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PREPARATION AND CHARACTERIZATION OF PEG-ALBUMIN-ANASTRAZOLE (PAA) NANOPARTICLES INTENDED TO TREAT BREAST CANCER

Ramchander Tadakapally[1*], Jithan Aukunuru[2] and Mohammad Habbibudin[3]
[1]Mother Teresa College of Pharmacy, Hyderabad, Telangana, India
[2] Omega College of Pharmacy, Hyderabad, Telangana
[3]Adept Pharma & Bio science Excellence Pvt. Ltd. Hyderabad, Telangana

***Corresponding Author Email: ramdeno@gmail.com**

ABSTRACT

The aim of present research is to prepare serum stable long- circulating polymeric nanoparticles for Anastrazole by NAB (Nanoparticle albumin bound) technology for breast cancer treatment. Nanoparticles were prepared by desolvation technique. After preparation, nanoparticles were characterized for particle size, scanning electron microscope (SEM), zeta potential, drug entrapment efficiency (EE), Fourier transform infrared spectroscopy (FTIR), and differential scanning calorimetry (DSC). Solubility and *in vitro* release of the drug from nanoparticles are determined. With optimized formulation In-vivo drug release issue, Kupffer cell uptake and tissue uptake was determined, cell viable assay was performed. *In vitro* studies were performed in phosphate buffer saline pH 7.4 at 37˚ ± 0.5˚C for 30 days. Sustained drug release from nanoparticles was observed for 18 days. Cell viability assay was determined with optimized formulation. Solubility of the drug is increased by pegylated nanoparticles. The entrapment efficiency was about 92.0 %, the mean particle size of obtained nanoparticles was 119 - 319 nm, and it was spherical with a smooth surface. The zeta potential is found to be -64 mV, and the nanoparticles are found to be stable. FT - IR studies conducted drug - excipient interaction studies, demonstrating that the drug was not changed during the fabrication process. The DSC results also showed no significant shift in the endothermic peaks confirming the drug's stability in the formulations. Polymeric peaks revealed that, the drug is in amorphous state in the formulations. The pegylated anastrazole polymeric nanoparticles released the drug with a small initial burst followed by a slower and controlled release with improved of 5- fold than pure drug.

KEY WORDS

Anastrazole, albumin, desolvation, breast cancer, PEG and nanoparticle.

INTRODUCTION

Anastrazole is non-steroidal aromatase inhibitor. It is an amorphous powder white in colour, with melting point 130^{O}C and chemical structure is 2,2'-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-phenylene] bis(2-methylpropanenitril) molecular weight 293.36 g/mol. The major limitation of anastrazole is low solubility and low permeability and belongs in class -IV of biopharmaceutical classification system[1]. Several techniques can be used to solve the delivery issues with anasatrozaole. Increase in bioavailabilty and aqueous solubility, enhanced stability, sustained and targeted delivery can be achieved with these delivery systems. Pegylation is a common technique that can be employed to further improve the delivery of intravenous nanoparticles. PEG can be used to promote steal these to the nanoparticles. PEG was introduced to the surface of the nanoparticles to improve pharmacokinetics of the nanoparticles after i.v. administration. PEG aids in *in-vivo* longevity to drug carrier. Surveillance of spleen, bone marrow and liver can be escaped with pegylation and therefore nanoparticles circulate in the blood for long time[2]. These are generally called as stealth nanoparticles. Stealth nanoparticles help in more accumulation at the tumour site and enhance tumour targeting. Polycaprolactone pegylated (PCL-PEG) based

nanoparticles and enhanced tumour retention was developed by Lupi *et al*., and this enhanced the stealth promotion to the nanoparticles. Many studies revealed the importance and success of pegylated stealth nanoparticle formulations for clinical use. Thus, similar technology has adopted for anastrazole. Abraxis Bio Science developed Nanoparticle albumin bound (NAB) technology [16], it's a novel patented technology, exploits the properties of albumin to achieve a solvent free, efficient, safe and targeted drug delivery system. Jithan *et al.,*[16]developed curcumin-albumin nanoparticles which enhanced drug solubility and formulation was more effective in breast cancer compared to solution form of the drug. In this study we aimed at improvement of the formulation by conjugating pegylation technique to Nanoparticle albumin bound technology. The objective of this study is to prepare and evaluate serum stable long circulating polymeric PEG-albumin-Anastrazole-nanoparticles to be administered in breast cancer, by increasing its solubility, permeability, therapeutic index and tumour accumulation[3,4,5].

