Development and *in vitro* evaluation of Letrozole loaded biodegradable nanoparticles for breast cancer therapy

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The objectives of our study were to prepare and evaluate a biodegradable nanoparticulate system of Letrozole (LTZ) intended for breast cancer therapy. LTZ loaded poly(lactide-co-glycolide) nanoparticles (LTZ-PLGA-NPs) were prepared by emulsion-solvent evaporation method using methylene chloride and polyvinyl alcohol. Percentage of drug (with respect to polymer) was selected as formulation variable. LTZ-PLGA-NPs were characterized by particle size, zeta potential, infrared spectra, drug entrapment efficiency and in vitro release. Sonication was done with an ultrasound pulse sonicator at 70 W, 30 kHz for 90 sec to produce stable NPs of mean size range from 64 nm to 255 nm with high entrapment efficiency (68% to 82%). Percentage of drug significantly influenced particle size, entrapment efficiency and release (p <0.05). The system sustained release of LTZ significantly and further investigation could exhibit its potential usefulness in breast cancer therapy.

Uniterms: Breast cancer/therapy. Letrozole. Biodegradable nanoparticles.

Os objetivos de nosso estudo foram preparar e avaliar o sistema de nanopartícula biodegradável de letrozol na terapia de câncer mamário. Nanopartículas de poli(lactídeo-co-glicolídeo) carregadas com LTZ (LTZ-PLGA-NPs) foram preparadas pelo método de emulsão-evaporação de solvente, utilizando dicloro metano e álcool polivinílico. A porcentagem do fármaco (com relação ao polímero) foi selecionada como variável da formulação. LTZ-PLGA-NPs foram caracterizadas pelo tamanho da partícula, potencial zeta, espectros no infravermelho, eficiência de inclusão e liberação in vitro. A sonicação foi realizada com sonicador de ultrassom, de pulso a 70W e 30 kHz por 90 segundos para produzir NPs estáveis, de faixa de tamanho médio de 64 nm a 266 nm, com alta eficiência de inclusão (68% a 82%). A porcentagem do fármaco foi significativamente influenciada pelo tamanho da partícula, eficiência de inclusão e liberação (p<0,05). O sistema controlou significativamente a liberação de LTZ e estudos posteriores poderiam mostrar sua utilidade potencial na terapia de câncer de mama.

Unitermos: Câncer de mama/terapia. Letrozol. Nanopartícula biodegradável.

INTRODUCTION

Worldwide, breast cancer is the second most common type of cancer after lung cancer. It is the primary cause of cancer death among women globally, responsible for about 40000 US women deaths in 2001 (James *et al*., 2002). The use of Letrozole, which inhibits estrogen biosynthesis, is an attractive treatment for postmenopausal women with hormone-dependent breast cancer (Geisler *et al*., 2008). The most important goal of cancer chemo-

therapy is to minimize the exposure of normal tissues to drugs while maintaining their therapeutic concentration in tumors. Interestingly, Nanoparticles (NPs) exhibit a significant tendency to accumulate in a number of tumors after intravenous injection (Bibby *et al*., 2005). Besides targeted delivery, drug-entrapped biodegradable NPs have unique property to control drug release for prolonged period of time, e.g., several weeks (Leroux *et al*., 1996). To increase patient compliance, to overcome the undesirable side effects, Letrozole could be entrapped into biodegradable NPs for sustained delivery so that it can inhibit estrogen biosynthesis for a prolonged time by virtue of increased local concentration of the drug at the receptor site. Biodegradable matrices would offer the advantage

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of disappearing gradually while releasing the drug to the site of action. Additionally, the administration of NPs will also provide the advantage of facilitating their injection through standard infiltration needles. So far, there was one published literature on Letrozole NPs prepared by direct precipitation technique (Mondal *et al*., 2008). These workers reported 146-267 nm particle size with very low entrapment efficiency (12-27%). The drawback of direct precipitation method is extremely low drug entrapment efficiency. On the other hand, emulsification-solvent evaporation method is interesting for many reasons: the use of pharmaceutically acceptable organic solvents, high yields, good reproducibility, and easy scaling up (Nguyen *et al*., 2006). It is a very popular method because it mainly allows efficient encapsulation of numerous compounds of lipophilic nature (Song *et al.,* 1997).

