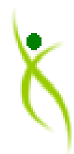

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Research Article

Pharmaceutics for effective drug dosage

Preparation, Process Optimization and Cytotoxicity Evaluation of Lyophilized Etoposide Loaded Nanoparticles

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Abstract: The purpose of this study was to develop freeze dried Polylactide-co-glycolide (PLGA) nanoparticles loaded with etoposide intended to be administered intravenously with improved therapeutic efficacy of the drug and thereby reduced dose related toxicity associated with it. The etoposide loaded nanoparticles were prepared using modified spontaneous emulsification solvent diffusion method (SESD).The *in Vitro* release study of etoposide loaded Polylactide-co-glycolide (PLGA) –Nanoparticles NP was carried by dialysis bag diffusion technique.The incorporation efficiency was found to be higher in modified SESD technique than emulsification solvent evaporation method. The release behavior of etoposide exhibited a biphasic pattern characterized by an initial burst release followed by a slow and continuous release. The nanoparticles were characterized by particle size, zeta potential, polydispersity index, DSC and FTIR. The long term stability was achieved by lyophilisation technique. The cell line study using XTT assay on LNCaP prostate tumor cell lines revealed that etoposide loaded nanoparticles showed greater cancer cell inhibition compared to plain nanoparticles and marketed conventional formulations.

Keyword: PLGA, Etoposide, Lyophilization, Cryoprotectant, cell line studies, cytotoxicity studies

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1. INTRODUCTION

Currently, cancer treatment has significant side effects due to the low tumor targeting specificity of chemotherapy drugs, which leads to systemic exposure. Systemic exposure of the body to chemotherapy drugs leads to higher cytotoxic effects in both cancerous and normal cells, making current chemotherapy techniques balancing game often restricted by dose-limiting toxicity[1-3]. Nanoparticles are especially suited for drug delivery applications because they have a higher drug loading capacity than larger particles (due to a higher surface area to volume ratio) and they can access a wider range of locations in the body which were previously inaccessible due to size cut-offs. These size-related properties of nanoparticles offer promising opportunities to exploit the properties of solid tumors for drug delivery [4-5]. For instance, tumors have inherently leaky and permeable vasculature, which results in the disproportionate accumulation of macromolecules and nanocarriers within the tumor. Combined with the tumor's dysfunctional lymphatic drainage system, a selective accumulation and retention of nanocarriers within the tumor interstitial space has been observed, an effect known as the enhanced permeability and retention effect (EPR)[6-8]. Particular attention has been paid to the development of parenteral therapeutic systems, made of biodegradable polymers, as potential formulations for site-specific drug delivery, including drug targeting. The study and technological development of these colloidal systems represent an important field in pharmaceutical research.In general, the methods for the preparation of nanoparticles can be classified into two main categories: polymerization of dispersed monomers and emulsification of natural macromolecules (such as albumin) or preformed polymers (Pseudolatexes)[9-10-11]

2. MATERIALS AND METHODS

Etoposide was a gift sample from Astron Research Ltd, Ahmedabad, India. Resomer RG509S with inherent viscosity1.4-1.8 dl/g and polymer composition: 47:53 to 53:47 molar ratios (D, L Lactide co glycolide) was purchased from Boegher Intellgam, Germany. LNCaP Cell line was purchased from National Cancer Research Institute, Pune. Various media like RPMI-1640, Bovine Serum Albumin, streptomycin and other antibiotics were purchased from M.P.Biogen. USA.

2.1 Quantification of Etoposide by HPLC method

The reversed Phase HPLC method for determination of content of etoposide in various solvents was developed. The chromatographic separations were performed using Phenomenex□ C_{18} (250 mm x 4.6 mm i.d, 5 µm particle sizes) column at 40 °C [12-14]. The sample chromatogram was shown in Fig:1. The optimum mobile phase consisted of acetonitrile and water in the ratio of 45:55 v/v. Auto sampler 20 µl was used and kept at 15 °C. Analysis was done with a flow rate of 1.0 ml/min at 254 nm (λ max of etoposide) wavelength by using photodiode array (PDA) detector.The HPLC was calibrated with standard solution of 2.5 ug/ml to 20 ug/ml by dissolving etoposide in Acetonitrile/water solution. A standard solution of 100ug/ml concentration was prepared and from that various stock solutions of 2.5 ug/ml, 5ug/ml, 10 ug/ml, 15 ug/ml and 20 ug/ml were prepared. A calibration plot was developed finally[15-17].