MATERIALS AND METHODS

Anastrazole was purchased from Natco pharmaceuticals Ltd. Hyderabad. PEG (1500,4000, and 6000) were purchased from S.D.Fine chemicals Pvt. Ltd. Bovine serum albumin (faction-v) specification-Albumin min 98.5% was purchased from S.D fine chem. Pvt. Ltd, Mumbai. Methanol HPLC grade and Glutaraldehyde was purchased from SDFCL, Hyderabad. Ethyl acetate was purchased from Qualikems Fine Chem. Ultracentrifuge, micro centrifuge, Magnetic stirrer,and cyclomixer from REMI Equipments were used. Waters HPLC used in the analysis of drug levels in *in vitro*. A Shimadzu UV- Visible spectrophotometer was used in the analysis of samples, *in vitro* drug release and in drug content assays. A bath sonicator from Fischer brand was used.

Preparation of Pegylated albumin Anastrazole (PAA)nanoparticles[10]:

PAA nanoparticles were prepared by desolvation technique. The Polymeric solution was prepared by weighing 200mg bovine serum albumin and polyethylene glycols (6000, 4000 and 1500) were dissolved in 2.0ml of distilled water (Table.1). Drug solution was prepared by taking required amount of anastrazole 100mg and dissolved in 8ml of ethanol. Prepared drug solution was added dropwise to the polymeric solution under magnetic stirring(500rpm). To this, glutraldehyde 0.11mlof 8%inwater(v/v) was added to cross-link the desolvated BS Anano particles and cross-linking process was performed for over 24h. The obtained suspension was subjected to differential centrifugation for 5cycles and redispered the pellets to the original volume in water. Each redispersion step was carried out using bath sonicator. The obtained suspension was lyophilized using freeze dryer. The particles were collected as PEG-albumin-Anastrazole (PAA) nanoparticles[7,8].

Table: 1 Preparation of serum stable long circulating polymeric nanoparticles of Anastrazole nanoparticles

Formulation	F1	F2	F3	F4
Anastrazole	100	100	100	100
BSA(mg)	200	200	200	200
PEG(6000) (mg)	-	20	-	-
PEG(4000) (mg)	-	-	20	-
PEG(1500) (mg)	-	-	-	20
Distilled water(ml)	2ml	2ml	2ml	2ml
Ethanol(ml)	8ml	8ml	8ml	8ml
Glutaraldehyde(8%v/v) (ml)	0.11ml	0.11ml	0.11ml	0.11ml

Table 2: Drug entrapment efficiency of Anastrazolenano particle formulations

FORMULATIONS	% DRUG ENCAPSULATED
F_1	80.9
F_2	82.9
F_3	86.5
F_4	92

Characterization of PEG -Albumin - Anastrazole Nanoparticles:

Particle size and surface morphology:

In order to examine the particle surface morphology and shape, scanning electron microscopy (SEM) (S-3700) was used. Anastrazole nanoparticles solution and formulations spread over a slab and dried under vacuum, images of the samples were captured[9]

Zeta Potential:

The electrophoretic mobility and zeta potential were measured using a zeta potentiometer (Malvern Zetasizer). nanoparticle formulationsF1, F2, F3 and F4 were diluted with KCl (0.1 mm) and placed in the electrophoretic cell where an electric field of 15.2 V/cm was applied. Each sample was analyzed in triplicate[10].

Drug entrapment efficiency:

Drug entrapment efficiency was determined by the dialysis method. Drug entrapment efficiency was calculated for the samples Anastrazole -BSA nanoparticles, Anastrazole – BSA – Pegylated -6000 nanoparticles, Anastrazole – BSA – Pegylated -4000 nanoparticles, Anastrazole – BSA - Pegylated 1500 nanoparticles, at the end of centrifugation, supernatants was obtained and analyzed the drug by UV spectrophotometer.

$$\text{Entrapment efficiency \%} = \frac{\text{Weight of the drug in nanoparticles}}{\text{Weight of the nanoparticles}} \times 100\ \%$$

Drug-excipient compatibility studies:

Fourier Transform Infrared (FTIR) spectroscopy (FTIR):

FT -IR is used for the Anastrazole and excipient compatibility studies. A sample of about 5mg was mixed thoroughly with 100mg KBr IR powder and compacted under pressure of about 12psi, for 3 min. The resultant disc was mounted in a suitable holder in Perkin Elmwer IR spectro photometer, and the IR spectrum was recorded from 4000cm^{-1} to 400cm^{-1} in a scan time of 12min. These studies were done for 1) Anastrazole pure drug 2) Anastrazole-BSA - nanoparticles 3) Anastrazole - BSA-PEG-4000 nanoparticles 4) Anastrazole -BSA-PEG 1500 nanoparticles[11].