We have prepared Letrozole-loaded poly(lactide-co-glycolide) nanoparticles (LTZ-PLGA-NPs) by emulsion-solvent evaporation technique utilizing dichloromethane as organic solvent and polyvinyl alcohol as colloid stabilizer to obtain smaller particle size with high entrapment efficiency and sustained release profile. Particle size, morphology, entrapment efficiency, drug-polymer interaction and *in vitro* release of LTZ-PLGA-NPs were evaluated. The influence of % of drug (relation to polymer mass) on formulation performance including particle size, zeta potential, entrapment efficiency, *in vitro* release were investigated.

MATERIALS AND METHODS

Materials

Poly (D, L-lactide-co-glycolide), PLGA 75:25 having molecular weight 15000 gifted by Sun Pharmaceutical Advanced Research Center (SPARC), Baroda, India. Letrozole was donated by Dr.Reddy's Laboratories Limited (Hyderabad, India). Polyvinyl alcohol (PVA) & Dichloromethane were supplied by S.D. Fine Chemicals Ltd (Mumbai, India) & Merck Ltd (Mumbai, India) respectively. All other chemicals and solvents used were of analytical grade.

Preparation of Nanoparticles

LTZ-PLGA-NPs were prepared by emulsion-solvent evaporation method (single emulsion technique) proposed first by Gurny *et al*. (1981) and based on the classical procedure patented by Vanderhoff *et al*. (1979). Briefly, LTZ (10–40% w/w) was dissolved in 2 mL of the Dichloromethane, containing 25 mg of PLGA. The organic phase solution was slowly poured into 10 mL of aqueous 0.75%

(w/v) PVA solution with moderate stirring by magnetic bar (Remi Magnetic stirrer, Mumbai, India). The formed o/w type emulsion was further sonicated using a pulse sonicator fitted with a round 3-mm diameter probe (model Labsonic M, B. Braun Biotech International; Melsungen, Germany) at 70% amplitude and 0.7 sec pulse cycle for 90 seconds. A following slow stirring by magnetic stirrer at room temperature for 4 hrs ensured the complete evaporation of the organic solvent. The resulting dispersion of LTZ-PLGA-NPs was centrifuged at 25,000 rpm (44,800 g) at 5 ^{0}C and freeze-dried for 24 hrs using sucrose (2% w/w) as cryoprotectant. Blank NPs without LTZ were also prepared using same technique.

Measurement of particle size and zeta potential

Mean diameter and polydispersity index of NPs were determined by photon correlation spectroscopy (PCS) using a Malvern Zetasizer 3000 (Malvern Instruments, GB) at a fixed angle of 90^0, and temperature of 27 ^{0}C. Aliquot samples in which LTZ-PLGA-NPs were uniformly dispersed in double distilled water was kept in cuvette and analyzed. Each reported value is the average of 30 measurements. A suitably diluted aqueous dispersion of NPs was mounted in a Malvern Easier 3000 (Malvern Instruments, GB) and mean zeta potential was calculated by the instrument software.

Scanning Electron Microscopy (SEM)

A drop of concentrated aqueous suspension (20 mg freeze-dried LTZ-PLGA-NPs in 10 mL double distilled water) was spread over a slab and dried under vacuum. The sample was shadowed in a cathodic evaporator with a gold layer 20 nm thick. The diameters of all the particles in each field were calculated using JSM-5200 scanning electron microscope (Tokyo, Japan) operated at 15 kV.

Field Emission Scanning Electron Microscopy (FE-SEM)

A concentrated aqueous suspension was spread over a slab and dried under vacuum. The coating was done with platinum. The diameters of all the particles in each field were calculated using JEOL JSM-6700F scanning electron microscope. (Tokyo, Japan) operated at 5.0 kV.

Transmission Electron Microscopy (TEM)

For the observation of morphology and size distribution, a drop of the sample solution was placed onto a 400 mesh copper grid coated with carbon. About 1 min

after deposition, the grid was tapped with a filter paper to remove surface water. The samples were air dried before measurement. Transmission electron microscopy (TEM) was performed on a TECNAI SPIRIT, model FE1 electron microscope, Netherlands.

Fourier transform infra-red spectroscopy (FTIR) study

The Fourier transform infrared (JASCO-FTIR, Model-8300) analysis was conducted to verify the possibility of chemical bonds between drug and polymer. Samples of pure LTZ, pure PLGA and LTZ /PLGA physical mixture 1:1 were scanned in the IR range from 400–4000 cm^{-1} with carbon black as reference. The detector was purged carefully by clean dry helium gas to increase the signal level and reduce moisture.

