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Development and Characterization of Temoxifen Loaded PLGA Nanoparticle Active against Breast Cancer Cell-Line.

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Abstract

Temoxifen citrate loaded PLGA particles prepared by oil-in-water single emulsion solvent evaporation method. The surface morphology was evaluated by scanning electron microscopy. The physical state of the drug in the formulation was analyzed by Differential Scanning Calorimetry (DSC). The cellular uptake and cytotoxicity of the nanoparticles was determined in MCF-7 and HEK-293T cell lines. The size of the nanoparticles ranged from 890 to 1148 nm. The PLGA nanoparticles had a negative surface charge. SEM pictures revealed spherical particles with a smooth surface. The % entrapment efficiency of formulation is between 44.65%, to 89.29 % for PLGA nanoparticles. The formulations FM-1 showed good drug release after 48, 72, 96, 120 and 144 hours was 44.37%, 52.89%, 57.24%, 58.54%, and 60.12% respectively from the polymer. *In-vitro* anticancer study revealed that the formulated nanoparticles were found to have good cidal activity on cancer cells in sustained manner against MCF-7 cell line.

Key Words

TMX, PLGA, Solvent evaporation, 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 localization 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, utilizing 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 capability to reverse multi drug resistance, a major problem in chemotherapy[1,2]. Temoxifen is an antagonist of the estrogen receptor in breast tissue via its active metabolite, hydroxytemoxifen. In other tissues such as the endometrium, it behaves as an agonist, hence temoxifen 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 inhibitors have been proposed[3,4]. 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 utilized for the delivery of therapeutic agents to wide array of cellular types and targets[4,5].

Materials and Methods

Temoxifen citrate was a gift sample from Sun Pharmaceuticals, Baroda, India. PVA (poly vinyl alcohol) was obtained from SD Fine Chemical, India. Cell line was obtained from NCCS, Pune. Cell Proliferation Kit (MTT and XTT) was obtained from Roche, Cat. No. 11 465 007 001., Trypan blue (Hyclone, Lot no: JRH27098, 100 ml), Liquid Paraffin and Glutaraldehyde (Loba Chemie Pvt Ltd, Mumbai, India), Penicillin and Streptomycin solution , DMSO cell culture grade (MP Biomedicals, Lot No: R20759),

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Preparation of drug loaded nanoparticles Method Oil-in-water single emulsion solvent evaporation technique: The TMX loaded nanoparticles were fabricated by oil-in-water single emulsion solvent evaporation technique. Briefly, known amounts of mass of PLGA and TMX (25 mg) were added into dichloromethane, which was sonicated to ensure that all material was dissolved. The solution of organic phase was slowly poured into aqueous solution containing PVA as surfactant under stirring at magnetic stirrer at stirring at 1800 rpm. This emulsion is broken down into nanodroplets by applying external energy through Probe-sonicator. Then solution was stirred magnetically for 3 h at room temperature to evaporate. The nanosuspension was then centrifuged at 18,000 rpm, 20°C, for 1 hour (Remi, Mumbai, India). Nanoparticles were collected and washed (three times) with distilled water using a previously described centrifugation approach. Sucrose (2% w/v) was added as cryoprotectant (Jeong, Y.I. et al., 2005) and freeze-drying was carried out yielding powdered nanoparticles[6-9].

Design of Experiment

A three factor, two-level full factorial design (2^3) was selected to study the main effects and interactions of three factors on entrapment efficiency and particle size. Design-expert version 6 software was applied for designing the experiment. The independent factors investigated were the polymer concentration, surfactant concentration and sonication cycle. The parameter level selection was based on literature. The two levels of independent factors for the screening design and experiment domain of each variable are summarized in Table No. 1, 2 and 3.

Characterization of Nanoparticles

Determination of loading/entrapment efficiency[9]

Freeze-dried nanoparticles were dissolved in 10 ml of dichloromethane (a common solvent for PLGA and drug). Quantity of TMX in the solution was measured by ultraviolet spectroscopy at 372 nm. Drug incorporation efficiency was expressed as drug entrapment (%); represented by Eq.: The individual values for three replicates were determined, and their mean values are reported[10-12].

Particle Size analysis

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[13,14].

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[15].

In vitro Drug Release

A modified dialysis method was used to evaluate the in vitro release of TMX-loaded PLGA-NPs. Milligrams of nanoparticles (corresponding to 10 mg of TMX) were placed in a dialysis bag (cellophane membrane, molecular weight cut off 10,000–12,000, Hi-Media, India), which was tied and placed into 30 ml of PBS (pH 7.4), maintained at 37 °C with continuous magnetic stirring. At selected time intervals, aliquots were withdrawn from the release medium and replaced with the same amount of phosphate buffer. The sample was assayed spectrophotometrically for TMX at 372 nm. The studies were performed in triplicate[16-18].

