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FORMULATION AND EVALUATION OF NANOPARTICLES CONTAINING CYCLOPHOSPHAMIDE

[1]*Pankaj Sharma, [1]Sarvesh Bhargava, [2]Deepesh Parashar and [3]Ashish Mangal

[1]Department of Pharmaceutics, Shri Ram College of Pharmacy, Morena M.P., India.

[2]Department of Pharmaceutical Chemistry, Shri Ram College of Pharmacy, Morena M.P., India.

[3]Department of Pharmacology, Shri Ram College of Pharmacy, Morena M.P., India.

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***Corresponding Author**
Pankaj Sharma
Department of Pharmaceutics, Shri Ram College of Pharmacy, Morena M.P., India.

ABSTRACT

Objectives of present study were- to maintain the therapeutic drug concentration in the site of action for a prolonged period, improve the drug's efficiency & reduce the dose related side effect of cyclophosphamide. Cyclophosphamide nanoparticles were prepared by salting out method. Nine (F1-F9) formulations of nanoparticles were developed in research. After development of formulations, various evaluation parameters were calculated. Percentage yield of nanoparticles were determined for all 9 formulations (F1 to F9). The result for all different formulated was obtained in the range of 26.09±0.95 to 39.8±1.4. Loading efficiency was determined for all nine formulations (F1 to F9). The results for all different formulations were obtained in the range from 40.56±0.85 to 45.29±2.1. Particle size determination was done by using Malvern mastersizer instrument for the optimized formulation F8. Particle size was found 730.9 nm with maximum intensity and volume. From this data, it could be said that the nanoparticles has been formed successfully by using ionic cross linking method. Polydispersibility index of the cyclophosphamide obtained nanoparticles was found to be 0.918 and hence shows a narrow size distribution and contributes to the stability of nanoparticles. Based on the observations, formulation F8can be concluded that the PLGA coated Nano particulate targeted delivery system of cyclophosphamide formulated using widely accepted and physiologically safe polymer was capable of exhibiting sustained release properties for 8hrs.

KEYWORDS: Nanoparticles, FTIR, Polydispersibility, Entrapment Efficiency.

INTRODUCTION

Cancer is a critical disease characterized by uncontrolled multiplication of immature cells and spread of abnormal forms of the body's own cells. The branch of medicine apprehensive with the study, diagnosis, treatment and prevention of cancer is Oncology. Cancer may affect people at all ages, but the risk of most assortments increase with age[1]. The use of nanoparticles in the field of cancer therapy is attractive for several purpose: they show unique pharmacokinetics, including minimum renal filtration; they have high surface to volume ratio facilitating modification with numerous surface functional groups that home, internalize, or stabilize; and they may be constructed from a wide range of materials used to encapsulate or solubilize therapeutic agents for drug delivery. The topology of a nanoparticle—core, coating, and surface functional groups makes it particularly acquiescent to modular design, where by features and functional moieties may be switched or combined. Although much functionality of nanoparticles have been demonstrated, including some clinically arrogated drug formulations and also imaging agents, the merging of these into multifunctional nanoparticles capable of targeting, imaging, and delivering therapeutics is an exciting area of research that holds great promise for cancer therapy in the future. To include many features such as the ability to target tumors, evade uptake by the reticuloendothelial system (RES), protect therapeutics that can be released on petition, act as sensors of tumor responsiveness, and provide image distinction to visualize sites of disease and monitor disease progression, so that is show many desirable features in cancer desease. Some of these useful features, such as targeting, influence biological mechanism. Others are derived synthetically and enable external curious or manipulation that is otherwise not realistic in biological systems. In this chapter, we review both bio-inspired and synthetic nanoparticle functionalities that have been used in cancer therapy and address current efforts and future opportunities to combine these into multifunctional devices for better resolution in cancer therapy[2-10].

Objectives of present study were- to maintain the therapeutic drug concentration in the site of action for a prolonged period, improve the drug's efficiency & reduce the dose related side effect.

MATERIALS AND METHODS

Materials: Cyclophosphamide API was kindly gifted by Glenmark Pharmaceuticals Pithampur Indore M.P.. Poly (lactide-co-glycolide) Coating agent, Poly (vinyl-pyrollidine) stabilizing agent by Fisher Scientific Mumbai. Acetone solvent agent obtained from

Qualikems, Vadodara and magnesium chloride Electrolyte agent obtained from Finar Reagent.

