Chapter 3

Analytical Methods

TABLE OF CONTENTS

3.1 METHOD OF ESTIMATION OF PACLITAXEL BY RP-HPLC.....67

3.2 METHOD OF ESTIMATION OF CYCLOPHOSPHAMIDE BY RP-HPLC 68

3.3 VERIFICATION OF METHODS.....69

3.3.1 Verification of HPLC method for determination of Paclitaxel 70

3.3.1.1 Linearity and Range.....70

3.3.1.2 Specificity.....72

3.3.1.3 Accuracy.....73

3.3.1.4 Precision (Repeatability) 74

3.3.1.5 Limit of Detection (LOD) and Limit of Quantification (LOQ).....74

3.3.2 Verification of HPLC method for determination of Cyclophosphamide 75

3.3.2.1 Linearity and Range.....75

3.3.2.2 Specificity.....77

3.3.2.3 Accuracy.....78

3.3.2.4 Precision (Repeatability) 78

3.3.2.5 Limit of Detection (LOD) and Limit of Quantification (LOQ).....79

REFERENCES.....81

ANALYTICAL METHODS

3.1 METHOD OF ESTIMATION OF PACLITAXEL BY RP-HPLC

Method for determination and estimation of Paclitaxel in Paclitaxel and Cyclophosphamide loaded NLCs and Microemulsion was adopted from USP Monograph of Paclitaxel Injection and same was evaluated for developed NLCs and Microemulsion [1].

Mobile Phase Preparation: To make 1L of mobile phase, 550 mL of water was mixed with 450 mL Acetonitrile into suitable bottle and degassed prior to use.

Diluent Preparation: Transferred 200µL of glacial acetic acid into 1L of volumetric flask containing about 500mL of methanol, mixed well and volume was made up to 1L with methanol.

Preparation of standard Solution: Accurately weighed and transferred about 12.0 mg of Paclitaxel API into a 20 mL of volumetric flask, added 14 mL of diluent, dissolved it followed by sonication for 15minutes and diluted to volume with diluent. Solution was mixed properly.

Sample preparation (~600 ppm): Accurately transferred 2.0 g of sample solution to a 20 mL volumetric flask, added about 14mL of diluent, dissolved it followed by sonication for 15minutes and further diluted to volume with diluent. The solution was filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial.

Chromatographic parameters

Instrument: HPLC, Shimadzu
Detection wavelength: 227nm
Column: Thermo FLUOPHASE PFP, (250 x 4.6) mm, 5µm, (Thermo)
Column temperature: 45°C
Flow rate: 1.5mL/minute
Injection volume: 100µL
Running Time: 15minutes

Procedure: The column was equilibrated with mobile phase till the stable baseline was obtained. After the stable baseline was obtained, chromatographic system was injected with blank (diluent), standard solution followed by samples of NLCs and microemulsion and responses were recorded.

3.2 METHOD OF ESTIMATION OF CYCLOPHOSPHAMIDE BY RP-HPLC

Method for determination and estimation of Paclitaxel in Paclitaxel and Cyclophosphamide loaded NLCs and Microemulsion was adopted from USP Monograph of Cyclophosphamide Injection and same was evaluated for developed NLCs and Microemulsion [2].

Mobile Phase Preparation: To make 1L of mobile phase, 700 mL of water was mixed with 300 mL Acetonitrile into suitable bottle and degassed prior to use.

Diluent Preparation: 10% in Methanol in chilled water for Injection was used as diluent.

Preparation of standard Solution: Accurately weighed and transferred about 10.0 mg of Cyclophosphamide API into a 20 mL volumetric flask, added 14 mL of diluent, dissolved it and diluted to volume with diluent. Solution was mixed properly.

Sample preparation (~500 ppm): Accurately transferred 2.0 g of sample solution to a 20 mL volumetric flask, added about 14mL of diluent, dissolved it and further diluted to volume with diluent. The solution was filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial.