Differential scanning calorimetry (DSC):

Differential scanning calorimetry (DSC) is a thermo analytical method used to study thermal transitions involving energy or heat capacity changes. Thermal properties of the powder samples were investigated with differential scanning colorimetry. Approximately 10mg of samples was analyzed in an open aluminium pan and heated at scanning rate of 10˚ c/min between 0˚C and 400˚C. Magnesia was used as the standard reference materials. The thermograms of anastrazole were obtained and the samples of anastrazole-BSA-Pegylated-6000 nanoparticles, anastrazole-BSA-Pegylated-4000 nanoparticles, anastrazole-BSA-Pegylated1500 nanoparticles[12]

***In vitro* drug release studies:**

In vitro drug release experiments were conducted for Anastrazole drug. About10mg of each sample anastrazole - BSA nanoparticles, anastrazole-BSA-Pegylated-6000 nanoparticles, anastrazole-BSA-Pegylated-4000 nanoparticles, anastrazole-BSA-Pegylated1500 nanoparticles were weighed and redispersed in 100ml of release medium (phosphate buffer pH 7.4)[11]. The release medium is phosphate buffer of pH7.4 which contains butylated hydroxyl toluene and ascorbic acid at a concentration of 0.1% and 1% respectively to prevent degradation. The beaker was kept at 37±0.5˚C under stirring at 100rpm. At the time intervals 1hr, 2hr, 3hr, 4hr, 5hr , 6hr, 7hr, 8hr, 9hr, 10hr, 24hr, 48hr till 30 days the samples 10ml was withdrawn and replaced with the fresh medium of buffer. Samples withdrawn were filtered by using 0.2µ sterile filter. The amount of drug in the release medium was determined by UV-Visible spectrophotometer at 225 nm. The cumulative amount of drug release over the time period was plotted. To determine the enhancement in the solubility with the formulation and solubility studies of anastrazole conducted and compared[13].

Solubility studies:

The solubility of pure anastrazole and anastrazole-BSA-PEG formulations was investigated by using the USP rotating paddle dissolution apparatus at 100rpm and temperature 37±0.5^0. A 100mg of the formulation was weighed and added to 500ml of release medium. 5ml of samples were withdrawn using a syringe filter at various time intervals such as 30 min, 1hr, 2hr, 4hr, 6hr, 8hr, 12hr and 24hr replaced with fresh dissolution medium, samples were analyzed, using UV spectrophotometer at 425nm[14].

***In-vivo* animal studies:**

Animal experiments were conducted with the institutional animal ethics committee of Geetanjali college of pharmacy, Hyderabad (IAEC No.1684/PO/a/12/CPCSEA). The study was conducted using 12 wistar rats divided into 3 groups (n=4), each mice was injected via lateral vein, Pure Anastrazole is injected for (group-1), controlled anastrazole nanoparticle is injected for (group -2), pegylated anastrazole nanoparticles is injected for (group-3), blood sample was collected from retro orbital plexus at different time intervals. Concentration of Anastrazole was determined by HPLC analysis (maiti k et al., int j Pharma 2007; 330(1-2), 155-63).

Cell viability assay:

The cell viability assay with pure anastrazole and optimized Pegylated-BSA-anastrazole nanoparticles was carried out in MDA-MB-231 cell lines following the protocol previously described [(4)]. The method is described in detail as below. MDAMB 231 and BT 549 cell lines were grown as adherent in DMEM medium supplemented with 10% fetal bovine serum, 100µg/ml pencillin, 200µg/ml, streptomycin, 2mML- glutamine, and culture was maintained in a atmosphere with 5% CO_2.

Preparation of samples for cytotoxicity:

Formulations and blank nanoparticles were dissolved in sterile PBS, PEG and ethanol to desired concentrations for the treatment as follows.

Cytotoxicity evaluation:

Cytotoxicity of optimized formulations was determined by MTT assay,based on reduction of yellow MTT tetrazolium dye to a highly colored blue formazan product. $1x10_4$ Cells (counted by Trypan blue exclusion dye method) in 96 - well plates, were incubated with optimized formulations and pure drug with series of concentrations for 48 h at 37˚ in DMEM with 10% FBS medium. The above media was replaced with 90µ1 of fresh serum free media and 10µ1 of MTT reagent (5 mg/ml) and plates were incubated at37˚ for 4hr, the above media was replaced with 200µ1 of DMSO and incubated. The absorbance was measured at 570 nm on a spectrophotometer (spectra max, Molecular devices).

Statistical analysis:

All experiments were done more than four times and the data were expressed as mean ± standard deviation and Tukey's post-hoc test was done to analyze significance of difference between different groups using the statistical analysis software package SPSS (version 16.0, IBM, USA)[15,16].