Drug entrapment efficiency

Drug entrapment efficiency was determined by centrifuging (with Centrifuge-3K30, Sigma Laboratory) aqueous dispersion of LTZ-PLGA-NPs at 25,000 rpm (44,800 g), 5 ^{0}C for 25 min and measuring the amount of LTZ in the supernatant with the help of double beam UV-VIS Spectrophotometer (UV-2450, Shimadzu, Japan), set at 238 nm. The amount of LTZ was subtracted from initial amount of LTZ taken to calculate drug entrapment efficiency of NPs. The experiment was performed in triplicate for each batch and average drug entrapment efficiency was calculated.

In vitro *release study*

In vitro release study of LTZ-PLGA-NPs was conducted in Franz Diffusion Cell. The diffusion cell model adapted to the spectrophotometer cuvette, with 1 cm of optic way and 1 mL of volume, was used for the *in vitro* release of LTZ. A cellulose acetate membrane (Dialysis membrane with Molecular weight cut off value of 5,000-10,000, Himedia-60) was adapted to the terminal portion of the glass cylinder of the Franz Diffusion cell by a rubber ring. 5 mL of LTZ-PLGA-NPs aqueous dispersion was loaded to cylinder and coupled to the diffusion cell containing the receptor phase (60 mL of 0.2 M Phosphate buffer solution pH 7.4) at 37 °C. The dissolution media was agitated at 25 rpm using magnetic stirrer. At different time intervals, aliquots of 2 mL were withdrawn and immediately restored with same volume of fresh Phosphate buffer. The amount of LTZ released was assessed by double beam UV spectrophotometer (UV-2450, Shimadzu, Japan) set at 238 nm *versus* a calibration curve prepared in the same buffer. Cumulative

percentage released at different time points were fitted into different release models: zero order, first order (Gibaldi *et al.*, 1967), Higuchi (Higuchi, 1963), Hixson-Crowell (Hixon *et al.*, 1931), Korsemeyer-Peppas (Korsemeyer *et al.*, 1983) semi empirical model. The release rate constants (k) and correlation coefficients (R^2) were calculated by different mathematical models. The model giving a correlation coefficient close to unity was taken as the order of release. From the presented data, it was evident that drug release best fitted with Zero order release model.

Statistical analysis

Experimental results were expressed as mean ±SD. Student's t-test was applied to determine the level of significance. One-way analysis of variance was also applied to check significant difference in formulations. Differences were considered statistically significant when p<0.05.
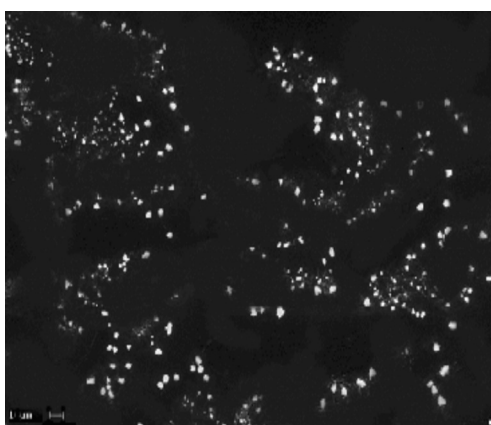
RESULTS AND DISCUSSION

