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Development and testing of novel temoxifen citrate loaded chitosan nanoparticles using ionic gelation method

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ABSTRACT

The goal of the present investigation was to formulate and evaluate chitosan nanoparticles of temoxifen citrate for cancer therapy. Nanoparticles of TMX were prepared using chitosan using ionic gelation method. The concentration of the polymers Chitosan were selected based on the results on preliminary screening. The nanoparticles prepared were evaluated for morphology, loading efficiency, invitro release and invitro anticancer activities. The particle shape and morphology of the prepared TMX nanoparticles was determined by SEM analysis. The amount of TMX entrapment in the nanoparticles was calculated by the difference between the total amount of drug added to the nanoparticle and the amount of non entrapped drug remaining in the aqueous supernatant. A diffusion cell was used to monitor TMX release from the nanoparticles. The formulations FM-1 showed good drug release from the polymer. The percentage cumulative drug release after 3,4,5,6,7 and 8 hours was 40.54, 48.68, 56.26, 65.84, 71.42 and 78.03% respectively. Among the all formulations FM 1 showed maximum drug release in 8 hours diffusion study and good entrapment efficiency.

Keywords: TMX, nanoparticles, chitosan, MCF-7.

INTRODUCTION

The substances with size ranges from 1 to 1000 nm are called nanoparticles. These materials are mainly used in oncology for early detection of malignancy and precise localisation of cancer therapeutics without or with minimal adverse effect to the somatic tissues. These carriers are used to protect drugs, vaccines, nutrients and cosmetics. Nanoparticles exerts its site specific drug delivery by avoiding the reticuloendothelial system, utilising enhanced permeability and retention effect and tumour specific targeting. The formation of nano particles and physicochemical parameters such as pH, monomer concentration, ionic strength as well as surface charge, particle size and molecular weight are important for drug delivery. Further, these

nanoparticles have the capabilityto reverse multi drug resistance, a major problem in chemotherapy[1].

Tamoxifen is an antagonist of the estrogen receptor in breast tissue via its active metabolite, hydroxytamoxifen. In other tissues such as the endometrium, it behaves as an agonist, hence tamoxifen may be characterized as a mixed agonist/antagonist. It has been the standard endocrine (anti-estrogen) therapy for hormone receptor-positive early breast cancer in pre-menopausal women, although aromatase inhibitorshave been proposed[2].

The limitation in conventional cancer treatment can be alleviated by targeted drug delivery, which is a vehicle that will preferentially carry the drug to the target site in the body and thereby reduce the amount of drug in the rest of the body that can cause undesired side effect. These would increase the range in which a drug is both safe and effective. The distinct capability of nanoparticles to provide access to virtually all cell types may be utilised for the delivery of therapeutic agents to wide array of cellular types and targets[3].

MATERIALS AND METHODS

Temoxifen citrate was a gift sample from Sun Pharmaceuticals, Baroda, India. Chitosan was procured from Central Institute of Fisheries Technology, Cochin, India. Sodium tripolyphosphate, Petroleum Ether and Acetic Acid was obtained from SD Fine Chemical, India.

Preparation of drug loaded nanoparticles

Method: Ionic gelation method:

Drug loaded chitosan nanoparticles were prepared by the method reported by Calvo et al. (1997b) with some modifications based on the ionic gelation of Tamoxifen with TPP anions. Chitosan was dissolved in acetic aqueous solution (6 % v/v) at various concentrations such as 1.0, 2.0, 3.0, 4.0 and 5.0 mg / ml. 10 mg of drug (Tamoxifen) was dissolved in 5 ml of 2 % w/v tween 80 solutions, which was added to the chitosan solution. Under magnetic stirring at room temperature, 5 ml and 10ml of 0.25 % sodium tripolyphosphate (TPP) aqueous solution was added drop wise into drug and polymeric mixture, respectively. The stirring was continued for about 20 – 25 min. The obtained nanoparticle suspension was centrifuged at 12000x rpm for 30 min. The formation of the particle was a result of the interaction between the negative groups of the TPP and the positively charged amino groups of chitosan (ionic gelation) [4,14-15].