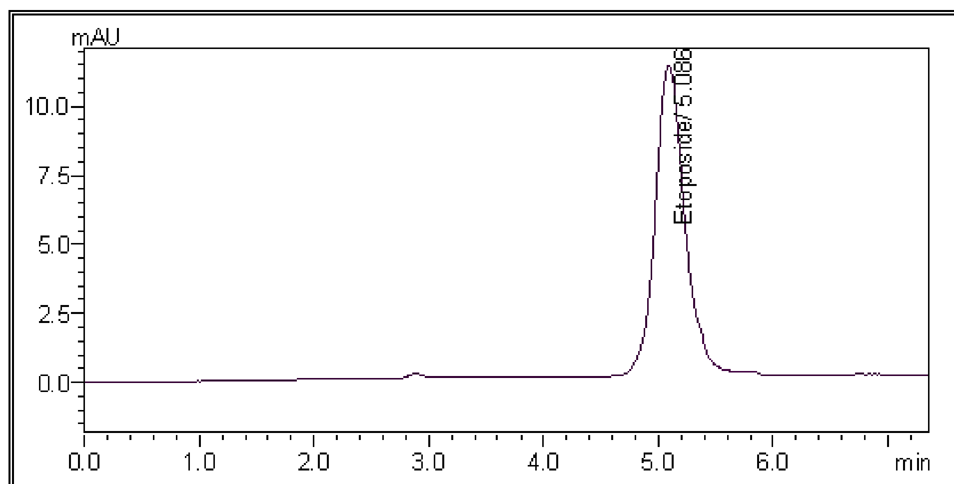

Fig 1. Chromatogram of etoposide (1µg/ml) standard with corresponding retention time at 254 nm by RP-HPLC method

2.2 Preparations of Etoposide loaded Polylactide co Glycolide based nanoparticles

2.2.1 Modified (SESD) spontaneous emulsification solvent diffusion

One promising technique was the spontaneous emulsification solvent diffusion method in which nano-sized particles of PLGA or PLA can be effectively obtained by pouring the polymeric organic solution into an aqueous phase with moderate mechanical stirring. Here etoposide loaded nanoparticles were prepared by SESD (spontaneous emulsification solvent diffusion technique). Various drug to polymer(PLGA) ratios like 1:1, 1:2 and 1:4 were selected. Here dichloromethane and acetone, a less water miscible organic solvent and water miscible organic solvent respectively were selected in different ratio like 1:1, 1:2, and 2:1, 4:1. In another modification, methanol, a water miscible organic solvent was taken with acetone in different ratio like1:1, 1:2, and 2:1, 4:1. Here 1%w/v aqueous PVA solution was used as a surfactant in order to prevent the aggregation and coalescence among the particles. The drug and polymer both were dissolved in biphasic organic solvent mixtures and then this solution was added slowly to aqueous solution

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containing PVA solution.Nowthis mixture was stirred constantly in order to evaporate the organic solvent for 5 hrs in order to complete removal of solvent. Nanoparticles were formed via the following mechanism. When a polymeric solution was added, emulsion droplets are formed in the aqueous phase, acetone quickly diffuses out from each emulsion droplet, drastically reducing its size to nano size. A consequent solvent – evaporation process, in which the remaining organic solvent like dichloromethane and methanol was removed from the system makes the droplets to solidify and finally form polymeric nanoparticles.[18]

2.2.2. *Separation of nanoparticles (Purification)*

The resultant nanoemulsion was subjected to ultra centrifugation at 2 °C to 5 °C in cooling centrifuge at around 15000 rpm for 30 mins. The settled nanoparticles were collected by separating supernatant containing free drug. The supernatant and nanoparticles both were dissolved in mobile phase solution and quantified by Re-Phase HPLC method. [19]

2.2.2 *Measurement of entrapment efficiency*

The encapsulation efficiency of the PLGA–NP was defined as the percentage of etoposide encapsulated in respect to the total amount of etoposide used to prepare the PLGA-NP .The amount of etoposide entrapped within the PLGA-NP was determined by measuring the amount of non-entrapped etoposide by RP-Phased HPLC quantification that was recovered in the supernatant after ultracentrifugation and wash of the etoposide loaded PLGA nanoparticles for three times at 15000 rpm for 30 min. Each sample was assayed in triplicate and the results were reported as average ±Standard deviation.[20]

2.2.3 *In vitro release study*

The in vitro release profiles were studied by dialysis tube diffusion method using phosphate buffer saline at pH: 7.4, as a release medium. The in vitro release experiment was carried out as follows:20 mg of lyophilized Etoposide loaded PLGA nanoparticles and 5 ml PBS (Phosphate Buffer Saline) (pH:7.4) was placed into dialysis bag with membrane with cutoff value of 100Kd immersed into 100 ml PBS solution and the system was placed in an orbital shaker maintained at 37±0.1^{0}Cand shaken horizontally at 100 min. At predetermined intervals, 5 ml of dissolution medium was taken out and assayed for drug content analysis along with replacement of 5 ml of fresh buffer at sampling point and agitation was continued. Etoposide release was quantified at 254 nm by reversed phase HPLC method. No interference of PLGA and PLA was observed at the same wavelength. All release experiments were performed in triplicate[21] The release of etoposide from the nanoparticles followed a Higuchi pattern(R^2=0.98)

2.2.4 *FTIR (Fourior Transform) Infrared Spectroscopy*

A Shimatzu FouriorTransform Infrared Spetrophotometer (FTIR) was used for IR analysis of samples. About 1–2 mg of sample were mixed with dry potassium dichromate and the samples were examined at transmission mode over wave number range of 4,000 to 400 cm^{-1}.[22]

