Research Article

Nano Biomed Eng

Formulation and Evaluation of Letrozole Nanoparticles by Salting Out Technique and Determination of Anti-cancer Activity by MTT Assay

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Received: Sep. 01, 2022; **Revised:** Nov. 18, 2022; **Accepted:** Nov. 25, 2022; **Published:** Nov. 30, 2022

Citation: A. Alekhya, A.K. Sailaja. Formulation and evaluation of letrozole nanoparticles by salting out technique and determination of anti-cancer activity by MTT assay. *Nano Biomedicine and Engineering*, 2022, 14(3): 246–253.

DOI: 10.5101/nbe.v14i3.p246–253

Abstract

Objective: Letrozole (LTZ) drug is an aromatase inhibitor used for the treatment of hormonally positive breast cancer in postmenopausal women. It has poor water solubility, rapid metabolism and a range of side effects. In this study, polymer-based nanoparticles (NPs) incorporating the drug have been formulated and evaluated, aimed to control the release, potentially maximize the therapeutic efficiency, and minimize the side effects of the drug.

Methodology: The drug is incorporated into the polymer (i.e., Eudragit S 100 and Ethyl cellulose) by employing the salting out technique. Total twelve formulations were prepared by varying drug-polymer concentrations and organic to aqueous phase ratios and evaluated for percentage yield, drug content and *invitro* drug release studies. Out of 12 formulations, the best formulations were selected based on drug content and *invitro* drug release studies and characterized for mean particle diameter and zeta potential.

Results: Among all the twelve formulations F5EC, 1:2 was considered to be the best formulation with minimum particle size of 194.55 nm, zeta potential value of –18.6 mV, drug content of 90.28%, entrapment efficiency of 92.36%, and *invitro* drug release of 95% within 12 h. The drug release kinetic studies of the best formulations indicated that the release of drug followed zero order kinetics and showed non-fickian diffusion mechanism. Based on the evaluation and characterization of the formulations, the best formulation prepared by salting out technique (F5EC 1:2) was considered for the determination of anti-cancer activity *invitro* in MCF-7 breast cancer cell line by MTT assay. The results indicated that the prepared formulation exhibited anti-cancer activity with an IC_{50} value of 49.63 ng.

Conclusion: Finally, by comparing results, Ethyl cellulose (EC) was considered to be most suitable for the preparation of LTZ NPs by salting out technique. The Entrapment Efficiency of LTZ NPs was improved up to 92.36% by using salting out technique.

Keywords: Letrozole; Ethyl cellulose; Eudragit S 100; Salting out technique; Nanoparticles

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women worldwide, with more than 2 million new cases in 2020 [1]. Its incidence and death rates have increased over the last three decades due to changes in risk factor profiles, better cancer registration, and better cancer detection. The number of risk factors for BC is significant and includes both modifiable and non-modifiable factors. Currently, about 80% of patients with BC are individuals aged >50. According to the WHO, malignant neoplasms are the greatest worldwide burden for women, estimated at 107.8 million Disability-Adjusted Life Years (DALYs),

of which 19.6 million DALYs are due to breast cancer [2]. In the United States, breast cancer alone is expected to account for 29% of all new cancers in women [3]. Besides being the most common, breast cancer is also the leading cause of cancer death in women worldwide. Although incidence rates were the highest in developed regions, countries in Asia and Africa shared 63% of total deaths in 2020. Current projections indicate that by 2030, the worldwide number of new cases diagnosed will reach 2.7 million annually, while the number of deaths will be 0.87 million [4]. Most women who develop breast cancer in a high-income country will survive; the opposite is true for women in most low-income and many middle-income countries [5]. In low- and medium-income countries, the breast cancer incidence is expected to increase further due to the westernization of lifestyles (e.g., delayed pregnancies, reduced breastfeeding, low age at menarche, lack of physical activity, and poor diet), better cancer registration, and earlier cancer detection [6].