RESULTS AND DISCUSSIONS:

Prior to formulation development, preformulation studies were performed. Anastrazole is a class-IV drug classified under BCS classification with low solubility and low permeability. The physicochemical properties were described. The drug is a poorly soluble compound. To enhance dissolution and achieve targeting several nanoparticles formulations can be developed and tested. In this study we employed the same technique to achieve enhanced dissolution and targeting to breast cancer. Nanoparticle technique is a novel technique for breast cancer as demonstrated for paclitaxel using NAB technology. Similar technology was used in this study. The drug nanoparticles were prepared by the polymer albumin and these nanoparticles were coated with PEG. SEM was used to determine the particle size of samples such as 1) anastrazole nanoparticles 2) anastrazole-BSA-Pegylated-6000 nanoparticles, 3) anastrazole-BSA-Pegylated-4000 nanoparticles, anastrazole-BSA-Pegylated1500 nanoparticles from (Figure.1 & 2). It was concluded that the average particle size for all formulations was found to be in nano range of 119nm to 319nm. Surface morphology and shape were visualized. The particles were appeared as spheres. From this study it has been concluded that size reduction of particle results in enhancing dissolution rate of anastrazole. Drug entrapment efficiency results are shown in the (table. 2), results show a proper distribution of anastrazole in the nanoparticles. The percentage entrapment efficiency was found to be 80.9 % to 92%. (Table. 2) A maximum of 92% drug entrapment efficiency was obtained in the Anastrazole-Pegylated-nanoparticles (F3-Formulation). Drug release from different formulations F1, F2, F3 and F4 were 11.5%,20.5%,17.3%,21.6% shows with-in 2hrs was observed as given in (table.3). The sustained release activity of the drug was slow due to drug entrapped inside the PEGylated nanoparticles.

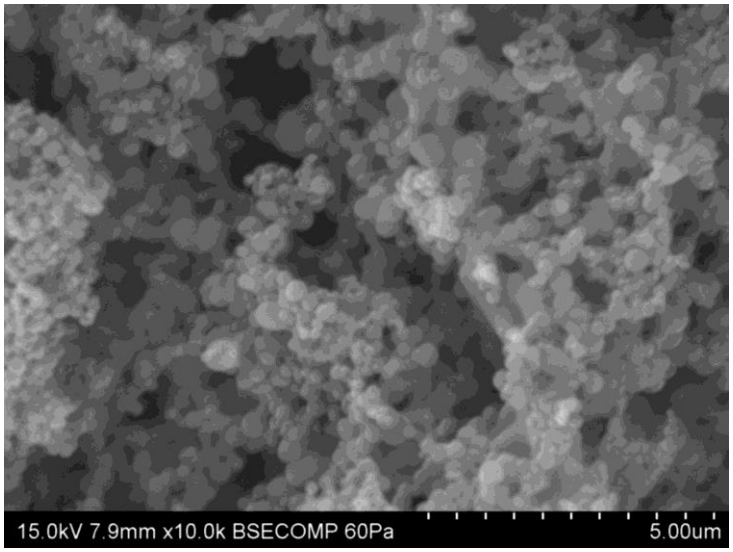

Figure: 1 SEM pictogram of optimized anastrazole nanoparticles F4 for surface morphology

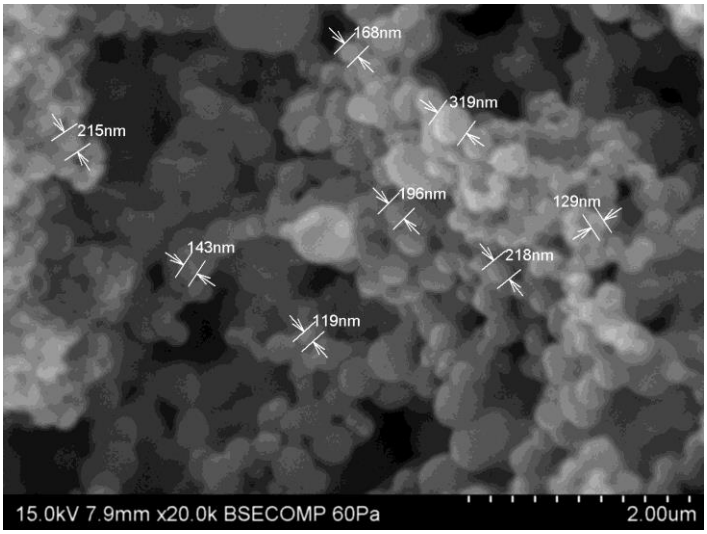

Figure: 2 SEM pictogram for optimized formulation of F4 anastrazole nanoparticles for particle size

Table: 3 *In vitro* drug release studies of Anastrazole from various formulations F1 to F4

Time in days	F1	F2	F3	F4
0	0	0	0	0
0.5	11.5	20.5	17.3	21.6
1	16.2	24.2	25.2	46.5
2	18.5	28.6	28.2	52.2
3	21.3	36.7	31.4	63.5
4	23.2	41.2	34.6	70.9
5	28.3	43.5	39.2	74.5
6	33.5	45.5	43.5	80.2
7	37.5	48.6	50.4	85.9
8	42.3	51.5	58.6	88.7
9	45.6	55.4	64.1	89.2
10	48.1	57.6	72.2	91.5
11	53.4	60.6	78.8	93.5
12	59.6	63.2	84.9	95.6
15	63.1	69.4	87.2	97.5
18	72.2	78.3	89.2	98.3