2.2.5 *DSC study of Etoposide*

Mixtures were obtained as dry lyophilized powders prior to DSC analysis. DSC instrument of Shimadzu (Japan) were used in at the temperature range of 25–300 ^{0}C under oxygen atmosphere, samples weighing approximately 3mg were heated in a hermetically sealed aluminum pan at a rate of 10 ^{0}C/min.[23]

2.2.6 *Size distribution and zeta potential*

Different batches of Nanoparticles were monitored for their morphological attributes with the help of scanning electron microscope (SEM) (JSM, JEOL, Tokyo, Japan). Size distribution and Zeta potential of nanoparticle were measured by dynamic light scattering method using Zetasizernano ZS (Malvern Instruments Ltd., UK).[24]

2.2.7 *Lyophilization and In vitro Plasma stability study of Etoposide Nanoparticles*

A common limitation of using polymeric nanoparticles in aqueous suspension was due to their poor chemical and physical stability when conserved for a long time. So freeze drying of these colloidal systems is an alternative method to achieve long-term stability. Here various parameter like cooling rate, cryoprotectant ratio were optimized and the particle size was determined before freezing and after freezing and final to initial size (S_F/S_I) was calculated. PBS stability assays for long term stability were carried out where the NPs were dialyzed in PBS over 120 hrs at 37^{0}C and withdrawn at 24 hour intervals. PBS stability assays for long –term stability were carried out where the NPs were dialyzed in Phosphate buffer saline (pH: 7.4) PBS over 120 hrs at 37^{0}C and withdrawn at 24 h intervals. 10% Human Plasma diluted with SWFI, Phosphate buffer saline were used as diffusion media. Human plasma was diluted with sterile water for injection (SWFI) in order to check the effect of plasma protein on nanoparticle size and whether the nanoparticles remain within its particle size range in blood stream. As plasma protein on long term shows the aggregation of nanoparticles, the size was improved to micron size[25]

2.2.8 *In vitro antitumor effect of Etoposide loaded Nanoparticle on LNCaP cell lines*

The cytotoxicity of PLGA plain nanoparticles, etoposide loaded nanoparticles and plain etoposide as a control were investigated using Prostate tumor carcinoma cell lines (LNCaP) by XTT assay. The primary culture of Prostate cell line was procured from National Cancer Research Institute, Pune. LNCaP cell line seeded and sub cultured in T flask in bio safety cabinet hood. Here growth media, 100 ml RPMI - 1640, with L-Glutamine. Na Carbonate and glucose were added. The media was supplemented with 10 ml fetal bovine serum for the purpose of generating monolayer. Addition of 1 ml of penicillin and amphotericin B prevented the fungal growth. T flasks were incubated for 15 days in co_2 incubator with every 3 to 4 days for media change and cell viability was checked by trypsinization. Then LNCaP cells were seeded and 96 well plate sat at a density of 10,000 cells per well in 100 µl RPMI supplemented with 10% fetal bovine serum along with 50 µl of cell culture. Cytotoxicity were carried out at three fold dilution with concentration range of 100mMolar to 0.41mMolar/ml. After twenty-four hours plating, incubation at 37^{0}C, 50 µl of XTT die was added into each well, after 24,48 and 72 hours , the well plates were read in microplate reader(Bio-Rad, Hercules,

CA,USA) at a wavelength of 490 nm. The experiments were repeated in triplicate .Cytotoxicity was expressed as % reduction cell viability.[26]

3. STATISTICAL ANALYSIS

The Result data were presented as mean ± standard deviation (SD). All data were generated in three independent experiments. Statistical analysis was performed with one-way analysis of variance (ANNOVA) .Difference were considered to be significant at a level of $P<0.5$.

4. RESULTS AND DISCUSSION

4.1 Particle size and Size Distribution

As a starting point for controlling nanoparticle size distribution, efforts have been made to study the effect of different types of organic solvents used to solubilize the drug and polymer. Studies have suggested that the miscibility of the organic solvent in water can impact NP size for a given solvent: water system .Generally the miscibility can be quantitatively expressed by comparing the solubility parameters of both the solvent and water. We chose to investigate the relationship of NP size and solvent miscibility with water using four organic solvent and observed the water miscibility of the organic solvents used in this study. .[24,25] Firstly in SESD method ,the different ratios of binary mixtures of water immiscible solvents like dichloromethane and water miscible solvent like acetone were taken in different ratios like 1:1,1:2 2:1 and 4:1.For each ratio ,drug to PLGA ratios were optimized like 1:1,1:2,1:4.It has been observed that, as there was increase of etoposide: PLGA ratios there was an increase in mean particle size along with increase in zeta potential values which indicate that shear plane was in the bulk of the phase. Even higher the amount of dichloromethane with acetone leads to definite raise in particle size[14]. Whereas, when with double the amount of acetone with dichloromethane, there is a decrease in particle size. No significant change was found on the polydispersity index along with the entrapment efficiency as shown in Table: 01. In modified SESD method, The optimized batch has a uniform particle size of 204 nm with zeta potential value of -7.5 mV, polydispersity index of 0.211 and entrapment efficiency of 71±3.2.Here Drug: Polymer ratio was 1:2 and Methanol :Acetone ratio was 1:2. Modified SESD process uses two water miscible organic solvents such as methanol and acetone instead of dichloromethane/acetone. This alteration helped to achieve lower sized nanoparticles with less chances of aggregation and avoidance of toxic solvents like dichloromethane. Trends toward lower NPs size were observed when methanol was used instead of dichloromethane which is probably due to the water miscibility. By this method we can avoid the use of toxic solvents also. Upon improving the polymer concentration, the particle size and zeta potential also improved with no significant polydispersity index change as shown in Table: 02.