Design of Experiment

Table-1: Formulation consideration of chitosan nanoparicles

Batch No.	Tamoxifen (TMX) (mg)	Concentration of chitosan (mg)	0.25 % TPP solution (ml)	TMX:Chitosan
F1	**30**	30	10	1.1
F2	**30**	60	10	1.2
F3	**30**	90	10	1.3
F4	**30**	120	10	1.4
F5	**30**	150	10	1.5

Characterization of Nanoparticles:-

Determination of Percent Entrapment Efficiency

Weighed quantity (10mg) of nanoparticles were crushed and suspended in 10 ml methanol to extract the drug from nanoparticles. After 24 h, the filtrate was assayed spectrophotometrically (Shimadzu-1700, Japan) for drug content against Methanol as blank (Patil *et al*., 2006) [5,16].

Physicochemical Characterization

Particle Size

Nanoparticle size and size distribution were determined using photon correlation spectroscopy (Zetasizer, HAS 3000; Malvern Instruments, Malvern, UK). The size distribution analysis was performed at a scattering angle of 90 degrees and at a temperature of 25°C using samples appropriately diluted with filtered water (0.2-µm filter; Minisart, Gottirgen, Germany). For each sample, the mean diameter ± standard deviation of three determinations was calculated applying multimodal analysis[6,17].

Zeta Potential

Zeta potential was determined by photon correlation spectroscopy (Zetasizer, HAS 3000; Malvern Instruments, Malvern, UK) using a disposable zeta cuvette. For each sample, the mean diameter/zeta potential ± standard deviation of six determinations was calculated applying multimodal analysis[7].

In vitro Drug Release

In vitro release of Temoxifen was conducted by dialysis in a dialysis sac (Sigma, 12000 MW cut off) with 200 ml of PBS (pH 7.4) at 37°C±1°C . Briefly, in a 350 ml conical flask, 300 ml of PBS was taken. One ml of formulation was taken into a dialysis bag and dipped into the buffer solution. The flask was kept on a magnetic stirrer. Stirring was maintained at 300 rpm and the temperature of the buffer was maintained at 37°C±1°C. Sampling was done by withdrawing 0.5 mL from the released medium with the help of micropipette and 0.5 mL of fresh buffer was added. Samples were analyzed using a spectrophotometer at a wave length of 274 nm. With the help of the standard curve prepared earlier, drug concentration was measured[8-11].

Stability Studies

Stability is defined as "the capacity of the drug product to remain within specifications established to ensure its identity, strength, quality and purity". The purpose of stability testing is to provide evidence on how the quality a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light and to establish a re-test period for the drug substance or a shelf for the drug product (ICH guideline, 2003) [12-13].

RESULTS AND DISCUSSION

In total Five formulations of Temoxifen citrate loaded nanoparticles were prepared and evaluated for various parameters such as particle size, morphology, drug entrapment efficiency, in-vitro release and in-vitro anticancer activity.

Preparation of drug loaded nanoparticles

Nanoparticles were prepared by ionic gelation technique. It is a laboratory method proved for the preparation of nanoparticles. The concentration of the polymers chitosan were selected based on the results on preliminary screening. The time taken to complete preparation was around 2 hours.

Yields of Production:- Total amount of nanoparticles obtained was weighed individually for each batch and the percentage yield was calculated taking into consideration the weight of drug and polymer (Yadav *et al*., 2008). Yields of production of different formulations were calculated by using the formula:

$$\text{Percent Yield of Production} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100$$

Table-2:-Percentage yield of TMX Nanoparticles

Formulation code	Total amount of Ingredient (mg)	Practical yield (mg)	Percentage yield (%)
FM – 1	32.5	19.89	61.2
FM – 2	42.5	23.41	55.1
FM – 3	52.5	31.08	59.2
FM – 4	62.5	37.68	60.3
FM – 5	72.5	42.05	58.0

Each data represents mean ±SD, (n=3)

The percentage yield of different formulation for chitosan nanoparticle were higher in FM-1 as compare to other Formulation. In FM-1 percentage yield is 61.2% compare to FM-2, FM-3, FM-4 and FM-5 is 55.1%,59.2%,60.3% and 58.0% respectively.

Percent Entrapment Efficiency

Weighed quantity (10mg) of nanoparticles were crushed and suspended in 10 ml methanol to extract the drug from nanoparticles. After 24 h, the filtrate was assayed spectrophotometrically (Shimadzu-1700, Japan) for drug content against Methanol as blank (Patil *et al*., 2006).

