Chapter 6

Formulation Development of Microemulsion

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FORMULATION DEVELOPMENT AND OPTIMIZATION OF PACLITAXEL AND CYCLOPHOSPHAMIDE LOADED MICROEMULSION

6.1. METHOD FOR MICROEMULSION MANUFACTURING

Oil phase was prepared by dissolving Paclitaxel in PEG-8 Caprylic/Capric Glycerides with heating at 35±2°C, cooled to room temperature and then followed by addition of Cremophor EL, PEG-400, and Cyclophosphamide. Aqueous phase was prepared by dissolving soluplus in water for injection. Thereafter, aqueous phase was added to oil phase under continuous stirring on a magnetic stirrer. This prepared microemulsion was filtered through 0.2µ PES filter, filled in amber glass vial, stoppered with coated rubber stopper under nitrogen headspace, sealed with flip-off seal and finished product was stored at 2 to 8°C, under light protection [1-15]. The manufacturing process flow is presented in Figure 6.1.

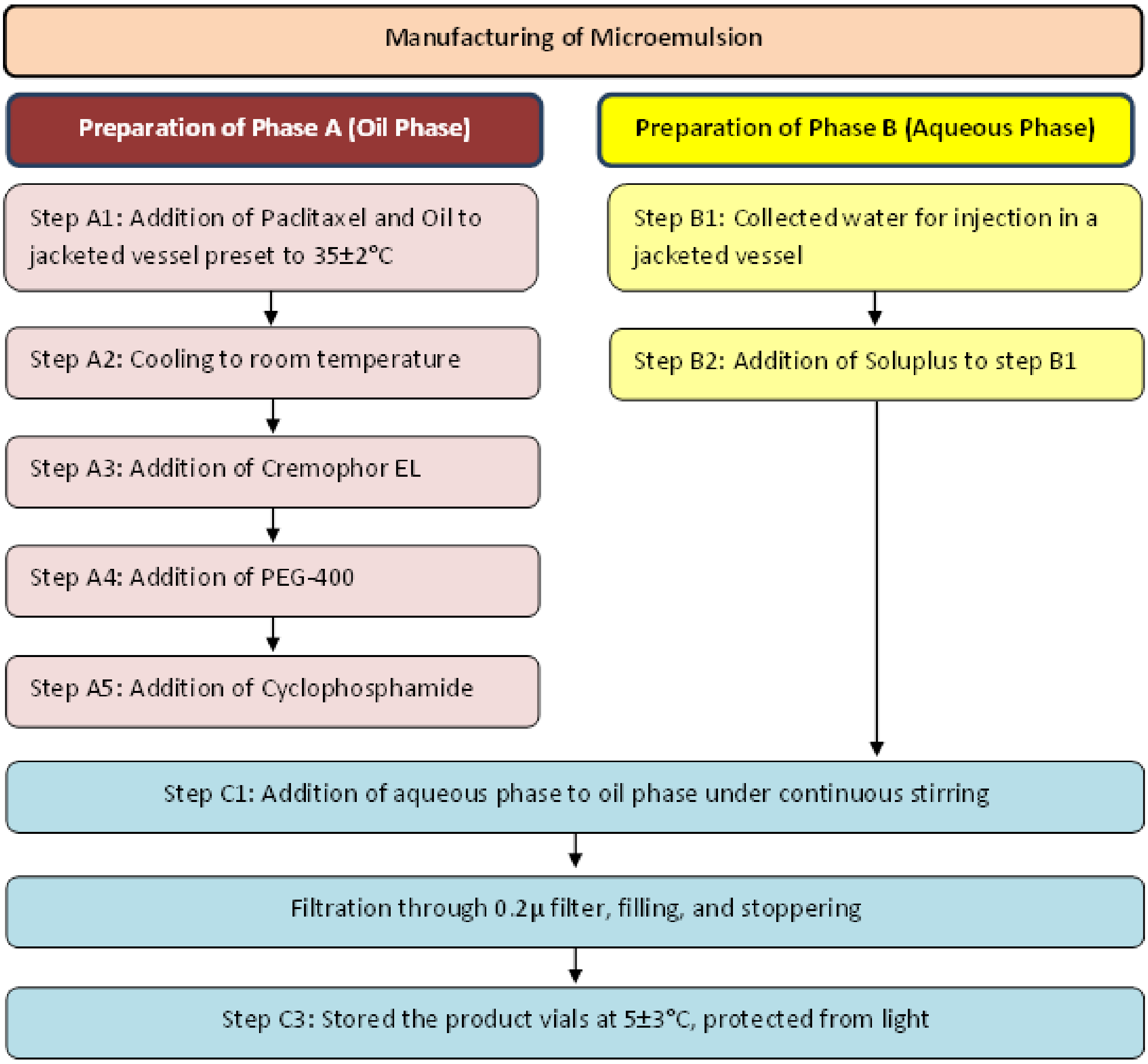

Figure 6.1: Manufacturing process of Paclitaxel and Cyclophosphamide Microemulsion

As a part of formulation development studies, quality by design (QbD) approach was adopted for the development of Paclitaxel and Cyclophosphamide Microemulsion. Formulation development contains following elements delineated in ICH Q8 (R2):

[A] Quality Target Product Profile (QTPP)
[B] Critical Quality Attributes (CQAs) of the drug product
[C] Qualitative risk assessment using Ishikawa diagram

The formulation development emphasizes a science and risk based approach to product, process development, and presents findings as a knowledge-based report, where relevant, supporting data have been summarized in appropriate tables or illustrations. Quality by Design is "A systematic approach to development that begins with predefined objectives and emphasizes product & process understanding and process control, based on sound science and quality risk management".

The scientific approach for pharmaceutical development begins with identification of desired dosage form and performance attributes through the QTPP. From this QTPP, a list of potential drug product CQAs was derived. A risk assessment was performed to identify the variables and unit operations which are most likely to impact the CQAs.

[A] Quality Target Product Profile (QTPP)

Quality Target Product Profile (QTPP) forms the basis of design for the development of a drug product. For development of Paclitaxel and Cyclophosphamide Microemulsion, QTPP was defined based on prior knowledge and literature review as presented in Table 6.1.

Table 6.1: QTPP for development of Paclitaxel and Cyclophosphamide Microemulsion

Sr. No.	QTPP Element	Target	Justification
1	Dosage Form	Injectable	Pharmaceutical equivalence: Same dosage form as per Innovator TAXOL (Paclitaxel Injection) and CYTOXAN (Cyclophosphamide Injection).
2	Route of administration	Injection, Intravenous	Pharmaceutical equivalence: Same dosage form as per Innovator TAXOL (Paclitaxel Injection) and CYTOXAN (Cyclophosphamide Injection)

Sr. No.	QTPP Element	Target		Justification
3	Stability	At least 6 Months at refrigerated condition (5±3°C)		ICH Q1A (R2) mandates to ensure that product maintains identity, quality, and purity upon storage.
4	Product Description	Clear transparent to translucent liquid with blue tint. Free from any visible particulate matter.		As per ICH Q6A, “A qualitative description of the dosage form should be provided. If any of these characteristics change during manufacture or storage, this change should be investigated and appropriate action taken”.
5	Assay of Paclitaxel	90.0 to 110.0 %		According to ICH Q6A, “A specific, stability-indicating assay to determine strength (content) should be included for all new drug products”.
6	Assay of Cyclophosphamide	90.0 to 110.0 %		
7	Sterility	Should be sterile		The dosage form is intended to be delivered by intravenous injection. Sterility of injectable is important for patient safety.
8	Globule size distribution	Z-average	< 150nm	Globule size distribution is a critical quality attribute particular to a microemulsion drug product.
		PDI	< 0.2	
9	Zeta Potential	To be reported		To ensure NLCs stability needed for patient safety and efficacy.
10	Entrapment efficiency	Not less than 90%		Higher entrapment efficiency is needed for patient safety and efficacy.
11	Filterability	Not less than 100 %		Filterability ensures particulate matter free product which is required for patient safety.

[B] Critical Quality Attributes (CQAs) of the drug product

Based on the QTPP, prior knowledge, literature review, and various guidance documents, drug product Critical Quality Attributes (CQAs) were defined. The list of CQAs identified is tabulated in Table 6.2.

Table 6.2: CQAs for development of Paclitaxel and Cyclophosphamide Microemulsion

Sr. No.	Critical Quality Attributes	Target		Is this a CQA? (Y/N)	Justification
1	Product Description	Clear transparent to translucent liquid with blue tint. Free from any visible particulate matter.		Yes	Description is an indicator of physical stability of dosage form. Hence, it was monitored throughout the drug product development.
2	Assay of Paclitaxel	90.0 to 110.0 %		Yes	Formulation parameters and process parameters may impact the assay of the drug substance. Also, there may be impact of storage conditions on the drug substance assay. Hence, this CQA was assessed throughout the drug product development.
3	Assay of Cyclophosphamide	90.0 to 110.0 %		Yes	
4	Sterility	Should be sterile		Yes*	The dosage form is intended to be delivered by intravenous injection. Sterility of injectable is important for patient safety. However, this CQA was assessed for the final formulation developed at the end of drug product development.
5	Globule size distribution	Z-average	< 150nm	Yes	Formulation parameters and process parameters may impact the particle size distribution. Also, there may be impact of storage conditions on the globule size distribution. Hence, this CQA was assessed throughout the drug product development.
		PDI	< 0.2		
6	Zeta Potential	To be reported		Yes*	This CQA will be assessed for final formulation developed at the end of drug product development. Also, there may be impact of storage conditions on the zeta potential. Hence, this CQA was assessed during the stability study.