Methods

Methods used for preformulation study-

1. UV spectroscopy (determination of λmax)

Stock solution (1000µg/ml) of cyclophosphamide in Phosphate buffer pH 7.4 was prepared. This solution was appropriately diluted with the same solution to obtain stock solution of (100µg/ml). The resultant solution was scanned in the range of 200 nm – 400 nm in UV-Visible Spectrophotometer. It showed λmax201 nm in phosphate buffer pH 7.4 for cyclophosphamide.

2. Determination of calibration curve

Spectrophotometric analysis of cyclophosphamide was carried out on double beam UV spectrophotometer.

3. Standard calibration curve of cyclophosphamide in water

Cyclophosphamide (25 mg) was dissolved in 25 ml water to obtain a stock solution of 1000µg/ml concentration. From the stock solution of sample 10ml of solution diluted with 100ml to obtained 100µg/ml. then this solution (100µg/ml) was further diluted with water to obtained solution of 2 to 10µg/ml. Absorbance of each solution was measured at 201 nm using UV-Visible spectrophotometer and water was taken as blank. The standard curve was generated for the entire range from 2 to10 µg/ml.

4. Standard calibration curve of cyclophosphamide in Phosphate buffer pH 7.4

Cyclophosphamide (25 mg) was dissolved in 25 ml phosphate buffer pH 7.4 to obtain a stock solution of 1000µg/ml concentration. From the stock solution of prepared sample 10ml of solution diluted with 100ml to obtained 100µg/ml. This solution (100µg/ml) was further diluted with phosphate buffer pH 7.4 to obtained solution of 20 to 100µg/ml. Absorbance of each solution was measured at 201 nm using UV-Visible spectrophotometer and phosphate buffer pH 7.4 was taken as blank. The standard curve was generated for the entire range from 20 to 100µg/ml[11].

5. Solubility study

Solubility of the drug sample was examined by saturation equilibrium method. Excess quantity of cyclophosphamide was added in to the china dish containing 5ml of water to make supersaturated solution of drug in water. China dish was kept on water bath to evaporate the water. A dry powder was collected and weighs to measure the amount of drug[12].

6. Melting point determination

Melting point of the drug sample was determined by capillary method using melting point Apparatus 41-45°C.

7. Drug – polymer compatibility study

Infrared spectra of pure drug, polymer, as well as for combination of drug-polymer were taken by KBr pellet technique and were recorded in the range of 4000 – 400cm^{-1} by using FT-IR Spectrophotometer Shimadzu.

8. Fourier Transformed Infrared Spectroscopy (FTIR)

Fourier transform infrared (FT-IR) spectra were obtained on a Nicolet AVATAR 360 FT-IR spectrometer. Samples of pure drug or nanoparticles (with or without drug) were prepared in KBr disks (2 mg sample in 200 mg KBr). The scanning range was 450–4000 cm^{-1} and the resolution was 1 cm^{-1}. Sample analysis was carried out by the E2 OMNIC software for analysis[13].

PREPARATION OF CYCLOPHOSPHAMIDE NANOPARTICLES

Salting out method was used for preparation of cyclophosphamide nanoparticles. Cyclophosphamide nanoparticles were prepared by salting out method. Salting out is based on the separation of a water miscible solvent from aqueous solution via a salting out effect. The salting out procedure can be considered as a modification of the appropriate emulsification/solvent diffusion. Polymer and cyclophosphamide were primarily dissolved in a solvent acetone, which is subsequently emulsified into an aqueous gel containing the salting-out agent magnesium chloride and a colloidal stabilizer such as polyvinylpyrrolidone. This oil/water emulsion is diluted with a sufficient volume of water or aqueous solution to enhance the diffusion of acetone into the aqueous phase, thus convincing the formation of nanospheres. The selection of the salting out agent is important, because it can play an important role in the encapsulation efficiency of the drug. Both the solvent and the salting out

agent are then eliminated by cross-flow filtration method. This technique used in the preparation of poly (lactide-coglycolide), poly (methacrylic) acid, Nano spheres leads to high efficiency and is easily scaled up. The main advantage of salting out is that it minimizes stress to protein encapsulates. Salting out does not require an increase of temperature and therefore, may be useful when heat sensitive substances have to be processed. The greatest disadvantages are exclusive application to lipophilic drugs and the extensive nanoparticles washing steps.