Table 1. Optimization of various parameters of etoposide loaded PLGA nanoparticles by SESD method

CH_2Cl_2/ Acetone Ratio	CODE	Drug: Polymer ratio	Particle size (nm)	Zeta potential (mV)	(PDI)	Entrapment efficiency± S.D
1:1	EP1	1:1	255	-7.9	0.206	65±3.4
	EP2	1:2	311	-8.1	0.301	68±3.5
	EP3	1:4	324	-8.4	0.231	67±2.3
1:2	EP4	1:1	243	-8.2	0.211	64±3.2
	EP5	1:2	305	-8.4	0.203	68±2.1
	EP6	1:4	376	-7.9	0.190	65±3.2
2:1	EP7	1:1	310	-9.7	0.231	61±2.4
	EP8	1:2	339	-8.9	0.211	68±1.7
	EP9	1:4	401	-10.1	0.190	66±2.4
4:1	EP10	1:1	442	-9.9	0.219	55±2.5
	EP11	1:2	513	-10.2	0.195	57±3.2
	EP12	1:4	556	-10.5	0.205	52±4.5

Table 2. Optimization of parameters of etoposide loaded PLGA nanoparticles by Modified SESD method.

Methanol/ Acetone Ratio	CODE	Drug: Polymer ratio	Particle size (nm)	Zeta potential (mV)	(PDI)	Entrapment efficiency ± S.D
1:1	EP13	1:1	203	-7.1	0.191	69±2.1
	EP14	1:2	267	-7.4	0.102	70±3.3
	EP15	1:4	306	-8.5	0.211	70±4.5
1:2	EP16	1:1	184	-6.9	0.171	68±1.2
	EP17	1:2	204	-7.5	0.211	71±3.2
	EP18	1:4	324	-7.3	0.209	71±3.5
2:1	EP19	1:1	254	-6.9	0.190	66±2.3
	EP20	1:2	286	-7.3	0.110	68±2.5
	EP21	1:4	324	-7.4	0.211	69±2.5
4:1	EP22	1:1	210	-6.9	0.119	62±4.5
	EP23	1:2	255	-6.2	0.171	61±2.7
	EP24	1:4	312	-7.2	0.201	64±4.3

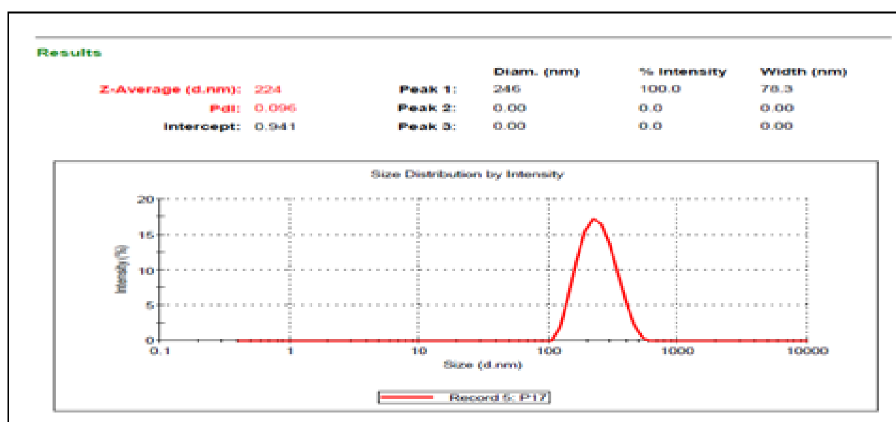

Fig 2. Particle size distribution of optimized batch

4.2 *Scanning Electron Microscopy*

Scanning electron microscopy image of etoposide loaded freeze dried PLGA based nanoparticles revealed the surface texture of freeze dried powder which shows the amorphous nature of the nanoparticles.

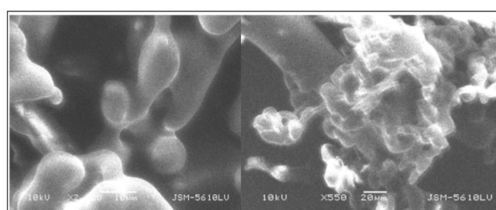

Fig 3. Scanning electron micrograph of freeze dried Etoposide nanoparticles.