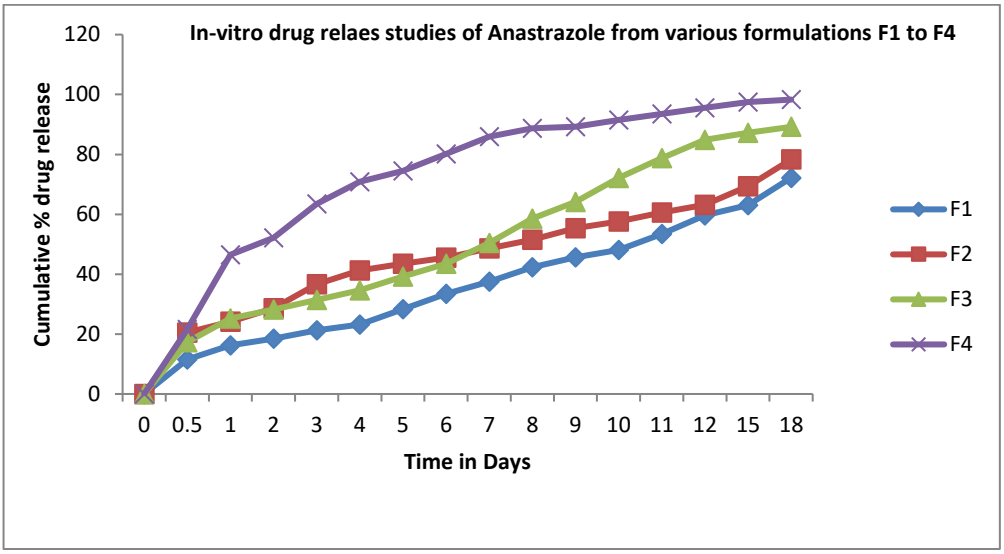

Figure: 3 *In vitro* drug release studies of Anastrazole from various formulations F1 to F4

Table: 4 Plasma drug concentration of Anastrazolewith various formulationspure drug Anastrazole and Anastrazole-BSA-Nanoparticles and F4 (Formulation)

Time in hr	Pure Anastrazole	Anastrazole-BSA Nanoparticles	Anastrazole-BSA-PEG-6000(F4)
0	420	980.4	1050.2
1	280	900.4	1048.6
2	100	800.2	958.8
4	0	740.3	760.1
6	0	630.5	656.1
8		600	610.4
10		584.5	592.2
12		570.2	583.6
24		560.5	583
48		540.5	575.4
96		540.4	560.8
144		536.7	550.3
192		534.7	550.2
240		525.5	542.6
288		500.3	510.7
336		480.5	505.5
384		350.2	410.6
432		220.6	245.5
504		0.9	12.6

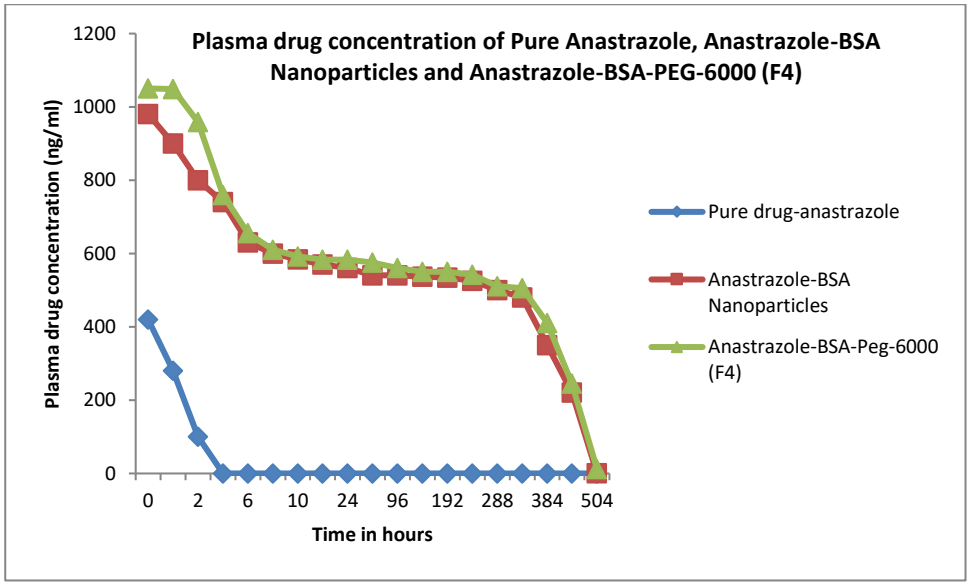

Figure: 4 Plasma drug concentration of Pure Anastrazole, Anastrazole-BSA Nanoparticles and Anastrazole-BSA-PEG-6000(F4)

