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Development and Assessment of Cyclophosphamide-Loaded Microspheres for Enhanced Topical Drug Delivery

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ABSTRACT

This research focuses on the development and validation of cyclophosphamide-loaded microspheres for drug delivery. Cyclophosphamide, which is widely used in cancer therapy and immunosuppression, was analyzed using a newly established method with water as a diluent. The maximum absorption wavelength (λ_{max}) was identified at 263 nm. Various analytical parameters, including linearity, precision, accuracy, limits of detection, robustness, and ruggedness, were assessed. The drug showed linearity over the concentration range of 0.4–1.4 µg/mL, with a correlation coefficient of 0.999. The formulated microspheres showed 99.3% drug content. Recovery studies at 80%, 100%, and 120% confirmed accuracy, with values ranging between 99% and 100%. The precision analysis showed an % RSD value within the acceptable range (< 2), confirming the reliability of the method. Additionally, robustness and ruggedness assessments were performed at different wavelengths and by multiple analysts. This UV-spectrophotometric technique provides a reliable, cost-efficient, and time-saving approach for validating cyclophosphamide microspheres in pharmaceutical applications.

Keywords: Cyclophosphamide, HPLC, Cancer therapy, Drug analysis, UV-spectrophotometry, Microspheres

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Introduction

Cancer is a group of diseases characterized by uncontrolled cell growth, which can spread to other parts of the body. Risk factors include tobacco use, which accounts for 25% of cancer-related deaths, as well as obesity, poor diet, physical inactivity, and excessive alcohol consumption, contributing to another 13% [1]. Other contributing factors include exposure to radiation, environmental pollutants, toxic chemicals, infections, and genetic predispositions. Any factor that disrupts normal cell proliferation can potentially lead to cancer.

Symptoms of cancer vary depending on the type and stage but often include unexplained weight loss, persistent pain, skin changes, abnormal bowel or bladder function, unusual bleeding, chronic cough, fever, and the presence of lumps or masses [2].

Skin cancer occurs when abnormal skin cells grow uncontrollably, potentially spreading to other parts of the body. The three primary types of skin cancer are melanoma, squamous cell carcinoma, and basal cell carcinoma. The latter two, along with some rarer forms, are categorized as non-melanoma skin cancers (NMSC). While basal cell carcinoma progresses slowly and may damage nearby tissues, it rarely spreads or causes fatality. Unlike malignant melanoma, most non-melanoma skin cancers are localized and seldom metastasize [3].

Cyclophosphamide, a chemotherapy drug, is widely used to treat various cancers, including lymphoma, leukemia, multiple myeloma, ovarian cancer, breast cancer, neuroblastoma, small-cell lung cancer, and sarcomas. It can be administered orally or intravenously and is also used as an immunosuppressant following organ transplants and for nephrotic syndrome. Common side effects include low white blood cell count, appetite loss, vomiting, hair loss, and hemorrhagic cystitis [4].

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Cyclophosphamide plays a key role in adaptive immunotherapy, where it enhances immune responses through:

- Elimination of regulatory T cells (CD4+CD25+ T cells) in tumor-bearing and healthy individuals.
- Facilitating the expansion of tumor-reactive T cells by creating immunological space.
- Promoting the activity of T-cell stimulatory agents, such as type I interferons.

Cyclophosphamide pre-conditioning has been used to enhance adoptive T-cell therapy, vaccination strategies, and tumor-specific immunity. Other medications used for skin cancer treatment include Aldara (Imiquimod), Efudex (5-FU), and Erivedge (Vismodegib) [5].

Microspheres are spherical particles, typically ranging from the micron to nanometer scale, composed of biodegradable polymers that encapsulate active substances such as drugs or antibodies for controlled release [6].

This study aims to develop a microsphere formulation containing cyclophosphamide for the treatment of skin cancer. Additionally, the physicochemical properties of the formulated microspheres were evaluated using spectroscopy, and the proposed method was validated following ICH guidelines.

Materials and Methods

Chemicals and solvents

Cyclophosphamide was obtained from Cipla Pharmaceutical PVT (Sigma Aldrich). Other essential chemicals, including starch, agar, acetic acid, ethanol, and ethyl cellulose, were provided by the institution. All chemicals and reagents used in this study were of analytical grade.

