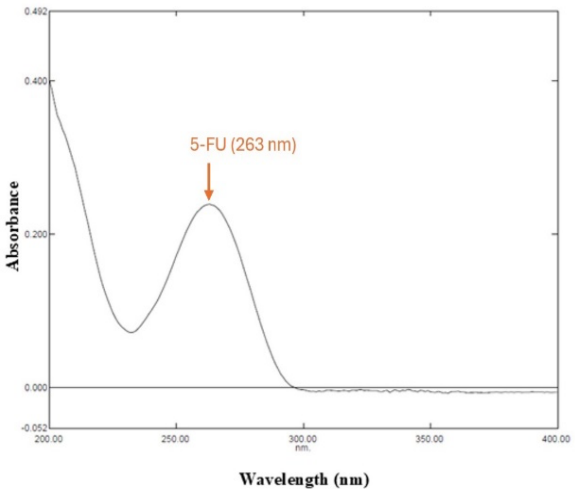

Figure 5. UV absorption spectra of 5-FU in methanol at 263 λ_{max}

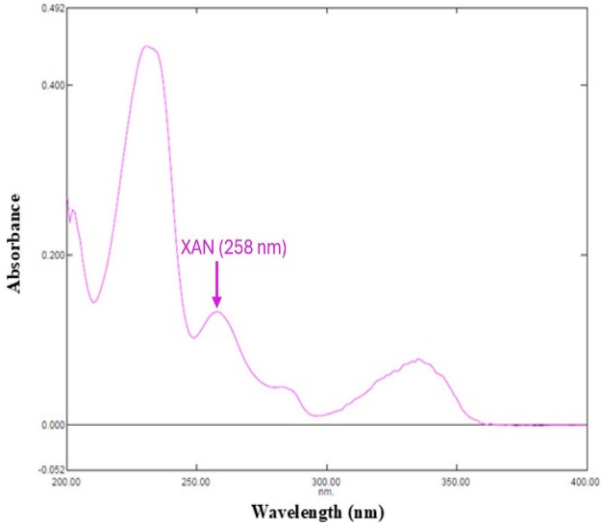

Figure 6. UV absorption spectra of XAN in methanol at 258 λ

max

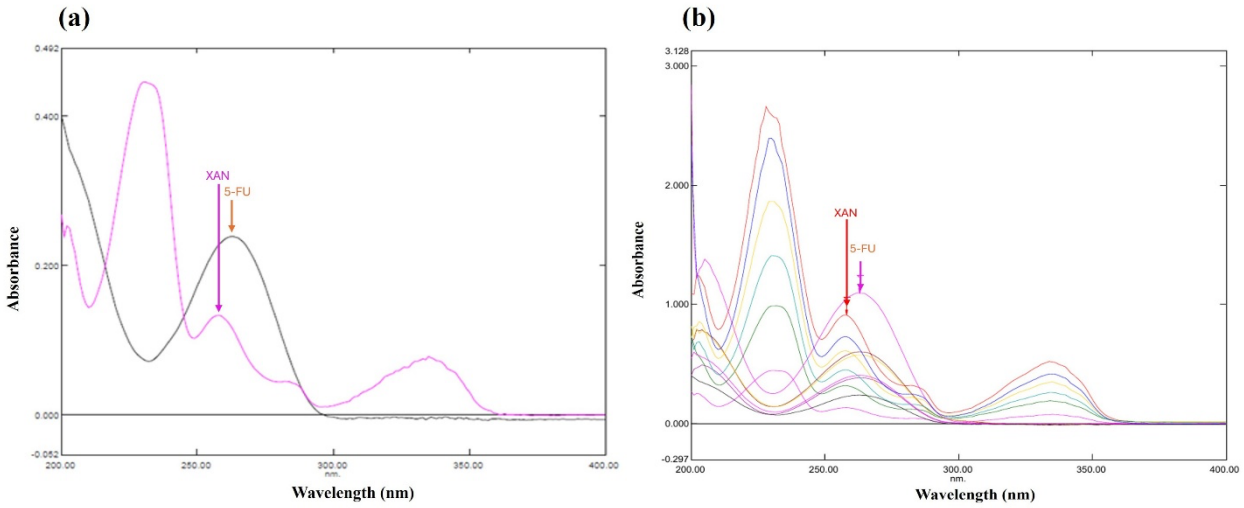

Figure 7. Comparative UV spectra of 5-FU and XAN in methanol: a) single concentration and b) multiple concentrations

