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STUDIES IN DEVELOPMENT OF BICALUTAMIDE NANOFORMULATIONS

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ABSTRACT
Bicalutamide (BIC) is an anti-cancer drug having poor water solubility and low bioavailability. The aim of present work was to increase solubility of BIC which may increase the bioavailability. Nanoformulations of BIC such as Nanoparticle, Solid Lipid Nanoparticle (SLNs) and Nanocrystals were prepared and evaluated for physicochemical characteristics. Finalised Nanoformulations were compared with respect to Solubility, *In-Vitro* drug release, *In-Vitro* cytotoxicity and Bioavailability study. From the physicochemical characterization and evaluation of all nanoformulations, it was observed that Polylactic-co-glycolic acid (PLGA) Nanoparticles prepared using high pressure homogenizer has higher increment in the solubility compare to SLNs and Nanocrystal of BIC. Cytotoxicity Study showed that PLGA Nanoparticles have higher bioavailability as well as higher % Cell inhibition. In a nutshell, Nanoformulation such as PLGA Nanoparticle is one of the promising approach to enhance the Bioavailability of BIC.

KEYWORDS: Bicalutamide (BIC), Nanoparticles, Solid Lipid Nanoparticles (SLNs), Nanocrystals, Cytotoxicity, Bioavailability, Poly lactic-co-glycolic acid (PLGA).

1. INTRODUCTION
Formulation of poorly water soluble molecule is one of the most difficult and challenging tasks for the formulation scientist. An enhancement in the solubility and dissolution rate can improve the oral bioavailability of such molecules which further improves the therapeutic efficacy as well. Bicalutamide (BIC) is a BCS Class II drug having very low solubility and high permeability.[1] Low aqueous solubility and poor dissolution of Bicalutamide (BIC) leads to poor bioavailability. Thus, limited aqueous solubility is the bottleneck for the therapeutic outcome of BIC. Animal data suggests that the absolute bioavailability of BIC is about 4% due to an extensive first-pass effect.[2]

Polylactic-co-glycolic acid (PLGA) is one of the most successfully developed biodegradable polymer among the different polymers developed to formulate polymeric nanoparticles. It gives sustained release as well as increase solubility of drug.[1,2] SLNs consist of a solid lipid matrix where the drug is incorporated. Nanocrystals are aggregates of molecules that combine in a crystalline form which contains pure drug and surfactant. To avoid aggregation and to stabilize the SLNs and Nanocrystal, surfactants are used in the present research work which have an accepted GRAS (Generally Recognized as Safe) status.[3]

2. MATERIALS AND METHODS
2.1 Materials
BIC was kindly supplied by Astron Research Centre, Ahmedabad, India as a gift sample. Tween 80 and Glyceryl monostearate were obtained as a gift sample from Croda and Gattefosse respectively. Poloxamer 188 was supplied by Sigma–Aldrich, Bangalore, India and PVPK-30 was supplied by Signet Chemicals, Mumbai, India as a gift sample. All remaining reagents and chemicals were of analytical grade. Purified water used for all experiments was MilliQ Plus, Millipore.

2.2 Analytical Method Development using UV Technique
Accurately weighed BIC (10 mg) was transfered into 100 ml of volumetric flask. 5 ml of methanol was added and volume was made up to mark with distilled water (100µg/ml). The aliquots of 0.1 to 0.7 ml were withdrawn and diluted up to 10 ml with distilled water (10 to 70µg/ml). The absorbance of these solutions were measured using UV spectrometer (Shimadzu 1800, Japan). The graph of absorbance vs. concentration was plotted.[4]

Table 1: UV Calibration Curve Data.