Sr. No.	Critical Quality Attributes	Target	Is this a CQA? (Y/N)	Justification
7	Entrapment efficiency	Not less than 90%	Yes	Formulation parameters and process parameters may impact the entrapment efficiency. Also, there may be impact of storage conditions on the drug substance assay. Hence, this CQA was assessed throughout the drug product development.
8	Filterability	Not less than 100 %	Yes	Formulation parameters and process parameters may impact the filterability. Hence, this CQA was assessed throughout the drug product development.

* Shall be assessed as and when required during the characterization of optimized product and stability analysis.

[C] Qualitative risk assessment using Ishikawa diagram

All possible variables which were linked with the development of Paclitaxel and Cyclophosphamide Microemulsion were demonstrated with the help of Ishikawa diagram. These factors were considered as ‘low, moderate, and high risk’ based on their predicted effect on the Critical Quality Attributes derive from the Quality Target Product Profile. Ishikawa diagram is presented in Figure 6.2.

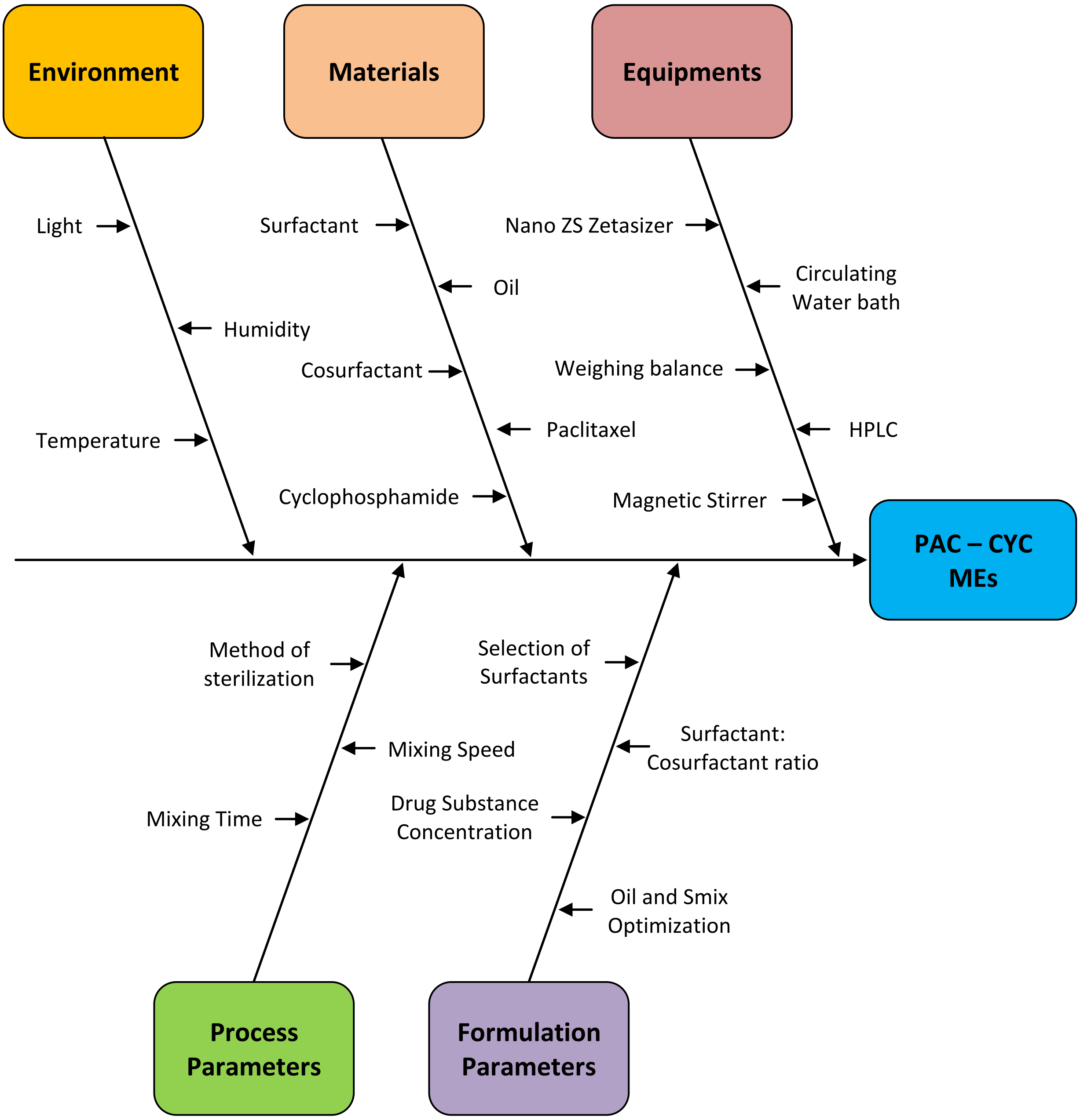

Figure 6.2: Ishikawa diagram of Paclitaxel and Cyclophosphamide Microemulsion

Based on the above Ishikawa diagram of Paclitaxel and Cyclophosphamide Microemulsion, the initial qualitative risk assessment is presented in Table 6.4, and justification for initial risk assessment is presented in Table 6.5. Risk assessment was performed to identify and rank parameters with potential to have an impact on the drug product CQAs. The relative risk that each attribute presents was ranked as high, medium, or low. The high risk attributes warranted further investigation whereas the low risk attributes required no further investigation. The medium risk is considered acceptable based on current knowledge. Further investigation for medium risk may be needed in order to reduce the risk.

Table 6.3: Overview of Risk Ranking System

Low	Broadly acceptable risk. No further investigation is needed.
Medium	Risk is acceptable. Further investigation may be needed or can be justified based on the literature review or prior knowledge in order to reduce risk.
High	Risk is unacceptable. Further investigation is needed to reduce the risk.

Table 6.4: Initial Risk Assessment

Drug Product CQAs	**Factor**				
	Environment	**Materials**	**Equipments**	**Process Parameters**	**Formulation Parameters**
Product Description	Low	Low	Low	High	High
Assay of Paclitaxel	Medium	Low	Low	High	High
Assay of Cyclophosphamide	Medium	Low	Low	High	High
Sterility	Low	Low	Low	Medium	Low
Globule size distribution	Low	Low	Low	High	High
Zeta Potential	Low	Low	Low	Medium	Medium
Entrapment efficiency	Low	Low	Low	High	High
Filterability	Low	Low	Low	High	High

Table 6.5: Justification for Initial Risk Assessment

Drug Product CQAs	**Factor**	**Justification**
Product Description	Environment	Environment condition does not impact the product description after reconstitution. Hence, the risk is Low.
	Materials	Materials used do not impact the product description after reconstitution. Hence, the risk is Low.
	Equipments	Equipments used do not impact the product description after reconstitution. Hence, the risk is Low.
	Process Parameters	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization directly impacts the product description. Hence, the risk is High.
	Formulation Parameters	

Drug Product CQAs	Factor	Justification
Assay of Paclitaxel	Environment	Based on literature review, Paclitaxel is sensitive to light conditions. Hence, the risk is medium.
	Materials	Materials used do not impact the assay of Paclitaxel. Hence, the risk is Low.
	Equipments	Equipments used do not impact the assay of Paclitaxel. Hence, the risk is Low.
	Process Parameters	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization directly impacts the assay of Paclitaxel. Hence, the risk is High.
	Formulation Parameters	
Assay of Cyclophosphamide	Environment	Based on literature review, Cyclophosphamide is sensitive to light conditions. Hence, the risk is medium.
	Materials	Materials used do not impact the assay of Cyclophosphamide. Hence, the risk is Low.
	Equipments	Equipments used do not impact the assay of Cyclophosphamide. Hence, the risk is Low.
	Process Parameters	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization directly impacts the assay of Cyclophosphamide. Hence, the risk is High.
	Formulation Parameters	
Sterility	Environment	Environment condition does not impact the product sterility. Hence, the risk is Low.
	Materials	Materials used do not impact the product sterility. Hence, the risk is Low.
	Equipments	Equipments used do not impact the product sterility. Hence, the risk is Low.
	Process Parameters	Process parameters (selection of method of sterilization) impact the sterility of drug product. Hence, the risk is Medium.
	Formulation Parameters	Formulation parameters do not impact the sterility of product. Hence, the risk is Low.
Globule size distribution	Environment	Environment condition does not impact the particle size distribution. Hence, the risk is Low.
	Materials	Materials used do not impact the particle size distribution. Hence, the risk is Low.
	Equipments	Equipments used do not impact the particle size distribution. Hence, the risk is Low.
	Process Parameters	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization directly impacts the globule size distribution. Hence, the risk is High.
	Formulation Parameters	

Drug Product CQAs	**Factor**	**Justification**
Zeta Potential	Environment	Environment condition does not impact the zeta potential. Hence, the risk is Low.
	Materials	Materials used do not impact the zeta potential. Hence, the risk is Low.
	Equipments	Equipments used do not impact the zeta potential. Hence, the risk is Low.
	Process Parameters	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization may impact the zeta potential. Hence, the risk is High.
	Formulation Parameters	
Entrapment efficiency	Environment	Environment condition does not impact the entrapment efficiency. Hence, the risk is Low.
	Materials	Materials used do not impact the entrapment efficiency. Hence, the risk is Low.
	Equipments	Equipments used do not impact the particle size distribution. Hence, the risk is Low.
	Process Parameters	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization directly impacts the entrapment efficiency. Hence, the risk is High.
	Formulation Parameters	
Filterability	Environment	Environment condition does not impact the filterability. Hence, the risk is Low.
	Materials	Materials used do not impact the filterability. Hence, the risk is Low.
	Equipments	Equipments used do not impact the filterability. Hence, the risk is Low.
	Process Parameters	Formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization impacts the filterability. Hence, the risk is High.
	Formulation Parameters	

Based on the above initial risk assessment, process parameters and formulation parameters were observed to have HIGH risk and hence were optimized using OFAT (One Factor At a Time) technique. The Critical Quality Attributes (Globule size distribution – Z-average and PDI, and Entrapment efficiency) were selected as criteria for optimization of formulation and process parameters.