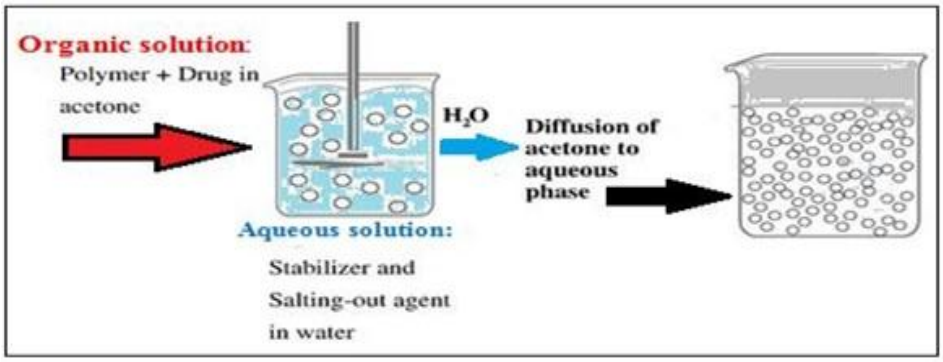


Fig1: Schematic representation of the salting out technique.

Table 1: Formulation of cyclophosphamide nanoparticles from F1 to F9.

Sr.no.	Ingredients	Formulations								
		F1	F2	F3	F4	F5	F6	F7	F8	F9
1	Cyclophosphamide	25mg	25mg	25mg	25mg	25mg	25mg	25mg	25mg	25mg
2	Poly lactide (co-glycolide)	100mg	200mg	300mg	400mg	500mg	100mg	200mg	300mg	400mg
3	Poly-vinyl pyrrolidone	400mg	300mg	200mg	100mg	100mg	300mg	300mg	200mg	100mg
4	Acetone	10ml	10ml	10ml	10ml	10ml	10ml	10ml	10ml	10ml
5	Magnesium	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
6	Distilled water	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

EVALUATION OF NANOPARTICLES

1. Percentage Yield

The yield of production of nanoparticles of various batches were calculated using the weight of the final product after drying with respect to the initial total weight of the drug and polymer used for preparation of nanoparticles and percent production yield were calculated as per the formula mentioned below.

%Yield= Practical mass×100 Theoretical mass

2. Drug content

The drug content of the prepared nanoparticles was determined by the formula:

$$\textbf{Drug content (\%)} = \frac{\text{Weight of the drug in nanoparticles} \times 100}{\text{Weight of NP}}$$

3. Drug entrapment efficiency

Separation of free drug: The separation was done by centrifugation at 12000 RPM for 30 minutes of nanoparticles significantly. Then, the nanoparticles pellets and supernatant were separated out.

Indirect method: In this method, analysis of drug from Nanoparticles was done by suitably diluting supernatant in water and absorbance was taken against water as a blank on UV-Visible Spectrophotometer at 201nm. To find out % entrapment following equation was used.

The percentage drug entrapment efficiency (DEE) was determined by the formula:

DEE% = Amount of drug actually present ×100 Theoretical drug load expected

4. Particle size and surface charge

The particle size analysis and zeta-potential measurement were analyzed by Zetasizer Nano ZS. For the analysis, NP sample of the desired concentration was flushed through a folded capillary cell (DTS1060) and the measurement was carried out on the second filling; a sufficient sample volume was used to completely cover the electrodes of the cell. Then the sample was injected slowly and analysis was carried out if there were no visible air bubble annexation present. After inspection, the cell was placed into the Zetasizer and equilibrated at 2 min. prior to the particle size measurement, of which there were six replicates[14].

5. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy is an instrument that produce largely magnified image by using electrons instead of light to form an image. Electron gun produces a beam of electrons which follows the vertical path through the microscope between electromagnetic field and lenses en route for the sample due to which electron and X-rays are ejected from sample. Selected method for estimation of NP morphology of Cyclophosphamide loaded NPs and was determined using scanning electron microscope (SEM). Prior to examination, the sample were mounted on to metal stubs using a double-sided adhesive tape under vacuum.

