



## FORMULATION, EVALUATION, AND VALIDATION OF MICROSPHERES OF CYCLOPHOSPHAMIDE FOR TOPICAL DELIVERY

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### ABSTRACT

Cyclophosphamide is a chemotherapy medication used to treat cancer. It is also used to suppress the immune system. The present investigation deals with the preparation of microspheres containing cyclophosphamide. The method has been developed and validated for the estimation of cyclophosphamide using water as a diluent. The  $\lambda_{max}$  of cyclophosphamide in water was found to be 263 nm. The parameters, range, linearity, accuracy, precision, LOD, LOQ, robustness, and ruggedness were determined. The drug cyclophosphamide showed linearity in the concentration range from 0.4-1.4  $\mu\text{g/ml}$  with a correlation coefficient value of 0.999. The proposed method was applied to pharmaceutical formulation and the percentage amount of drug estimated was found to be 99.3%. The accuracy of the method was checked by a percentage recovery experiment at three levels, i.e., 80%, 100%, and 120%. The percentage recovery was found to be in the range of 99 $\pm$ 100%. The precision of the method was studied as day variation. The % RSD value within the official limit (<2) indicates that the method is precise and accurate. The robustness and ruggedness of the proposed methods were studied on two different wavelengths with the help of analysts. The UV-spectrophotometric method was found to be secure and useful for the calculation of microsphere-loaded Cyclophosphamide. The novelty of this research is based on a safe, simple, and effective method that is cost-effective and time-saving for the validation of cyclophosphamide.

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### Introduction

A set of illnesses known as cancer involve abnormal cell growth and have the potential to spread to other body areas. 25% of cancer fatalities are attributable to cigarette usage. A further 13% is brought by obesity, a bad diet, inadequate exercise, and heavy alcohol use [1]. Some diseases, radiation exposure, and environmental contamination are additional concerns. Toxic or chemical compound exposures, ionizing radiation, some infections, human genetics, etc. are major categories of cancer-related or causative agents. Anything that might cause a normal proliferation of cells to develop abnormally potentially could cause cancer.

Cancer symptoms and indicators vary depending on the exact grade of cancer, but individuals with various malignancies may experience the following: weight loss, pain, skin changes, altered bowel or bladder function, unusual bleeding, persistent cough or fever, lumps or tissue masses, etc [2].

Skin cancer is the growth of abnormal cells with the potential to invade or spread to other body parts. Three main categories of skin cancer exist; Melanoma, Squamous-cell carcinoma, and Basal-cell carcinoma.

Non-melanoma skin cancer is also referred to as the first two, along with a handful of relatively uncommon skin cancers (NMSC).

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Basal-cell cancer grows slowly, and can harm nearby tissue, but is not expected to spread to surrounding areas or cause death. Skin cancers are typically malignant (cancerous) skin growths that cause localized destruction. They are produced from the epidermal cells of the skin's uppermost layer. Contrary to cutaneous malignant melanoma, the vast majority of these skin tumors rarely metastasize and are rarely life-threatening [3].

A chemotherapy drug called cyclophosphamide is used to treat many malignancies. Cyclophosphamide (CP), commonly known as cytophosphane among other names, is a drug that is employed in chemotherapy and immune system suppression. Lymphoma, multiple myeloma, leukemia, ovarian cancer, breast cancer, small cell lung cancer, neuroblastoma, and sarcoma are all treated with it during chemotherapy. It is taken orally or administered intravenously after organ transplantation and is used in nephritic syndrome as an immune suppressant. The majority of people have side effects. Low white blood cell counts, loss of appetite, vomiting, hair loss, and bladder hemorrhage are typical side effects [4].

In adaptive immunotherapy, cyclophosphamide produces positive immunomodulatory effects. The following processes have been proposed:

- Deletion of T regulatory cells (CD4+CD25+ T cells) in tumor-bearing and naive hosts
- Extended grafting of adoptively transferred, tumor-reactive effector T cells by the construction of an immunologic space niche;
- Promising T cell growth agents, such as type I IFNs.