Table 5: Solubility of Pure Anastrazole and PEG Anastrazole Nanoparticles

Concentration	Pure Anastrazole	PAA Nanoparticles
0	0	0
0.2	0.2	0.6
0.4	0.3	0.8
0.6	0.35	0.9
0.8	0.35	0.9
1	0.3	0.9
1.2	0.3	0.9
1.4	0.3	0.9
1.6	0.3	0.9
1.8	0.3	0.9
2	0.3	0.9

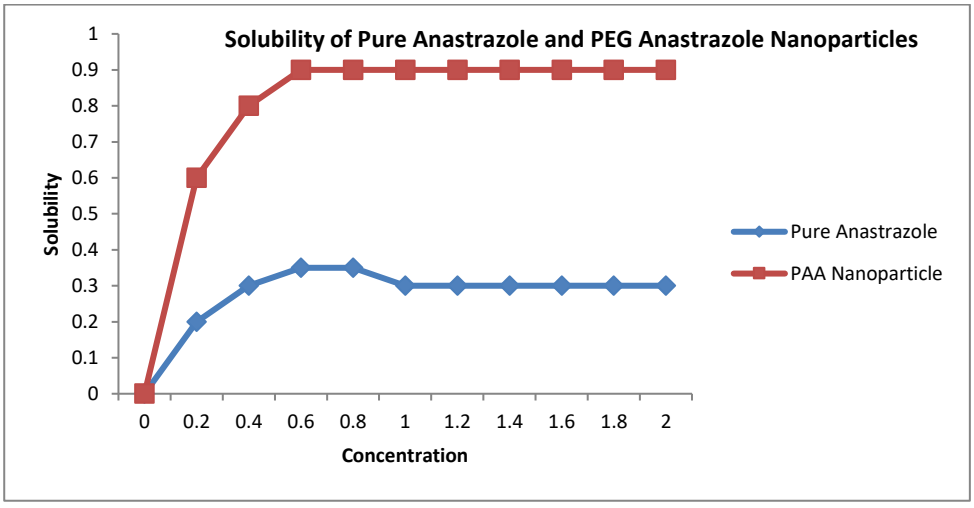

Figure 5: Solubility of pure Anastrazole and PEG Anastrazole nanoparticles

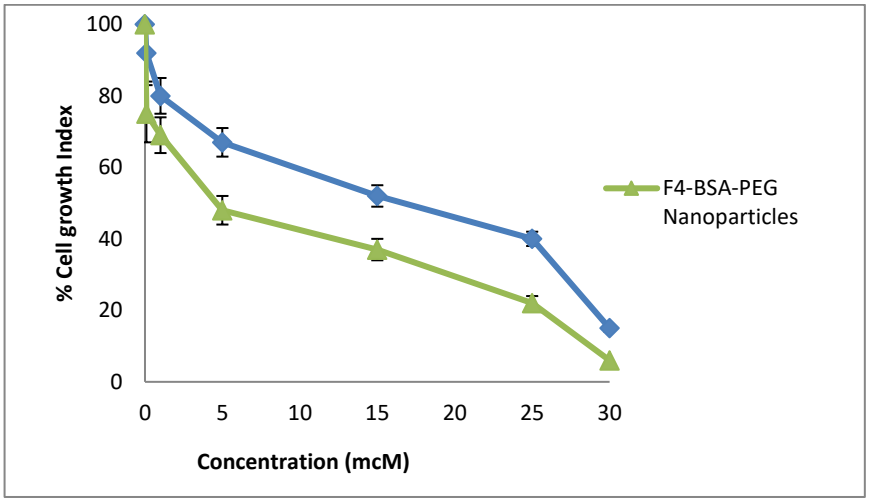

Figure: 6 Cell viability assay with various samples native Anastrazole and F4-BSA-PEG nanoparticles