6. *In-vitro* drug release

In-vitro drug release of cyclophosphamide nanoparticles in present research work is carried out by dialysis bag diffusion method. A 4–5 cm long portion of the dialysis bag was made into a dialysis sac by folding and tying up one end of the bag with thread. It was filled up with phosphate-buffer pH 7.4 and examined for the leaks. The sac was then emptied and NPs dispersion (equivalent to 10 mg drug) was precisely transferred into sac, which served as the donor compartments. The sac was once again inspected for leak and then suspended in the glass beakers containing 50 ml phosphate-buffer pH 7.4, which become the receptor compartment. Aliquots were taken at 1,2,3,4,5,6,7 and 8 hours and analyzed spectrophotometrically at 201 nm. Fresh buffer was used to replenish the receptor compartment at each time to maintain sink condition.

RESULT AND DISCUSSION

PREFORMULATION

1. Colour, Odour, and Appearance

Table 2: Colour, Odour and Appearance of cyclophosphamide.

Sr. No	Parameter	Observation
1	Colour	White
2	Odour	Odourless
3	Appearance	Crystalline powder

2. Determination of λmax

Table 3: λmax (nm) of cyclophosphamide.

Drug	λmax (nm)
Cyclophosphamide	201

3. Determination of Calibration curve

Table 4: Calibration curve of cyclophosphamide in water at λmax 201 nm.

Sr. no.	Conc. (µg/ml)	Absorbance			Mean SD*
		I	II	III	
1	2	0.115	0.116	0.116	0.116±0.004
2	4	0.250	0.2512	0.2512	0.251±0.003
3	6	0.408	0.408	0.408	0.408±0.001
4	8	0.516	0.516	0.516	0.516±0.002
5	10	0.637	0.635	0.635	0.636±0.001

n = 3

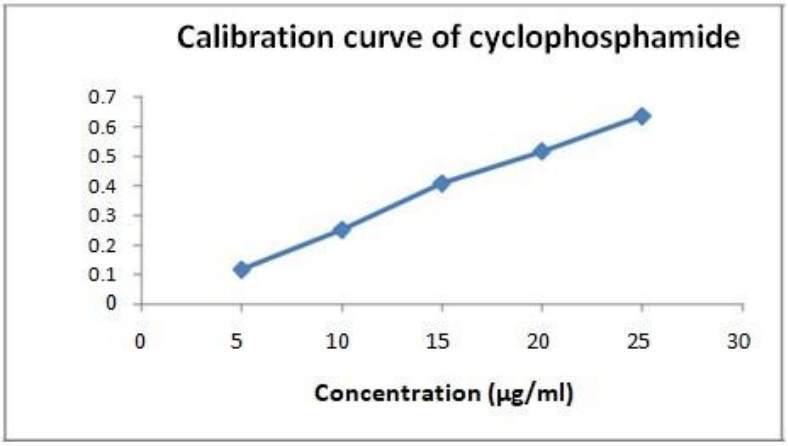


Figure 2: cyclophosphamide calibration curve in water at λmax 201 nm.

Table 5: Calibration curve of cyclophosphamide in phosphate buffer pH 7.4 at λmax 201nm.

Sr. no.	Conc. (µg/ml)	Absorbance			Mean ± SD*
		I	II	III	
1	20	0.216	0.214	0.215	0.215±0.003
2	40	0.331	0.332	0.331	0.331±0.002
3	60	0.405	0.406	0.404	0.405±0.001
4	80	0.528	0.529	0.527	0.528±0.001
5	100	0.634	0.636	0.635	0.635±0.002