To boost adoptive T cell immunotherapy regimens, active vaccination techniques, and immunity in naive hosts, cyclophosphamide pre-conditioning of recipient hosts (for donor T cells) has been used. This stimulates objective antitumor immunity.

Aldara (Imiquimod) and Efudex 5-FU Erivedge (Vismodegib) (Fluorouracil—Topical) are medications used to treat skin cancer (Fluorouracil-Topical).

Microspheres are defined as spherical shells with very small diameters, typically in the micron or nanometer range [5]. They are typically made of a biodegradable or reasonable plastic polymer and are frequently filled with a substance (such as a drug or antibody) for release as the shell degrades [6].

The goal of the current study was to create a cyclophosphamide-containing microsphere formulation to treat skin cancer. Additionally, utilizing spectroscopy, the physicochemical characteristics of the generated microspheres were assessed, and the proposed method's validity was carried out under ICH recommendations.

## Materials and Methods

### *Chemical and Solvents*

Cyclophosphamide (drug) was purchased from Cipla Pharmaceutical PVT. [Sigma Aldrich]. Starch, agar, acetic acid, ethanol, ethyl cellulose, and other chemicals were provided by the Institution. All the chemicals and reagents used in the study were of analytical grade.

### *Apparatus*

Burette stand, capillary ignition tube, beaker, ultra bathsonicator machine, hot air oven, digital melting point apparatus, partial size zeta potential, UV-visible spectrophotometer 1800 double beam with 1cm matched quartz cell.

### *Experimental Work*

- *Pre-formulation Study of Cyclophosphamide*

#### *UV-Spectroscopy*

#### *Stock Solution Preparation*

10 mg cyclophosphamide was weighed & transferred into a 25 ml volumetric flask, then a small amount of solvent was added into a volumetric flask and sonicated for 5-10 minutes, and the final volume up to 10 ml was adjusted with water to obtain the 100µg/ml.

#### *Preparation of Working Standard Solution & Dilution*

From the standard stock solution, 1 ml was transferred into a volumetric flask and diluted up to 100 ml to get a concentration of about 10µg/ml. From the working stock, five dilution solutions were prepared of any one of the concentrations (0.6µg/ml) selected from the 0.2-1.4µg/ml concentration range.

#### *Determination of Maximum Wavelength ( $\lambda_{max}$ )*

The working standard solution of the drug was scanned in the UV range of 200 to 400 nm in normal mode, using water as blank. Then obtained peaks were noted and the absorption maxima were determined.

#### *Determination of Melting Point, Solubility, and Partition Coefficient*

The melting point of cyclophosphamide was calculated using digital melting point equipment. The solubility of any medicine is defined as the highest concentration of the drug that can completely dissolve in a given solvent at a specific temperature and pressure level. Shaking equal amounts of two immiscible liquids (one is the organic layer, and the second is an aqueous layer,

which is saturated with water, and the aqueous drug solution) until equality is achieved can be used to estimate a drug's partition coefficient. The value was estimated based on the amount of the substance in one layer [7].

- *Preparation of Microspheres*

Microspheres are widely used in treatment strategy and therapy for cancer as they are formulated in a way using technology that leads to maximum therapeutic activity with minimum side/adverse effects. To date, various chemotherapeutic and cytotoxic drugs are available as microspheres delivery systems. In the present work; microspheres of cyclophosphamide were prepared and characterized. For the preparation; 1.5gm of ethyl cellulose was dissolved in 15ml of ethyl acetate in a 100ml beaker. In another beaker, 0.5 ml of tween 80 was dissolved in 300ml of water with continuous stirring at 700 RPM. Then solutions of ethyl cellulose were added into the water phase drop by drop with proper mixing for 30-40 minutes followed by continued stirring. After that solution was kept aside for 48 hours and then observed under the optical microscope [8].