3.1 Validation

3.1.1 Simultaneous estimation of 5-FU and XAN in methanol

A standard stock solution was prepared by mixing 5-FU and XAN at a concentration of 100 µg/ml. Using this stock solution, drug samples were prepared at concentrations ranging from 2 to 12 µg/ml. Next, the maximum absorption wavelengths of the 5-FU and XAN samples were measured at 263 (λ_{max} 1) and 258 (λ_{max} 2), respectively. The absorptivity coefficients of both drugs were calculated at these two wavelengths. For 5-FU, the absorptivity values were 0.0953 (a_{x1}) at λ_1 and 0.0774 (a_{x2}) at λ_2, whereas for XAN, the corresponding values were 0.0657 (a_{y1}) at λ_1 and 0.0763 (a_{y2}) at λ_2 were found to be 0.06571 (a_{y1}) and 0.0763 (a_{y2}), respectively. These values were subsequently converted into molar concentrations. The concentrations of 5-FU (C_x) and XAN (C_y) in the combination were determined to be 6.369 µg/ml and 9.076 µg/ml, respectively, using the simultaneous mathematical equations (**Table 1**) [17].

Table 1. UV spectrophotometer linearity data of 5-FU and XAN

S. No.	Conc. (µg/ml)	5-FU		XAN	
		263 nm	258 nm	263 nm	258 nm
1	2	0.243	0.221	0.132	0.153
2	4	0.404	0.389	0.271	0.313
3	6	0.558	0.474	0.408	0.471
4	8	0.696	0.544	0.531	0.616
5	10	0.839	0.56	0.62	0.724
6	12	1.025	0.684	0.776	0.908

*Data are expressed as mean ± SD

n=3; three repeated absorbance; SD: Standard deviation; D1: Drug 1; D2: Drug 2; 5-FU: 5-Fluorouracil; XAN: Xanthone; λ1: FU; λ2: XAN; A1: concentration of 5-FU; A2: concentration of XAN.

3.1.2 Linearity and range

Linearity was analyzed using standard solutions at six distinct concentrations ranging from 2 to 12 µg/ml for both 5-FU and XAN, in accordance with Beer's law. The absorbance of 5-FU and XAN for each solution was measured at λ_1 (263 nm) and at λ_2 (258 nm) for XAN. The calibration curves exhibited linearity with regression coefficients (R^2) of 0.9929 for 5-FU (**Figure 8**) and 0.9977 for XAN (**Figure 9**), signifying a robust relation within the examined range. The LoQ was determined as 3.765 µg/ml for 5-FU and 3.778 µg/ml for XAN, while the LoD were 1.2418 µg/ml and 1.2467 µg/ml, respectively, in accordance with the ICH guidelines. Additional validation parameters are summarized in **Table 2** [23].

Table 2. Analytical validation parameters of the UV spectrophotometric method for 5FU and XAN

Parameters	5-FU	XAN
Beer's Law limit (µg/ml)	2-12 µg/ml	2-12 µg/ml
λ_{max} (nm)	263	258
Correlation Coefficient (R^2)	0.9929	0.9977
Slope	0.0495	0.0083
Intercept	0.0814	0.0744
LoD (µg/ml)	1.2418	1.2467
LoQ (µg/ml)	3.765	3.778

5-FU: 5-Fluorouracil; XAN: Xanthone; λ_{max}: absorbance maxima; LoD: Limit of Detection; LoQ: Limit of Quantification.