Evaluation of *in-vitro* anticancer activity

The invitro anticancer activity of formulated nanoparticles was carried out in MCF-7 cell line. The viability of the cells were checked using trypan blue (cell viability should above 98%) and different dilutions of 10-1, 10-2, and 10-3 were made[19]. The number of cells in the 10-3 dilutions was counted using a haemocytometer and the numbers of cells were adjusted to 1× 10-6 cells/ml. The experiment was set up by incubating different formulations of nanoparticles (100µg/ml) with 1×106 cells/ml. The final volume of the assay mixture was made up to 1 ml using phosphate buffered saline and was incubated at 37°C for 3 hrs. 0.1 ml of trypan blue solution was added after incubation period and the number of dead cells was counted using a haemocytometer. The percentage cytotoxicity was calculated[19].

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[20].

Results and Discussion

In total 8 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 oil-in-water single emulsion solvent evaporation technique. It is a laboratory method proved for the preparation of nanoparticles. The concentrations of the polymers PLGA were selected based on the results on preliminary screening. The time taken to complete preparation was around 2 hours.

Entrapment efficiency and loading capacity

Weighed quantity (10mg) of nanoparticles was suspended in 10 ml methanol to extract the drug from nanoparticles. After 24 h, the filtrate was assayed spectrophotometrically. The data of drug entrapment efficiency and drug loading capacity for drug loaded nanoparticles were as shown in the table 4. 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 encapsulation efficiencies for the formulations were expressed as percentage of the drug in the polymer. The % entrapment efficiency of FM-1, FM-2, FM-3, FM-4, FM-5, FM-6, FM-7 and FM-8 is 56.61%, 47.26%, 81.26%, 84.04%, 44.65%, 81.29%, 46.25% and 89.29 % respectively for PLGA nanoparticles.

The particle size of FM-1, FM-2, FM-3, FM-4, FM-5, FM-6, FM-7 and FM-8 is 890.02 nm, 840.17nm, 1089.58nm, 1148.07nm, 870.76nm, 950.64nm, 918.21nm and 983.33 nm respectively. The formulation batch 1 showed around 56% of drug entrapment efficiency and 890 nm particle size as compare to other seven formulation (Table No. 2).

Zeta Potential

Zeta potential was determined by photon correlation spectroscopy (Zetasizer, HAS 3000; Malvern Instruments, Malvern, UK) using a disposable zeta cuvette. For seleceted batch number-1 sample, the mean diameter/zeta potential ± is -4.71mV (Fig.3).

In-vitro diffusion study of optimized nanoparticle batch-1

In vitro release of Temoxifen citrate 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 372 nm. With the help of the standard curve prepared earlier, drug concentration was measured. The percentage cumulative release of Temoxifen citrate loaded PLGA nanoparticles is shown in table 5. The release of Temoxifen citrate appeared to be dependent on the drug load for PLGA particles. The percentage release of drug increased as the drug load increased in the nanoparticles for the formulations.

The percentage cumulative drug release after 48, 72, 96, 120 and 144 hours was 44.37%, 52.89%, 57.24%, 58.54%, and 60.12% respectively. As per different kinetic models regression (R^2) value is near to 0.99 and its follows better release pattern as per zero order, first order and peppas plot (Table No. 9).

***In-vitro* anti cancer studies**

Cell viability and Density determination by trypan blue dye exclusion method.

This procedure has been carried for determination of % viability and density (cells/ml) of the cells in the suspension medium. From the above results it revealed that, given cell lines have excellent conditions in terms of their % viability and density, and should be the choice of further in vitro cytotoxicity experiment.

Cytotoxicity Screening

The In Vitro cytotoxicity Screening of Formulation-1 had been carried out against MCF-7 and HEK293T cell lines. FM-1 batch were subjected for cytotoxicity screening because of their high drug loading efficiency, sphericity and smallest particle size. The particle size ranging lower is more suitable for the cytotoxicity screening.

Results of various formulations & Temoxifen citrate against MCF-7, and HEK293T cell line by XTT assay

Formulation-1 was subjected to XTT cytotoxicity screening against MCF-7 and HEK293T cell lines. In XTT, cells were treated with different dilutions of drug formulation-1 and absorbance were taken for each formulations followed by determination of % growth inhibition. According to results, Formulation-1 showed 59.11 % and 53.66 % of growth inhibition against the MCF-7 and HEK293T cell lines as compared to the % growth inhibition by Temoxifen citrate alone after 24 hours. Formulation-1 gives sustained release of Temoxifen citrate and shows increasing effect of % growth inhibition against the cell lines depending upon time

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 three months. Samples were withdrawn monthly and were determined for drug content by the method discussed previously in entrapment efficiency section. As per results the stability of optimized batch-1 after 3 months shows better release at 4^0c as compare to room temperature. But formulation also shows good release pattern at room temperature. After stability studies particle size of formulation-1 was measure and as per shown in Table No. 10 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 (PVA) had better drug release and entrapment efficiency. So the formulations were designed with that polymer concentration and surfactant.