Chromatographic condition

Instrument: HPLC

Detection wavelength: 195nm

Column: C18, 10µ 125 A°, 300 × 3.9mm ID, L41 stainless steel column

Column temperature: 25°C

Flow rate: 1.5mL/minute

Injection volume: 100µL

Running Time: 15 minutes

Procedure: The column was equilibrated with mobile phase till the stable baseline was obtained. After the stable baseline was obtained, chromatographic system was injected with blank (diluent), standard solution followed by samples of NLCs and microemulsion and responses were recorded.

3.3 VERIFICATION OF METHODS

The verification of both the methods was considered using only PAC-CYC NLCs. NLCs contain solid lipid and liquid lipid/ oil whereas microemulsion contain only liquid lipid /oil. Therefore, NLCs turn out to be worst case due to the presence of solid lipid and liquid lipid/ oil in NLCs posing a probable higher risk of interference during the estimation of Paclitaxel and Cyclophosphamide by HPLC when compared to microemulsion.

Following parameters were performed for verification of the method:

(1) Specificity: Specificity denotes the capacity of an analytical method to distinctly evaluate analytes amidst potential interfering substances [3-5]. It is crucial for ensuring that the method can accurately measure the target analytes without interference from other substances [3-5].

(2) Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [3-7].

(3) Precision (Repeatability): The precision of an analytical technique denotes the degree of agreement (scatter) across a set of measurements derived from multiple samples of the same identical sample under specified conditions [3].

(4) Linearity and Range: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analytes in the sample [10]. The range of an analytical technique is the range between the highest and lowest concentrations (amounts) of analytes in the sample (including these values) for which the preciseness, accuracy, and linearity of the analytical procedure have been shown to be adequate [3].

(5) LOD and LOQ: The detection limit of a specific analytical method refers to the minimum concentration of analytes present in a sample that can be identified, although it may not be quantified with precision [3]. The quantitation limit of an individual analytical procedure is the lowest amount of analytes in a sample which can be quantitatively determined with suitable precision and accuracy [3].

LOD and LOQ were measured as follows:

$$\text{LOD} = \frac{3.3 \times \sigma}{S} \quad \text{and LOQ} = \frac{10 \times \sigma}{S}$$

Where,

σ = standard deviation of y intercept of three calibration curves

S = mean of slope of three calibration curves

3.3.1 Verification of HPLC method for determination of Paclitaxel

3.3.1.1 Linearity and Range

Procedure: The linearity of Paclitaxel was studied from 0.1µg/mL to 100µg/mL and Linearity equation was derived. PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL was used for determining the linearity and range. Different volumes of PAC-CYC NLCs were diluted to 10mL in a volumetric flask with the diluent followed by sonication for 15minutes to achieve the sample concentration as 0.1, 0.5, 1, 10, 20, 50 and 100µg/mL for determining the linearity and range. The sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded.

Results and Discussion: The results of Linearity of Paclitaxel are presented in Table 3.1 and graphical representation of Linearity is presented in Figure 3.1 whereas overlay of linearity chromatograms is presented in Figure 3.2.

Table 3.1: Results of Linearity of Paclitaxel

Concentration (mcg/mL)	Average	SD
0.1	13747	681
0.5	71008	1956
1	139698	1691
10	1415680	21035
20	2820252	33065
50	7080795	112464
100	14050009	198847