Table-3: Drug Entrapment Efficiency of TmxNanoparticles Optimized Formulations

Formulation code	Absorbance	Concentration (µg/ml)	Amount of drug present in 10 mg of nanoparticles (mg)	Drug content (%)	Drug Entrapment Efficiency (%)	Particle size (nm)
FM – 1	0.4752	54.63	5.463	54.63	10.86	750
FM – 2	0.4445	51.10	5.110	51.10	11.96	780
FM – 3	0.5537	63.65	6.365	63.65	19.78	766
FM – 4	0.4592	52.79	5.279	52.79	19.89	758
FM - 5	0.5739	65.97	6.597	65.97	27.74	767

Each data represents mean ±SD, (n=3)

The efficiency of encapsulation was determined by measuring the total amount of Temoxifen citrate present in a known amount of the nanoparticulate sample, and comparing the measured value to the expected amount of Temoxifen citrate in the sample. The drug load was expressed as percentage of the drug in the polymer. The % drug content were 65.97 % for FM-5 and FM-1, FM-2, FM-3, and FM-4 and is 54.63%, 51.10%, 63.65% and 52.79 % respectively for chitosan nanoparticles.

The encapsulation efficiencies for the formulations were 27.74 % for FM-5 and FM-1, FM-2, FM-3, and FM-4 and is 10.86%, 11.96 %,19.78 % and 19.89 % respectively for chitosan nanoparticles.

The particle size for the formulations were 753nm for FM-5 and FM-1, FM-2, FM-3, and FM-4 and is 750nm, 780nm, 766nm and 758nm respectively for chitosan nanoparticles.
(Table 3)