NPs formation by emulsion-solvent evaporation is due to the partitioning of the organic solvent from the emulsion droplet into the continuous phase, followed by the evaporation of the organic solvent and simultaneous inward diffusion of the solvent into the droplet (Birnbaum *et al.,* 2000). The polymer concentration and organic solvent selection are critical. High concentration of polymer in organic phase results in producing larger size microparticles. Since nanoparticles are formed from the emulsion droplets after organic solvent diffusion, their size is dependent on the stability of the emulsion droplets which is affected by miscibility of organic phase with water. Dichloromethane, a water immiscible solvent, forms NPs by a true emulsification mechanism in which the larger emulsion droplets are broken into smaller droplets by the application of external energy. Mean diameter of LTZ-PLGA-NPs varied from 64 nm to 255 nm, when the theoretical amount of LTZ was increased from 10–40% in relation to polymer mass (Table I). It can also be observed that the particle size distribution became progressively wider with the increase in LTZ amount as shown by polydispersity index in Table I. At higher solid (PLGA+LTZ) concentrations, the energy applied through ultrasonication is insufficient to overcome the resistive viscous forces provided by the dissolved solid in the organic phase and the dissolved colloid stabilizer (PVA) in the aqueous phase, leading to heterogeneous droplets and a wider size distribution (Polydispersity index 0.27 to 0.66). During emulsification process, the lower the viscosity of the dispersed phase, the smaller the mean diameter.
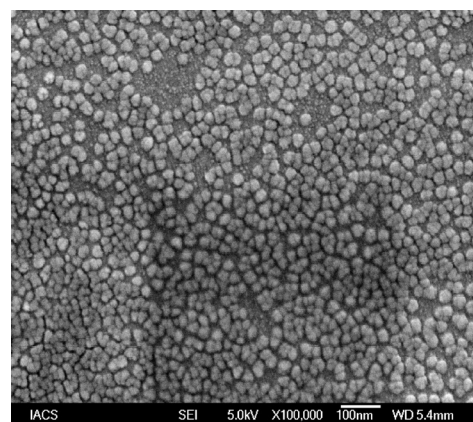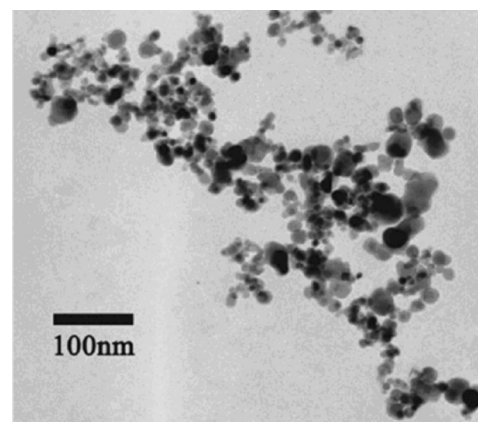
TABLE I. Characterization of blank NPs and LTZ-PLGA-NPs

Formulation Drug (wt% of polymer)	Encapsulation efficiency (%)	Mean diameter (nm)	Poly-dispersity index	Zeta Potential (mV)
Blank	---------	60.4±25.1	0.27±0.01	-28.4±1.1
10%	68.43±0.22	64.0±15.4	0.34±0.03	-25.0 ± 0.4
20%	74.14±3.1	101.9±39.4	0.42±0.01	-22.5 ± 2.5
30%	79.52±4.3	145.5±80.4	0.45±0.07	-19.8 ± 0.7
40%	82.38±6.2	255.3±40.5	0.66±0.11	-14.3 ± 0.5

A more viscous organic phase provides a higher mass transfer resistance, the diffusion of polymer-solvent phase into external aqueous phase is reduced and larger NPs are formed. Moreover, evaporation of organic solvent using magnetic stirrer should be slow for 4 h. Otherwise, rapid evaporation under vigorous stirring resulted in formation of fiber like agglomerates, which eventually separated from aqueous phase. Figure 1, Figure 2 and Figure 3 showed the Scanning Electron microscopy (SEM), Field Emission (FE-SEM) Transmission Electron Microscopy (TEM) images of LTZ-PLGA-NPs respectively. There were no significant differences in morphology, as can be observed in batches prepared with different percentage of drug (images of all batches not shown). All NPs were almost irregularly shaped, polymorphic in nature, without any significant aggregation or adhesion.

FIGURE 2 - FE-SEM of LTZ-PLGA-NPs with 10% LTZ.

FIGURE 3 - TEM of LTZ-PLGA-NPs with 10% LTZ.

FIGURE 1 - SEM picture of LTZ-PLGA-NPs (40% LTZ).

All formulations showed a negative zeta potential value and no significant statistical differences were observed among formulations (Table I). Negative zeta potential values are due to free carboxyl end groups of PLGA exposed on NP surface. Compared to blank NPs, the presence of LTZ into NPs did not influence the zeta potential in a significant way. Zeta potential value is directly proportional to electrophoretic mobility (ratio of velocity of migration over potential gradient), as described by Helmholtz-Smoluchowski equation **(Egorova *et al.*, 2005).** Therefore, higher the average NP size, slower the velocity of migration of charged particles in a known applied electric potential and thus resulted in decreased zeta potential value compared to smaller mean size NPs, which has higher velocity of migration and higher zeta potential value.