6.2. OPTIMIZATION OF FORMULATION PARAMETERS

6.2.1 Screening of Surfactants

Various surfactants which can be used for i.v. route viz. Polysorbate 20, Polysorbate 80, Poloxamer 188, Soluplus, and Cremophor EL were evaluated for microemulsion as per Table 6.6.

Table 6.6: Surfactants, type, HLB value, and maximum allowable concentration

Sr. No.	Surfactant	Type of Surfactant	Nature of Surfactant	CMC value	HLB Value	Maximum allowable Conc.	Reference
1	Polysorbate 20	Hydrophilic	Non-ionic	0.06mg/mL	16.7	1.00 %	16-18
2	Polysorbate 80	Hydrophilic	Non-ionic	0.012mg/mL	15	69.33 %	16, 19-21
3	Poloxamer 188	Hydrophilic	Non-ionic	0.41mg/mL	29	2.0 %	22-24
4	Soluplus	Amphiphilic	Non-ionic	0.0076mg/mL	14	75.0 %	25-30
5	Cremophor ELP	Amphiphilic	Non-ionic	0.02mg/mL	13	65.0 %	16, 31-34

6.2.2 Optimization of Surfactant: Co-surfactant (S_{mix}) ratio

Construction of pseudo-ternary phase diagrams was done which helped to identify and determine the oil-in-water microemulsion forming compositions and region [35-41]. Pseudo-ternary diagrams provide a clear visual representation of phase behaviour of microemulsions, allowing researchers to identify the regions where microemulsions are stable. This is essential for understanding the interactions between the components and optimizing the formulation for specific applications [42, 43]. These diagrams help in determining optimal ratios of surfactant to co-surfactant, which is critical for maximizing solubilization capacity and stability of microemulsion [44].

Constructing pseudo-ternary diagrams can significantly reduce experimental workload by providing a systematic approach to identify the conditions under which microemulsions form. This is achieved by using mathematical modelling and surface modelling techniques to predict phase behaviour with fewer experimental data points [45]. For construction of phase diagrams, water titration method was used. Water titration provides a high level of precision and repeatability, which is crucial for accurately determining phase boundaries in ternary systems [46, 47].

Table 6.7: Ratio of Surfactants and Co-surfactant

Sr. No.	Ratio of Surfactant to Co-surfactant	Surfactants		Co-surfactant
		Soluplus	Cremophor EL	PEG-400
1	2:1	1	1	1
2	3:1	1	2	1
3	5:1	1	4	1

A combination of surfactants and co-surfactants were mixed in fixed weight ratios i.e. 2:1, 3:1, and 5:1 as mentioned in Table 6.7 with oil at room temperature. In each phase diagram, proportion of oil to Smix was adjusted at different ratios, ranging from 9:1 to 1:9 (% v/v). Water was carefully added to each oil-Smix mixture while stirring vigorously. Following establishment of equilibrium, samples underwent a visual inspection to determine whether they exhibited characteristics of a clear microemulsion, emulsion, or gel. The oil, surfactants, and co-surfactant were utilized to establish boundaries of microemulsion region [35-41]. Once microemulsion region was identified in phase diagrams, appropriate microemulsion formulations were chosen based on desired Surfactant: Co-surfactant (Smix) ratios [46-49]. The percentages of components were determined experimentally, and diagrams were constructed by using “Ternary plot” software.

6.2.3 Optimization of Drug Substance Concentration

Based on preformulation studies, Paclitaxel showed solubility of > 120mg/gram ≈ 0.12mg/mg in PEG-8 Caprylic/Capric Glycerides whereas Cyclophosphamide showed solubility of > 1200mg/gram ≈ 1.2mg/mg. Based on construction of pseudo ternary diagram, incorporation of 15 % oil phase in aqueous phase was accommodated with 10% S_{mix} ratio, which means 150mg of oil phase is present in 1mL of microemulsion. Therefore, each mL of microemulsion can accommodate 18mg of Paclitaxel and 180mg of Cyclophosphamide.

However to avoid overloading of microemulsion, Paclitaxel and Cyclophosphamide were studied at 1.5mg, 3mg and 4.5mg and 13.125mg, 26.25mg and 39.375mg respectively which fulfils our requirement of Paclitaxel: Cyclophosphamide ratio of 1: 8.75 in formulation [50]. High drug concentrations can lead to phase separation, as observed in studies where increasing drug load altered the stability of the microemulsion [51, 52].

Maintaining a lower concentration helps preserve the isotropic nature of the microemulsion which is crucial for stability and effectiveness as a drug delivery system [52]. Lower drug concentrations ensure that drug remains solubilized within microemulsion, facilitating better absorption and bioavailability [53, 54]. The effect of amount of drug on Globule Size, PDI, Entrapment Efficiency, stability, and filterability of Microemulsions was evaluated.

6.2.4 Optimization of Oil and S_{mix}

Optimizing concentration of oil and S_{mix} in microemulsions is crucial for achieving desired physicochemical properties and enhancing the efficacy of the microemulsion system. The balance between oil and Smix determines microemulsion's ability to solubilize active ingredients, maintain stability, and perform effectively in its intended application. The concentration of oil and Smix affects the stability and phase behaviour of microemulsions.

Formulations F1 – F9 were prepared containing Cremophor EL (CEL) and Soluplus (SOL) as surfactants and PEG-400 (PEG) as cosurfactant to optimize concentration of oil and S_{mix} as presented in Table 6.8 [48]. Three different oil concentrations i.e. 10%, 12.5%, and 15% and three different concentrations of S_{mix} i.e. 7.5%, 10%, and 15% were used. These concentrations were chosen after preliminary research and a pseudoternary phase diagram were taken into consideration [48]. Total 9 formulations of microemulsions containing three different oil concentrations and three different concentrations of S_{mix} were prepared and appearance before and after 100 times dilution was evaluated.

Table 6.8: Compositions of Microemulsion for Optimization of Oil and S_{mix}

Formulation No.	Oil (%)	S_{mix} (%)
F1	10	7.5
F2	10	10
F3	10	15
F4	12.5	7.5
F5	12.5	10
F6	12.5	15
F7	15	7.5
F8	15	10
F9	15	15

6.3. OPTIMIZATION OF PROCESS PARAMETERS

Mixing speed and mixing time are crucial process parameters required to be optimized for microemulsion formation using OFAT (One factor at a time) technique. Based on the literature review, it was concluded that optimization of process parameters is critical for the development of microemulsion. The levels for evaluating the mixing speed and mixing time are presented in Table 6.9.

Table 6.9: Process parameters of mixing speed and mixing time

Levels	Mixing Speed	Mixing Time
1	250 RPM	5 minutes
2	500 RPM	10 minutes
3	1000 RPM	15 minutes

6.4. CHARACTERIZATION OF MICROEMULSION

6.4.1 Dilution test

The dilution method helps determine distribution of cosurfactants between interface and bulk phases, which is critical for understanding stability and formation of microemulsions [55, 56]. Microemulsion was diluted with water at 1:10, 1: 100, and 1: 250 ratios. Clarity of solution upon dilution was evaluated. If water was easily dispersed in continuous phase, microemulsion was defined as oil-in-water microemulsion and if microemulsion becomes hazy upon dilution, it was defined as bicontinuous [55-57].

6.4.2 Viscosity

Viscosity of Microemulsion was measured using Anton Paar Rheometer, Model: MCR 102 in rotational mode with spindle plate of Parallel plate (PP50), rotational speed of 50 RPM and gap of 0.1mm. 0.5 to 1.0mL of Microemulsion sample was placed on cleaned and dry plate of Rheometer, spindle was lowered down and excess sample was trimmed down and measurement was started [58, 59].

6.4.3 Globule size distribution and Zeta Potential

Globule size distribution (Z-Average) and Zeta potential were obtained using Malvern Zetasizer (Nano ZS) for Paclitaxel-Cyclophosphamide loaded microemulsion. The temperature for sample testing was selected as 25°C and equilibrium time of 60 seconds was given to each sample [60, 61].

6.4.4 Morphology by Transmission Electron Microscopy

The optimized Microemulsion's shape was analyzed using a JEM-2100 TEM at the Sophisticated Analytical Instrument Facility, North Eastern Hill University, Shillong. A single drop from each sample was placed onto a carbon film-coated copper grid (200-mesh). After duration of five minutes, a filter paper positioned at the periphery of the copper grid was employed to eliminate any surplus liquid that remained. Samples were air-dried at room temperature and subsequently investigated at 200 kV power [62].