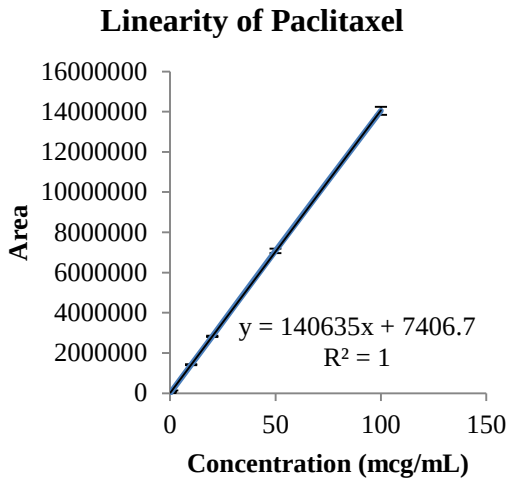

Figure 3.1: Graph of Linearity of Paclitaxel

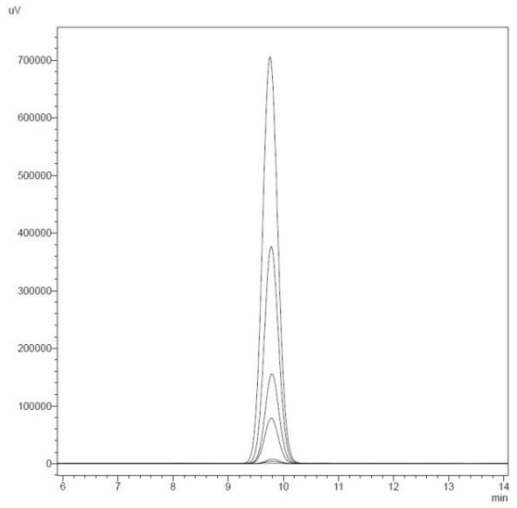

Figure 3.2: Overlay of Linearity chromatograms of Paclitaxel

Based on the results of linearity of Paclitaxel, Paclitaxel followed linear equation at 0.1µg/mL to 100µg/mL with the linearity coefficient (R^2) as 1. The linearity equation generated has slope of 140635 and intercept of 7406.7. Linearity is a critical parameter in method validation as per ICH guidelines, which require that the method's response is directly proportional to the analytes concentration [3].

A high degree of linearity with an R^2 of 1 supported the reliability and reproducibility of the method, ensuring consistent results across different batches and conditions [10, 11]. The range is crucial for establishing the linearity of the method, which is the ability to obtain test results that are directly proportional to the concentration of analytes in the sample [9]. The method was able to produce proportionate results from 0.1µg/mL to 100µg/mL of Paclitaxel.

3.3.1.2 Specificity

Procedure:

PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL and PAC-CYC microemulsion containing Paclitaxel at 3mg/mL and Cyclophosphamide 26.25mg/mL were used for determining the specificity. PAC-CYC NLCs, Placebo NLCs, PAC-CYC microemulsion, and Placebo microemulsion were diluted to 10mL in a volumetric flask respectively with the diluent followed by sonication for 15 minutes for determining the specificity.

The concentration of Paclitaxel was achieved as 50µg/mL for NLCs and microemulsion samples. All the sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded.