Concentration (µg/ml)	Absorbance
10	0.117 ± 0.029
20	0.327 ± 0.038
40	0.543 ± 0.045
60	0.787 ± 0.057
70	0.854 ± 0.065

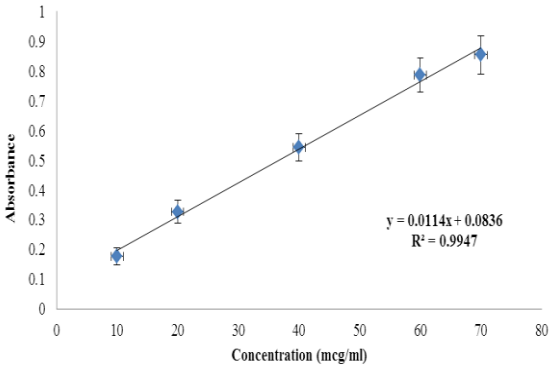

Figure 1: UV Calibration Curve at 270 nm (Absorbance vs. Concentration).

2.3 Analytical Method Development using HPLC Technique

The mobile phase consisting of phosphate buffer (pH 2.8 adjusted with orthophosphoric acid) and acetonitrile (HPLC grade) were filtered through 0.45µ membrane filter before use, degassed and were pumped from the solvent reservoir in them ratio of 50:50 v/v which was pumped into the column at a flow rate of 1.0 ml/min. The detection was monitored at 270 nm and the run time was 6 min. The volume of injection loop was 12 µl prior to injection of the drug solution the column was equilibrated for at least 30 min. with the mobile phase flowing through the system.[5] Weigh 10 mg of BIC standard and transferred to a 50 ml volumetric flask, dissolved in 30 ml of diluent and diluted to 50 ml with diluent. Sonication to be done for 10 min. By using the working standard, aliquots of 5, 10, 15, 20 and 25 µg/ml were prepared with diluent. 10 µl of each dilution was injected into the column with a flow rate of 1.0 ml/min. The retention time of BIC was found to be 5.281. The calibration curve was constructed by plotting concentration vs peak area ratio. The amount of BIC present in sample was calculated through the standard calibration curve.

Table 2: HPLC Calibration Curve Data.

Concentration(µg/ml)	Peak Area Ratio
5	277295 ±28906
10	354296 ± 32586
15	561358 ±36874
20	677450 ±44879
25	833570 ±53587
Y=28714X + 11008	**R^2= 0.986**

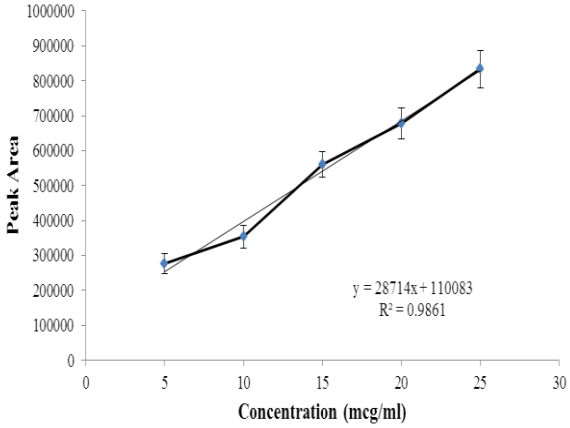

Figure 2: HPLC Calibration Curve.

2.4 Formulation Development of PLGA Nanoparticles/SLNs/Nanocrystals

Nanoformulations were prepared by precipitation and solvent evaporation method. Weighed BIC was dissolved in 3 ml acetone to form organic solution of drug. Add PLGA/GMS in to organic Phase. Specified quantities of stabilizers were dissolved in 25 ml water (anti-solvent system). Both solutions were passed through a 0.45µm filter (Gelman Laboratory, Mumbai, India). The anti-solvent was kept below 8°C in an ice-water bath. Then, organic solution was added drop by drop with the use of syringe in a continuous **(A)** Homogenization condition under high speed homogenizer (Kinematica, polytron. Pt 2500E). Initially nanoparticles was characterized by bluish tint of the resulting solution after homogenization. Homogenization speed of 10000 RPM was maintained for 30 minutes followed by sonication for 10 minutes. **(B)** Using High Pressure Homogenization at 2000 PSI Pressure for 10 cycles. All process parameters were optimized and found to be same as mentioned in above process. Nanoparticles containing ampoules with the addition of D-mannitol (5%w/v) as cryoprotectant were frozen in deep freezer at -95°C for 8h for primary freezing. The influence of different experimental parameters such as polymer to drug ratio, organic to aqueous phase ratio and sonication time on the encapsulation efficiency of BIC in the nanoparticles was evaluated.