- *Characterization of Microspheres*

- Particle Size and Zeta Potential*

An inner field (Stern layer), where the ions are tightly linked, and an outer (diffuse) region, where they are less strongly coupled, make up the liquid layer that surrounds the particle. There is a hypothetical border within the diffuse layer where the ions particle and other particles come together to form a stable entity. Ions within the border move when a particle moves (for instance, due to gravity). Beyond the range, the ions remain with the bulk dispersion. Zeta potential is the name given to the potential at this boundary (surface of hydrodynamic shear) [9]. Malvern Zetasizer was used to determine the microspheres' zeta potential.

- FTIR*

To ascertain the structure of specific molecules and the makeup of molecular mixtures, academic laboratories, and the pharmaceutical sector use Fourier Transform Infrared (FTIR) spectroscopy [10]. To examine a sample, FTIR spectroscopy used modulated mid-infrared energy. At particular frequencies that are precisely related to the atom-to-atom vibrational energy in the molecule, infrared light is absorbed. The bond can absorb that energy when the bond energy of the vibration and the energy of the mid-infrared light are equal. A molecule's many bonds vibrate at various energies, which causes them to absorb IR radiation at various wavelengths. The frequency and strength of each of these separate absorption bands contribute to the overall spectrum, giving the molecule a unique fingerprint. The FTIR spectra of microspheres were recorded by KBr pellet technique and scanning from 400 – 4000cm<sup>-1</sup>

- Validation of Microspheres*

The prepared microspheres were validated as per ICH guidelines.

- *Linearity and Calibration Curve*

The dilutions were prepared in the range of 0.4 to 1.4µg/ml from the working standard solution. The samples were scanned for absorbance by the use of the UV-Visible spectrophotometer using purified water as a blank and solvent at 260±265 nm. The graph between the concentration X-axis and mean response Y-axis was plotted for the drug. The r<sup>2</sup>-value, Y-intercept, and correlation coefficient were calculated [11].

- *Accuracy*

The degree to which the test result is close to its genuine value defines how accurate a method of analysis is. By analyzing the known additional amount of analyte, accuracy can be given as a percentage of recovery. The percentage of analyte recovered by the test can be used to calculate the analytical method's accuracy by adding a sample with a known, fixed concentration to the standard [12].

- *Precision*

According to ICH Q2 (R1) precision test was to be performed to get compatible results with changes in time. In this parameter, the sample was measured at least 3 times on the same day at intervals of an hour and on 3 different days for inter- and intra-day study. The standard deviation (SD) and relative standard deviation (RSD) were calculated using the formula. The precision value is determined by:

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

- *Limit of Detection & 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 & LOQ are calculated by:

$$\text{LOD} = 3.3\sigma / S \text{ \& LOQ} = 10\sigma / S$$

(2)

Where;  $\sigma$  = the standard deviation of response

S = the slope of the calibration curve.

- *Ruggedness and Robustness*

The robustness and ruggedness of an analytical method is the ability to produce consistent findings under a wide range of typical test settings, including those involving various labs, analysts, instruments, mobile phases, reagents, temperatures, days of the week, etc.

- *Range*

The interval between the upper and lower concentration of analyte in the sample for which it has been established that the analytical technique has a sufficient level of accuracy, precision, and linearity is referred to as an analytical procedure's range following the ICH Q1 (R2) recommendation.

- *Assay of Marketed Formulation*

Average weights of 10 tablets of cyclophosphamide (Cytoxan 500mg) were taken. Then small amounts of the drug were taken from average wt. which was equivalent to 500mg of the drug and dissolved with a small amount of water into a 25 ml volumetric flask. The volume was made up to 25 ml. 2.5 ml from this stock solution was taken and the volume was made up to 25 ml with water. Finally, 0.8 ml was taken from the working stock solution and the volume was made up to 10 ml with methanol. Three dilutions were prepared then the absorbance of the solutions was measured.

## Results and Discussion

- *Pre-Formulation Study*

The absorption maximum of CP as determined by UV was found to be 263 nm. The solubility of cyclophosphamide was found in water, ethanol, and methanol; and slightly soluble in chloroform, acetone, and ether. Therefore, for the entire analytical study purified water was selected as the solvent. The melting point was found to be 43 C<sup>0</sup>. A two-phase system hydrophobic (top) and hydrophilic (bottom) was used for measuring the partition coefficient of CP and it was found to be 3.13. These results complied with official pharmacopeia.