Apparatus

Various laboratory instruments were used, including a burette stand, capillary ignition tube, beaker, ultra bath sonicator, hot air oven, digital melting point apparatus, particle size zeta potential analyzer, and a UV-visible spectrophotometer (1800 double beam) with a 1 cm matched quartz cell.

Experimental procedures

Pre-formulation Study of cyclophosphamide

UV-spectroscopy

Stock solution preparation

A 10 mg sample of cyclophosphamide was weighed and placed in a 25 mL volumetric flask. A small amount of solvent was added, and the solution was sonicated for 5–10 minutes. The volume was then adjusted to 10 mL with water, resulting in a stock concentration of 100 µg/mL.

Preparation of working standard solution and dilution

To prepare the working solution, 1 mL of the stock solution was diluted to 100 mL, yielding a final concentration of 10 µg/mL. From this solution, five different dilutions were prepared, with one concentration (0.6 µg/mL) selected from a range of 0.2–1.4 µg/mL.

Determination of maximum wavelength (λ_{max})

The working standard solution was scanned in the UV spectrum (200–400 nm) using water as a blank. The peak absorption wavelength (λ_{max}) was then recorded.

Determination of melting point, solubility, and partition coefficient

The melting point of cyclophosphamide was determined using a digital melting point apparatus. Solubility was assessed by determining the maximum concentration of the drug that dissolves in a specific solvent under controlled conditions. The partition coefficient was measured by equilibrating equal volumes of an organic solvent and an aqueous drug solution and then analyzing the drug distribution between the two phases [7].

Microsphere preparation

Microspheres are a widely used drug delivery system in cancer treatment, enhancing therapeutic effects while reducing adverse reactions. Cyclophosphamide microspheres were prepared using an emulsion solvent evaporation method.

In this process, 1.5 g of ethyl cellulose was dissolved in 15 mL of ethyl acetate in a 100 mL beaker. Separately, 0.5 mL of Tween 80 was dissolved in 300 mL of water under continuous stirring at 700 RPM. The ethyl cellulose solution was then added drop by drop into the aqueous phase while stirring for 30–40 minutes. The mixture was left undisturbed for 48 hours, and the resulting microspheres were analyzed under an optical microscope [8].

Characterization of microspheres

Particle size and zeta potential

Microspheres are surrounded by a liquid layer, consisting of an inner Stern layer (where ions are tightly bound) and a more diffuse outer region. The zeta potential represents the electrostatic potential at the boundary of these layers and plays a crucial role in particle stability [9]. The zeta potential of the prepared microspheres was determined using a Malvern Zetasizer.

FTIR analysis

Fourier transform infrared (FTIR) spectroscopy was employed to analyze the molecular structure and chemical composition of the microspheres. This technique detects infrared light absorption at specific frequencies corresponding to molecular vibrations. Each bond in a molecule absorbs at distinct frequencies, producing a characteristic spectrum. The FTIR spectra of the microspheres were recorded using the KBr press pellet method and scanned within a wavelength range of 400–4000 cm^{-1} [10].

Microsphere validation

The developed microspheres underwent validation following ICH guidelines to ensure their accuracy, reliability, and reproducibility.

Linearity and calibration curve

To evaluate linearity, a series of dilutions (0.4–1.4 µg/mL) were prepared from the working standard solution. Absorbance measurements were taken using a UV-visible spectrophotometer at 260 ± 265 nm, with purified water serving as the blank and solvent. A calibration curve was generated by plotting concentration (X-axis) against absorbance (Y-axis). The correlation coefficient, Y-intercept, and coefficient of determination (r^2) were calculated to confirm the linearity of the method [11].

Accuracy assessment

Accuracy refers to how closely the measured results align with the actual concentration of the analyte. This was determined through a recovery study, where a known amount of analyte was added to the sample, and the percentage recovered was calculated. The method's accuracy was verified by assessing the percentage recovery, ensuring the validity of the analytical technique [12].

Precision analysis

Precision testing, based on ICH Q2 (R1) standards, was performed to confirm the consistency of results over time. Two types of precision studies were conducted:

- Intra-day precision: The sample was analyzed three times within a single day at hourly intervals.
- Inter-day precision: The same procedure was repeated over three separate days to check for consistency.