2.5 Characterization

2.4.1 *FT-IR studies*

FT-IR studies were carried out for pure drug alone and along with excipients. BIC was mixed with each Poloxamer-188 in ratio of 1:1. The pure drug and a physical mixture of drug with excipients were crushed differently with KBr and their IR spectra were recorded over the region 400 to 4000 cm^{-1} in FTIR 8400S, Shimadzu instruments.[6]

2.4.2 Differential scanning calorimetry (DSC) analysis

Differential Scanning Calorimetry (DSC) is among the most useful tools of thermal analysis available for the determination of various thermal parameters of a

formulation, which allows for the detection of phase transition of the samples under study. DSC scans of the powdered sample of BIC and Poloxamer-188 were recorded using DSC- Shimadzu 60 with TDA trend line software. The thermal traces were obtained by heating from 50°C to 300°C at heating rate of 10°C under inert N_2 dynamic atmosphere (100 ml/min) in open crucibles. Aluminium pans and lids were used for all samples. Pure water and indium were used to calibrate the DSC temperature scale and enthalpy response.

2.4.3 Particle size

The average diameter, polydispersity index (PI) and zeta potential of dry nanoparticles were determined by Malvern nano HSA 3000 (Malvern Instruments Ltd., U.K) at room temperature. The samples were adequately diluted with deionized water and placed in a cell. The average particle size was measured after performing the experiment in triplicates.[7]

2.4.4 *In-vitro* drug release study

In vitro release studies were performed using dialysis bag method having molecular weight of 12,000–14,000 Daltons. PLGA Nanoparticles dispersion equivalent to 50 mg of drug was filled into a dialysis membrane bag and tied at both the ends and placed in a basket containing 900 ml of diffusion medium; temperature and speed were maintained at 37 ± 2°C and 100rpm respectively, using dissolution tester (TDT-06P Tablet Dissolution Rate Test App Electro. Lab, Mumbai) by USP apparatus I in 900 ml of 1% sodium lauryl sulphate aqueous solution. At fixed intervals of 0.5, 1, 2, 4, 6, 1, 15, 20, 25, 30, 35, 40 hrs, 10 ml of sample was withdrawn from release media and analyzed by using HPLC. Cumulative percentage release was then calculated from the amount of drug release. The release kinetics were determined by following kinetic equations such as zero order (cumulative % release vs. time), first order (log % drug remaining vs. time), Hixon Crowell Cube root (Cube root of Drug release vs. Time), Higuchi's model (cumulative % drug release vs. square root of time) and Korsmeyer-Peppas model (log drug release vs. log time). Values of $r2$ were calculated from the linear curve obtained by regression analysis of the plots. In case of Korsmeyer-Peppas model, n value was calculated.[8]

2.4.5 Powder X-ray diffraction (PXRD)

PXRD diffractograms of the pure drug and spray dried PLGA nanoparticles were recorded. The theta scan range was 0 to 50 theta.

2.4.6 Solubility Study

Accurately weigh pure BIC drug and equivalent amount of PLGA nanoparticles. Transfer it in 100 ml of volumetric flask and dissolve it with distilled water. Sonicate for 5min. Dilute the solutions as per need. Take absorbance by using UV-spectrophotometer. Determine the solubility of pure BIC drug & PLGA nanoformulations. Find out % increment in Solubility.[8]

2.4.7 Zeta Potential

The zeta potential of nanoparticles is commonly used to characterize the surface charge property of nanoparticles. It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed.