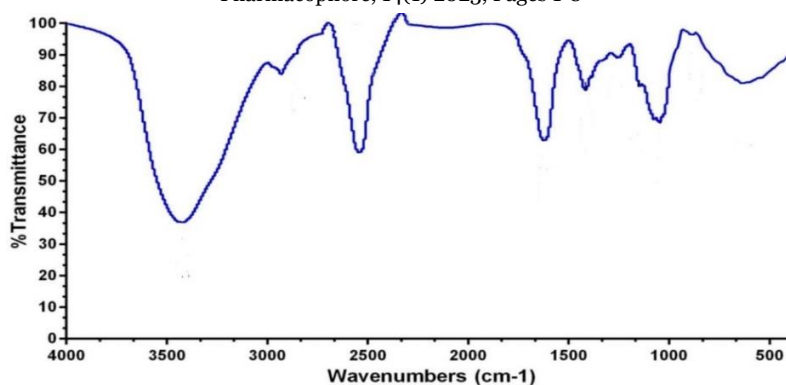
- *Preparation and Characterization of Microspheres*

The CP microspheres that were created were uniformly spherical, non-aggregated, and on average 102  $\mu\text{m}$  in size. Zeta potential is a crucial sign of colloidal dispersions' stability. The size of the zeta potential represents the strength of electrostatic attraction between nearby particles with identical charges in dispersion. Zeta potential was discovered to be -27 mV. **Table 1** is a list of the materials used in the preparation of microspheres.

**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 peak were 3450 $\text{cm}^{-1}$ , 1150 $\text{cm}^{-1}$ , 1440 $\text{cm}^{-1}$ , 875 $\text{cm}^{-1}$ , and 2670 $\text{cm}^{-1}$  (**Figure 1**). This suggest the presence of functional group like N-H, P=O, CH<sub>2</sub>-Cl. FTIR spectrum was recorded between 4000 and 400  $\text{cm}^{-1}$



**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 were shown in **Table 2**.

**Table 2.** Optimized and validated parameters of Cyclophosphamide

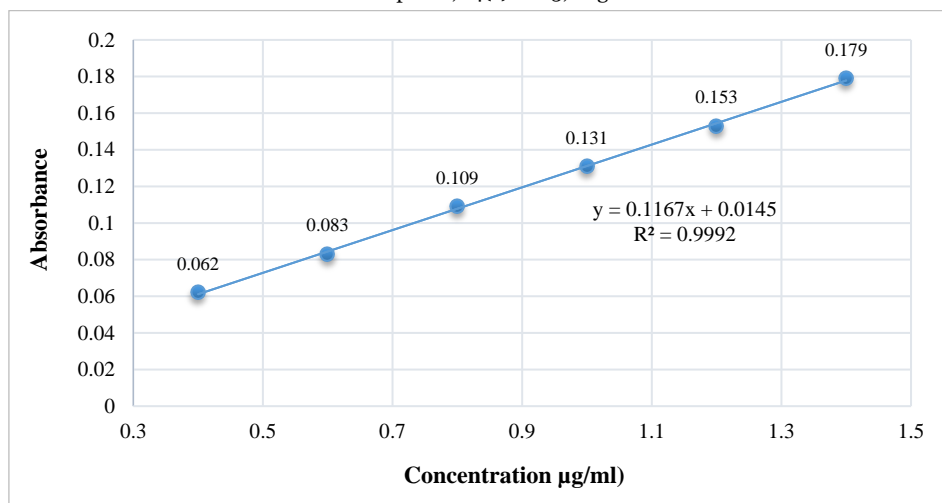
Sr.No	Parameters	Results	
1	Linearity	0.999	
2	Accuracy	0.64	
3	Precision	Repeatability	0.67
		Intraday	0.86
		Interday	0.97
4	LOD	0.047938766	
5	LOQ	0.145268988	
6	Robustness (% RSD<2%)	1.48	
7	Ruggedness (% RSD<2%)	0.88	
8	Standard Regression Equation	$y = 0.1167x + 0.0145$	
9	Slope	0.1167	
10	Intercept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

#### • Linearity

The linearity of cyclophosphamide was found to be in the range of 0.4 to 1.4  $\mu\text{g/ml}$  with a correlation coefficient of 0.999. The calibration curve of Cyclophosphamide in water at 263 nm is shown in **Figure 2**. The linearity of Cyclophosphamide was shown in **Table 3**.