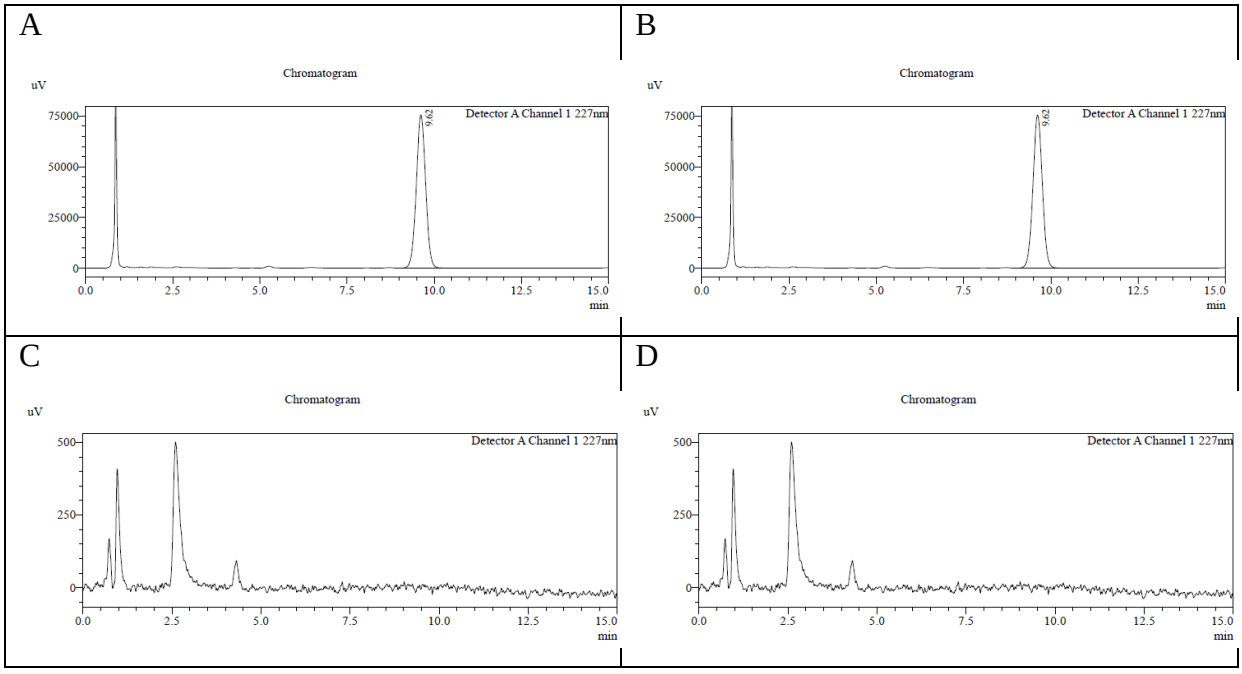

Figure 3.3: Chromatograms for Specificity [A] Paclitaxel in NLCs [B] Paclitaxel in Microemulsion [C] Placebo NLCs [D] Placebo Microemulsion

Results and Discussion:

The chromatogram in Figure 3.3 clearly indicates that no supplementary peaks were observed at the retention time of Paclitaxel in both NLCs and microemulsion [6]. Therefore method was specific to Paclitaxel and was able to identify Paclitaxel in both NLCs and microemulsion [5].

3.3.1.3 Accuracy

The accuracy of the method was determined by calculating the recoveries of Paclitaxel by the standard addition method [7, 8].

Procedure:

PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL was used for determining the accuracy. In a volumetric flask containing 3mL of diluent, PAC-CYC NLCs was added, further standard solutions of pure Paclitaxel were spiked in known quantities at 50%, 100%, and 150% levels were ultimately diluted to 10mL respectively with the diluent followed by sonication for 15 minutes for determining the accuracy. All the sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded.

Results and Discussion:

The results of accuracy and % recovery are presented in Table 3.2.

Table 3.2: Results of accuracy and % recovery of Paclitaxel

Amount Taken (µg/mL)	Amount added (% of original amount)	Total drug content (µg/mL)	Area obtained	Concentration obtained (µg/mL)	%Recovery
20	0	0	2829447	20.00	N.A.
20	50	30	4234173	29.93	99.8
20	100	40	5668994	40.07	100.2
20	150	50	7026509	49.67	99.3
				Accuracy	**99.8**
				Standard Deviation	**0.4**
				Relative Standard Deviation (RSD) (%)	**0.4**

Accuracy is typically expressed as a percentage of recovery, with acceptable ranges often between 98% and 102% for pharmaceutical applications [6, 7]. The results in Table 3.2 indicate that the method demonstrated high accuracy, with a % recovery of 100%, falling within the anticipated range of 98.0 to 102.0%, and an RSD below 1.

3.3.1.4 Precision (Repeatability)

Procedure:

PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL was used for determining the precision (repeatability). In a volumetric flask containing 3mL of diluent, PAC-CYC NLCs was added and further diluted to 10mL respectively with the diluent followed by sonication for 15 minutes. All the sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded. Repeatability was assessed using 6 determinations at 50µg/mL of the test concentration of Paclitaxel.