2.4.8 Measurement of drug Entrapment Efficiency and Loading Capacity

Samples from each PLGA nanoparticles were centrifuged at 10,000 rpm and 4°C for 20 minutes using an Optima MAX-E ultracentrifuge (Beckman Coulter, Inc., Nyon, Switzerland). The amount of non-entrapped drug in the supernatant obtained after centrifugation was determined using UV. The percent entrapment efficiency (%EE) and percent drug loading (%LC) were calculated according to Equations.[9]

$$\%\,EE = \frac{W_{TotalDrug} - W_{FreeDrug}}{W_{TotalDrug}} X\ 100$$

$$\%\,LC = \frac{W_{TotalDrug} - W_{FreeDrug}}{(W_{TotalDrug} + W_{TotalLipid} - W_{FreeDrug})} X\ 100$$

2.4.9 SEM Study

SEM Study is mainly done to get idea about surface morphology of the solid Particles. SEM study is very useful to observe nano/micro size particulate structure.

2.4.10 MTT Assay for Cytotoxicity Screening

Pre incubate cells at a concentration of 1×10^6 cells/ml in culture medium for 3 h at 37°C and 6.5% CO_2. Seed cells at a concentration of 5×10^4 cells/well in 100 µl culture medium and various concentrations of BIC and BIC PLGA nanoparticles (final concentration *e.g.* 150µM - 0.005µM) into microplates (tissue culture grade, 96 wells, flat bottom). Incubate cell cultures for 24 h at 37°C and 6.5% CO_2. Add 10 µl MTT labelling mixture and incubate for 4 h at 37°C and 6.5% CO_2. Add 100 µl of solubilization solution each well & incubate for overnight. Measure the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the product is between 450 and 600 nm according to the filters available for the ELISA reader. The reference wavelength should be more than 650 nm. Measure the spectrophotometrical absorbance of the nanoparticles samples after 24 hrs using a microplate (ELISA) reader and find out % cell inhibition.

2.4.11 *In-Vivo* Bioavailability Studies

BIC has very poor aqueous solubility & good permeability. The absolute bioavailability of BIC is approximately 4%. Formulation of solid lipid nanoparticles gives nano size drug particles which can increase permeability and bioavailability by increasing surface area. To check these effects, *in vivo* pharmacokinetic study was planned. Male Wistar rats

(weighted 180–200g, 7–9 weeks old) were used as experimental animals and procured from Central Animal Facility of Shree S. K. Patel College of Pharmaceutical Education and Research (Animal House Registration number 197/CPCSEA). All experiments and protocols described in this study were approved in accordance with the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India with the project number SKPCPER/IAEC/2014-01/03. The animals were maintained in controlled temperature of 24±1°C as well as humidity of 55±5% RH with 12-h light and dark cycle and with standard diet and tap water provided. All animals were acclimatized for minimum period of 3 days prior to the beginning of study. The rats were deprived of food 24 h before the experiment and food was reoffered 4 h post-dosing. The rats were divided into 5 groups of 2 animals each. Each group was administered orally 1 ml of 0.5% w/v plain BIC suspensions, (equivalent to 25 mg/kg body weight as Bicalutamide), BIC PLGA nanoparticles and suspensions containing without drug respectively. Blood samples (0.5 ml) was collected by eye vein; predose, 30, 60, 120, 240 and 360 minutes post-dosing. The collected blood samples were placed in sodium EDTA 2µl containing Eppendorf tube and then plasma was separated immediately by centrifugation at 10000 rpm for 10 mins. Protein precipitation was carried out by adding methanol and centrifuged at 10000 rpm for 10 mins. The supernatant was collected and stored at -20°C until analysis. To estimate amount of BIC present in rat plasma, HPLC analysis was done on plasma sample. Calculation of concentration was done using linear regression equation. *In-vivo* study was carried out two times on interval of week to reduce the variation. Pharmacokinetic parameters C_{max}, T_{max} and $AUC_{0\text{-}48h}$ were calculated using software Graph pad prism 5.