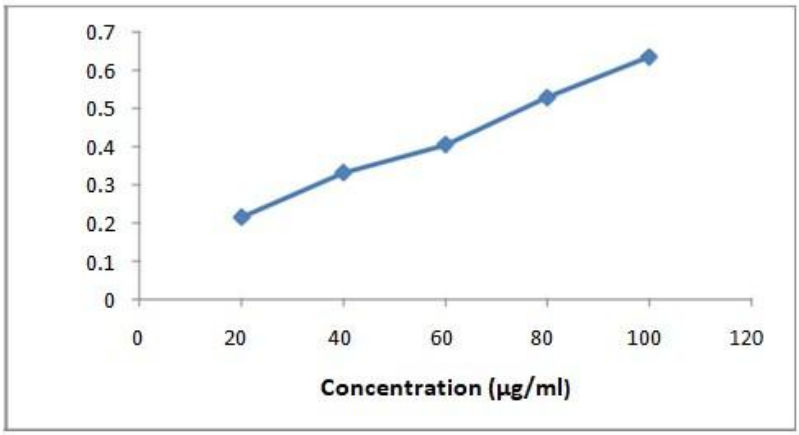


Figure 3: cyclophosphamide calibration curve in phosphate buffer pH 7.4 at λmax201 nm.

4. Determination of Solubility study

Table 6: solubility in water.

Solvent	Solubility	Terms
Water	9.56±0.27 mg/ml	Freely soluble

5. Melting point

Table 7: Melting point of cyclophosphamide.

Reported Melting Point	Observed Melting Point
41-45^{0}C	43 - 44^{0}C

6. Drug Polymer compatibility Study

FT-IR studies

Plain drug cyclophosphamide

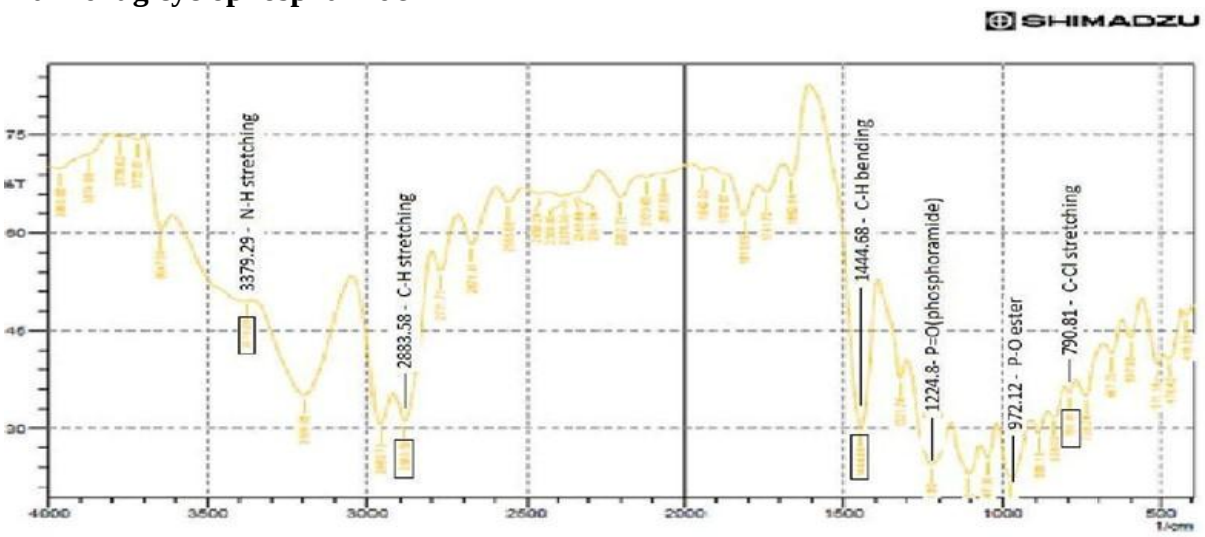


Figure 4: FT-IR Spectrum of pure drug in range 4000 to 400 c m $^{-1}$.

Table 8: functional group and wave no observed in pure drug and mixture of drug and polymer.

Functional group	Pure drug wave no.(cm)$^{-1}$	Drug+polymer wave no.(cm)$^{-1}$
N-H stretching	3378.29	3273.57
C-Cl stretching	791.81	792.81
C-H stretching	2884.58	2885.58
-C-H bending	1445.68	1442.79
P=O(phosphoramide)	1225.8	1223.94
P-O(ester)	973.12	978.84

EVALUATIONS OF FORMULATION

Data of % yield, %E.E and % drug loading of cyclophosphamide NPs

Table 9: Data of % yield, %E.E and % drug loading of cyclophosphamide NPs.