4.3 *In vitro release study*

The cumulative percentage release is shown in Figure: 3. after an initial burst release of 4 hrs, the etoposide release reduced and became almost zero order release rate (R^2=0.987). The etoposide released in the first 4 hrs was equivalent to 29.84±5.5% of the initial drug load of nanoparticles. The burst release of etoposide may be due to the dissolution and diffusion of the drug that was poorly entrapped in the polymer matrix, while the slower and continuous release may be attributed to the diffusion of the drug localized in the PLGA core of the nanoparticles[18]. The controlled release of etoposide loaded nanopartlicles continued for over 4 days almost more than 90% drug got released , whereas the standard conventional etoposide injection showed an almost entire release within 3 to 4 hrs.[21]

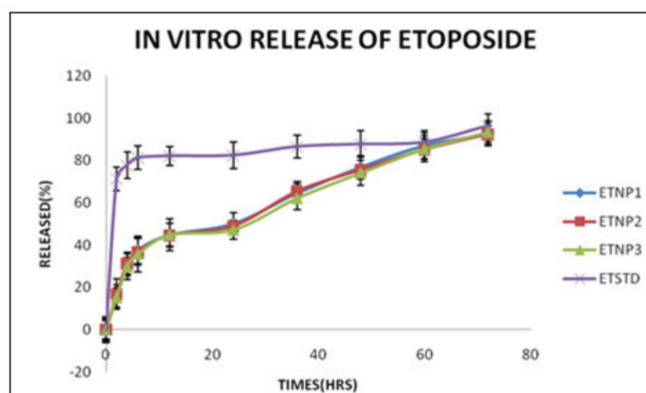

Fig 4. In vitro release pattern of Freeze dried nanoparticle of Etoposide.

4.4 *FTIR study of Etoposide Nanoparticles*

In plain etoposide spectra(Fig:5), characteristic bands were carbonyl stretch vibration of the lactone ring at 1775 cm^{-1},the OH stretch vibration of the phenolic and sugar OH groups at 3400 cm^{-1} ,the aromatic band at 1610 , and the c-o stretch vibration at1250 cm^{-1}. In FTIR of etoposide encapsulated nanoparticles (Fig: 5A), the corresponding peaks of lactone ring and OH group peaks were disappear or buried which indicating etoposide entrapment in PLGA polymer matrix

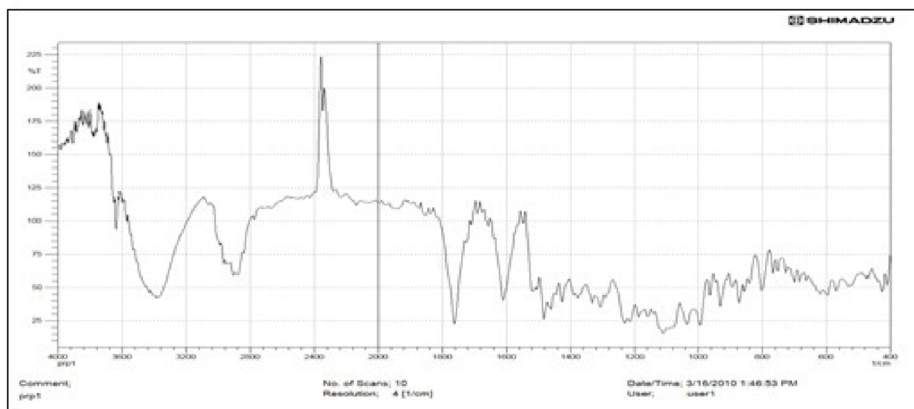

Fig 5. FTIR spectra of plain Etoposide Drug.

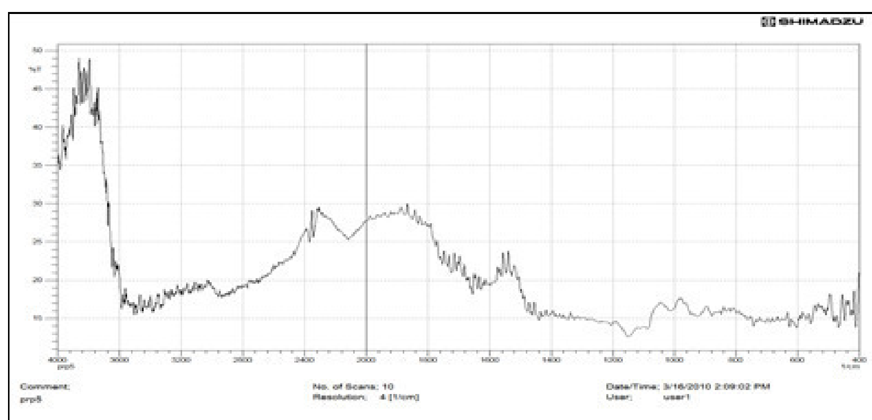

Fig 5(A). FTIR spectra of Etoposide loaded nanoparticles.

4.5 DSC study of Etoposide Nanoparticles

In DSC of plain etoposide (Fig:6), a strong endothermic peak was shown at 383.83°C.In individual DSC of PLGA polymer, endothermic peaks was shown at around 297.33° C(Fig:7). In (Fig:8) the DSC of etoposide loaded PLGA nanoparticles, the change in endothermic peak attributed to dissolution of etoposide in PLGA polymer matrix and decrease in melting temperature of nanoparticle formulated etoposide compared to bulk has been indicating to their small size.