In the present work, sample of pure PLGA, pure LTZ and LTZ/PLGA physical mixture 1:1 were characterized by the FTIR. The obtained spectra were illustrated in Fig. 4. It showed that no significant differences on shape and

position of the absorption peaks could be clearly observed for A, B and C. LTZ showed major peaks at 2250 cm^{-1} for C≡N stretching, 3010 cm^{-1} for sp^2 CH stretching, 690-900 cm^{-1} for out-of-plane CH bending. PLGA sample showed peaks such as –CH, –CH$_2$, –CH$_3$ stretching (2850-3000 cm^{-1}), carbonyl –C=O stretching vibrations (1700-1800 cm^{-1}), C–O stretching (1050-1250 cm^{-1}) and –OH stretching vibrations (3200-3500 cm^{-1}) which were broad. Most of the absorption peaks from pure LTZ & pure PLGA overlapped with the absorption peaks from LTZ-PLGA-NPs. It can be concluded that no strong chemical interaction occurred between drug and polymer.
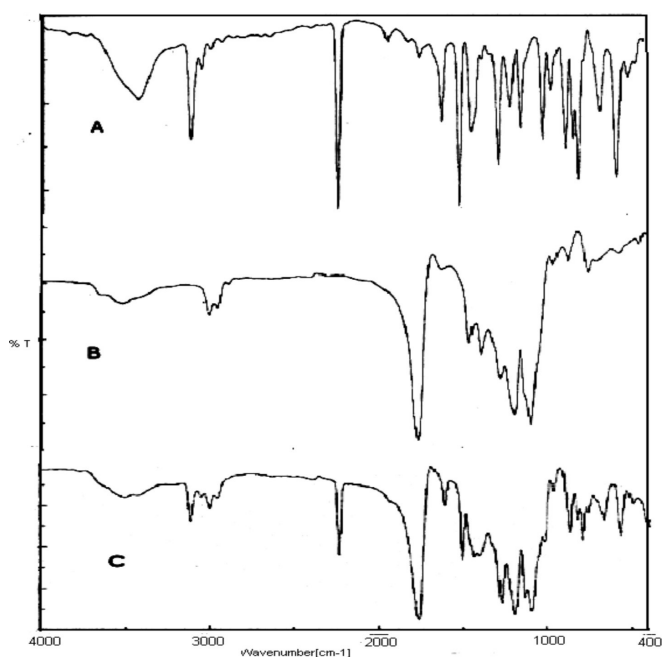

FIGURE 4 - Infrared spectra of pure LTZ (A), pure PLGA (B), LTZ /PLGA physical mixture 1:1 (C).

It has been reported that the encapsulation efficiency of LTZ-PLGA-NPs increases from about 68% to 86% with the increment of their mean diameter from 64 nm to 255 nm). The lower encapsulation efficiencies obtained with the smaller particles could be explained by the larger surface area of smaller droplets for a given volume of organic phase. Hence, during the emulsification step, a more direct contact between internal and external phases occurred, resulting in a higher drug loss by diffusion towards the external medium. Similarly, for a given volume of organic phase, there was comparatively smaller surface area for larger droplets, resulted in lesser drug loss towards aqueous phase and higher drug entrapment efficiency.

Batches of four different compositions (10%, 20%, 30% and 40% of LTZ) were studied and the results of

cumulative percentage released over 15 h were shown in Fig. 5. The results showed that there was a pronounced time prolongation of drug release from LTZ-PLGA-NPs. Only 10, 13, 15 and 18% of LTZ were released only after 15 hrs from batches containing 10, 20, 30 and 40% of drug, respectively. Batch with 10% LTZ showed the slowest release and batch with 40% LTZ showed highest release. Hence, a progressive increase of drug release rate was observed as a function of LTZ (% wt of polymer). Different release profiles can be correlated with entrapment efficiency. Formulation (40% drug) with highest LTZ entrapment (82.38%) showed highest drug release rate and cumulative drug release (18% after 15 h) while formulation with 10% drug and lowest LTZ entrapment (68.43%) showed lowest release rate and only 10% cumulative drug release after 15 h. The increase in drug entrapment increases the amount of drug close to the surface as well as the drug in the core of NPs and is responsible for an increased release of drug from NPs (Buchman *et al*., 2008). Lower amount of drug in polymer matrix is more homogenously distributed than higher amount of drug distributed in same amount of polymer. Thus difference in drug release profiles of four batches of LTZ-PLGA-NPs can be explained.
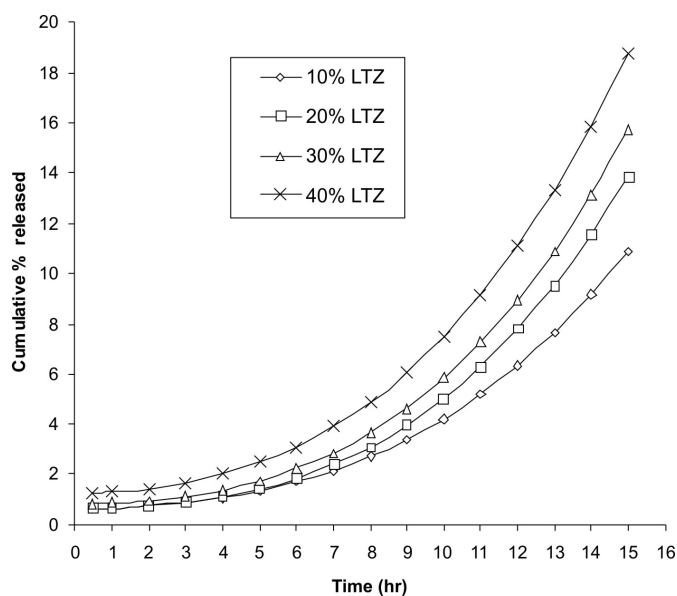