6.4.5 Assay of Paclitaxel

HPLC method from USP 43 NF 38 was adopted for determination of Paclitaxel wherein Shimadzu Chromatograph, Prominence-I LC2030C plus was used. The quantitation of Paclitaxel was done using (250 x 4.6) mm, 5µm, L43 column with flow rate of 1.5mL/minute, Injection volume of 100µL in isocratic mode with column oven temperature of 25°C and detection wavelength of 227nm. Water and Acetonitrile in the ratio of 5.5:4.5 was used as mobile phase with run time of 15minutes [63].

6.4.6 Assay of Cyclophosphamide

HPLC method from USP 43 NF 38 was adopted for determination of Cyclophosphamide wherein Shimadzu Chromatograph, Prominence-I LC2030C plus was used. The quantitation of Cyclophosphamide was done using (250 x 4.6) mm, 5µm column with flow rate of 1.5mL/minute, and Injection volume of 100µL in isocratic mode with column oven temperature of 5°C and detection wavelength of 195nm. Water and Acetonitrile in the ratio of 30:70 was used as mobile phase with run time of 15minutes [64].

6.4.7 Entrapment efficiency

Dilution of microemulsion was done with pH 7.4 phosphate buffer (PBS) containing 2 % w/v Tween 80 by adding 2.5mL of Tween 80-buffer system to 5mL of microemulsion, followed by addition of 1gram of sodium sulphate (anhydrous). Tween 80 was added to PBS to avoid the precipitation of drugs in PBS alone. The mixture was then vortexed for 5 minutes. The dispersion was centrifuged for 30 minutes at 15000 RPM (Centrifuge model 2-16P, Sigma, Germany), supernatant was suitably diluted, and the amount of the free PAC and CYC in the dispersion medium was estimated by HPLC. The entrapment efficiency was calculated using following equation:

$$\%\ \text{Entrapment efficiency}= \frac{W_{total}\text{-}W_{free}}{W_{total}}\times 100$$

Here, the W_{total} is the weight of total drug added and W_{free} is the free drug obtained upon centrifugation [65, 66].

6.4.8 In-Vitro release studies

The in-vitro release of pure Paclitaxel, pure Cyclophosphamide, and Paclitaxel & Cyclophosphamide loaded microemulsion was evaluated by the dialysis method. INTAXEL contained Paclitaxel dissolved in a mixture of Ethanol and Cremophor EL (1:1), while ENDOXAN (Cyclophosphamide) was dissolved in Ethanol for the in-vitro release studies. The dialysis membrane was soaked for 24 hours before the experiment in the release medium (phosphate buffer, pH 7.4, containing Tween-80).

Accurately measured microemulsion was placed in dialysis membrane. No leakage was ensured by properly tying the membrane. The dialysis membrane was placed gently in a beaker consisting of fixed volume of release medium under continuous stirring at 200 RPM and temperature of release medium was ensured as 37.5 ± 2.0°C [67, 68]. At predetermined intervals, a fixed volume of medium was withdrawn and fresh quantity was added by the same volume. The samples were analyzed immediately using HPLC method for quantification of Paclitaxel and Cyclophosphamide [67, 68].

6.4.9 % Filterability

% filterability test is to monitor flow decay and gradual pore plugging caused by the product and to determine the maximum volume of product that can be filtered through a 0.2µ filter. Fixed volume of microemulsion was filtered through 0.2µm PES filter and the % volume filtered was recorded. [69]

6.4.10 Sterility

Membrane Filtration Method was used to determine the sterility of optimized Microemulsion as it is a regulatory method of choice for filterable pharmaceutical products. The microemulsion was passed through 0.45µm membrane filter under laminar air flow to maintain sterile conditions [70]. Post filtration, 0.45µm membrane filter was added to Fluid Thioglycolate Medium (FTM) and Soybean casein digest medium (SCDM) culture media and kept for 14 days incubation [70]. Sterile water for injection was used as negative control whereas growth media exposed to atmospheric conditions was used as positive control [70].

6.5. STABILITY STUDIES

Paclitaxel and Cyclophosphamide loaded microemulsion was manufactured as per the optimized process and further the microemulsion was stored in USP type-I glass vials, stoppered with rubber stoppers and loaded in stability for 12 months at 2 to 8°C and for 15 days at 25±2°C/60±5%RH. Batches were evaluated for globule size distribution, PDI, zeta potential, assay of Paclitaxel, assay of Cyclophosphamide, and sterility. Sterility was evaluated only at the end of 12 months stability.

RESULTS & DISCUSSION

6.6. OPTIMIZATION OF PRODUCT PARAMETERS

6.6.1 Screening of Surfactants

Elevated concentrations of surfactants may lead to hemolysis, resulting in damage to erythrocytes, as well as other adverse toxicological responses. Consequently, employing them at reduced concentrations mitigates this risk while preserving their efficacy as solubilizers and stabilizers [71, 72]. Surfactants have potential to influence functionality of cell membranes and biological barriers, which may result in disruption of normal physiological processes and the emergence of complications. The use of efficient surfactants and cosurfactants can help maintain stability while minimizing the required concentration, thus reducing potential toxicity [71-73]. Elevated levels of surfactants may influence pharmacokinetic profile of drug, impacting absorption, distribution, metabolism, and excretion processes [74]. This may result in unforeseen effects or potential toxicity. In certain instances, elevated concentrations of surfactants could potentially augment the immunogenic response to biologics, resulting in the formation of antibodies targeting either the therapeutic agent or the surfactant itself. Consequently, the research focused on employing the least quantity of surfactant to develop stable microemulsions [75-78]. Therefore, preliminary experiments to manufacture microemulsion for the selection of surfactants were performed with 1.0 % concentration for all the surfactants. The results of feasibility trials for the screening of surfactants are mentioned in Table 6.10.

Table 6.10: Results of evaluation of Surfactants on formulation of Microemulsion

Sr. No.	Surfactant	Z-Average (nm)	PDI	% Entrapment efficiency	
				PAC	CYC
1	Polysorbate 20	275.0 ± 8.1	0.203 ± 0.047	72.6 ± 2.0	77.4 ± 1.2
2	Polysorbate 80	303.5 ± 11.7	0.280 ± 0.073	69.7 ± 0.9	83.9 ± 1.0
3	Poloxamer 188	412.7 ± 16.2	0.0265 ± 0.040	65.1 ± 1.5	80.0 ± 0.7
4	Soluplus	127.7 ± 6.2	0.087 ± 0.010	97.0 ± 0.2	98.3 ± 0.4
5	Cremophor EL	130.6 ± 5.8	0.100 ± 0.015	95.5 ± 0.3	96.7 ± 0.1

Polysorbate 20 containing microemulsion resulted in a globule size above 200 nm and a PDI greater than 0.2. Polysorbate 20, a non-ionic surfactant, was not sufficient at 1% w/v to stabilize the microemulsion effectively, leading to larger droplet sizes [75, 76]. The use of Polysorbate 80 resulted in globules (Z-Average) of more than 300 nm and a PDI above 0.25.

Polysorbate 80 is a common surfactant used in microemulsions, but its concentration and interaction with other components can significantly affect droplet size and stability. At 1% w/v, Polysorbate 80 did not provide sufficient stabilization for the oil-water interface, leading to larger droplet sizes and increased polydispersity [79, 81-83].

The CMC value of Poloxamer 188 is 0.41mg/mL and therefore higher concentration is required for the stabilization of a formulation. Due to higher CMC value, Poloxamer 188 is not able to stabilize the formulation upon dilution. This was confirmed by Han J. Et Al wherein Paclitaxel emulsion manufactured using Poloxamer 188 as surfactant precipitated upon dilution [84].

The Soluplus-containing microemulsion achieved average globule size below 150 nm with a PDI below 0.1 and an entrapment efficiency of more than 95% due to the unique properties of Soluplus as a surfactant. Soluplus, a graft amphipathic copolymer, enhanced solubility and stability of poorly soluble drugs (Paclitaxel & Cyclophosphamide) as it formed nano-sized micelles with a narrow size distribution, which is crucial for achieving desired globule size & PDI in microemulsions [85].

Cremophor EL acts as a surfactant, facilitating formation of stable microemulsions by reducing surface tension and allowing for encapsulation of hydrophobic drugs like Paclitaxel [86, 87]. The high entrapment efficiency (>95%) is achieved through effective encapsulation of drugs within microemulsion, which is facilitated by use of appropriate surfactants and co-surfactants that stabilize emulsion and prevent drug leakage [88, 89].

Considering the benefits of both Soluplus and Cremophor EL, combination of these surfactants was evaluated. The results are presented in Table 6.11.