8 formulations were evaluated and among them FM1 was found to have good results. Formulations FM1 showed maximum drug release in 144 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 and shows better activity against MCF-7 cancer cell line. And FM-1 also shows better stability after 3 months at all temperature.

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Table No. 1: Values and Coded Units of 2^3 Factorial Design for Preparation of TMX Loaded PLGA Nanoparticles.

Independent variable	**Coded unit**	**Levels**	
		-1	**+1**
PLGA (mg)	A	50	200
PVA (% w/v)	B	1	2
Sonication cycle	C	1	3

Table No. 2: Full Factorial 2^3design Layout for Preparation of TMX Loaded PLGA Nanoparticles.

Batch No	A	B	C
1.	-1	-1	+1
2.	-1	+1	+1
3.	+1	+1	-1
4.	+1	-1	-1
5.	-1	+1	-1
6.	+1	+1	+1
7.	-1	-1	-1
8.	+1	-1	+1

Table No. 3: Full Factorial (2^3) Design Layout for PLGA nanoparticle.

Batch number	PLGA(mg)	B (% w/v)	C (cycle)
1	50	1	3
2	50	2	3
3	200	2	1
4	200	1	1
5	50	2	1
6	200	2	3
7	50	1	1
8	200	1	3

Table No.4: Full Factorial (2^3) Design Layout for PLGA nanoparticles.

Batch number	Drug(mg)	B (% w/v)	C (cycle)	EE (%)±S.D	Particle size (nm) ±S.D
1	50	1	3	56.61±1.76	890.02±8.72
2	50	2	3	47.26±3.55	840.17±11.24
3	200	2	1	81.26±2.36	1089.58±7.65
4	200	1	1	84.04±1.49	1148.07±8.40
5	50	2	1	44.65±1.61	870.76±5.64
6	200	2	3	81.29±2.94	950.64±4.12
7	50	1	1	46.25±2.73	918.21±2.07
8	200	1	3	89.29±1.45	983.33±6.39

Values are expressed in Mean± S.D., n = 3.

Table No. 5: In Vitro Release Data of Optimized Nanoparticles of TMX.

T (h)	√T	Log T	% X	Log % X	(% Y)	Log % Y	3√ % Y
0	0.00	-	0.00±0.00	-	100.00	2.00	4.64
1	1.00	0.00	6.93±1.97	0.84	93.07	1.97	4.53
2	1.41	0.30	9.18±1.29	0.96	90.82	1.96	4.49
4	2.00	0.60	13.06±1.62	1.12	86.94	1.94	4.43
6	2.45	0.78	17.65±1.18	1.25	82.35	1.92	4.35
8	2.83	0.90	18.26±1.76	1.26	81.74	1.91	4.34
10	3.16	1.00	20.83±1.57	1.32	79.17	1.90	4.29
12	3.46	1.08	23.49±1.48	1.37	76.15	1.88	4.25
24	4.90	1.38	30.32±1.86	1.48	69.68	1.84	4.11
48	6.93	1.68	44.37±2.09	1.65	55.63	1.75	3.82
72	8.49	1.86	52.89±1.42	1.72	47.11	1.67	3.61
96	9.80	1.98	57.24±2.59	1.76	42.76	1.63	3.50
120	10.95	2.08	58.54±3.15	1.77	41.46	1.62	3.46
144	12.00	2.16	60.12±1.76	1.78	39.88	1.60	3.42

Table No. 6: % Cell viability & Density of various cell lines by Trypan blue method.

Cell Line	% Viability	Density Cells/ml	Microbial Contamination	Cross Contamination	pH
MCF7	69.02	$4.8x10^4$	NO	NO	7.0
HEK-293T	70.61	$3.91x10^4$	NO	NO	7.5

Table No. 7: % growth Inhibition of chitosan formulations & Temoxifen citrate against MCF7 cell line.

Time(hr)	% Cell Inhibition	
	Formulation-1	Temoxifen citrate
0	0	0
2	4.12	68.02
4	7.69	68.04
8	12.22	68.03
12	22.19	68.03
24	48.6	68.07
36	59.11	6807

Table No. 8: % growth Inhibition of chitosan formulations & Temoxifen citrate against HEK293T cell line.

Time(hr)	% Cell Inhibition	
	Formulation-1	Temoxifen citrate
0	0	0
2	6.9	55.93
4	12.91	55.91
8	21.21	55.94
12	31.22	55.94
24	46.73	55.96
36	53.66	55.96

Table No. 9: Effect of Temperature on % Drug Content of Optimized Nanoparticles of TMX during Storage.

Time (months)	Drug content (%) at 4°C	Drug content (%) at Room Temperature
0	100.00±0.00	100.00±0.00
1	98.75±0.59	98.06±0.67
2	97.46±0.52	96.74±0.36
3	96.33±0.33	95.13±0.21

Table No. 10: Particle size after stability studies

Batch No.	Particle size After stability studies	
	4°C	Room Temperature
1	894 nm	899 nm

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