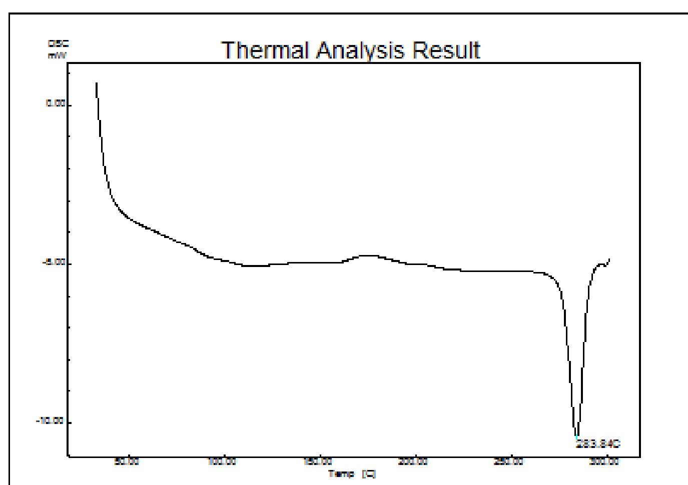

Fig 6. DSC thermogram of Plain etoposide drug

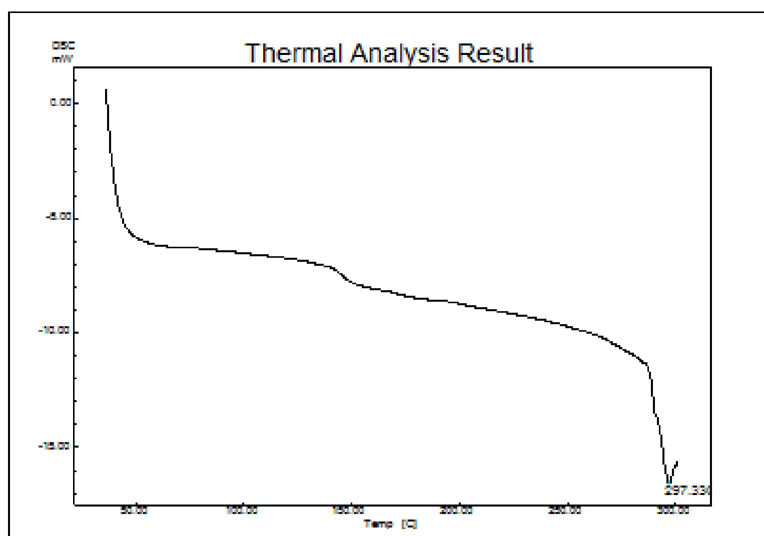

Fig 7. DSC thermogram of PLGA polymer

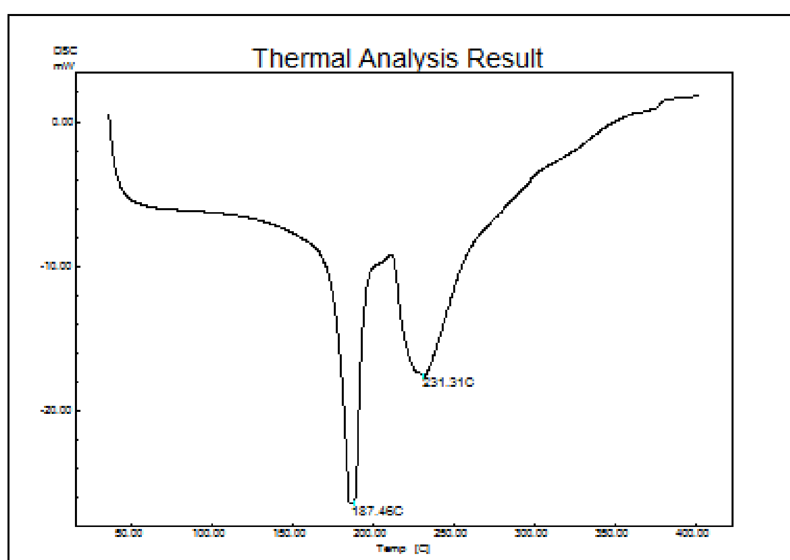

Fig 8. DSC thermogram of etoposide loaded PLGA freeze dried nanoparticles

4.6 Lyophilization of Etoposide nanoparticles

In this study Etoposide nanoparticles prepared by Modified SESD method were subjected to various cooling rate and different cryoprotectant ratios. Sucrose was chosen as a cryoprotectant at three different concentration 0,5 and 15%(w/v).Here we selected the batch EP17 from Modified SESD method, the concentration of 1%(w/v) PVA as a stabilizer(surfactant) present which has a influence on the particle size stability. The results in Table: 3 showed clearly that etoposide nanoparticles with 15%w/v cryoprotectant(Sucrose) and cooling procedure in liquid Nitrogen seems very stable due to the three procedures of freezing as the ratio S_F/S_I is very near from 1 confirming a high stability. Here fast cooling /freezing in liquid nitrogen leads to very small stress time on particle size and integrity as compared to freezing in deep freezer followed by shelf freezing which take considerable stress of freezing on nanoparticles[24]. Presence of Surfactant in nanoparticle formulation leads to development of protective envelope over the nanoparticle, which also provide a protective shield over the nanoparticles[1] So it can be said that cooling procedure and cryoprotectant ratio has an influence on the size and polydispersity of nanoparticles.it was observed an increase in the size and polydispersity when a slow cooling procedure , and low cryoprotectant ratio applied, whereas a flash cooling(-150°C) using liquid nitrogen reduces the ratio S_F/S_I about 1.24.in Table :3. The Table: 3, 4, 5 shown the optimization of various cooling procedure and different cryoprotectant ratios on the particle size.