Formulation no.	% yield	%EE	% drug loading
F1	39.8±1.4	71.6±1.5	45.29±2.1
F2	31.37±1.21	72.6±1.25	43.4±1.2
F3	26.09±0.95	78.91±1.023	44.82±2.3
F4	41.3±2.06	81.01±2.53	44.86±2.15
F5	32.1±1.3	82.35±1.32	43.67±1.1

F6	27.9±0.96	82.50±2.5	44.87±3.22
F7	41.19±2.5	84.93±2.21	40.56±0.85
F8	32.71±1.11	89.78±2.36	42.9±0.91
F9	29.08±0.93	86.49±3.21	43.03±2.13

1. Percentage yield

Percentage yield was determined for all 9 formulations (F1 to F9). The result for all different formulated was obtained in the range of 26.09±0.95 to 39.8±1.4.

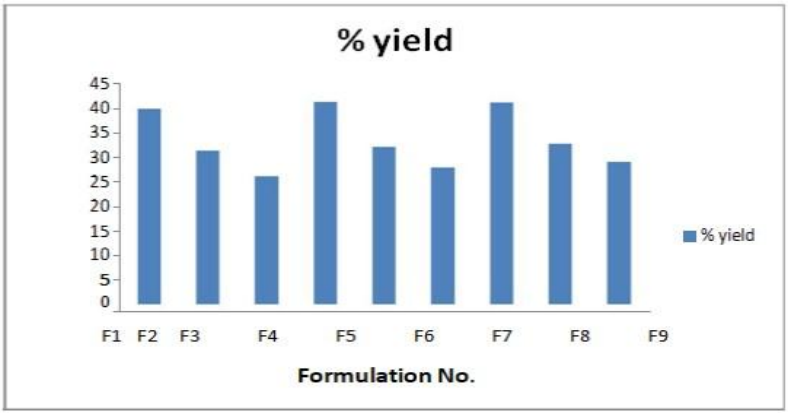
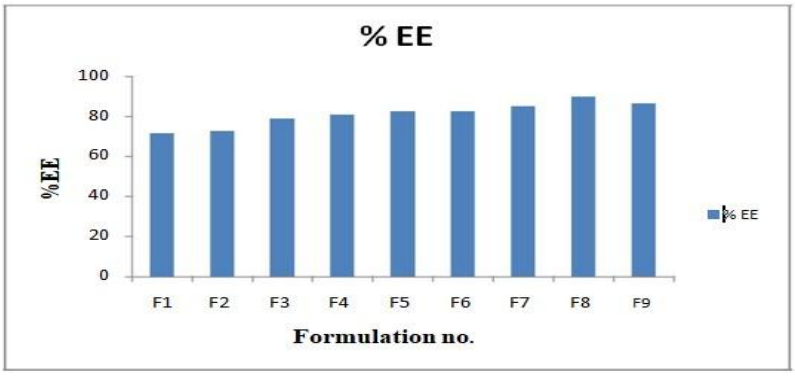


Figure 5: Column diagram for % yield.

2. Entrapment efficiency

Entrapment efficiency was determined for all 9 formulations (F1 to F9). The result for all different formulated was obtained in the range of 71.6±1.5 to 86.49±3.21. The maximum entrapment 89.78±2.36 was found for the Formulation 8. It was observed that as in formulation F1 to F7 and F9, entrapment was less as compared to formulation 8. The reason may be concentration of polymer. As concentration of polymer was increased, the entrapment was increased. But as concentration was further increased, the entrapment was decreased. Due to more cross linking occurs in between polymers, might be leads to decrease in entrapment.



n = 3

Figure 6: Column diagram for Entrapment efficiency.

3. Loading efficiency

Loading efficiency was determined for all nine formulations (F1 to F9). The results for all different formulations were obtained in the range from 40.56±0.85 to 45.29±2.1.