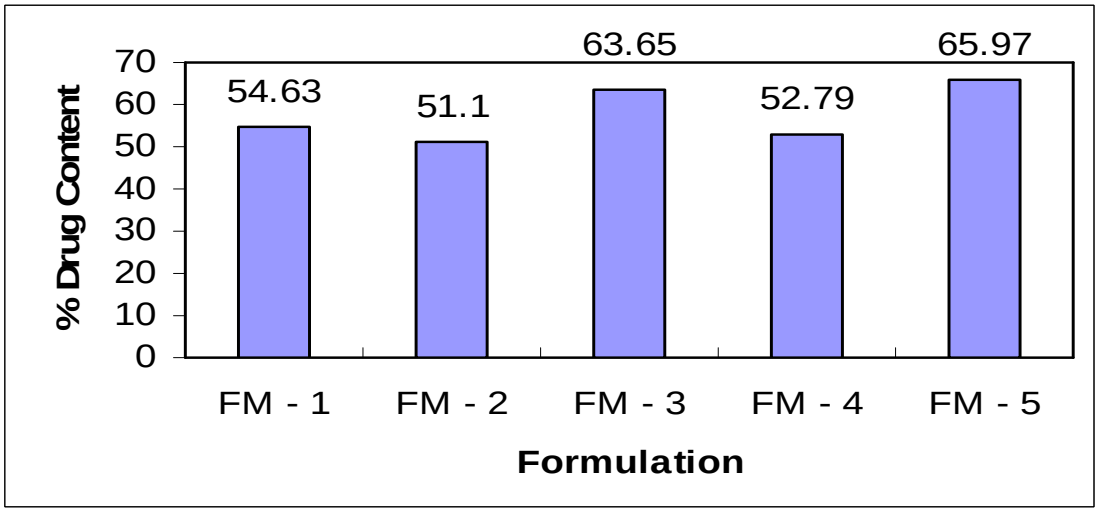

Figure-1: Drug Content of TMX Nanoparticles

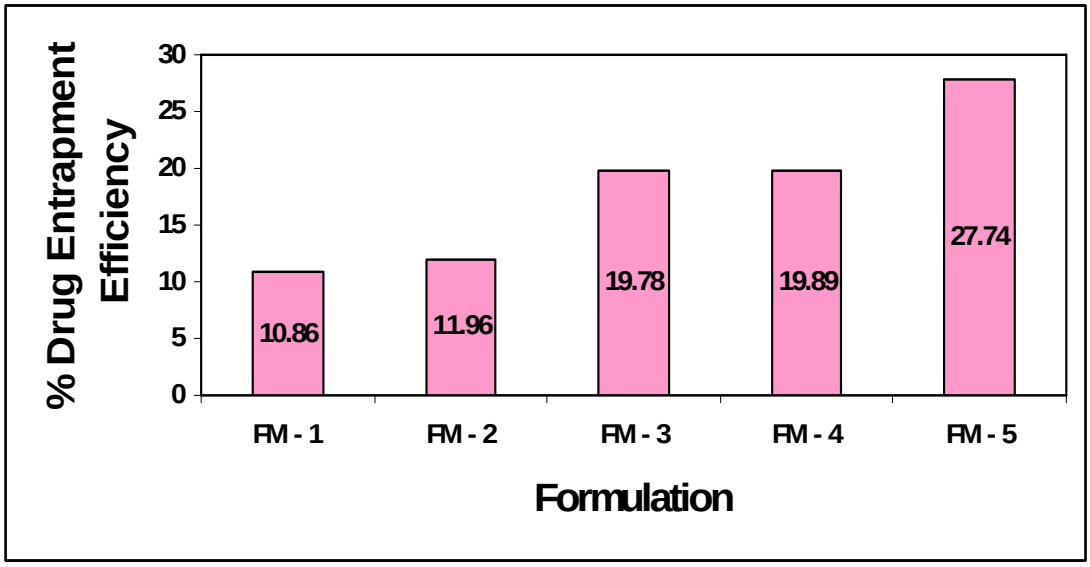

Figure-2: Drug Entrapment Efficiency of TMX Nanoparticles

Scanning Electron Microscopy (SEM):-Scanning electron photomicrographs of pure-drug and optimized drug loaded Chitosan nanoparticles were taken by using Scanning Electron Microscope (Jeol, JSM 5600, and JAPAN). A small amount of nanoparticles were spread on aluminium stub. Afterwards, the stub containing the sample was placed in the scanning electron microscopy (SEM) chamber. The photomicrographs were taken at the acceleration voltage of 20 Kv (Gavini et al., 2006).

The surface morphology of nanoparticles is shown in Figure 3. The chitosan nanoparticles had a spherical shape with a smooth surface The surface morphology of the nanoparticles was the same for the drug loading.

In vitro release studies:- In vitro release of Tmx was conducted by dialysis in a dialysis sac (Sigma, 12000 MW cut off) with 200 ml of PBS (pH 7.4) at 37°C±1°C . Briefly, in a 350 ml conical flask, 300 ml of PBS was taken. One ml of formulation was taken into a dialysis bag and dipped into the buffer solution. The flask was kept on a magnetic stirrer. Stirring was maintained at 300 rpm and the temperature of the buffer was maintained at 37°C±1°C. Sampling was done

by withdrawing 0.5 mL from the released medium with the help of micropipette and 0.5 mL of fresh buffer was added. Samples were analyzed using a spectrophotometer at a wave length of 274 nm. With the help of the standard curve prepared earlier, drug concentration was measured.

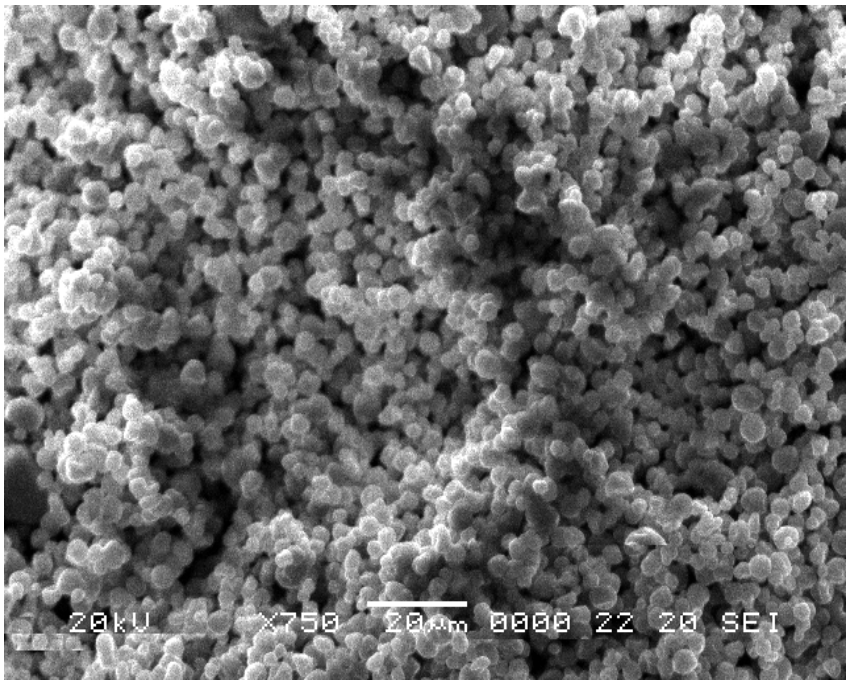

Figure 3: SEM of Chitosan with drug

Table-4 : *In vitro* release profile of TMX Nanoparticles optimized Formulation FM – 1