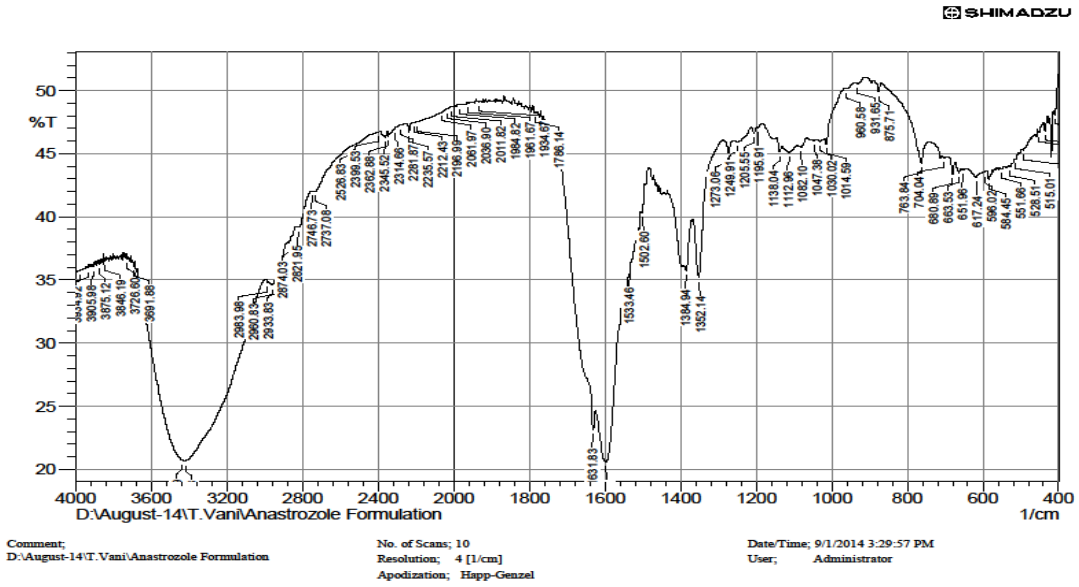

Figure: 6FTIR Spectra of Anastrazole-Pure drug

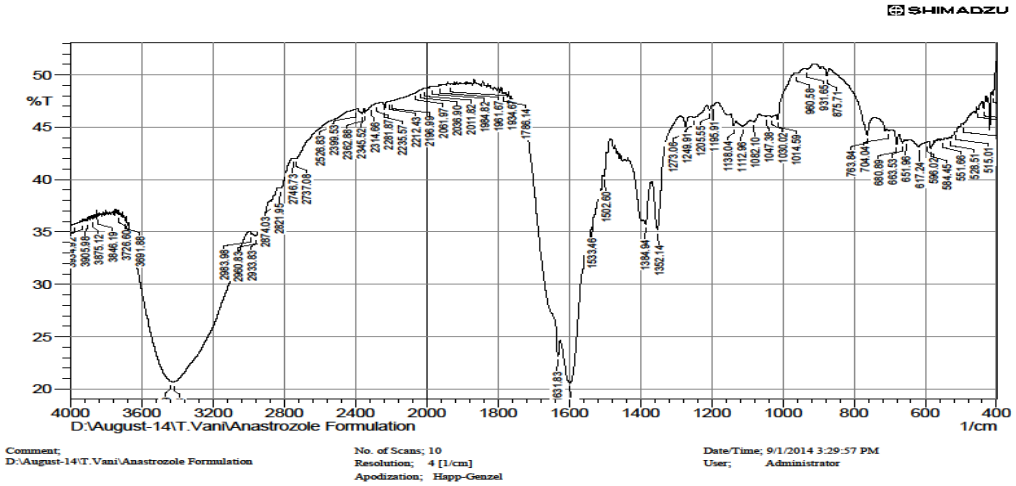

Figure: 7 FTIR Spectra of Anastrazole-Bovine Albumin-PEG 6000

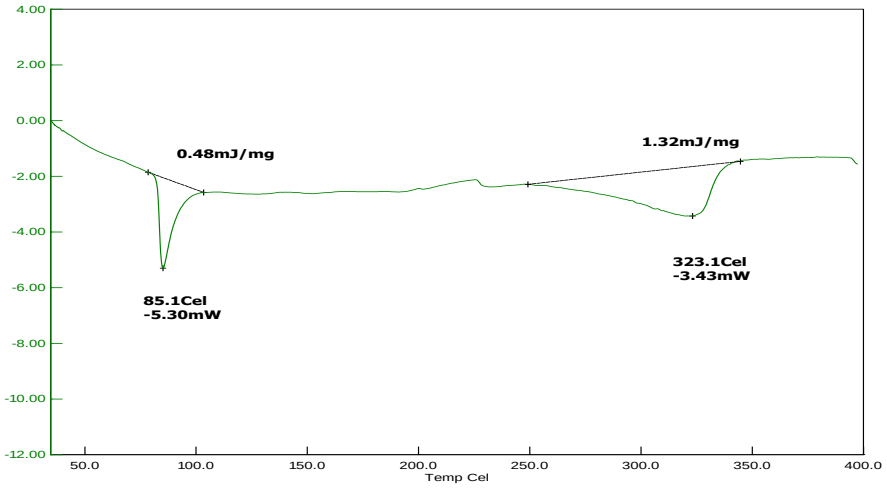

Figure: 8DSC thermogramof Anastrazole-Pure drug

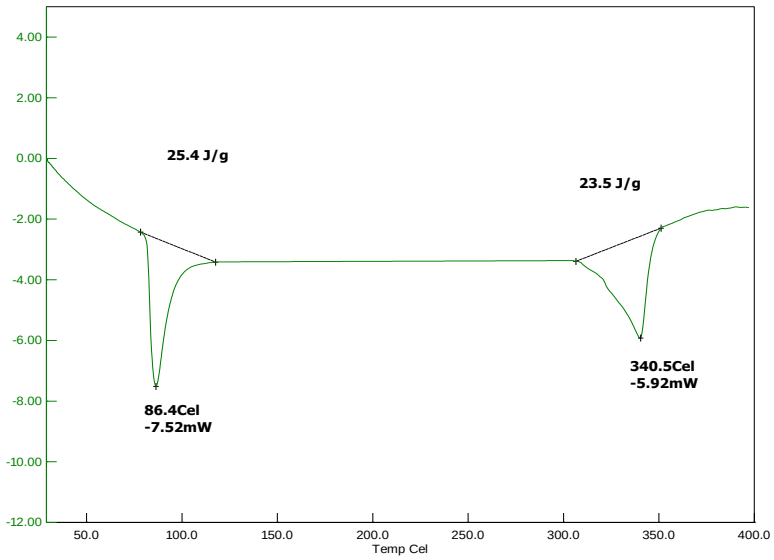

Figure:9 DSC thermogram of Anastrazole Nanopaticles optimized formulation (F4).

(FT-IR) spectroscopy has been performed to know the drug excipient compatibility and to check the presence of drug and polymer in the nanoparticle formulations. The chemical interaction between the drug and the polymer can be observed by the change in the infrared profile of nanoparticles of anastrazole. Compatibility can be interpreted by careful study of spectra from (fig7, 8 and 9) FT-IR is done for 1)anastrazole-Pure drug 2) anastrazole-Bovine serum Albumin-PEG1500 3) anastrazole-Bovine serum Albumin-PEG-4000 4) anastrazole-Bovine serum Albumin-PEG-6000. FT-IR spectra obtained for anastrazole shows characteristic peaks at 3427cm^{-1} (NH_3 group, stretching vibrations are seen at 3726 cm^{-1}). The spectra of anastrazole loaded polymer blend were not characteristic different from the spectra of the anastrazole. The peaks appearing at 3427 cm^{-1}, 2874.03 cm^{-1}, 1786.14 cm^{-1}, 1631.83 cm^{-1}, 1384.94 cm^{-1}, 1030.2 cm^{-1}, 515 cm^{-1} for Anastrazole and their polymers were also appearing in anastazole-loaded polymer blend, indicating the chemical stability of anastrazole in the blend. FT-IR studies demonstrated that the drug was not changed in the Formulation during the fabrication process.

DSC is done for the stability test for samples such as pure drug-anastrazole, physical mixture and optimized Formulation of nanoparticle. DSC thermograms obtained for the pure drug (60˚C) and for the Formulation showed no significant shift in the endothermic peaks in physical mixture (70˚C) and optimized nanoparticle F4 (40˚C) confirming the stability of the drug in the formulations and only polymer peak was observed, which revealed that is in amorphous state in the formulations as shown in Figure from 11,12 and13. The % drug release was determined