3. RESULTS AND DISCUSSION

3.1.1 FT-IR Study

FT-IR spectroscopy was carried out to study the drug-polymer interaction. The Spectra of BIC & prepared Nanoformulations showed in Figure 3. Characteristic peaks of the BIC was identified by absorption peaks at 3345 cm^{-1} (secondary amine N-H stretch), 3065 cm^{-1} (=C-H aromatic ring), 2947 cm^{-1} (C-H stretch), 2242 cm^{-1} (C=N stretch), 1699 cm^{-1} (C=O stretch) and 1595 cm^{-1} (C=C stretch, aromatic ring). For PLGA, one broad peak is observed at ~2100 cm^{-1}. Physically mixed sample of drug and PLGA proved that PLGA & Poloxamer 188 is not affecting the characteristic peaks of the Drug. Also in SLNs due to Glyceryl monostearate (GMS), drug peak did not change. The same thing is further proven by DSC thermogram of same physical mixture.

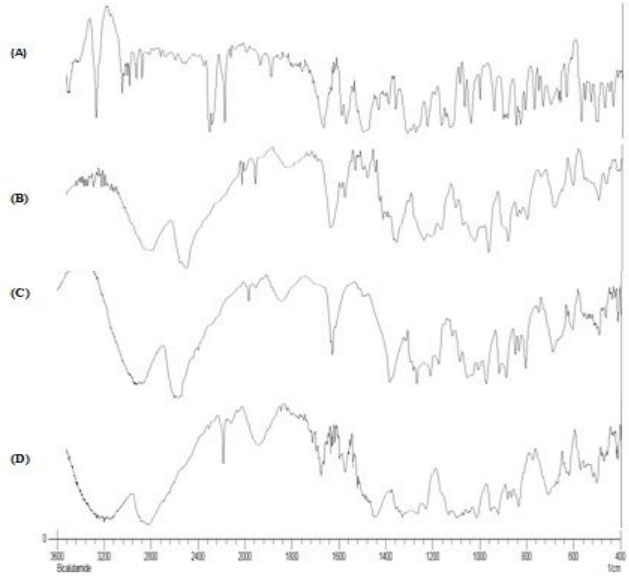

Figure 3: FT-IR of (A) Bicalutamide Drug (B) PLGA nanoparticles (C) SLNs (D) Nanocrystals.

3.1.2 DSC Study

DSC thermogram study was carried out for checking interaction between excipients. Pure drug showed endothermic peak at 196.05˚C while PLGA nanoparticles showed peak at 58.0˚C. In case of SLNs there was no interaction between Glyceryl monostearate & BIC. Drug peak was disappeared in case of SLNs which is due to incorporation of the drug in the lipid. In Nanocrystals, there was no interaction between drug peak & Poloxamer-188.

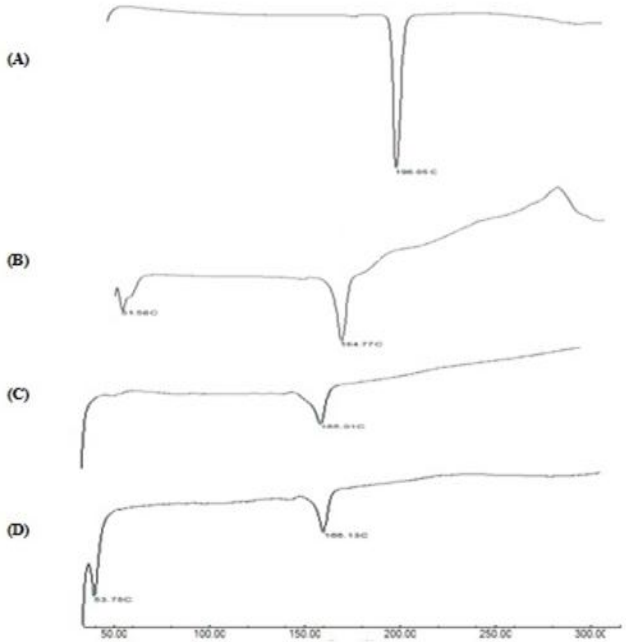

Figure 4: DSC spectra of (A) Bicalutamide Drug (B) PLGA nanoparticles (C) SLNs (D) Nanocrystals.

3.1.3 Particle Size Analysis

Droplet size analysis was done by Malvern nano HSA 3000 (Malvern Instruments Ltd., UK). It measures the size of particles typically in the sub micron region. It measures Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surrounds them. The measurement by Malvern is done by Laser Scattering Principle as per Mie Theory and Fraunhofer Approximation. Malvern measure the intensity of light scattered by nanoparticles.