Table 6.11: Results of evaluation of Soluplus-Cremophor EL combination

Sr. No.	Surfactant (%)		Z-Average (nm)	PDI	% Entrapment efficiency	
	Soluplus	Cremophor EL			PAC	CYC
1	1	1	158.2 ± 10.1	0.116 ± 0.010	98.9 ± 0.2	96.5 ± 0.3
2	1	2	142.7 ± 14.5	0.123 ± 0.010	99.0 ± 0.5	99.6 ± 0.3
3	1	4	120.2 ± 4.2	0.138 ± 0.009	100.1 ± 0.7	99.7 ± 0.7

Based on the results presented in Table 6.7, Cremophor EL at 4% resulted in reduced globule size in comparison to other levels of Cremophor EL. A higher concentration of Cremophor EL increases the solubilization capacity, leading to a more stable microemulsion with smaller droplet sizes and improved drug entrapment [90, 91]. Increasing the concentration of Cremophor EL from 1% to 4% enhances the hydrophilic-lipophilic balance (HLB), which is crucial for stabilizing the microemulsion and achieving optimal droplet size [92, 93]. Therefore, combination of Soluplus at 1% and Cremophor EL at 4% was considered for further optimization of microemulsion.

6.6.2 Optimization of Surfactant: Co-surfactant ratio

The system of Paclitaxel Cyclophosphamide Microemulsion composed of oil (PEG-8 Caprylic/Capric Glycerides), Surfactants (Soluplus and Cremophor EL), Co-surfactant (PEG-400), and purified water was evaluated for optimization of surfactant: co-surfactant ratio by water titration and spontaneous emulsification method. The readings for water titration spontaneous emulsification method for preparation of microemulsions are presented in Table 6.12.

Table 6.12: Water titration readings for Phase diagram

Oil (mL)	**Smix (mL)**	**Volume of water until system remained clear (mL)**		
		S:CoS::2:1	**S:CoS::3:1**	**S:CoS::5:1**
0.6	5.4	300.0	400.0	500.0
1.2	4.8	122.0	114.0	254.0
1.8	4.2	54.0	60.0	76.0
2.4	3.6	14.0	14.0	22.0
3.0	3.0	12.0	12.0	16.0
3.6	2.4	12.0	10.0	12.0
4.2	1.8	10.0	8.0	10.0
4.8	1.2	10.0	6.0	8.0
5.4	0.6	8.0	6.0	6.0

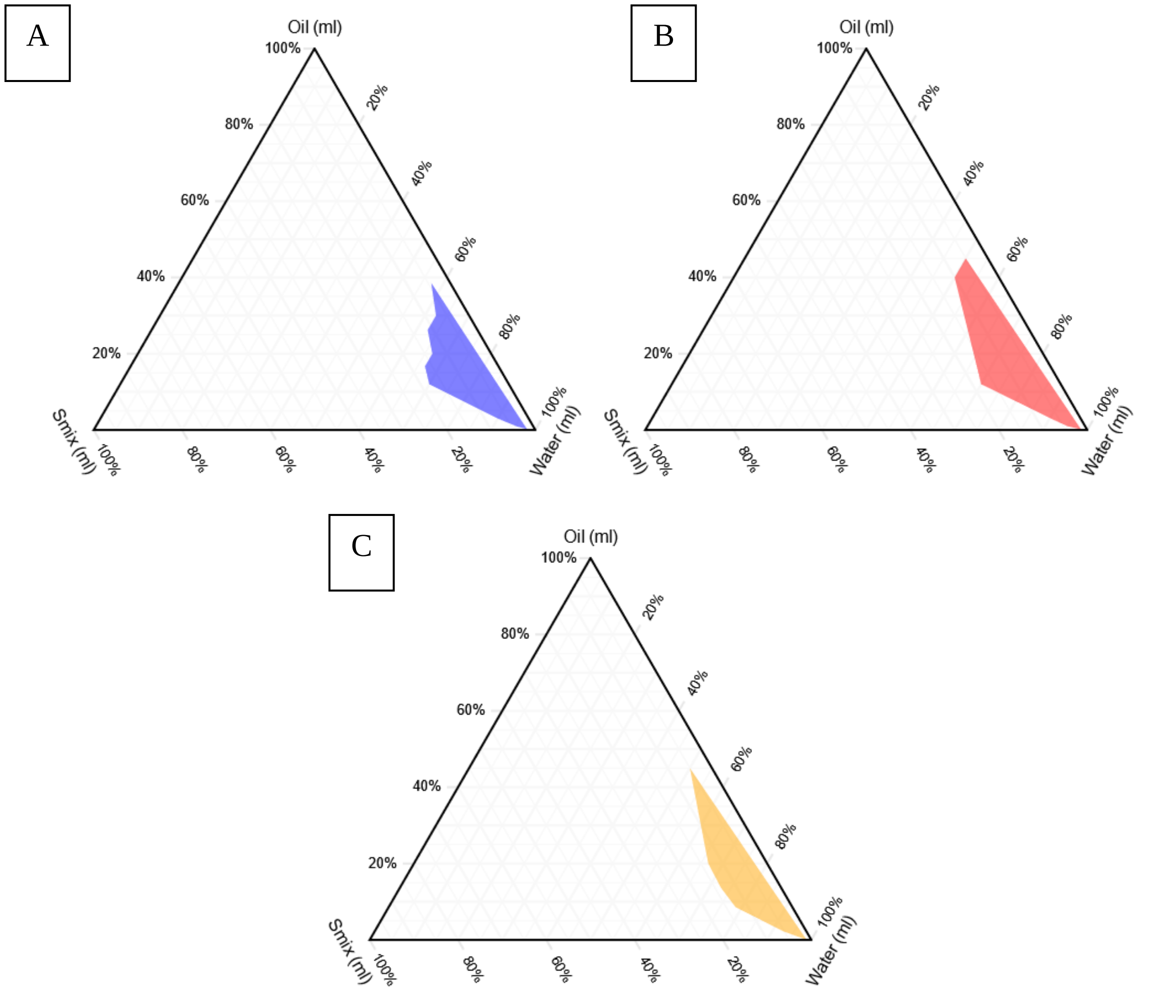

Figure 6.3: Pseudo ternary diagram [A] S:CoS::2:1 [B] S:CoS::3:1 [C] S:CoS::5:1

The selection of oil and surfactant, as well as the combination ratio of oil to S/CoS, plays a crucial role in the process of microemulsion formation. The differentiation between the microemulsion region and the macroemulsion region can be achieved through the use of a pseudo-ternary phase diagram [46, 47]. Table 6.12 presents the results of the water titration for the phase diagram. The microemulsion region is discernible within the pseudo-ternary phase diagram. Figure 6.3 demonstrates that the region of microemulsion existence broadened as the S: CoS concentration ratio increased.

As per the Figure 6.3 the S/ CoS mixture (S_{mix}) in ratio of 5:1 was able to incorporate highest amount of oil at lower concentration of S_{mix} when compared to ratio 2:1 and 3:1 with better stability in terms of appearance and clarity. Thus maintaining the ratio of S_{mix} as 5:1 should produce stable and clear microemulsion. Based on the pseudo ternary diagrams for all the 3 ratios, 10% of S_{mix} ratio 2:1 could accommodate only 6% of oil, 10% of Smix ratio 3:1 could accommodate 7% of oil whereas 10% of S_{mix} ratio 5:1 could accommodate 15% of oil which is the highest as compared to other two ratios. Hence, S_{mix} ratio of 5:1 was finalized for further optimization of Oil and S_{mix}.

6.6.3 Optimization of Drug Substance Concentration

The results of optimization of drug substance concentration are presented in Table 6.13.

Table 6.13: Results of optimization of drug substance concentration

Sr. No.	Drug substance Conc. (mg/mL)		Z-Average	PDI	% Entrapment efficiency		Stability	% Filterability
	PAC	CYC			PAC	CYC		
1	1.5	13.125	97.8 ± 4.1	0.092 ± 0.090	100.0 ± 1.2	100.5 ± 1.1	Stable	100.0 ± 0.0
2	3	26.25	88.5 ± 7.6	0.080 ± 0.005	99.1 ± 1.7	98.7 ± 1.7	Stable	100.0 ± 0.0
3	4.5	39.375	161.3 ± 2.1	0.252 ± 0.042	96.0 ± 1.2	97.5 ± 1.4	Stable	95.0 ± 5.0

At PAC concentrations of 1.5mg/mL, 3mg/mL, and CYC concentrations of 13.125mg/mL, 26.25mg/mL, microemulsion was observed to have globule size below 100nm and PDI less than 0.100. Also, the % entrapment efficiency and filterability were 100% and microemulsion was stable. With increase in the concentration of PAC to 4.5mg/mL and CYC to 39.375mg/mL, Z-average increased to more than 150nm and PDI was observed to be more than 0.200. Also, there was slight decrease in % entrapment efficiency and % filterability. However, the microemulsion was observed to be stable.

Increase in concentration of PAC to 4.5mg/mL and CYC to 39.375mg/mL resulted in formation of slightly larger globule dimensions and increased polydispersity, as indicated by a higher PDI. This phenomenon led to a marginal decline in entrapment efficiency and filterability, although the overall system maintained stability [90-92]. Increased concentrations of PAC and CYC lead to larger droplet sizes and higher polydispersity index (PDI), as the surfactant system becomes less effective at stabilizing the increased number of droplets, resulting in a broader size distribution [93, 94]. As droplet size increased with higher concentrations of PAC and CYC, the surface area-to-volume ratio decreased, leading to reduced entrapment efficiency and filterability [95]. Despite the changes in droplet size and PDI, the overall stability of microemulsion system was maintained due to inherent properties of surfactant and co-surfactant system, which continued to provide a stable interface [96, 97].

Based on above results, Paclitaxel concentration as 3mg/mL (0.3 %) and Cyclophosphamide as 26.25mg/mL (2.625 %) was finalized.