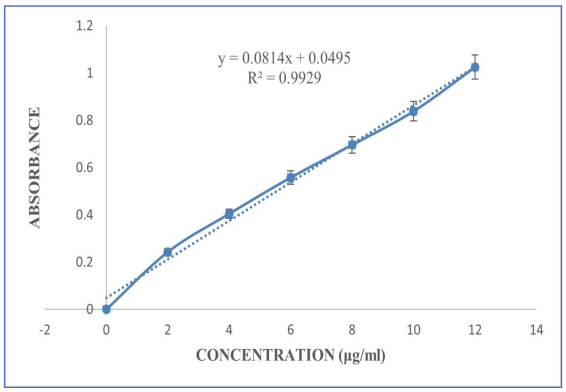

Figure 8. Calibration curve for 5-FU at 263 nm in methanol

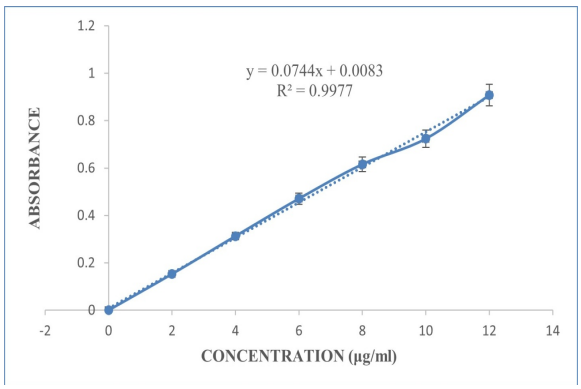

Figure 9. Calibration curve for XAN at 258 nm in methanol

3.1.3 Accuracy in recovery

The proposed analytical methods have demonstrated remarkable accuracy, with recovery rates of 98% and a low standard deviation (SD). Recovery of 5-FU and XAN was evaluated using the standard addition method. The percentage recovery was found to be 98-102.1 % for 5-FU and 82.3-90 % for XAN (**Table 3**) [39].

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Table 3. Accuracy as recovery data of 5-FU and XAN

Drug	True Concentration (µg/ml)	Absorbance	Experimental value	% Accuracy
5-FU	6	0.558	6.13	102.1
	8	0.696	7.94	98.75
	10	0.839	9.8	98
XAN	6	0.408	5.3	90
	8	0.531	7.06	89
	10	0.62	8.23	82.3

Data are expressed as mean ± SD (n=3)

3.1.4 Robustness

Robustness was evaluated by measuring absorbance at different wavelengths for 5-FU (263 nm), and XAN (258 nm). The %RSD values for samples with 10 µg/ml of 5-FU and XAN were determined to be 0.075241 and 0.733747, respectively, indicating the method's robustness (**Table 4**) [23].

Table 4. Robustness data of 5-FU and XAN

Drug	Conc. (µg/ml)	262	263	264	Mean	SD	% RSD
5-FU	6	0.563	0.564	0.562	0.563	0.001	0.17762
	8	0.684	0.685	0.684	0.684333	0.000577	0.084367
	10	0.767	0.768	0.767	0.767333	0.000577	0.075241

Different wavelength data for 5-FU at 263 nm

Drug	Conc. (µg/ml)	257	258	259	Mean	SD	% RSD
XAN	6	0.476	0.477	0.473	0.475333	0.002082	0.437938
	8	0.66	0.66	0.652	0.657333	0.004619	0.702658
	10	0.832	0.833	0.822	0.829	0.006083	0.733747

Different wavelength data for XAN at 258 nm

3.1.5 Precision

Precision data was evaluated in terms of repeatability, intraday and interday variations. The %RSD values for 5-FU and XAN at concentrations ranging from 6–10 µg/ml were within the permissible limits established by the ICH guidelines (>2%), indicating satisfactory repeatability (**Table 5**). In samples with a concentration of 10 µg/ml, the intraday precision exhibited a %RSD of 0.761464 for 5-FU and 0.39858 for XAN. While interday precision showed %RSD values of 1.434567 for 5-FU and 0.668467 for XAN. The results demonstrate that the proposed approach is accurate and reproducible (**Table 6**) [29].