FIGURE 5 - In vitro release data of LTZ –PLGA-NPs (n=3, Std. Deviation not shown).

The release rate constant (k) calculated by different mathematical models and correlation coefficient (R^2) between observed release data and fitted profiles were summarized in Table II and Table III. From the presented data, it was evident that drug release best fitted with zero order release model as evident from correlation coefficient value. Corresponding release constant (k) and release

TABLE II - Release rate constants k, Correlation coefficients (R^2), mean ± SD, (n=3) calculated after fitting the release profiles to mathematical models

LTZ (% of PLGA)	Zero order		First order		Higuchi		Hixon-Crowell	
	k_0	R^2	k_1	R^2	k_H	R^2	k_{HC}	R^2
10%	0.629±0.021	0.881±0.021	0.002±0.0005	0.872±0.032	2.441±0.521	0.713±0.016	0.010±0.001	0.875±0.051
20%	0.802±0.034	0.864±0.032	0.003±0.0009	0.853±0.061	3.088±0.725	0.689±0.054	0.012±0.007	0.857±0.036
30%	0.908±0.064	0.868±0.018	0.004±0.001	0.855±0.037	3.504±0.850	0.695±0.028	0.014±0.008	0.859±0.021
40%	1.088±0.095	0.886±0.043	0.005±0.002	0.871±0.051	4.230±0.675	0.720±0.31	0.017±0.006	0.876±0.045

TABLE III - Fittings of release profiles to Korsemeyer-Peppas semi-empirical model

Drug (% wt of polymer)	n	k	R^2	Release Mechanism
10%	0.8849	0.2675	0.8091	Non-fickian diffusion
20%	0.9677	0.2795	0.8058	Non-fickian diffusion
30%	0.917	0.1612	0.7980	Non-fickian diffusion
40%	0.8487	0.0173	0.8016	Non-fickian diffusion

exponent (n) for the four formulations were calculated. From Table II, it was observed that release rate constant gradually increased when drug in formulation was increased from 10% to 40% in formulation. All four batches of polymeric matrix NP systems of LTZ followed non-Fickian diffusion or anomalous mechanism of drug release (0.5 < n < 1) as shown in Table III. Anomalous transport occurs due to a coupling of Fickian diffusion through hydrated layers of the matrix and polymer chain relaxation or erosion. Drug release from LTZ-PLGA-NPs takes place by several mechanisms including surface and bulk erosion, disintegration, diffusion, and desorption. In this experiment, release of drug from PLGA matrix has been found to occur predominantly by its diffusion from the polymer matrix. During the later phases, the release is mediated through both diffusion of therapeutic agent and degradation of polymer matrix itself. The acidic monomers and the oligomers formed catalyzed the further degradation of the polymer. The later phase was not observed during our only 15 hrs of *in vitro* release study. It is generally anticipated from a bulk eroding polymer such as PLGA 75:25 to give an initial burst release followed by a controlled release. In contrast, there was no initial burst effect observed with all four formulations, which indicated a homogeneous encapsulation of drug in PLGA matrix. In this case, the release pattern can be explained by the dissolution of this poorly soluble drug from the polymer-drug matrix, which involves not only the erosion of the polymer entity but also the solubility of the drug into the surrounding media before its diffusion out of the dialysis membrane.

CONCLUSIONS

These findings demonstrate that Letrozole can be entrapped into PLGA NPs, which can provide sustained drug release with high drug entrapment efficiency. NPs were successfully developed by emulsion/ solvent evaporation method using dichloromethane and polyvinyl alcohol resulting in smaller mean particle size range. Size distribution, entrapment efficiency, release characteristics were influenced by drug to polymer ratio in formulation. However, determination of long-term stability and *in vivo* studies are in progress in order to determine the feasibility of drug delivery system in clinical practice.

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