for 18 days, and all the formulations are compared. The plots of cumulative percentage drug release v/s time for four formulations (F_1,F_2,F_3 and F_4,) initially all the formulations shown a burst in release and shows sustained with respectively 11.5%, 20.5%, 17.3% and 21.6% were shown in the (table.3). F4 formulation showed highest drug release, which was selected as optimized Formulation. Plasma drug concentrations from the animal studies concluded there is huge release by BSA-Pegylated- nanoparticle compared to BSA nanoparticle and pure drug anastrazole and showed an increase in drug release was observed 5-fold times than the pure anastrazole.

The anti-proliferation efficacy of free anastrazole and pegylated-Anastrazole-BSA nanoparticles on breast cancer cell line (MDA-MB-231) were determined at different concentrations 0,10,30,50,70, 90 and110 mc M for about 72 hours. The results showed that PEGylated anastrazole- bovine serum albumin nanoparticles were more anti-proliferative than that free anastrazole, blank nanoparticles did not show any cytotoxic effects this suggests that improved Formulation against breast cancer has been developed in the form of PEGylatedanastrazole-BSA nanoparticles.

CONCLUSION:

In conclusion, serum stable long-circulating PEGylated anastrazole-BSA nanoparicles were prepared physicochemical characteristics were studied. Our investigation suggests that albumin nanoparticles may act as a useful and safe carrier for anastrazole with 5 folds increase in drug release.

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***Corresponding Author:**
Ramchander Tadakapally
Email: ramdeno@gmail.com