Table 5. Displays the acceptable percentage % RSD for 5-FU and XAN, which were tested at different concentrations on three distinct days

Drug	Conc. (µg/ml)	Day 1	Day 2	Day 3	Mean	SD	% RSD
5-FU	6	0.569	0.573	0.575	0.571	0.003055	0.535035
	8	0.703	0.689	0.697	0.696	0.007024	1.009162
	10	0.764	0.775	0.773	0.7695	0.005859	0.761464
XAN	6	0.47	0.472	0.478	0.471	0.004163	0.883935
	8	0.647	0.65	0.662	0.6485	0.007937	1.22394
	10	0.806	0.807	0.812	0.8065	0.003215	0.39858

Three separate samples of three concentrations of a standard solution of XAN and 5-FU, A total of nine determinations were analyzed throughout three separate days. The first derivative absorbance was measured at 258 and 263 nm, and the % RSD was calculated.

Table 6. Displays the acceptable % RSD for 5-FU and XAN, which were tested at different concentrations on three distinct time intervals

Drug	Conc. (µg/ml)	12 PM	3 PM	6 PM	Mean	SD	% RSD
5-FU	6	0.54	0.53	0.549	0.539667	0.009504385	1.761158
	8	0.67	0.671	0.687	0.676	0.009539392	1.411153
	10	0.777	0.756	0.761	0.764667	0.010969655	1.434567
XAN	6	0.455	0.457	0.457	0.456333	0.001154701	0.253039
	8	0.633	0.632	0.649	0.634	0.009539392	1.504636
	10	0.762	0.769	0.772	0.767667	0.005131601	0.668467

Three separate samples of three concentrations of a standard solution of 5-FU and XAN, a total of nine determinations, were analyzed at different time intervals. The first derivative absorbance was measured at 258 and 263 nm, and the % RSD was calculated.

3.1.6 Applications of the validated method

The suggested approach was found to be adequate in analytical validation parameters. Entrapment efficiency studies of UDLs containing 5-FU and XAN were performed. The concentration of both drugs in the supernatant containing the entrapped drug was determined using the proposed simultaneous estimation method, and it was estimated to be 90 ± 0.56% for 5-FU and 75.77 ± 1.2%

for XAN in the formulation, respectively. These results suggest that the developed UDLs have a high capacity to simultaneously deliver both anticancer drugs effectively [40]. The optimized UDLs were analyzed for mean particle size, zeta potential, and surface morphology using TEM. The average particle size was 95.77 ± 1.12 nm, with a polydispersity index of 25.86 ± 1.5% (**Figure 10(a)**) [33]. The Zeta potential of the optimized formulation was -12.56 mV ± 0.98 (**Figure 10(b)**). TEM results revealed that the UDLs were spherical and nanoscale in size. **Figure 10(c)** displays the TEM images of the formulations, verifying the spherical morphology of the multidrug-loaded UDLs with no surface irregularities [41,42,43].