6.6.4 Optimization of Oil and Smix

Based on the above preliminary studies, S_{mix} ratio of 5:1 was finalized and a series of formulations were prepared with varying concentrations of S_{mix} as 7.5 %, 10 %, and 15 %with three different oil concentrations i.e. 10 %, 12.5 %, and 15%. The optimized concentration of Paclitaxel at 0.3 % and Cyclophosphamide at 2.625 % was incorporated in the formulated microemulsions to understand the impact of drug incorporation on the stability of microemulsion. Impact of dilution was evaluated for all the 9 formulations to determine the physical stability of formulated microemulsions.

Clarity upon dilution is a critical factor for intravenous microemulsions due to its implications on stability, safety, and efficacy of drug delivery systems. Formulated microemulsions require dilution prior to administration to attain the appropriate drug concentration for safe intravenous delivery [98, 99]. Drug bioavailability and therapeutic efficacy may be compromised if the microemulsion becomes unstable when diluted and precipitates or aggregates. This clarity is indicative of the microemulsion's ability to remain thermodynamically stable, which is crucial for intravenous applications where any instability could lead to complications such as embolism or reduced therapeutic efficacy. A clear microemulsion ensures that drug remained dissolved, facilitating absorption and therapeutic action [98-101].

The choice and level of oil, surfactants, and co-surfactants plays a significant role in maintaining clarity upon dilution. These components help stabilize the microemulsion and prevent phase separation, which is crucial for maintaining clarity [102, 103]. Therefore, clarity upon dilution was evaluated to optimize the concentrations of Oil and S_{mix}. The results are presented in Table 6.14.

Table 6.14: Trials for optimization of components (Oil and S_{mix})

Formulation No.	Oil (%)	Conc. of S_{mix} (%)	Appearance before 100 times dilution	Appearance after 100 times dilution
F1	10	7.5	Clear	Hazy
F2	10	10	Clear	Hazy
F3	10	15	Clear	Clear
F4	12.5	7.5	Clear	Hazy
F5	12.5	10	Clear	Clear
F6	12.5	15	Clear	Clear
F7	15	7.5	Hazy	Hazy
F8	15	10	Clear	Clear
F9	15	15	Clear	Clear

Formulations with lower S_{mix} concentrations (F1, F2, F4, and F7) were "hazy" after dilution, indicating that these concentrations were insufficient to stabilize the microemulsion when diluted. Formulations F3, F5, F6, F8, and F9, which contained either 10% or 15% S_{mix}, remained "clear" after dilution. This suggests that higher concentrations of S_{mix} maintained the stability and clarity of the microemulsion even after dilution. Higher oil concentrations (15%) appeared more challenging to stabilize, especially with lower S_{mix} concentrations [104, 105]. Based on the above results, Oil at 15% and Smix at 10% was finalized for further manufacturing of microemulsion.

6.7. OPTIMIZATION OF PROCESS PARAMETERS

The results of mixing speed and mixing time are presented in Table 6.15 and 6.16. The mixing speed directly impacts the size of the droplets formed in a microemulsion. Optimum mixing speeds can lead to smaller droplet sizes, which are desirable for stable microemulsions due to increased surface area and reduced coalescence [106].

Table 6.15: Results of mixing speed

Mixing Speed (RPM)	Z-Average	PDI	% Entrapment efficiency	
			PAC	CYC
250	151.4 ± 9.5	0.103 ± 0.05	89.2 ± 1.7	100.1 ± 1.3
500	92.1 ± 6.8	0.090 ± 0.07	99.7 ± 1.3	99.8 ± 1.1
1000	116.2 ± 10.6	0.115 ± 0.09	98.5 ± 1.5	97.9 ± 1.6

At 250 RPM, Z-average was observed to be on higher side as compared to results of 500RPM and 1000RPM. For entrapment efficiency, while CYC had near-perfect efficiency, PAC showed slightly lower at 89.2%. 500 RPM seemed to strike the best balance between small particle size, uniform distribution, and maximum entrapment efficiency. While at higher speeds (1000 RPM), slightly decreased efficiency and uniformity was observed.

The distribution of droplet sizes is also affected by mixing time. Adequate mixing time ensures uniform distribution of droplets, which is critical for stability and homogeneity of microemulsion [106-108]. Optimum mixing time is necessary to ensure proper entrapment of drugs in oil phase of microemulsion. It has been studied that optimal mixing times can enhance entrapment efficiency by ensuring thorough mixing and interaction between components [107-109].

Table 6.16: Results of mixing time

Mixing time (minutes)	Z-Average	PDI	% Entrapment efficiency	
			PAC	CYC
5	197.2 ± 15.9	0.336 ± 0.05	77.3 ± 1.4	96.4 ± 1.5
15	92.1 ± 6.8	0.090 ± 0.07	99.7 ± 1.3	99.8 ± 1.1
30	107.1 ± 11.0	0.052 ± 0.08	100.3 ± 1.2	95.0 ± 1.7

At 5 minutes, Z-Average indicated a large particle size, which indicated insufficient mixing time to reduce the globule size. PDI showed a wide globule size distribution and lacked uniformity. At lower mixing time, the results indicated relatively poor entrapment efficiency, particularly for PAC. The optimal mixing time appears to be 15 minutes, where the smallest particle size was achieved, the narrowest globule size distribution, and the highest entrapment efficiency for both PAC and CYC. Longer mixing time (30 minutes) provided marginally better uniformity (PDI), but entrapment efficiency for CYC dropped slightly.

The product and process parameters optimized for the manufacturing of microemulsion are presented in Table 6.17.

Table 6.17: Optimized Product and Process parameters for PAC-CYC Microemulsion

Sr. No.	Parameter		Concentration
1	Drug Concentration	Paclitaxel	0.3%
		Cyclophosphamide	2.625%
2	Amount of oil		15 %
3	Smix ratio		Surfactant 5:1 Co-surfactant
4	Amount of Smix		10 %
5	Mixing speed		500 RPM
6	Mixing time		15 minutes

6.8. CHARACTERIZATION OF MICROEMULSION

Paclitaxel and Cyclophosphamide loaded microemulsions were characterized for morphology by TEM, Entrapment efficiency, Assay of Paclitaxel, Assay of Cyclophosphamide, Globule size distribution, Zeta potential, Viscosity, and Dilution test.

6.8.1 Dilution test

The results of dilution test for formulation microemulsion are presented in Table 6.18.

Table 6.18: Results of dilution test of Optimized PAC-CYC Microemulsion

Sr. No.	Dilution ratio	Results
1	1:10	Clear
2	1:100	Clear
3	1:250	Clear

The microemulsion was easily diluted with water at 1:10, 1: 100, and 1: 250 ratios. The microemulsion demonstrated excellent stability upon dilution at ratios of 1:10, 1:100, and 1:250, exhibiting no indications of phase separation, precipitation, or breaking. The incorporation of additional water seamlessly integrated into the system, proving that the emulsion was classified as oil-in-water [56, 110].

6.8.2 Viscosity

The viscosity of optimized formulation was reported as 1.09 ± 0.06 cps. Microemulsions are defined by their remarkably small droplet size, generally falling within the range of 10 to 100 nanometres [111, 112]. The system exhibits characteristics akin to a homogeneous solution, indicating that the viscosity remains low and comparable to that of water. [112-115]. In oil-in-water microemulsions, the continuous phase consists of water [112-115]. The predominance of water in the system serves as the medium facilitating the movement of dispersed oil droplets, resulting in an overall viscosity of the microemulsion that closely resembles that of water. The limited quantity of the dispersed oil phase has a negligible impact on the overall viscosity. [111-116].

6.8.3 Zeta Potential

The zeta potential of microemulsion was reported as -2.6 ± 0.2 mV. The graph of Zeta potential as presented in Figure 6.4 revealed that there is no significant charge on the manufactured microemulsion. Paclitaxel and Cyclophosphamide are neutral compounds in its pure form, meaning they do not possess an inherent charge. [117-121]. Cremophor EL is a polyethoxylated castor oil, which is a non-ionic surfactant, meaning it does not carry a net charge [122, 123].

Oil used in formulating microemulsion does not have any charge on itself [124]. However, Soluplus solutions possess charge which is typically neutral to slightly negative [125]. Thus, the observed zeta potential can be attributed to use of Soluplus as surfactant in formulation of microemulsion.