Table 3 Procedure of freezing at -150°C in liquid nitrogen with ramp shelf temp.of -50°C for 24 hrs for sublimation (EP17)

Cooling Procedure	Cooling rate	Sucrose (%w/v)	Before freeze drying Particle size(nm)	After freeze Drying size(nm)	S_F/S_I
In liquid Nitrogen	-150°C	0	204	1102	5.40
		5	204	787	3.8
		15	204	254	1.24

Table 4 Procedure of freezing at -80°C in deep freezer for 45 minutes with ramp shelf temperature ramp -50°C for 24 hrs for sublimation (EP17)

Cooling Procedure	Cooling Rate	Sucrose (%w/v)	Before freeze drying Particle size(nm)	After freeze Drying size(nm)	S_F/S_I
Prechilled deep freezer	-80°C	0	204	1212	5.90
		5	204	702	3.4
		15	204	263	1.28

Table 5 Procedure of freezing at 1°C to -50°C in freezer condenser ramp for 45 minutes with shelf temperature ramp -50°C for 24 hrs for sublimation (EP17)

Cooling Procedure	Cooling Rate	Sucrose (%w/v)	Before freeze drying Particle size(nm)	After freeze drying size(nm)	S_F/S_I
Freeze dryer shelf	-50°C	0	204	1171	5.74
		5	204	693	3.39
		15	204	301	1.47

4.7 In vitro Plasma stability studies

We performed *invitro* assays of long term stability and protein binding on optimized formulation from emulsification solvent diffusion and nanoprecipitation method. Plasma buffer saline (PBS) stability assays for long-term stability were carried PBS stability assays for long –term stability were carried out where the NPs were dialyzed in PBS and Human plasma (10%) diluted with Sterile water for injection over 120 hrs at 37^{0}C and withdrawn at 24 h intervals. Particle size measurement demonstrated that the NPs remain stable over 5 days for the batches manufactured through emulsification solvent diffusion with no significant change in size and polydispersities for etoposide and there was no significant drop in size of particles due to steric stabilitization over a course of 120 hours, it could also be due to polymeric monolayer did not peel off over a long period, where as for the batches from nanoprecipitation shows relatively less stability of 3 days with with significant change in size and polydispersities, as it didn't contain any surfactant so due to intermolecular force of attraction between like molecules leads higher size apon storage in both in human plasma and PBS.These results suggest the steric stabilization of surfactant concentration in the formulation play important roles shown in Figure: 09 and 09A .Here it was observed that plasma protein do not show detrimental effect on the particle size stability so after IV infusion the nanoparticle remain in stable size form in blood stream.

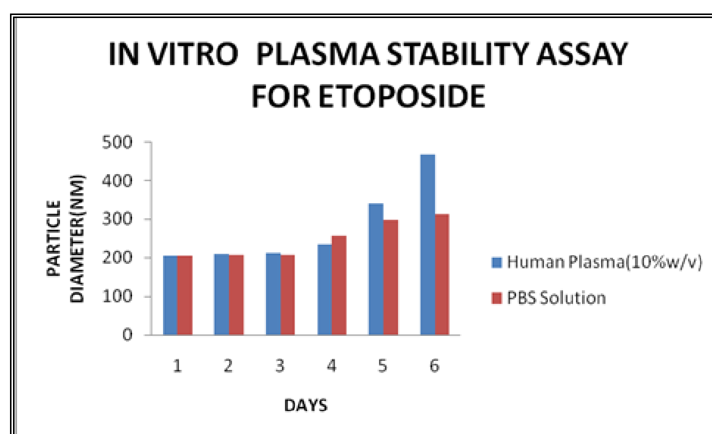

Fig 9. In vitro plasma stability assays of Etoposide nanoparticles prepared by emulsification solvent diffusion method

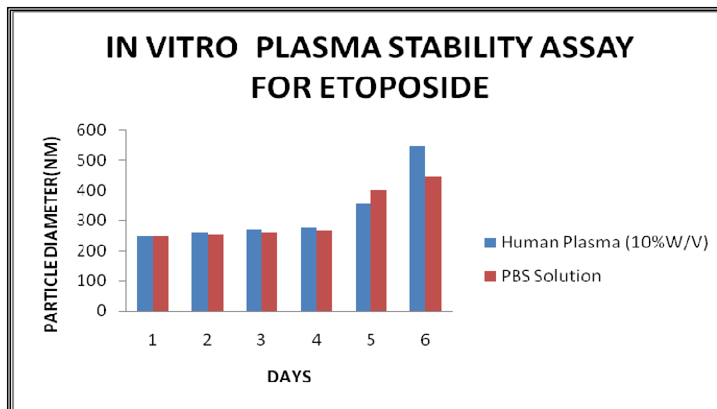

Fig 9A. In vitro plasma stability assays of etoposide nanoparticles prepared by nanoprecipitation

4.8 *Long term stability studies of Etoposide loaded nanoparticles*

The Etoposide based lyophilized nanoparticles were subjected to accelerated stability for 6 months at ICH temperature (4^0 C to 8^0 C)and (25^0C ±2) .After 6 months period , the freeze dried nanocarrier was redispersed again with PBS and SWFI and evaluated for various parameters. Lyophilized nanoparticles were showed better stability in terms of mean particle size diameter, polydispersity index and redispersibility.