Time T (hrs)	% Cumulative Drug Release (mg / ml)	% Cumulative Drug Retained (mg / ml)	Log Cumulative % Drug Released	Log Cumulative % Drug Retained	√ T	Log T
0.5	27.70	72.30	1.4424	1.8591	0.707	-0.301
1.0	34.83	65.17	1.5419	1.8140	1.000	0.000
2.0	39.12	60.88	1.5923	1.7844	1.414	0.301
3.0	40.54	59.46	1.6078	1.7742	1.732	0.477
4.0	48.68	51.32	1.6873	1.7102	2.000	0.602
5.0	56.26	43.74	1.7501	1.6408	2.236	0.698
6.0	65.84	34.16	1.8184	1.5335	2.449	0.778
7.0	71.42	28.58	1.8538	1.4560	2.645	0.845
8.0	78.03	21.97	1.8922	1.3418	2.828	0.903

Each data represents mean ±SD, (n=3)

The percentage cumulative release of Temoxifen citrate loaded chitosan nanoparticles is shown in table 4. The release of Temoxifen citrate appeared to be dependant on the drug load for chitosan particles. The percentage release of drug increased as the drug load increased in the nanoparticles for the formulations.

The percentage cumulative drug release after 3,4,5,6,7 and 8 hours was 40.54, 48.68, 56.26, 65.84, 71.42 and 78.03% respectively. Among the all formulations FM 1 showed maximum drug release in 8 hours diffusion study**.** (shown in graph).(table:4)