Results and Discussion:

The results of repeatability of Paclitaxel with 6 replicates are presented in Table 3.3.

Table 3.3: Results of Repeatability of Paclitaxel

Replicate No.	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
Area	7073618	7061325	7010105	7005772	7033265	7029320
Average	**7035567.42**					
SD	**27172.39**					
RSD (%)	**0.39**					

The repeatability data in Table 3.3 indicate an RSD of 0.39, which is below 2.0%. The RSD is a statistical metric employed to evaluate repeatability. It is derived from the S.D. of repeated measurements divided by the mean of those measurements, represented as a percentage. A low RSD value, particularly below 2%, indicated high precision in the analytical method. This precision is essential for ensuring that repeated measurements under unchanged conditions yield consistent results [8, 9]. Therefore method was concluded to be precise.

3.3.1.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Procedure:

The intercepts for three calibration curves in linearity were used to determine the LOD and LOQ of the method.

Results and Discussion:

Standard deviation of the intercepts for three calibration curves in Linearity was calculated and LOD and LOQ results are tabulated in Table 3.4.

Table 3.4: Results of LOD and LOQ of Paclitaxel

Parameter	Intercepts				Standard deviation	LOD (µg/mL)	LOQ (µg/mL)
	Curve 1	Curve 2	Curve 3	Average			
Results	16205	15509	21526	17747	3291	0.08	0.23

The LOD and LOQ are critical parameters in the validation of analytical methods, particularly in HPLC. LOD denotes the minimal concentration of an analyte that can be identified, though it may not be quantifiable, whereas LOQ signifies the minimal concentration that can be quantitatively assessed with acceptable levels of precision and accuracy [3]. Based on the results presented in Table 3.4, the Limit of Detection for Paclitaxel was obtained as 0.08µg/mL and Limit of Quantification was obtained as 0.23µg/mL which confirms that method is sensitive enough to detect low levels of Paclitaxel in formulation [6, 9].

3.3.2 Verification of HPLC method for determination of Cyclophosphamide

3.3.2.1 Linearity and Range

Procedure:

The linearity of Cyclophosphamide was studied from 0.5µg/mL to 100µg/mL and Linearity equation was derived. PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL was used for determining the linearity and range. Different volumes of PAC-CYC NLCs were diluted to 10mL in a volumetric flask with the diluent followed by sonication for 15 minutes to achieve the sample concentration as 0.5, 1, 2.5, 5, 10, 20, 50 and 100µg/mL for determining the linearity and range. The sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded.

Results and Discussion:

The results of Linearity of Cyclophosphamide are presented in Table 3.5. Graphical representation of Linearity is presented in Figure 3.4 whereas overlay of linearity chromatograms is presented in Figure 3.5.

Table 3.5: Results of Linearity of Cyclophosphamide

Concentration (mcg/mL)	Average Area	SD
0.5	6295	173
1	14257	392
2.5	37322	1026
5	70910	1949
10	146811	4036
20	304007	8358
50	766890	21083
100	1515191	41656