4.9 *In vitro antitumor effect on LNCaP cell lines*

The in vitro anticancer cytotoxic activity of Etoposide and Etoposide -loaded PLGA nanoparticle and plain nanoparticles on LNCaP cells, expressed as % reduction of cell viability. The 50% growth inhibitory concentration(IC_{50}) values for free Etoposide and etoposide loaded nanoparticles were estimated from the available cytotoxicity Data in Table :6.The nanoparticles loaded with Etoposide exhibited more *invitro* anticancer activity comparable to that of free etoposide. The activity of both free and nanoparticle-entrapped Etoposide increased with increasing drug concentration and incubation Time. Whereas plain nanoparticles showed no change in viability of cancer cells.

Table 6 IC_{50} values (mMolar/ml) for free etoposide and etoposide loaded PLGA nanoparticles

Formulation	24 HRS	48HRS	72 HRS
Free etoposide formulation	89.98	79.94	74.26
Etoposide based PLGA nanoparticles	67.06	41.08	33.10

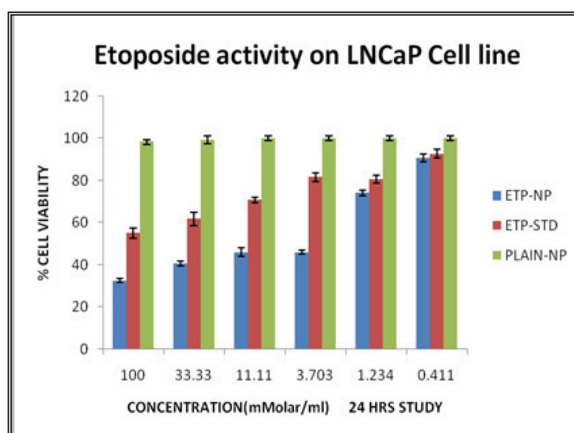

Fig 10. Cytotoxicity study on LNCaP cell line in 24 hrs.

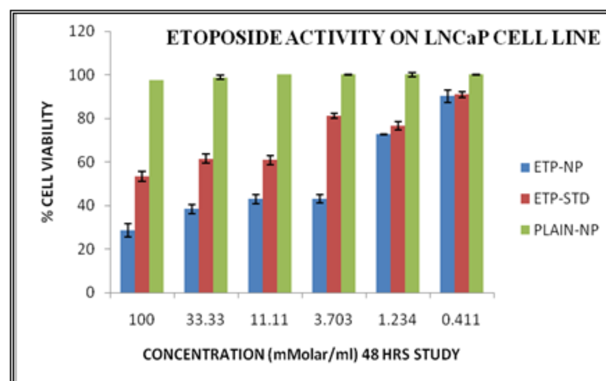

Fig 11. Cytotoxicity study on LNCaP cell line in 48 hrs.

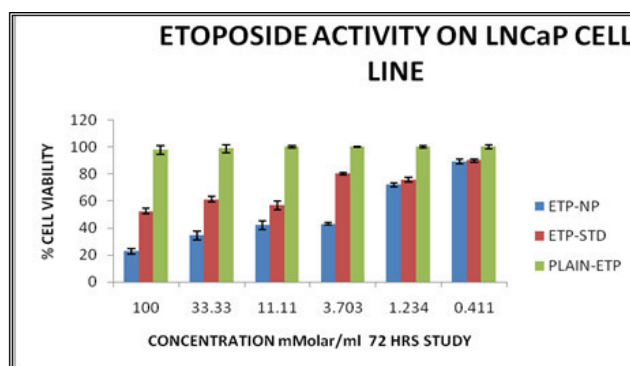

Fig 12. Cytotoxicity study on LNCaP cell line in 72 hrs.

5. CONCLUSION

The present study demonstrates that both modified SESD and nanoprecipitation methods are feasible, viable and advantageous methods to get nanoparticles of desired property. The size and polydispersity index of nanoparticles can be controlled by various parameters like drug: polymer ratio, binary mixture of organic solvents and organic to aqueousphaseratios. The lyophilization technique provides us an excellent source of getting a stable nanoparticle formulation for long a period of time. *In vitro* antitumor activity indicated a nanoparticulate formulation is therapeutically more effective compared to conventional system. s Study on vero cell line shows that drug loaded nanoparticles are less toxic to normal healthy cells compared to plain conventional formulation.

6. AUTHORS CONTRIBUTION STATEMENT

Dr.Priyal worked on project where as Dr.Jayvadan actively suggested about characterization and stability studies and Dr.falgun and Dr.Shailesh involved in HPLC method development. Dr.Mahajan involved in drafting the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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