Standard deviation (SD) and relative standard deviation (RSD) were computed to evaluate the precision of the method. A low RSD value indicated minimal variability, confirming the method's reliability.

$$\% \text{ Relative standard deviation} = \text{Standard deviation/mean} \times 100 \quad (1)$$

Limit of detection and limit of quantification

The limit of quantitation (LOQ) is the lowest amount of drug in a sample that can be quantified, and the limit of detection (LOD) is the lowest amount of drug in a sample that can be detected, according to ICH recommendations. LOD and LOQ are calculated by:

$$LOD = 3.3\sigma / S \ \& \ LOQ = 10\sigma / S \quad (2)$$

Validation of microspheres

The microsphere formulations were validated according to ICH guidelines to ensure their reliability and consistency across various analytical conditions.

Robustness and ruggedness

Robustness and ruggedness evaluate the method's ability to yield consistent and reproducible results under varying laboratory conditions. This includes different operators, instruments, reagents, environmental conditions, and days of testing. A robust method ensures stability and reliability across all these variables.

Range determination

The analytical range is defined as the interval between the lowest and highest analyte concentrations where the method demonstrates acceptable accuracy, precision, and linearity. The range was established according to ICH Q1 (R2) guidelines to confirm the method's suitability for analyzing cyclophosphamide.

Assay of marketed cyclophosphamide formulation

To determine drug content in commercial formulations, 10 tablets of Cytoxan (500 mg) were weighed, and an amount equivalent to 500 mg of cyclophosphamide was dissolved in a small quantity of water in a 25 mL volumetric flask. The volume was adjusted to 25 mL. From this solution, 2.5 mL was taken and further diluted to 25 mL with water. Finally, 0.8 mL of the working solution was taken and diluted to 10 mL with methanol. The absorbance of three separate dilutions was then recorded using a UV-visible spectrophotometer.

Results and Discussion

Pre-formulation study

The maximum absorption wavelength (λ_{max}) of cyclophosphamide was recorded at 263 nm using UV spectroscopy. Solubility analysis showed that cyclophosphamide dissolves well in water, ethanol, and methanol while exhibiting limited solubility in chloroform, acetone, and ether. Based on these findings, purified water was selected as the solvent for analytical studies. The melting point was determined to be 43 °C. The partition coefficient of cyclophosphamide, measured using a biphasic hydrophobic-hydrophilic system, was found to be 3.13, aligning with official pharmacopeial standards.

Microsphere preparation and characterization

The formulated cyclophosphamide microspheres exhibited a spherical, non-aggregated morphology, with an average particle size of 102 µm. Zeta potential analysis, a key parameter in evaluating colloidal stability, was conducted to determine electrostatic interactions within the dispersion. The zeta potential value was measured at -27 mV, indicating stable dispersion.

Table 1 provides a comprehensive list of materials used in the microsphere preparation process.

Table 1. Preparation of microspheres

S. no.	Name of ingredient	Quantity
1.	Cyclophosphamide	50 mg
2.	Ethyl acetate	15 ml
3.	Acetone	5 ml
4.	Tween 80	0.5 ml
5.	Distilled Water	300 ml

The FTIR spectra of microspheres revealed the major peaks were 3450, 1150, 1440, 875, and 2670 cm^{-1} **(Figure 1)**. This suggests the presence of functional groups like N-H, P=O, CH_2-Cl. FTIR spectrum was recorded between 4000 and 400 cm^{-1}.

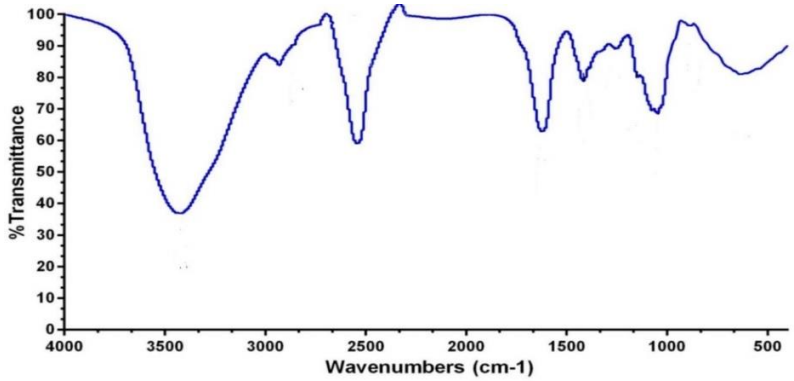

Figure 1. FTIR spectra of microspheres of CP

Method validation

The developed method was validated as per ICH guidelines Q2 (R1) for its linearity, precision, limit of detection, limit of quantification, accuracy, ruggedness, and robustness. The results of all validation parameters are shown in **Table 2**.