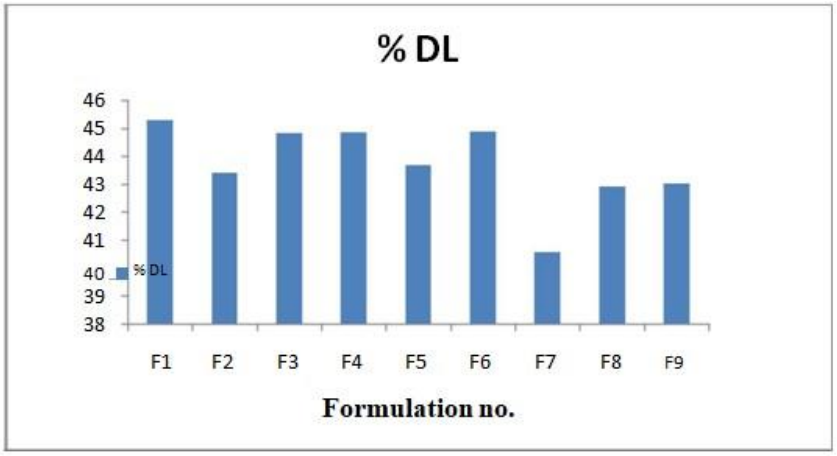


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Figure 7: Column diagram for % drug loading.

4. Particle size of formulation F8

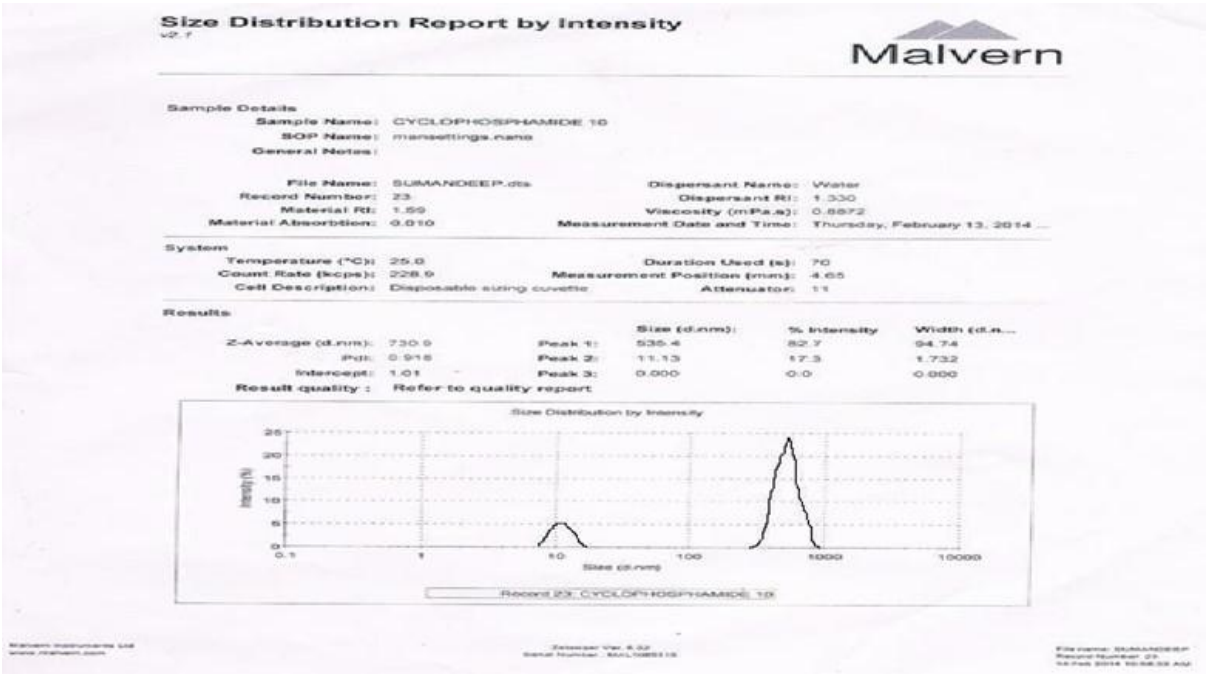

Figure 8: particle size of formulation F8.

Particle size determination was done by using Malvern mastersizer instrument for the optimized formulation F8. Particle size was found 730.9 nm with maximum intensity and volume. From this data, it could be said that the nanoparticles has been formed successfully by using ionic cross linking method.

5. PDI (polydispersibility index)

PI of the cyclophosphamide obtained nanoparticles was found to be 0.918 and hence shows a narrow size distribution and contributes to the stability of nanoparticles.