For postmenopausal women with hormone-dependent breast cancer, Letrozole, which suppresses estrogen biosynthesis, is an appealing therapy option. The most fundamental aim of cancer chemotherapy is to keep medication concentrations in tumors therapeutic while minimizing drug exposure to normal organs. After intravenous injection, nanoparticles (NPs) have a significant tendency to accumulate in a variety of malignancies. Aside from targeted delivery, drug-entrapped polymeric NPs offer the unique capability of controlling drug release over a lengthy period of time. Letrozole might be encapsulated in NPs for sustained administration to suppress estrogen production for a longer period of time by virtue of enhanced local concentration of the medication at the receptor site, increasing patient compliance and reducing unpleasant side effects. Furthermore, the administration of NPs will have the benefit of allowing them to be injected using ordinary infiltration needles. There was a published paper on Letrozole NPs prepared by direct precipitation method. These workers reported 146—267 nm particle size with very low entrapment efficiency. On the other hand, salting out technique has an advantage of increasing entrapment efficiency of the nanoparticles [7–35].

In this work, Letrozole loaded polymeric nanoparticles by salting out technique was prepared by utilizing acetone as organic solvent and polyvinyl alcohol as colloid stabilizer to obtain smaller particle size with high entrapment efficiency and sustained release profile. Particle size, entrapment efficiency, zeta potential and in vitro release of LTZ NPs were evaluated. The influence of percentage of drug on formulation performance including particle size, zeta potential, entrapment efficiency, and in vitro release was investigated.

Materials

Drug: Letrozole

Reagents and chemicals

(1) Eudragit S100 [ED], SD Fine Chem. Limited, Mumbai.

(2) Ethyl Cellulose [EC], SD Fine Chem. Limited, Mumbai.

(3) Polyvinyl Alcohol [PVA], SD Fine Chem. Limited, Mumbai.

(4) $ZNSO_4 \cdot 7H_2O$, SD Fine Chem. Limited, Mumbai.

(5) Acetone, SD Fine Chem. Limited, Mumbai.

(6) Distilled water.

Method of Preparation of Letrozole Nanoparticles

Saling out Technique

Letrozole (LTZ) and polymer (Eudragit S 100 and Ethyl cellulose) were dissolved in ethanol at various drug-polymer ratios (1:1, 1:2, 2:1) as given in Tables 1 and 2. Then the organic dispersion was added drop wise to the aqueous phase containing 5% PVA and 41% (mass fraction) $ZnSO_4 \cdot 7H_2O$. The stirring of oil/water emulsion was continued for 3 h. After 3 h, excess of distilled water was added to the formed dispersion. Then stirring was continued for 3 h. The resultant dispersion was subjected for centrifugation to about 15 min. The obtained dried product was subjected for 3-time washing with distilled water.

Characterization and Evaluation of Letrozole Nanoparticles

Measurement of particle size and zeta potential

Mean diameter and polydispersity index of NPs were determined by photon correlation spectroscopy (PCS) using a Litesizer 500 analyser at a fixed angle of 90° and temperature of 27 °C. Aliquot samples in

Table 1 Formulation of LTZ nanoparticles by salting out technique of 1:10 ratio (organic: aqueous)

Formulation	Batch code	Amount of drug (mg)	Amount of polymer (mg)	Drug: polymer ratio
F1	F1ED 1:1	100	100	1:1
F2	F2ED 1:2	100	200	1:2
F3	F3ED 2:1	200	100	2:1
F4	F4EC 1:1	100	100	1:1
F5	F5EC 1:2	100	200	1:2
F6	F6EC 2:1	200	100	2:1

Table 2 Formulation of LTZ nanoparticles by salting out technique of 1:5 ratios (organic: aqueous)

Formulation	Batch code	Amount of drug (mg)	Amount of polymer (mg)	Drug: polymer ratio
F7	F7ED 1:1	100	100	1:1
F8	F8ED 1:2	100	200	1:2
F9	F9ED 2:1	200	100	2:1
F10	F10EC 1:1	100	100	1:1
F11	F11EC 1:2	100	200	1:2
F12	F12EC 2:1	200	100	2:1

which LTZ-NPs were uniformly dispersed in double distilled water was kept in cuvette and analysed. Each reported value is the average of 30 measurements. A suitably diluted aqueous dispersion of NPs was mounted in a Litesizer 500 analyser and mean zeta potential was calculated by the instrument software.