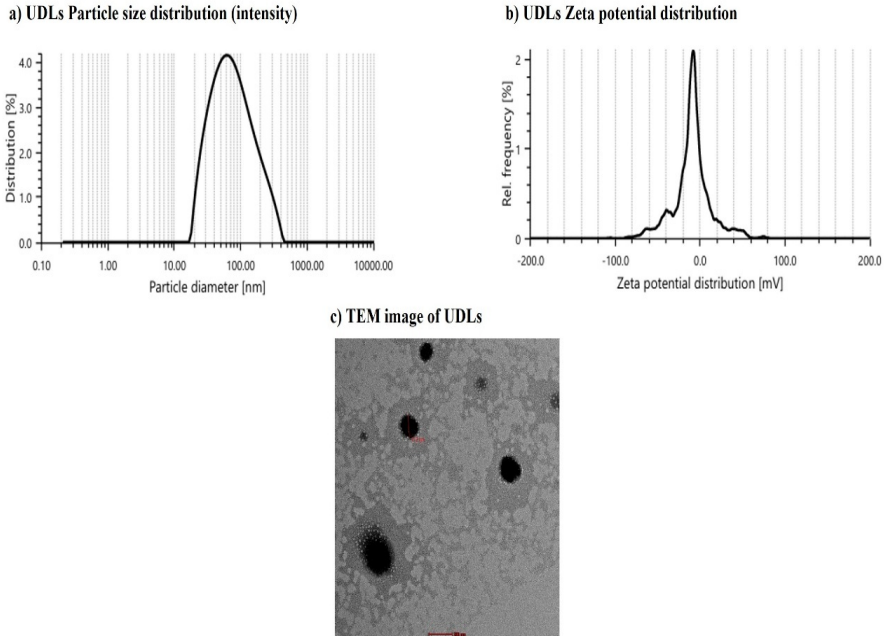

Figure 10. Fig 10. (a) Particle size of multidrug-loaded UDLs (XAN-5-FU-UDLs), (b) Zeta potential of multidrug-loaded UDLs (XAN-5-FU-UDLs) and (c) TEM image of multidrug-loaded UDLs (XAN-5-FU-UDLs)

4. DISCUSSION

The findings of this study include significant implications for pharmaceutical analysis, particularly in the context of skin cancer therapeutics as a multidrug treatment approach. A UV spectrophotometric technique for the simultaneous estimation of 5-FU and XAN was successfully developed and validated. The proposed method was simple, accurate, precise, robust, and reliable for measuring both active constituents in the combined sample solution. The low values of calculated SD and %RSD confirm the method's suitability, with all results remaining within the acceptable limit of less than 2% as per ICH guidelines. This approach enhances analytical efficiency by enabling the simultaneous estimation of drugs when administered in combination, thereby ensuring robustness for quality control and regulatory compliance. The successful application of this approach in characterizing UDL formulation highlights the method's adaptability in modern drug delivery research. Future investigations could explore the robustness of this method under manufacturing conditions to evaluate its applicability to additional combined formulations. Overall, the established UV spectrophotometric method's reliability, efficacy and versatility make it an essential tool for routine assessment and regulatory compliance. Moreover, the ability to apply this method to UDLs underscores its practical importance in advancing innovative formulations for effective skin cancer care.

5. CONCLUSION

The developed spectrophotometric approach proved to be linear, precise, specific, and accurate for estimating 5-FU and XAN in combination therapy. The approach was validated as per ICH Q2(R1) guidelines and successfully quantified both drugs. The method application in multidrug-loaded UDLs was established, demonstrating precision and accuracy in determining the %EE of both drugs. The low limits of detection and quantification for 5-FU and XAN confirmed the appropriateness of this method for assessing drug entrapment efficiency and formulation stability. The prepared UDL formulation is under investigation for the synergistic anticancer efficacy when given in combination. The validated method enables the simultaneous quantification of 5-FU and XAN in multidrug-loaded UDLs, supporting the in vitro characterization of drug release as one of the key parameters of physicochemical characterization and stability studies. This analytical technique can be effectively applied to pharmaceutical research and quality control for the routine evaluation of advanced drug delivery systems in the treatment of skin cancer.

Author contributions

Shalini Singh: Writing - original draft, Methodology. **Sanjeev Kumar Patel:** Writing - review & editing, formatting, graphical representation. **Swati Dubey and Geetika Sharma:** Data curation, Validation, Formal analysis, **Sunita Minz:** Validation Supervision, Software, Resources, Project administration, Methodology, Data curation, Conceptualization. This study is part of the research work conducted by the author, Shalini Singh, for her PhD thesis. All authors examined and approved the final version of the manuscript for publication.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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