Table 2. Optimized and validated parameters of cyclophosphamide

Sr. no.	Parameters		Results
1	Linearity		0.999
2	Accuracy		0.64
3	Precision	Repeatability	0.67
		Intraday	0.86
		Interday	0.97
4	LOD		0.047938766
5	LOQ		0.145268988
6	Robustness (% RSD < 2%)		1.48
7	Ruggedness (% RSD < 2%)		0.88
8	Standard regression equation		y = 0.1167x + 0.0145
9	Slope		0.1167
10	Intercept		0.0145
11	% Assay of CP		99.3

Linearity

The linearity of cyclophosphamide was observed in the concentration range of 0.4 to 1.4 µg/ml, with a correlation coefficient of 0.999. The calibration curve for cyclophosphamide in water at 263 nm is displayed in **Figure 2**. The detailed linearity data of cyclophosphamide is presented in **Table 3**.

Table 3. Linearity of cyclophosphamide at λ_{max} of 263 nm

Sr. no.	Concentration (µg/ml)	Absorbance (263 nm)
1	0.4	0.062
2	0.6	0.083
3	0.8	0.109
4	1	0.131
5	1.2	0.153
6	1.4	0.179
Standard regression equation		y = 0.1167x + 0.0145
R^2		0.9992

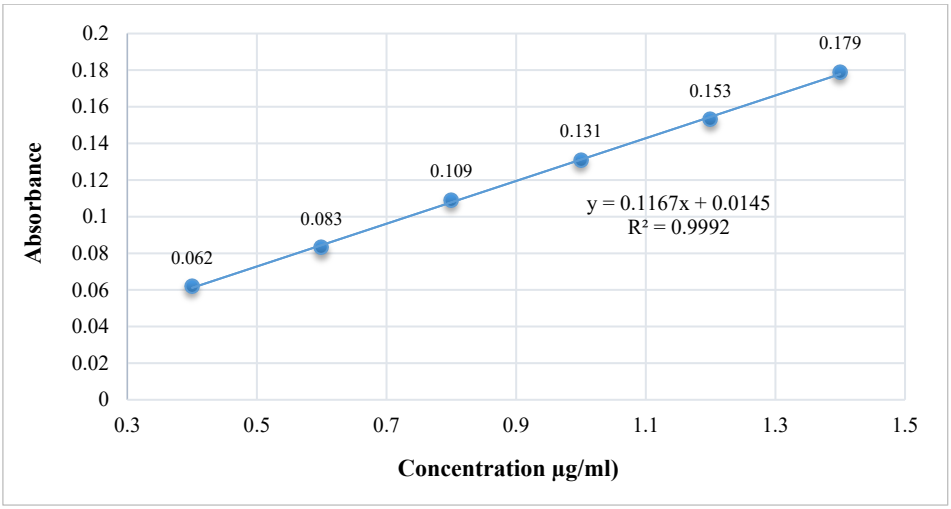

Figure 2. Calibration curve of cyclophosphamide

Accuracy

The accuracy of the developed method was evaluated by calculating the percentage recovery at three different levels of addition: 80%, 100%, and 120%. The recovery percentage for cyclophosphamide ranged from 99% to 100% **(Table 4)**.