It was seen that PLGA nanoparticles had desired particle size (Batch P7 & P8) in Nano range. In other batches, particle size increase with increase in drug loading. SLNs have larger particle size than PLGA nanoparticles. The best particle size was seen in batch S4 (High Speed Homogenization) & S9 (High Pressure Homogenization) Batches. For Nanocrystal, High pressure homogenization batches had good particle size in nano range than high speed homogenization. Batch C2 had high Particle Size of 105.0 nm.

Table 3: Particle size of BIC nanoformulations Prepared by High Speed Homogenization.

Batch No	Drug	Stabilizers	PLGA polymer/ GMS Lipid	Particle Size (nm)	PDI
P8	150	Poloxamer-188 (1 % W/W)	50	118.00±0.4	0.321
P11	150	Poloxamer-188 (1 % W/W)	50	100.00±0.1	0.231
S4	100	Poloxamer-188 (1 % W/W)	50	123.00±0.5	0.759
S9	100	Poloxamer-188 (1 % W/W)	50	119.15±0.8	0.321
C1	100	Poloxamer-188 (1 % W/W)	….	118.50	0.428
C2	100	Poloxamer-188 (1 % W/W)	….	105.00	0.238

3.1.4 Drug Entrapment Efficiency and drug Loading

Drug loading & Entrapment efficiency were measured in PLGA nanoparticles & SLNs. In Case of PLGA nanoparticles, P11 Batch have high % drug loading as well as entrapment efficiency which is 72.27% & 86.0% respectively. In SLNs, batch S9 had high % drug loading capacity & Entrapment efficiency which is 60.93% & 78% respectively. There was high % drug loading/entrapment efficiency in case of High Pressure Homogenization batches than high speed homogenization batches.

Table 4: % Drug Loading & Entrapment efficiency of Nanoformulations.

Batch No:	Total polymer (A)	Total drug (B)	Free Drug (C)	Total-Free (D)	Total drug+ Total Polymer- free drug (E)	% LC (D)/(E)*100	EE (D)/(B)*100
P8	50	150	22	128	178	71.91±0.4	85.33±0.3
P11	50	150	21	129	179	72.07±0.7	86.00±0.7
S4	50	100	28	72	122	59.01±0.25	72±1
S9	50	100	22	78	128	60.93±0.37	78±1.5

3.1.5 *In-Vitro* Drug Release Study

In vitro drug release study shows that PLGA nanoparticles (P11) have sustained effect than SLNs & nanocrystals. SLNs have cumulative % drug release of 66.87 & 73.27 for S4 & S9 respectively, while for nanocrystals it was 81.27 & 79.27 for C1 & C2 respectively. Nanoformulations have higher % Cumulative Drug Release than pure BIC drug. PLGA nanoparticles have nearer to zero order release kinetics. This was due to slow degradation of PLGA Polymer.

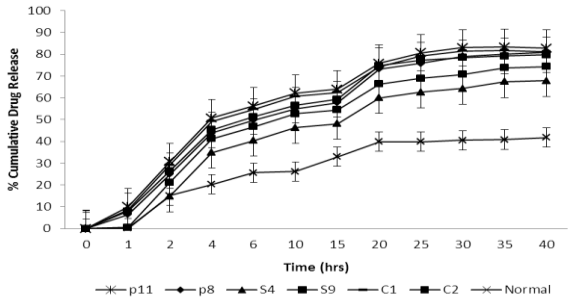

Figure 5: *In-Vitro* Drug release of nanoformulations.

3.1.6 Solubility Study

Solubility Study data was listed in table 5. Pure BIC drug has 16.32 mg/100 ml of solubility. P11 (PLGA nanoparticles) shows the higher solubility compare to other batches. Nanocrystals have the good solubility increments than SLNs. SLNs have poor Solubility increments than PLGA nanoparticles.

Table 5: Solubility of Nanoformulations.