6. Scanning Electron microscopy

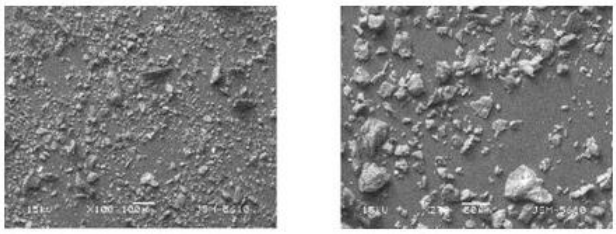

Figure 9: Scanning Electron microscopic photograph of optimized formulation F8.

7. Formulation *In-vitro* Diffusion Study

Table 10: % CDR of formulation from F1 to F9.

S. No.	% CDR								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	10.1	18.0	5.5	2.5	14.3	5.2	4.0	3.6	3.0
2	10.5	15.5	7.5	4.5	11.0	7.0	5.5	6.0	6.5
3	35.0	39.0	29.4	22.8	42.0	27.0	23.0	22.0	25.4
4	35.6	37.0	32.3	29.5	38.0	44.0	37.0	35.2	42.0
5	41.0	39.0	37.0	33.7	44.5	55.0	49.0	45.0	52.0
6	49.0	47.1	43.2	39.7	52.3	76.0	72.0	69.5	74.0
7	61.0	59.0	55.8	50.0	64.0	86.0	81.3	80.0	83.2
8	85.0	82.3	75.7	70.3	89.0	94.0	92.1	90.2	93.0

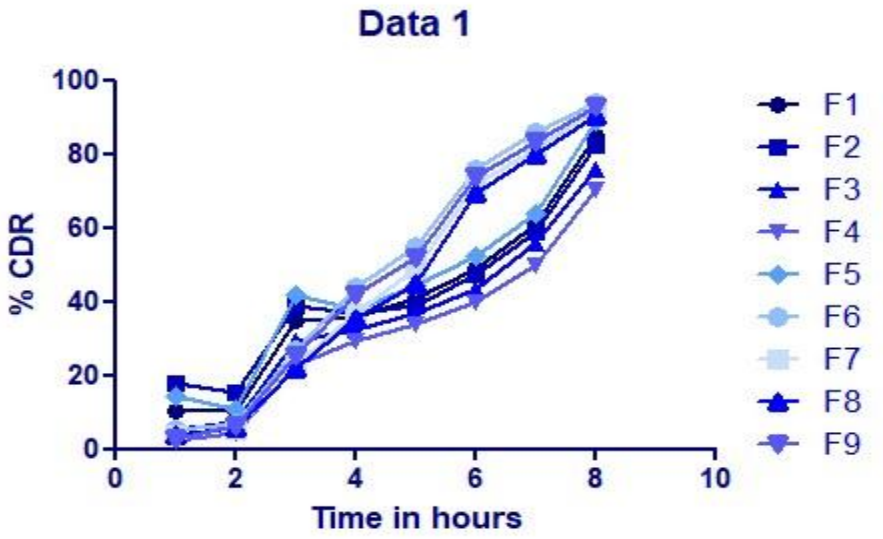

Figure 10: *In-vitro* diffusion profile of formulations F1 to F9.

The *in-vitro* drug release of drug cyclophosphamide from the many nanoparticles formulations was carried out by using dialysis method in phosphate buffer pH 7.4 for 8 hrs. Formulation no F8 have been selected as optimized formulation.

CONCLUSION

Based on the observations, formulation F8can be concluded that the PLGA coated Nano particulate targeted delivery system of cyclophosphamide formulated using widely accepted and physiologically safe polymer was capable of exhibiting sustained release properties for 8hrs. It was also concluded that the concentration of polymer and cross-linking agent that is polyvinyl pyrollidone was highly influence the formulation characteristics. From research articles, it was found that cancer cells contain folate receptors on the surface and folic acid have over expression on cancer cells. By considering this, we have prepared Cyclophosphamide loaded CS-NPs have been coated with PLGA that shows better coating on NPs which is useful for targeting the cancer cells.

By administering such formulation, it may reduce the amount of drug to be administered along with frequency of dosing, thereby minimizing the occurrence of the side effects and may improve the effectiveness of the drug.

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