Determination of drug content

The formulations' free drug was first measured in the supernatant by using a solvent that only dissolved the free drug and not the other components. To assess the drug content, 50 mg of formulation-equivalent medication was carefully weighed and put into a 100 mL beaker containing 50 mL of dichloromethane. Using a magnetic stirrer, the solution was swirled at 700 r/min for 3 h. The resulting solution was filtered, and the quantity of medication in the filtrate was determined using an ultraviolet (UV) spectrophotometer set to 240 nm after appropriate dilution.

$$\text{Drug content} = \frac{\text{Amount of drug present in the sample}}{\text{Total amount of drug loaded initially}} \times 100\%$$

Determination of entrapment efficiency

The amount of drug entrapped in the formulation is measured by entrapment efficiency. Separation of free drug by ultra centrifugation, followed by quantitative analysis of the drug from the formulation, is the method of choice for determining entrapment efficiency. The samples were centrifuged for 40 min at –4 °C using an ultracentrifuge at 17000 r/min. The following formula can be used to compute percentage entrapment efficiency:

$$\text{Entrapment efficiency} = \frac{\text{Amount of drug entrapped in the formulation}}{\text{Total amount of drug in the formulation}} \times 100\%$$

$$\text{Loading capacity} = \frac{\text{Total amount of drug} - \text{amount of free drug concentration}}{\text{Nanoparticle's weight}} \times 100\%$$

Invitro drug release study

An Orbital shaker (Orchid Scientifics) was used to undertake in vitro drug release experiments. In a 250 mL conical flask containing 50 mL pH = 7.4 phosphate buffer, 50 mg of each properly weighed formulation was transferred. They were shaken at 100 r/min at 37 °C in an orbital shaker. At predetermined intervals, aliquots of 5 mL solution were removed from the medium and replaced with the same volume of buffer. The removed samples were centrifuged for 15 min at 3000 r/min. A sample of the supernatant was taken. This research was carried out over a 12 h period using all of the produced formulations for both ratios. Using an Elico UV spectrophotometer, the concentration of drug release was calculated by measuring the absorbance at 240 nm (model No: 164).

Results

In the preparation of Letrozole nanoparticles, the technique adopted is salting out technique. The process parameters, such as type and concentration of stabilizer, salting out agent, stirring speed, stirring time, and organic: aqueous phase ratios, were optimized.

By employing salting out technique, 12 formulations were prepared by varying organic to aqueous phase ratios with two polymers (Ethyl cellulose and Eudragit S 100) at various drug polymer ratios of 1:1, 1:2 and 2:1. Six formulations are with 1:10 organic to aqueous phase ratio, and another six formulations are with 1:5 organic to aqueous phase ratio. The prepared formulations are evaluated for drug content, product yield, entrapment efficiency, loading capacity and *invitro* drug release studies. They are characterized for mean particle diameter and zeta potential, and the obtained results are discussed below.

The best formulations of 1:10 and 1:5 organic to aqueous phase ratios are further characterized for particle size and zeta potential (Table 6).

- Particle size analysis was determined by Litesizer 500 analyzer.

Table 3 Percentage yield, drug content and *invitro* drug release of 1:10 organic to aqueous phase ratio formulations

Formulations	Percentage yield (%)	Drug content (%)	Invitro drug release (%)
F1	84.0	91.92	94.71
F2	87.6	94.36	88.73
F3	72.6	65.42	74.92
F4	61.5	87.40	97.86
F5	82.6	90.28	95.00
F6	71.6	78.44	87.45

Table 4 Percentage yield, drug content and *invitro* drug release of 1:5 organic to aqueous phase ratio formulations

Formulations	Percentage yield (%)	Drug content (%)	Invitro drug release (%)
F7	86.5	**96.95**	72.42
F8	92.0	79.52	98.63
F9	**94.6**	77.16	94.31
F10	81.0	78.44	81.07
F11	77.3	86.66	91.23
F12	71.3	71.62	73.31

Table 5 Entrapment efficiency and loading capacity of best formulations of 1:10 and 1:5 organic to aqueous phase ratios

Formulations	Entrapment efficiency (%)	Loading capacity (%)
F2	82.06	22.77
F4	80.82	29.23
F5	**92.36**	**52.75**
F8	85.60	32.33
F9	78.68	24.57
F11	89.36	42.28

Table 6 Particle size and zeta potential of the best formulations of organic to aqueous phase ratios

Formulations	Mean particle size (nm)	Zeta potential (mV)
F2	412.0	−10.7
F5	194.6	−18.6
F8	404.8	−15.8
F11	404.9	−12.6