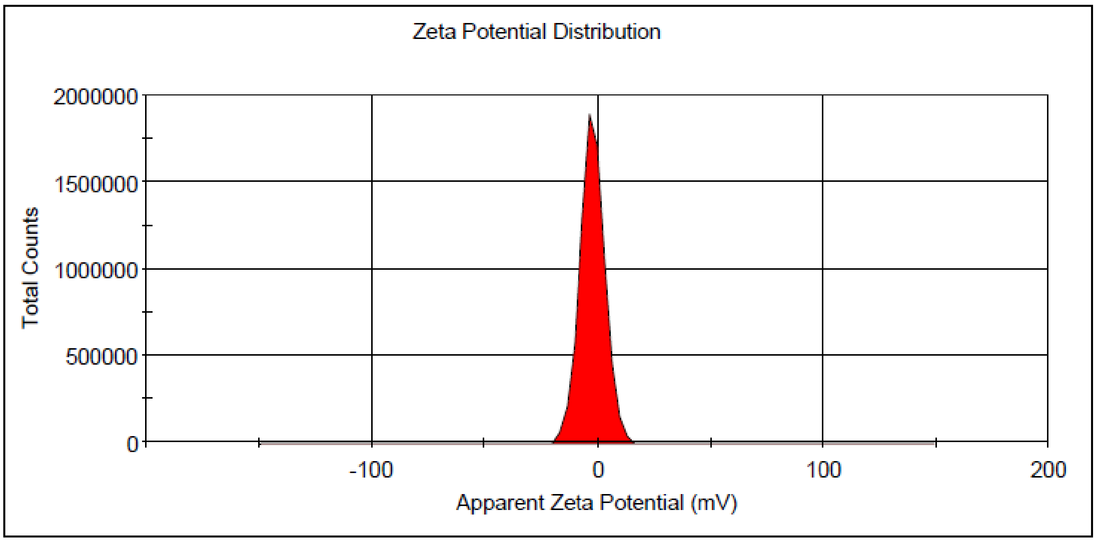

Figure 6.4: Graph of Zeta Potential of microemulsion

6.8.4 Globule size distribution

The globule size distribution of optimized microemulsion revealed Z-average as 62.8 ± 1.0nm with PDI of 0.142 ± 0.031, indicating that size distribution is narrow. Smaller globules tend to aggregate less and maintain their dispersed state over a longer period, ensuring long-term stability of the microemulsion [126, 127]. A PDI of 0.072 suggests that the microemulsion has a highly homogeneous particle size distribution, which is desirable for stability and reproducibility in formulations. A narrow size distribution often correlates with increased physical stability [105, 127, 128]. The absence of a wide range of particle sizes reduces the likelihood of instability phenomena like Ostwald ripening, which can compromise the longevity of the microemulsion [128]. The graph of globule size distribution is presented in Figure 6.5.

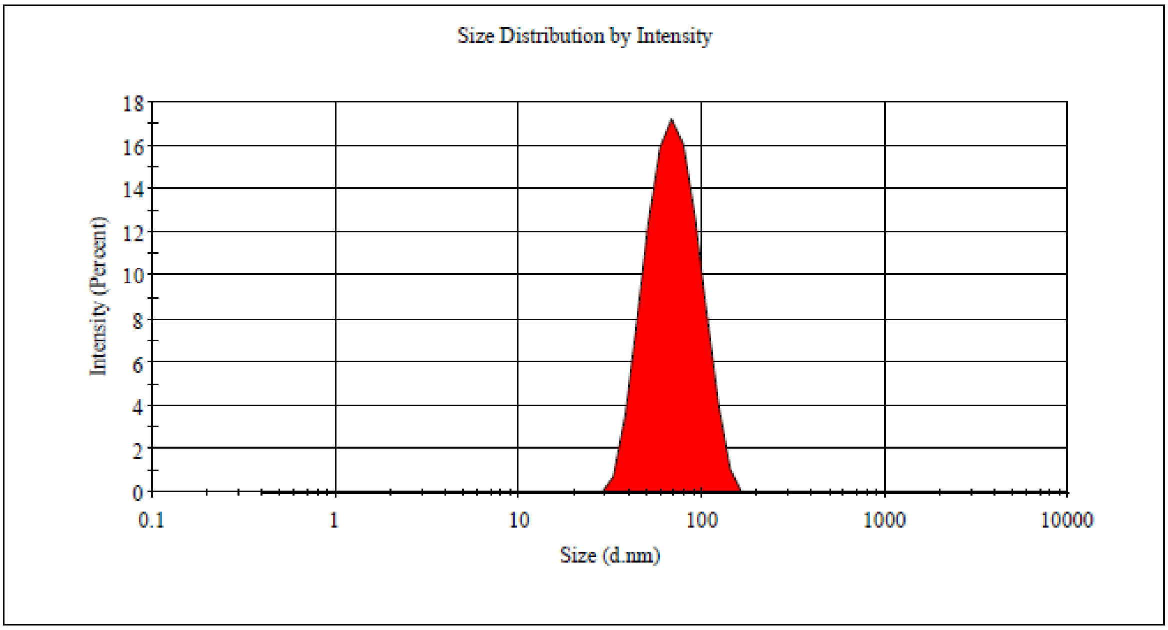

Figure 6.5: Graph of Particle size distribution of Microemulsion

6.8.5 Morphology by TEM

The images obtained by TEM shown in Figure 6.6 revealed that optimized microemulsion were discrete and spherical oil globules dispersed in continuous phase of microemulsion with droplet size ~ 100 nm, which was similar to results obtained by dynamic light scattering (DLS) method.

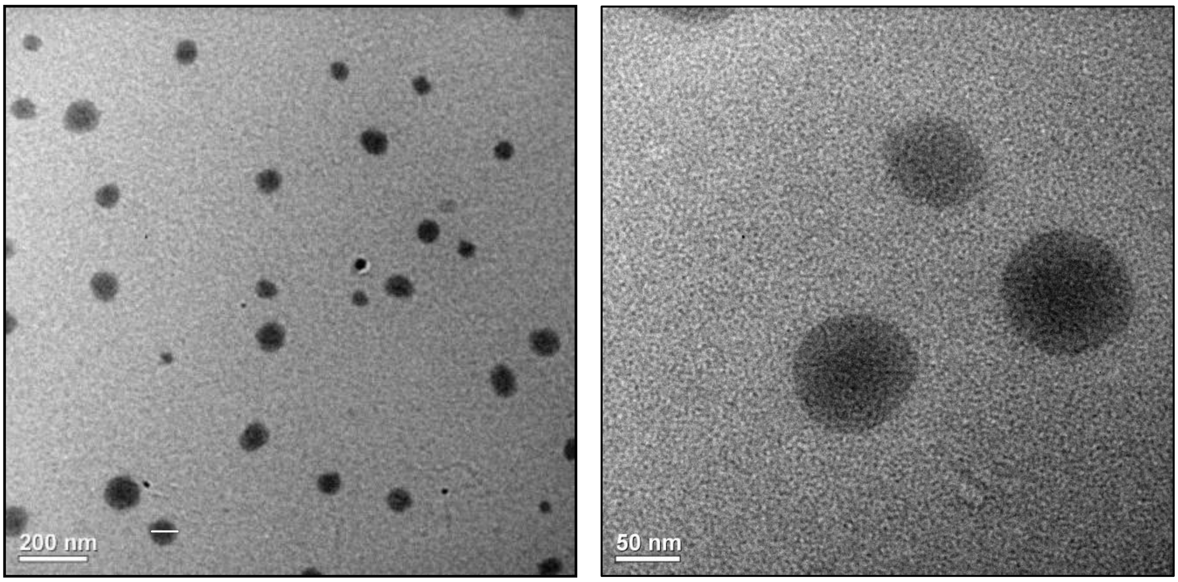

Figure 6.6: TEM Image of Optimized Microemulsion

6.8.6 Entrapment efficiency, Assay of Paclitaxel, and Assay of Cyclophosphamide

The entrapment efficiency of Paclitaxel was obtained as 99.7±0.2 % whereas for Cyclophosphamide it was obtained as 100.6±0.8 %. Assay of Paclitaxel in microemulsion was determined as 99.0 ± 0.4 % and assay of Cyclophosphamide in microemulsion was reported as 100.8 ± 0.3 %. As per the USP monograph of Paclitaxel Injection and Cyclophosphamide Injection, the acceptable specification for assay is 90.0 to 110.0 % and reported results adhere to the specification limit.

6.8.7 In-vitro drug release study

In-vitro release studies were performed for Reference Standard of Paclitaxel, Reference Standard of Cyclophosphamide, and Paclitaxel & Cyclophosphamide loaded microemulsion. The graph of % cumulative release versus time in hours is as presented in Figure 6.7.

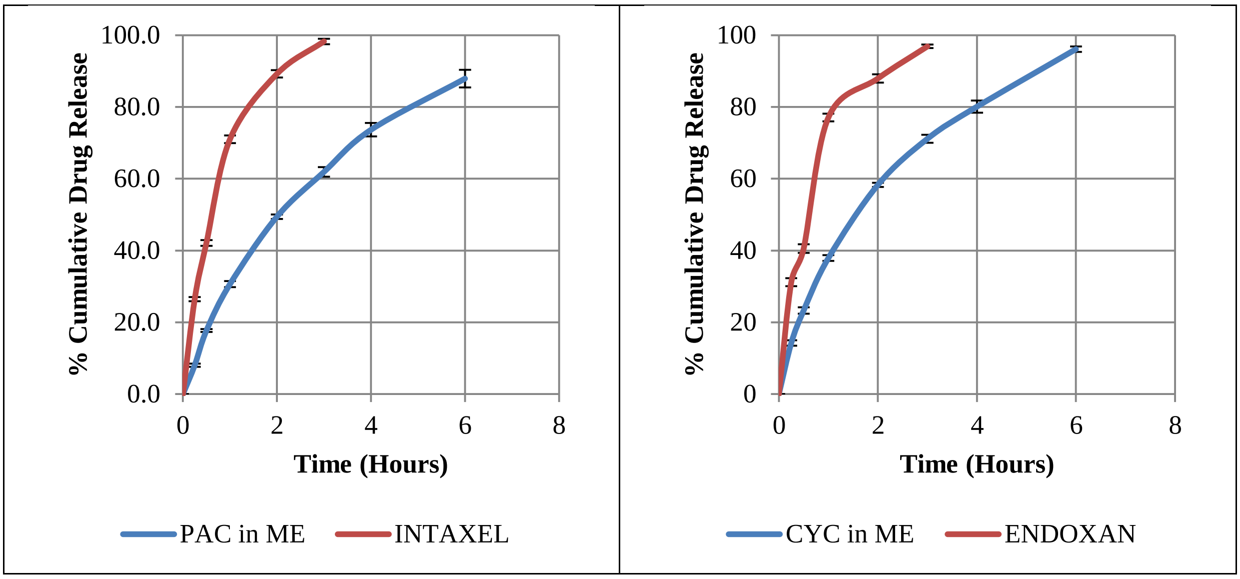

Figure 6.7: Graph of % cumulative drug release vs. time in hours for Paclitaxel & Cyclophosphamide loaded microemulsion, INTAXEL and ENDOXAN