Batch No.	Solubility (mg/ml)	% Solubility Increment
P8	146.0±0.09	64.1±0.4
P11	148.52±0.4	89.98±0.6
S4	45.2±0.35	63.89±0.5
S9	47.3±0.29	65.50±0.9
C1	114.0±0.6	83.43±0.7
C2	109.0±0.5	85.96±0.4

3.1.7 Zeta Potential

The zeta potential of nanoparticles is commonly used to characterize the surface charge property of

nanoformulations. It reflects the electrical potential of particles and is influenced by the composition of the particle and the medium in which it is dispersed. All nanoformulations P11, S9 & C2 have good zeta potential values. So it indicated that all prepared nanoformulations had comparatively effective stability.

Table 6: Zeta Potential of Optimized Nanoformulations.

Batch No:	Zeta Potetial
P11	**-25.0**
S9	-32.7
C2	-27.2

3.1.8

3.1.9 X-Ray Diffraction (XRD) Study

XRD was done to check crystalline transition of nanoformulations and it showed in Figure 6. Spectra shows that pure BIC (A) has sharp intense peak which indicates the crystalline nature. Nanocrystals shows less intense than pure drug which shows that conversion of crystalline to amorphous form. PLGA nanoparticles have very broad peak and it shows high conversion amorphous form.

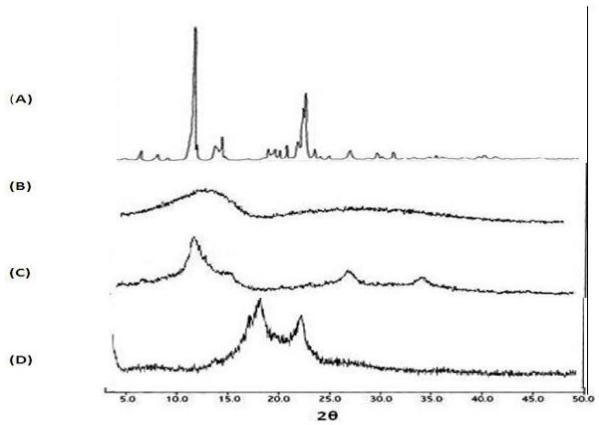

Figure 6: XRD Spectra of (A) Bicalutamide (B) PLGA Nanoparticles (C) SLNs (D) Nanocrystals.

3.1.10 Scanning Electron Microscope (SEM) Study

Prepared Nanoformulations were evaluated for SEM and its showed in figure 7. The PLGA nanoparticles shows spherical shape. The SEM of the SLNs shows that the nanoparticles have a solid dense structure with smooth spherical shape. Nanocrystals have irregular crystals structure.

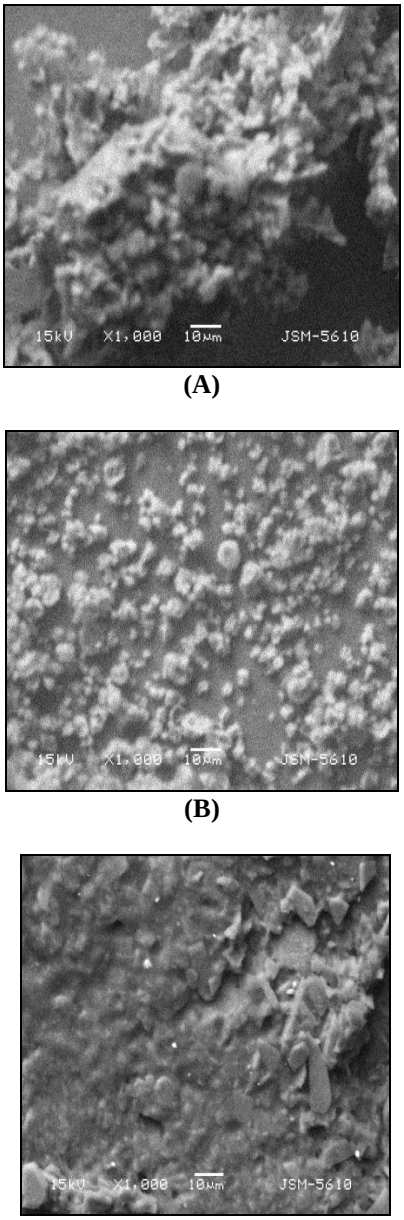

Figure 7: SEM images of (A) PLGA nanoparticles (B) SLNs (C) Nanocrystals.