**Table 3.** Linearity of Cyclophosphamide at  $\lambda_{\text{max}}$  of 263 nm

S.N.	Concentration( $\mu\text{g/ml}$ )	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 estimated by % recovery of the method at the three-level of percentage addition 80%, 100%, and 120%. The % recovery of Cyclophosphamide was found to be in the range of 99% - 100% and shown in **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	99.5	0.06	0.76
	6.0924	2(80%)	8.01			
	5.9129	2(80%)	7.89			
6	5.9621	4(100%)	9.923	99.52	0.93	0.94
	6.1923	4(100%)	10.024			
	6.0249	4(100%)	9.934			
6	5.9126	6(120%)	11.924	99.36	0.029	0.24
	6.1924	6(120%)	11.874			
	5.9942	6(120%)	11.992			
Mean of % RSD				0.64		
Average % Recovery				100%		

#### Precision

The %RSD of Cyclophosphamide during inter-day, intra-day, and repeatability precision study were found to be; 0.86, 0.97, and 0.67 %, respectively, and these values were found to be within the official limit (<2%). The precision study was measured by HPLC analysis. The results of inter-day and intra-day precision are depicted in **Table 5**.

#### LOD and LOQ

The detection limit (LOD) is defined by the Analytical Methods Validation as the concentration of drug analyst corresponds to the signal equal to the blank mean plus three times the blank standard deviation. The quantification limit (LOQ) of a drug analysis is the concentration that corresponds to the blank mean plus 10 times the blank standard deviation. Cyclophosphamide's LOD and LOQ values were determined to be 0.0479 and 0.01452 g/ml, respectively.

#### Ruggedness and Robustness Study

The evaluation data for the ruggedness and robustness study (single Analyst) at 0.8µg/ml concentration under two different wavelengths. The result is shown in **Table 5**.

**Table 5.** Evaluation of Precision, Robustness and Ruggedness

Intraday precision							
S.N	Concentration (µg/ml)	Peak Area			Mean	SD	% RSD
		10am	2 pm	4pm			
1	0.4	0.062	0.065	0.062	0.063	0.001	2.75%

S.N	Concentration (µg/ml)	Peak Area			Mean	SD	% RSD
		1day	2nd day	3rd day			
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
		1day	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, RSD - Relative Standard Deviation

#### Assay of Marketed Formulation

The marketed cyclophosphamide tablet formulations were analyzed for percentage purity by assay. The formulation label claim meets with the claim as the performed accuracy shows a percentage recovery of 99.3 %.

#### Conclusion

Through UV Spectroscopy and HPLC, the current analytical method was validated per ICH Q2 (R1) guidelines, leading to unique approval criteria. The analysis approach was found to be precise, accurate, linear, detached, and usable for standard quality control analysis of cyclophosphamide as per the results of the aforementioned methodologies. The relative standard deviation (RSD) for every parameter was found to be less than one, indicating the validity of the method and that the assay results are also within the allowable range. As a result, the proposed method can be used for routine quantitative simultaneous estimation of the drugs in pharmaceutical preparation. The work may also be investigated for an in vivo animal investigation to estimate anticancer activity, followed by kinetics and dynamics measurements. Additionally, it can be expanded to include routine CP analysis in pharmaceutical firms, hospitals, and research labs. The UV-visible spectrophotometer apparatus is simple and inexpensive, in contrast to the LC/MS and HPLC techniques. However, because the method is simpler and user-friendly, it may be considered superior to the previously published methods.

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**Ethics statement:** None

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