In-vitro drug release profile was studied for Paclitaxel and Cyclophosphamide loaded microemulsion, INTAXEL, and ENDOXAN. Cumulative drug release of 98.27 ± 0.75 % and 96.87 ± 0.50 % was observed for both the Paclitaxel and Cyclophosphamide marketed formulations within 3 hours respectively whereas, Paclitaxel and Cyclophosphamide loaded in microemulsion required 6hours for cumulative drug release of 87.89 ± 2.49 % and 96.10 ± 0.75 %respectively.

The rapid release of Paclitaxel from marketed formulation could be attributed to Cremophor EL and Ethanol. Ethanol is known to improve solubility of hydrophobic drugs like Paclitaxel due to its ability to engage in hydrogen bonding and its relatively high polarity compared to water. This property allows ethanol to dissolve paclitaxel more effectively than aqueous solutions [128, 129]. Cremophor EL, a non-ionic surfactant, also enhances solubility of paclitaxel, leading to rapid release [129-131]. Cyclophosphamide's solubility in ethanol ensured that it remained in a dissolved state, which is crucial for its diffusion through dialysis membrane. This solubility characteristic led to a high release percentage in a short time frame [129, 132].

Paclitaxel and Cyclophosphamide loaded microemulsion exhibited slower release of drug as compared to INTAXEL and ENDOXAN. The slower release can be attributed to structure of microemulsion, which incorporates an oil phase inside continuous aqueous phase that facilitated slower release [130-135].

6.8.8 Sterility

At the end of incubation period of 14 days, the test tube containing negative control, positive control, and test sample (microemulsion) were observed for any signs of microbial growth. No growth was observed in Negative control and test sample, whereas growth was observed for Positive control. Therefore, the optimized microemulsion passed the test for sterility.

Figure 6.8: Sterility testing [A] Negative Control (Water for Injection) [B] Positive Control for Soybean casein digest medium (SCDM) [C] Test Microemulsion in SCDM [D] Positive control for Fluid Thioglycolate Medium (FTM) [E] Test Microemulsion in FTM

6.9. STABILITY STUDIES

Stability testing is a critical component in the pharmaceutical industry, ensuring that drug products maintain their safety, efficacy, and quality throughout their shelf life. This process involves evaluating how various environmental factors such as temperature, humidity, and light affect a drug's chemical and physical properties over time [136].

Results and Discussion:

The results of stability studies are presented in Table 6.19.

Table 6.19: Results of Stability study batch 01 for PAC-CYC microemulsion

Sr. No.	**Stability Condition**	**2 to 8°C**				**25±2°C/ 60±5%RH**
	Tests	**Initial**	**3 Months**	**6 Months**	**12 Months**	**15 days**
1	Visual description	Clear with blue tint	Clear with blue tint	Clear with blue tint	Clear with blue tint	Clear with blue tint
2	Z-average (nm)	64.1 ± 0.7	61.8 ± 2.1	60.9 ± 1.3	66.6 ± 1.9	60.4 ± 0.9
3	PDI	0.101 ± 0.020	0.092 ± 0.011	0.105 ± 0.034	0.086 ± 0.016	0.113 ± 0.024
4	Zeta potential (mV)	-2.6 ± 0.2	-2.1 ± 0.6	-2.8 ± 0.4	-2.4 ± 0.3	-2.3 ± 0.5
5	Viscosity (cPs)	1.11 ± 0.02	1.08 ± 0.04	1.03 ± 0.05	1.09 ± 0.02	1.04 ± 0.07
6	Assay of Paclitaxel (%)	99.6±0.4	100.1±0.8	98.9±0.7	98.5±0.7	99.0±0.4
7	Assay of Cyclophosphamide (%)	100.3±0.7	99.9±0.4	98.0±0.8	97.2±0.5	96.6±0.9
8	Sterility	Sterile	NA	NA	Sterile	NA

PAC-CYC loaded microemulsions were observed to be stable for 12 months when stored at 2 to 8°C and stable for 15 days when stored at 25±2°C/60±5%RH. There was no significant change observed in the visual description, globule size (Z-average), PDI, and Zeta potential during the storage. Assay of Paclitaxel and Cyclophosphamide was observed to be constant during 12 months storage. Viscosity also remained constant during 12 months, which confirmed the stability of microemulsion upon storage. Sterility of the product was maintained even after 12 months storage at 2 to 8°C.

Updated Risk Assessment

Based on the optimization of various factors impacting the formulation development and characterization of PAC-CYC Microemulsion, the updated risk assessment is presented in Table 6.20 and justification for updated risk assessment is presented in Table 6.21.

Table 6.20: Updated Risk Assessment

Drug Product CQAs	Factor				
	Environment	Materials	Equipments	Process Parameters	Formulation Parameters
Product Description	Low	Low	Low	Low*	Low*
Assay of Paclitaxel	Low*	Low	Low	Low*	Low*
Assay of Cyclophosphamide	Low*	Low	Low	Low*	Low*
Sterility	Low	Low	Low	Low*	Low
Globule size distribution	Low	Low	Low	Low*	Low*
Zeta Potential	Low	Low	Low	Low*	Low*
Entrapment efficiency	Low	Low	Low	Low*	Low*
Filterability	Low	Low	Low	Low*	Low*

Table 6.21: Justification for Updated Risk Assessment

Drug Product CQAs	Factor	Justification
Product Description	Process Parameters (Previously a High risk) Formulation Parameters (Previously a High risk)	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization which directly impacts the product description was optimized during the formulation development of PAC-CYC Microemulsion. Hence, the risk is assessed to be Low.
Assay of Paclitaxel	Environment (Previously a Medium risk)	All the development studies and characterization of PAC-CYC microemulsion was executed at controlled room temperature (25±5°C) with relative humidity below 55%RH and either sodium vapor light or no light condition to ensure minimal exposure of room light. No separate experimentation was required as necessary precaution was in place throughout the development and stability. Hence, the risk is assessed to be Low.

Drug Product CQAs	Factor	Justification
Assay of Paclitaxel	Process Parameters (Previously a High risk)	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization was optimized during development studies to achieve the assay of Paclitaxel in the range of 90.0 to 110.0% for optimized PAC-CYC microemulsion. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Assay of Cyclophosphamide	Environment (Previously a Medium risk)	All the development studies and characterization of PAC-CYC Microemulsion was executed at controlled room temperature (25±5°C) with relative humidity below 55%RH and either sodium vapor light or no light condition to ensure minimal exposure of room light. No separate experimentation was required as necessary precaution was in place throughout the development and stability. Hence, the risk is assessed to be Low.
	Process Parameters (Previously a High risk)	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization was optimized during development studies to achieve the assay of Cyclophosphamide in the range of 90.0 to 110.0% for optimized PAC-CYC microemulsion. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Sterility	Process Parameters (Previously a Medium risk)	Sterilization of PAC-CYC microemulsion was ensured using sterile filtration technique. The filterability of developed product was observed to be 100% when filtered through 0.2µ pore size sterile PES filters. Drug product was observed to be sterile upon tested for sterility testing. Hence, the risk is assessed to be Low.
Globule size distribution	Process Parameters (Previously a High risk)	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization was optimized during development studies to achieve Z-average below 150nm and PDI below 0.2 for the optimized formulation. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	

Drug Product CQAs	Factor	Justification
Zeta Potential	Process Parameters (Previously a High risk)	Process parameters viz. temperature of phases, mixing speed, mixing time, and formulation parameters viz. selection of surfactant, total lipid concentration, drug substance concentration, and Solid lipid: liquid lipid concentration impacts the final formulation were optimized during development studies which led to zeta potential in between -10mV and + 10mV for final optimized product. The zeta potential was also observed to remain stable during storage stability of optimized product. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Entrapment efficiency	Process Parameters (Previously a High risk)	Process parameters viz. mixing speed and mixing time and formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization was optimized during development studies to achieve the entrapment efficiency of not less than 90.0%. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	
Filterability	Process Parameters (Previously a High risk)	Formulation parameters viz. selection of surfactants, surfactant: cosurfactant ratio (S_{mix}), drug substance concentration, and oil: S_{mix} optimization was optimized during development studies to achieve the filterability of 100% for the optimized PAC-CYC Microemulsion. 100% filterability also ensures that filtration achieved sterile drug product suitable for intravenous administration. Hence, the risk is assessed to be Low.
	Formulation Parameters (Previously a High risk)	

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