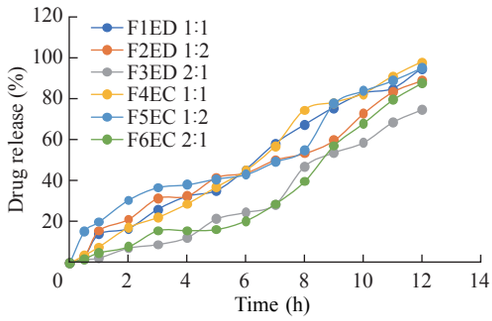

Fig. 1 *Invitro* drug release profile of formulations F1 to F6

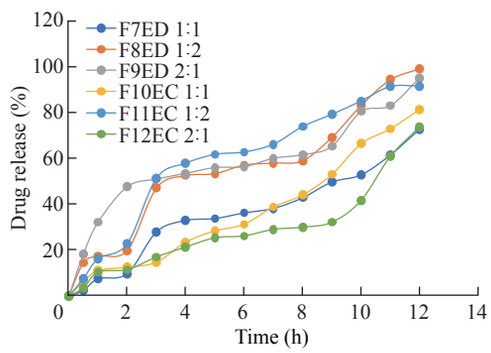

Fig. 2 *Invitro* drug release profile of formulations F7 to F12

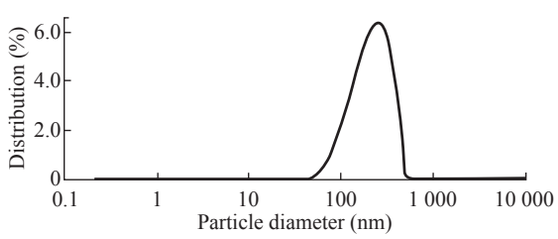

Fig. 3 Particle size distribution report of F5 formulation

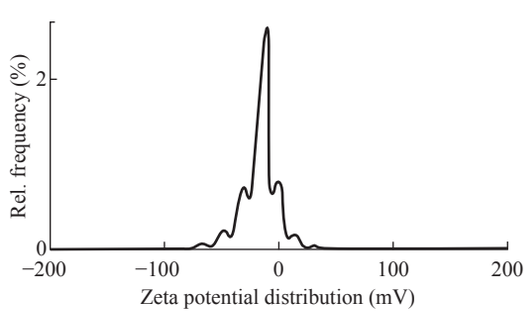

Fig. 4 Zeta potential report of F5 formulation

Procedure: For the size measurement, LTZ NPs were dispersed in distilled water. The particle size was measured at 25 °C with a scattering angle of 90°.

- Zeta potential is an import ant parameter to evaluate and establish for stability of colloidal/ dispersion system. It was determined by Litesizer 500 analyzer.

Procedure: For zeta potential measurement, LTZ NPs were dispersed in distilled water. The zeta potential was measured at 25 °C.

Comparison of best formulations of 1:10 organic to aqueous phase ratio and 1:5 organic to aqueous phase ratios with various kinetic plots

Several plots (zero order plot, first order plot, Higuchi plot and peppas plots) were drawn in order to know the release kinetics and drug release mechanism.

From the results in Table 7, it was concluded that the drug release was following zero order kinetics with non-fickian diffusion mechanism.

Table 7 Kinetic parameters of Letrozole nanoparticles by salting out technique

Formulations	Zero order R^2	First order R^2	Higuchi's R^2	Korsmeyer peppas R^2	N
F5EC 1:2	0.990	0.872	0.963	0.951	0.627
F8ED 1:2	0.978	0.720	0.982	0.984	0.692

Invitro cytotoxicityassay

Based on the evaluation and characterization of the formulations, the formulation prepared by salting out technique, i.e., F5EC 1:2, was considered to be the best formulation. It was considered for the determination of anti-cancer activity in the MCF-7 breast cancer cell line.

The anti-cancer activity was determined *invitro* by MTT assay. Cisplatin was taken as a standard and its IC_{50} value was observed to be 5.57 ng (Table 8).

The IC_{50} value of the given Letrozole nanoparticle formulation F5EC 1:2 were found to be 49.63 ng.