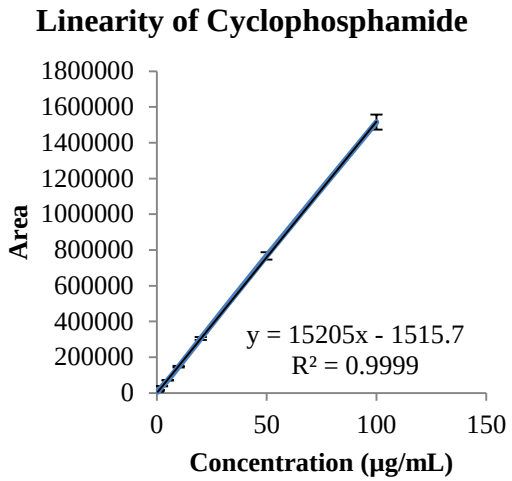

Figure 3.4: Graph of Linearity of Cyclophosphamide

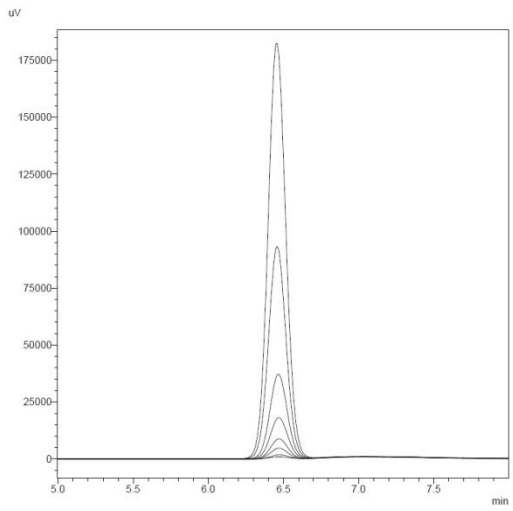

Figure 3.5: Overlay of Linearity chromatograms of Cyclophosphamide

Based on the results of linearity, Cyclophosphamide followed linear equation from 0.5µg/mL to 100µg/mL with the linearity coefficient (R^2) as 0.9999. The linearity equation generated has slope of 15205 and intercept of 1515.7. Linearity is a critical parameter in method validation as per ICH guidelines, which require that the method's response is directly proportional to the analytes concentration [3].

A high degree of linearity with an R^2 of 0.9999 supported the reliability and reproducibility of the method, ensuring consistent results across different batches and conditions [10, 11]. The range is crucial for establishing the linearity of the method, which is the ability to obtain test results that are directly proportional to the concentration of analytes in the sample [9]. The method was able to produce proportionate results from 0.1µg/mL to 100µg/mL of Paclitaxel.

3.3.2.2 Specificity

Procedure:

PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL and PAC-CYC microemulsion containing Paclitaxel at 3mg/mL and Cyclophosphamide 26.25mg/mL were used for determining the specificity. PAC-CYC NLCs, Placebo NLCs, PAC-CYC microemulsion, and Placebo microemulsion were diluted to 10mL in a volumetric flask respectively with the diluent followed by sonication for 15 minutes for determining the specificity.

The concentration of Cyclophosphamide was achieved as 50µg/mL for NLCs and microemulsion samples. All the sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded.