3.1.10 Cell-Line Study By using DU-145

In-Vitro Cytotoxicity data was showed in Table 7. PLGA nanoparticles had also effective % cell inhibition than SLNs & Nanocrystals. Also IC50 value is less in case of P11 than S9 & C2. Normal drug has high IC50 Value 740.0 mcg/ml.

Table 7: % Cell Inhibition of Nanoformulations.

Concentration 1.5 mg/ml	Log concentration	Formulation Qty (µl)	P11	S9	C2	Normal
0.01	-1.931	150.00	42.52	35.84	40.02	32.04
0.02	-1.630	75.00	28	29.06	25.5	25.26
0.05	-1.329	37.50	45.77	44.94	43.27	41.14
0.09	-0.028	18.75	31.77	28.55	29.27	24.75

0.19	-0.727	9.38	23.18	15.15	20.68	11.35
0.38	-0.426	4.69	36.22	21.17	33.72	17.37
0.75	-0.125	2.34	19.02	25.61	16.52	21.81
1.50	0.176	1.17	77.22	63.24	74.72	59.44
3.00	0.447	0.59	69.7	57.01	67.2	53.21
3.00	0.477	0.29	68.41	53.35	65.91	49.55

3.1.11 *In-Vivo* Bioavailability Studies

The Pharmacokinetic Parameters Showed in Table 8. *In-Vivo* Bioavailability study was performed and Calculated for C_{max}, T_{max} & AUC. C_{max} was high in P11 (283.59 ± 4.25) (PLGA Nanoparticles). S9 & C2 had low Cmax than P11. Also T_{max} was same (12 hrs) for all Nanoformulations. AUC was high in P11 (5248.0 ± 250.5) than S9 & C2. PLGA nanoparticles had good Pharmacokinetic parameters than other nanoformulations. It had high absorption rate. Onset of action was low for PLGA nanoparticles which seem effective & efficient formulation for treatment of Prostate Cancer. All Nanoformulations had high AUC, C_{max} & low in T_{max} than Bicalutamide drug. In SLNs & Nanocrystals, Cmax was 271.09 ± 5.18 mg & 275.19 ± 8.28 mg respectively. Both had same T_{max} Value (≈ 12 hrs) than Bicalutamide (≈ 16 hrs). AUC was 4500 ± 217.2 mg.hr/L & 4757 ± 257.9 mg.hr/L for S9 & C2 respectively which indicates that Nanocrystals had better Pharmacokinetic action than Bicalutamide SLNs. Pure BIC had C_{max} of 154.41 ± 6.23 mg, which was almost half than nanoformulations.

Table 8: Pharmacokinetic Data of Nanoformulations.

Pharmacokinetic Parameters	P11	S9	C2	Normal
Dose (mg)	300	300	300	300
C_{max} (mg)	283.59 ± 4.25	271.09 ± 5.18	275.19 ± 8.28	154.41 ± 6.23
T_{max} (Hrs)	12	12	12	16
AUC (mg.hr/L)	5248.0 ± 250.5	4500 ± 217.2	4757 ± 257.9	2500 ± 220.3

4 CONCLUSION

In the present investigation, BIC Nanoformulations were prepared for increasing its Solubility and Bioavailability. Prepared PLGA nanoparticles by high pressure homogenizer shows desired Particle size, Zeta potential, % Drug loading, Entrapment efficiency. *In-vitro* Cell-line study, XRD Study, Solubility study as well as *In-vivo* study shows that PLGA nanoparticles have more therapeutic effectiveness than SLNs & Nanocrystals to treat prostate cancer. The study opens the chances of manufacturing of the PLGA nanoparticles by competitive cost at commercial level.

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