Table 8 IC_{50} values of F5EC 1:2 and standard (Cisplatin)

SR. No	Samplename	IC_{50} (ng)
		MCF-7
01	F5EC 1:2	49.63
02	Cisplatin	5.57

Table 9 Cytotoxicity effect of F5EC 1:2 on growth of MCF-7 breast cancer cell line

Concentration (µg)	Absorbance at 570 nm	Inhibition (%)	Viability (%)
5	0.553	11.80	88.20
10	0.467	25.51	74.49
25	0.348	44.49	55.51
50	0.261	58.37	41.63
100	0.159	74.64	25.36

Discussion

Letrozole is a non-steroidal inhibitor of estrogen synthesis with anti-neoplastic activity. As a third-generation aromatase inhibitor, Letrozole selectively and reversibly inhibits aromatase, which may result in growth inhibition of Estrogen-dependent breast cancer cells in post-menopausal women. Letrozole has many adverse effects like osteoporosis. Increased risk of osteoporosis is a major concern of aromatase inhibitor treatment. Delivery of these molecules at their site of action is desired to reduce problems. So, in order to avoid adverse effects of letrozole like osteoporosis and to improve the efficiency of letrozole, there is a need to develop site specific targeted system like nanoparticles drug delivery system.

The objective of this research work was to formulate, characterize and evaluate Letrozole loaded nanoparticles. Letrozole nanoparticles were prepared by salting out method. By increasing the concentration of polymer, two formulations are prepared. The effects of polymer concentration upon final evaluation parameters were studied. By increasing the polymer concentration, drug release was decreased. By increasing the concentration of drug, there was no significant improvement in the evaluation parameters (i.e., drug content and *invitro* drug release). Further studies were not conducted by varying drug and polymer concentrations.

By employing salting out technique, 12 formulations were prepared by varying organic to aqueous phase ratios with two polymers (Ethyl cellulose and Eudragit S 100) at various drug polymer ratios of 1:1, 1:2 and 2:1.

Six formulations with 1:10 organic to aqueous phase ratio were prepared with various drug — polymer ratios (1:1, 1:2 and 2:1). The formulations are coded as F1ED 1:1, F2ED 1:2, F3ED 2:1, F4EC 1:1, F5EC 1:2 and F6EC 2:1 respectively. Among the 6 formulations, F5EC 1:2 was considered to be the best formulation with percentage yield of 82.6%, drug content of 90.28%, entrapment efficiency of 92.36%, mean particle diameter of 194.55 nm, zeta potential value of –18.6 mV and *invitro* drug release of 95% which was sustained up to 12 h. Kinetic plots were drawn for the best formulation, i.e., F5EC 1:2, indicated the release of the drug followed zero order kinetics and showed non-fickian diffusion mechanism.

Six formulations with 1:5 organic to aqueous phase ratio were prepared with various drug-polymer ratios (1:1, 1:2 and 2:1). The formulations are coded as F7ED 1:1, F8ED 1:2, F9ED 2:1, F10EC 1:1, F11EC 1:2 and F12EC 2:1 respectively. Among the 6 formulations, F8ED 1:2 was considered to be the best formulation with percentage yield of 92%, drug content of 79.52%, entrapment efficiency of 85.60%, mean particle diameter of 404.8 nm, zeta potential value of –15.8 mV and *invitro* drug release of 98.63% which was sustained up to 12 h. Kinetic plots were drawn for the best formulation, i.e., F8ED 1:2, indicated the release of the drug followed zero order kinetics and showed non-fickian diffusion mechanism.

Based on the characterization and evaluation parameters, the best formulation prepared by salting out technique using Ethyl cellulose as the polymer (F5EC 1:2) was considered for the determination of anti-cancer activity of the formulation *invitro* by MTT assay in MCF-7 breast cancer cell line. IC_{50} value of the given formulation was observed as 49.63 ng.

Conclusion

The objective of present research was to prepare and evaluate Letrozole nanoparticles by salting out technique. The objects have been fulfilled, and letrozole nanoparticles by salting out technique have been successfully formulated and evaluated.

Based on all parameters, F5EC 1:2 was considered to be the best formulation with drug content of 90.28%, entrapment efficiency of 92.36%, loading capacity of 52.75% and *invitro* drug release of 95% which was sustained up to 12 h. Mean particle diameter was found to be 194.55 nm and zeta potential value of –18.6 mV which is considered to be stable formulation. The drug release kinetic studies of the best formulations indicated the release of drug followed Zero order kinetics and showed non-fickian diffusion mechanism.

The best formulation (F5EC 1:2) was considered for the determination of anti-cancer activity by *invitro* MTT assay. Cisplatin was taken as a standard and its IC_{50} value was observed to be 5.57 ng. IC_{50} value of the given formulation was found to be 49.63 ng.

Finally, by comparing results, Ethyl cellulose (EC) was considered to be most suitable for the preparation of LTZ NPs by salting out technique. And the entrapment efficiency of letrozole nanoparticles by salting out technique was improved up to 92.36%.

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