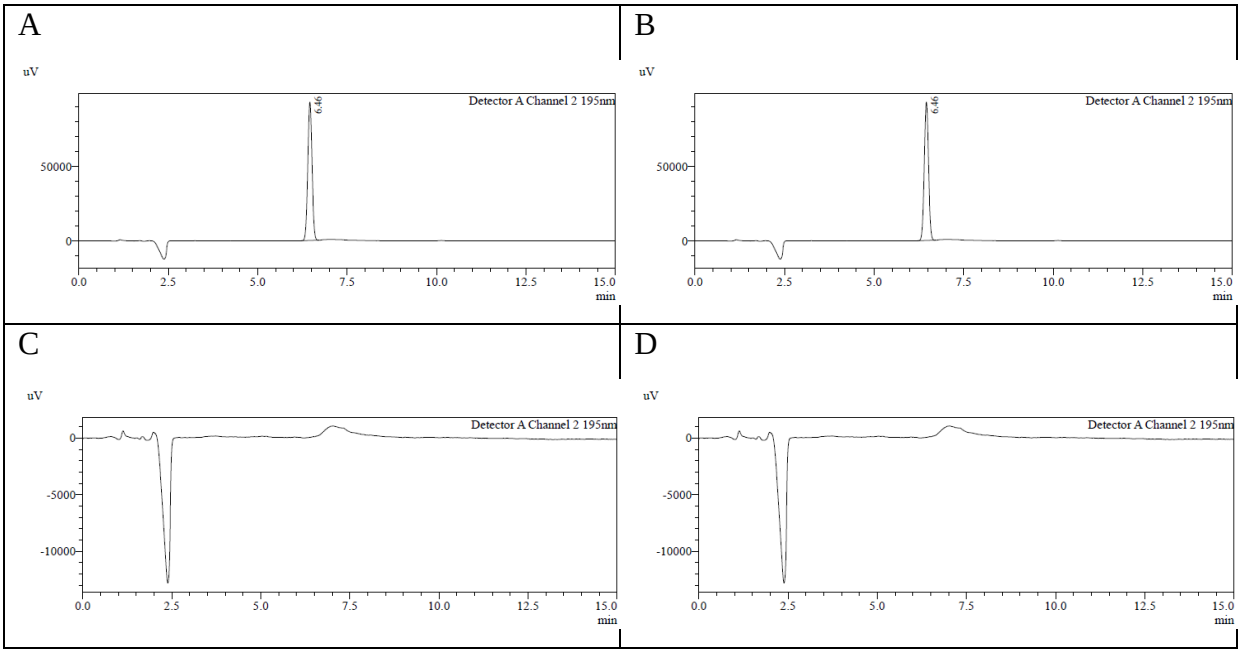

Figure 3.6: Chromatograms for Specificity [A] Cyclophosphamide in NLCs [B] Cyclophosphamide in Microemulsion [C] Placebo NLCs [D] Placebo Microemulsion

Results and Discussion:

The chromatogram in Figure 3.6 clearly indicates that no supplementary peaks were observed at the retention time of Cyclophosphamide in both NLCs and microemulsion. Therefore method was specific to Cyclophosphamide and was able to identify Cyclophosphamide in both NLCs and microemulsion [5].

3.3.2.3 Accuracy

Procedure:

PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL was used for determining the accuracy. In a volumetric flask containing 3mL of diluent, PAC-CYC NLCs was added, further standard solutions of pure Cyclophosphamide were spiked in known quantities at 50%, 100%, and 150% levels were ultimately diluted to 10mL respectively with the diluent followed by sonication for 15 minutes for determining the accuracy. All the sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded.

Results and Discussion:

The results of accuracy and % recovery are presented in Table 3.6.

Table 3.6: Results of accuracy and % recovery of Cyclophosphamide

Amount Taken (µg/mL)	Amount added (% of original amount)	Total drug content (µg/mL)	Area obtained	Concentration obtained (µg/mL)	%Recovery
20	0	0	303501	20	N.A.
20	50	30	454252	29.93	99.8
20	100	40	616089	40.60	101.5
20	150	50	765614	50.45	100.9
				Accuracy	**100.7**
				Standard Deviation	**0.9**
				Relative Standard Deviation (RSD) (%)	**0.9**

Accuracy is typically expressed as a percentage of recovery, with acceptable ranges often between 98% and 102% for pharmaceutical applications [6, 7]. The results in Table 3.6 indicate that the method demonstrated high accuracy, with a % recovery of 100%, falling within the anticipated range of 98.0 to 102.0%, and a relative standard deviation below 1.

3.3.2.4 Precision (Repeatability)

Procedure:

PAC-CYC NLCs containing Paclitaxel at 1mg/mL and Cyclophosphamide 8.75mg/mL was used for determining the precision (repeatability). In a volumetric flask containing 3mL of

diluent, PAC-CYC NLCs was added and further diluted to 10mL respectively with the diluent followed by sonication for 15 minutes. All the sample solutions were filtered by 0.45µ PTFE syringe filter (MDI: SY25TG). Initial 3 mL sample was discarded and then collected in HPLC vial. Samples were injected in HPLC and the responses were recorded. Repeatability was assessed using 6 determinations at 50µg/mL of the test concentration of Cyclophosphamide.

Results and Discussion:

The results of repeatability of Cyclophosphamide with 6 replicates are presented in Table 3.7.