Table 4. Characteristics of accuracy study

Concentration (µg/ml)	Conc. before add	Conc. of std.	Conc. spiking	% Recovery	SD	RSD
6	5.9624	2(80%)	7.99			
	6.0924	2(80%)	8.01	99.5	0.06	0.76
	5.9129	2(80%)	7.89			
6	5.9621	4(100%)	9.923			
	6.1923	4(100%)	10.024	99.52	0.93	0.94
	6.0249	4(100%)	9.934			
6	5.9126	6(120%)	11.924			
	6.1924	6(120%)	11.874	99.36	0.029	0.24
	5.9942	6(120%)	11.992			
Mean of % RSD			0.64			
Average % Recovery			100%			

Precision

The precision of cyclophosphamide was assessed through inter-day, intra-day, and repeatability studies, yielding % RSD values of 0.86%, 0.97%, and 0.67%, respectively. These results are well within the acceptable limit of < 2%. The precision tests were conducted using HPLC analysis, with inter-day and intra-day results shown in **Table 5**.

LOD and LOQ

The limit of detection (LOD) represents the concentration where the signal is equal to the blank mean plus three times the blank's standard deviation. The limit of quantification (LOQ) corresponds to the concentration where the signal equals the blank mean plus ten times the blank's standard deviation. For cyclophosphamide, the LOD was 0.0479 µg/ml, and the LOQ was 0.01452 µg/ml.

Ruggedness and robustness study

The ruggedness and robustness of the method were assessed by a single analyst at a 0.8 µg/ml concentration using two different wavelengths. The study results are summarized in **Table 5**.

Table 5. Evaluation of precision, robustness, and ruggedness

Intraday precision							
S.N	**Concentration (µg/ml)**	**Peak area**			**Mean**	**SD**	**% RSD**
		10 am	2 pm	4 pm			
1	0.4	0.062	0.065	0.062	0.063	0.001	2.75%
2	0.6	0.083	0.082	0.083	0.082	0.0005	0.70%
3	0.8	0.109	0.108	0.109	0.108	0.0005	0.53%
4	1	0.131	0.132	0.131	0.131	0.0005	0.44%
5	1.2	0.153	0.153	0.154	0.153	0.0005	0.38%
6	1.4	0.179	0.18	0.179	0.179	0.0005	0.35%
	Std. deviation				0.00058		
	% RSD				0.86%		
Interday precision							
S.N	**Concentration (µg/ml)**	**Peak area**			**Mean**	**SD**	**% RSD**
		1 day	2nd day	3rd day			
1	0.4	0.062	0.061	0.063	0.062	0.001	1.61%
2	0.6	0.083	0.084	0.083	0.083	0.0005	0.69%
3	0.8	0.109	0.108	0.107	0.108	0.001	0.93%
4	1	0.131	0.135	0.132	0.131	0.002	1.57%
5	1.2	0.153	0.154	0.153	0.153	0.0057	0.38%
6	1.4	0.179	0.181	0.181	0.18	0.0011	0.64%
	Std. deviation					0.0018	
	%RSD					0.97%	

Robustness and ruggedness							
Wavelength (nm)	1	**2**	**3**	**Mean**	**SD**	**RSD**	**Mean % RSD**
263	0.062	0.054	0.053	0.06	0.001	2.53	1.48%
	0.083	0.081	0.083	0.082	0.001	1.4	
	0.109	0.108	0.108	0.108	0.0005	0.53	
265	0.131	0.134	0.131	0.132	0.001	1.31	0.88%
	0.153	0.154	0.153	0.153	0.0005	0.38	
	0.179	0.182	0.182	0.181	0.001	0.96	

*SD = mean recovery, and RSD = relative standard deviation

Assay of marketed formulation

The marketed cyclophosphamide tablets were assessed to determine the percentage of active ingredients. The analysis revealed that the formulation's label claim was accurate, with the percentage recovery found to be 99.3%, demonstrating the reliability of the method used.

Conclusion

The developed analytical technique, validated using UV Spectroscopy and HPLC as per ICH Q2 (R1) standards, proved to be robust and accurate. The method showed high precision and linearity, with low relative standard deviations, indicating that it can be used for reliable and consistent cyclophosphamide analysis in pharmaceutical preparations. This approach is suitable for routine quality control in pharmaceutical manufacturing and testing. The method can also be adapted for further studies, including in vivo investigations to explore cyclophosphamide's anticancer potential. Additionally, this UV-visible spectrophotometry method offers a more cost-effective and simpler alternative to more complex techniques like LC/MS and HPLC, making it ideal for widespread use in both research and clinical settings.

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Conflict of Interest: None

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Ethics Statement: None

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