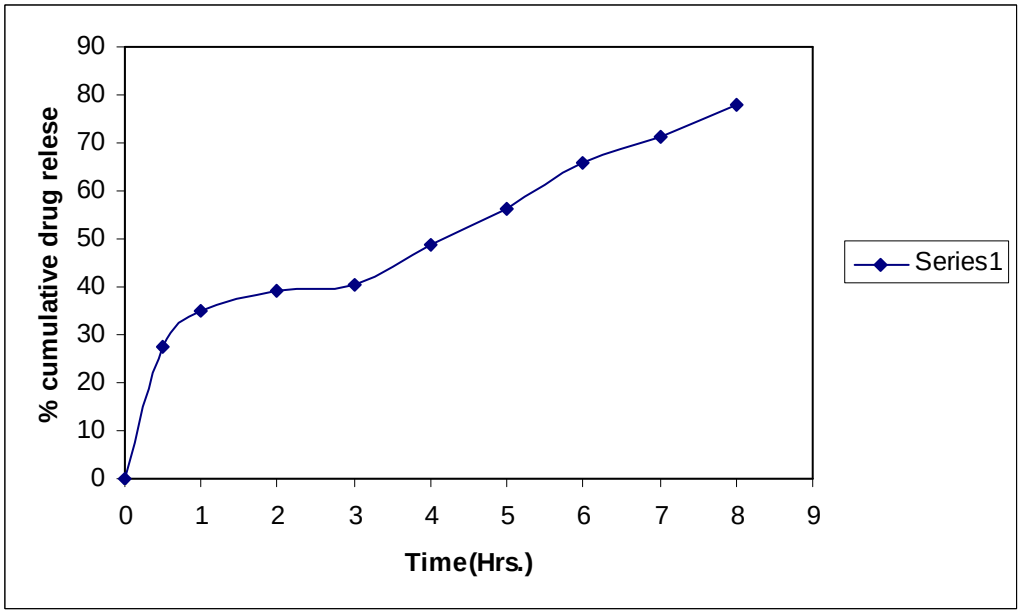

Figure-4: *In vitro* drug release profile of FM-I TMX Nanoparticles

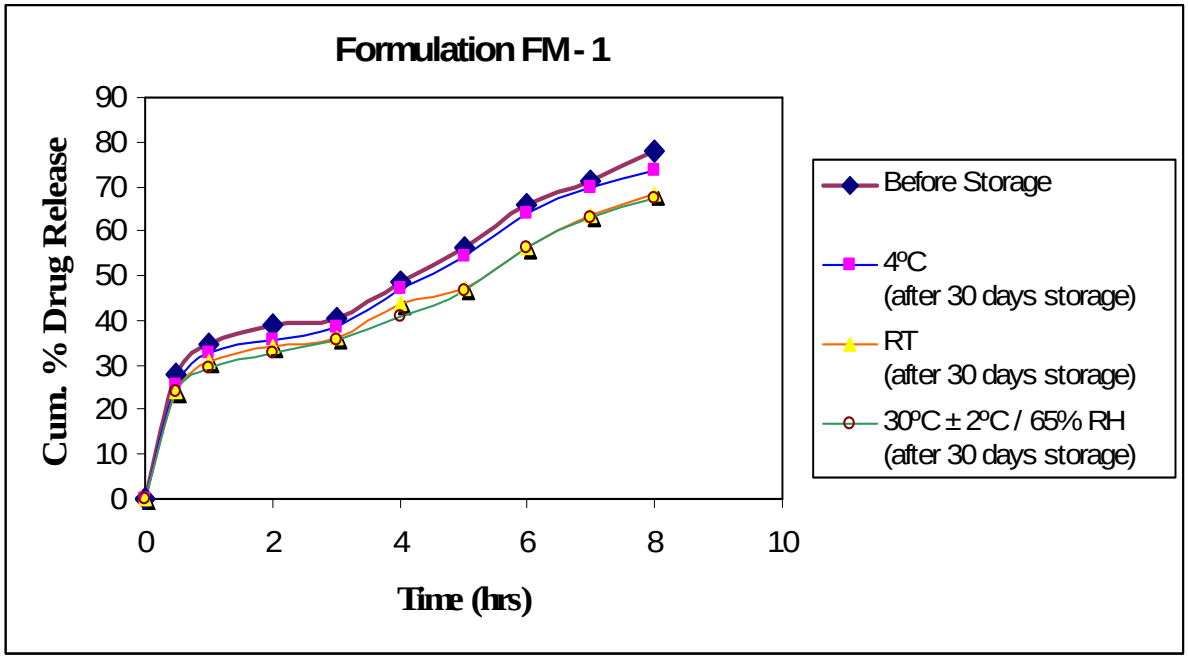

Figure-5:Comparison of Stability Studies by *In vitro* drug release profile of Formulation FM–1
As per results the stability of Fm-1 after 30 days shows better release on 4^0c as compare to 30^0c and room temperature. But formulation also shows good release pattern at room temperature.

Stability Study:-

The stability study of optimized formulation was carried out as per ICH (International Conference on Harmonization) guidelines at 4° C and at room Temperature for one months. Samples were withdrawn and were determined for drug content by the method discussed previously in entrapment efficiency section.

Table-5: Comparison of Stability Studies by *In vitro* drug release profile of Formulation FM–1

Time (hrs)	Cumulative % Drug Release before storage	Cumulative % Drug Release after 30 days storage at		
		4ºC	Room Temperature	Humidity 30ºC ± 2ºC / 65% RH
0.5	27.70	25.434	24.277	24.154
1	34.83	32.863	30.633	29.424
2	39.12	35.669	34.078	32.755
3	40.54	38.724	36.310	35.583
4	48.68	47.145	43.889	41.125
5	56.26	54.419	47.402	46.871
6	65.84	64.203	56.100	56.206
7	71.42	69.873	63.680	62.945
8	78.03	73.821	68.553	67.368

Table-6: Particle size After stability studies

Batch No.	Particle size After stability studies		
	4ºC	Room Temperature	Humidity 30ºC ± 2ºC / 65% RH
FM-1	754	752	754

After stability studies particle size of formulation-1 was measure and as per shown in table no.-6 at all temperature FM-1 shows stable particle size.

CONCLUSION

On preliminary screening different formulations were developed with various ratios of polymers and different surfactants. It revealed that formulations with the polymer concentration and surfactant (TPP) had better drug release and entrapment efficiency. So the formulations were designed with that polymer concentration and surfactant.

Five formulations were evaluated and among them FM1 was found to have good results. Formulations FM1 showed maximum drug release in 8 hours diffusion study and good entrapment efficiency. The work on formulation development of Temoxifen citrate nanoparticle was very much advantageous than the existing dosage forms as the drug is targeting to the cancerous cells, hence better action. And FM-1 also show better stability after 30 days at all temperature.

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