Table 3.7: Results of Repeatability of Cyclophosphamide

Replicate No.	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
Area	765614	749281	750362	770832	759116	749888
Average	**757515.50**					
SD	**9193.85**					
RSD (%)	**1.2**					

The repeatability data in Table 3.7 indicate an RSD of 1.2, which is below 2.0%. The RSD is a statistical metric employed to evaluate repeatability. It is derived from the S.D. of repeated measurements divided by the mean of those measurements, represented as a percentage. A low RSD value, particularly below 2.0 %, indicated high precision in the analytical method. This precision is essential for ensuring that repeated measurements under unchanged conditions yield consistent results [8, 9]. Therefore method was concluded to be precise.

3.3.2.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Procedure:

The intercepts for three calibration curves in linearity were used to determine the LOD and LOQ of the method.

Results and Discussion:

Standard deviation of the intercepts for three calibration curves in Linearity was calculated and LOD and LOQ results are tabulated in Table 3.8.

Table 3.8: Results of LOD and LOQ of Cyclophosphamide

Parameter	Intercepts				Standard deviation	LOD (µg/mL)	LOQ (µg/mL)
	Curve 1	Curve 2	Curve 3	Average			
Results	16205	15509	21526	17747	3291	0.01	0.03

The LOD and LOQ are critical parameters in the validation of analytical methods, particularly in HPLC. LOD denotes the minimal concentration of an analyte that can be identified, though it may not be quantifiable, whereas LOQ signifies the minimal concentration that can be quantitatively assessed with acceptable levels of precision and accuracy [3]. Based on the results presented in Table 3.8, the Limit of Detection for Cyclophosphamide was obtained as 0.01µg/mL and Limit of Quantification was obtained as 0.03µg/mL which confirms that method is sensitive enough to detect low levels of Cyclophosphamide in formulation [6, 9].

REFERENCES

1. USP 43 NF 38, Monograph of Paclitaxel Injection
2. USP 43 NF 38, Monograph of Cyclophosphamide Injection
3. ICH Harmonised Tripartite Guideline Q2 (R1): Validation of Analytical Procedures: Text and Methodology, November 2005.
4. N., A., Épshtein. (2022). 3. Validation of the Specificity of Chromatographic Methods: Key Points and Practical Recommendations. Pharmaceutical Chemistry Journal, doi: 10.1007/s11094-022-02698-9
5. G, Rama, Devi., K., Nagaraju. (2018). 4. Analytical method development and validation for the estimation of chlorthalidone and atenolol by RP-HPLC. International Journal for Advance Research and Development
6. P., Shanmugasundaram. (2024). 1. Analytical method validation for related substances of Ibuprofen by HPLC. African Journal of Biological Sciences, doi: 10.48047/afjbs.6.3.2024.407-414
7. Vaibhav, Tavare. (2024). 2. Analytical Method Development and Validation of Faricimab Injection by RP-HPLC. International Journal of Advanced Research in Science, Communication and Technology, doi: 10.48175/ijarsct-19181
8. Ronald, Steel. (2023). 5. Analytical Method Development and Validation for Determination of Assay of Antibacterial Drugs Besifloxacin Hydrochloride and Phenoxyethanol in Gel Formulation. Anti-infective agents, doi: 10.2174/2211352521666230331111623
9. Vaibhav, Tavare. (2024). 1. Analytical Method Development and Validation of Faricimab Injection by RP-HPLC. International Journal of Advanced Research in Science, Communication and Technology, doi: 10.48175/ijarsct-19181
10. Salakhet dinov, D.K., Dedania, Zarna., Dedania, Ronak., Jain, Vineet, C. (2024). 2. Analytical QbD approach to HPLC method development and validation for pregabalin. doi: 10.58532/v3bgpn19p1ch6
11. Oindrila, Mazumder., R., Sundararajan. (2022). 8. Analytical Method Development and Validation for Determination of Acalabrutinib by Using RP-HPLC. Ymer, doi: 10.37896/ymer21.07/75