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FORMULATION AND EVALUATION OF POLAXOMER COATED RETROVIRAL NANOPARTICLES

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ABSTRACT

Nanoparticulate delivery systems were found to be attractive mean in the treatment for various chronic diseases. The nanosize facilities improved bioavailability of poorly soluble drugs targeting to cancer to cancer cell to HIV infected cells. The main objective of this work was to investigate the ability of macromolecular protein. Bovine serum albumin (BSA) for functional carrier property to develop into nanoparticles. Lamivudine (LV) is an anti-retroviral drug and effectively to treat hepatitis B viral infections and also in the treatment of acquired immunodeficiency syndrome (AIDS) caused by Human immunodeficiency virus (HIV). In this study.LV was loaded into BSA solvent desolvation technique and the effect of process variable such as drug carrier ration, volume of cross linking agent, gluteraldehyde (GA) on the particle size, zeta- potential and in vitro release characteristics were studied. The particle size distribution of the formulation was in the range from 469-163nm. The entrapment efficiency and loading capacity of total of nine formulations (F1-F6) were in the range of 28-52% and 14-26%. The zeta potential of the formulation F5- was -27.8 than F6-21.4. the decreased surface charge could be attributed to high GA volumes whereas the in vitro release characteristics of F4-F6 extended for 64 hrs. From the study it was concluded that the method selected was able to effectively load the drug. The in vitro release characteristics found to exhibit better release retardation and prolonging the drug therapy and minimization of multiple doses.

KEYWORDS: Lamivudine, Bovine Serum Albumin, Nanoparticles.

INTRODUCTION

The global impact of viral infections, the development of resistance to current drugs and the emergence of new viruses all translate into the incessant scientific challenge of drug discovery and formulation development.[1] Over the past few decades there has been considerable interest in developing biodegradable nanoparticles (NPs) as effective drug delivery devices. Various polymers have been used in drug delivery as they can effectively deliver the drug to target site and thus increase the therapeutic benefit while minimizing side effects.[2] Nanoparticles generally vary in the size from 10-100 nm. The drug is dissolved, entrapped, encapsulated or attached to a NP matrix and depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are vesicular structures in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed.[4]

They can be defined as system which contain active ingredient dissolved, encapsulated or adsorbed in matrix material which are used as target delivery system.

To see the effect of drug in target tissue, to increase stability against degradation through enzymes and for solubilization at intra-vascular route nanoparticles have been used. For the safe administration of nanoparticle through intravenous route they are formulated in the form injection which consist spherical amorphous particle. Nanoparticles are sub nanosized colloidal structures composed of synthetic or semisynthetic polymers.[5]

Dispersion of preformed polymers is a common technique used to prepare biodegradable nanoparticles from poly (lactic acid) (PLA); poly (D,L-glycolide), PLG; poly (D, L-lactide-co-glycolide) (PLGA) and poly (cyanoacrylate)(PCA). This technique can be used in various ways as described below. Solvent evaporation method In this method, the polymer is dissolved in an organic solvent such as dichloromethane, chloroform or ethyl acetate which is also used as the solvent for dissolving the hydrophobic drug.[6]

Much research has been focused on the preparation of nanoparticles using biodegradable hydrophilic polymers such as chitosan, gelatin and sodium alginate developed a method for preparing hydrophilic chitosan

nanoparticles by ionic gelation. The method involves a mixture of two aqueous phases, of which one is the polymer chitosan, a di-block co-polymer ethylene oxide or propylene oxide (PEO-PPO) and the other is a polyanion sodium tripolyphosphate. In this method, positively charged amino group of chitosan interacts with negative charged tripolyphosphate to form coacervates with a size in the range of nanometer. Coacervates are formed as a result of electrostatic interaction between two aqueous phases, whereas, ionic gelation involves the material undergoing transition from liquid to gel due to ionic interaction conditions at room temperature.[7]

Emulsification-solvent evaporation involves two steps: The first step requires emulsification of the polymer solution into an aqueous phase, during the second step polymer solvent is evaporated, inducing polymer precipitation as nanospheres. The nano particles are collected by ultracentrifugation and washed with distilled water to remove stabilizer residue or any free drug and lyophilized for storage. Modification of this method is known as high pressure emulsification and solvent evaporation method. This method involves preparation of a emulsion which is then subjected to homogenization under high pressure followed by overall stirring to remove organic solvent. The size can be controlled by adjusting the stirring rate, type and amount of dispersing agent, viscosity of organic and aqueous phases and temperature. However this method can be applied to liposoluble drugs and limitation are imposed by the scale up issue. Polymers used in this method are PLA, PLGA, EC, cellulose acetate phthalate, poly (caprolactone)\ (pcl) poly (β- hydroxybutyrate).[8]

MATERIALS AND METHODS

Materials

Lamivudine was received as a gift sample from Aurobindo pharma, Hyderabad. Bovine serum Albumin was obtained from Merck, Mumbai, ethanol from Yangshuyangyuam chemical, sodium hydroxide was purchased from Qualigens fine chemicals, hydrocholic acid and gluteraldehyde was purchased from Loba chemi Ltd. Mumbai. Polaxomer was received as gift sample from Hetero drugs ltd., Hyderabad.

Methods

Pre-formulation studies

FT IR

In this study individual samples of pure drug Lamivudine, the carrier BSA, physical mixture i.e. Lamivudine and BSA in 1:1 ratio and the prepared formulation i.e. Lamivudine loaded BSA NPs were analyzed by IR spectroscopy using FT-IR module and the obtained spectra were recorded and depicted in the respectively. As there was no change in the frequency in the frequency range of the respective functional groups of the drug and carrier and no shifts of the peak, it indicates that there was no interaction between the drug and the carrier and it is suitable for nanoparticles formulation.

Analytical methodology[9]

Determination of bovine serum absorption maxima (λ_{max})

10 µg/ml of BSA solution was prepared by taking 1ml of 100 µg/ml of BSA Solution and to this 8ml of Bradford's reagent was added and finally made to 10ml with distilled water this prepared solution was scanned between 400-800nm using UV-visible spectrophotometer. an absorption maximum (λ_{max}) of 590nm was observed.

Construction of calibration curve of BSA

A stock solution of 100 µg/ml of BSA was prepared in distilled water. From this stock solution a series of dilutions were prepared of different concentrations i.e. 2, 4, 6, 8, 10 µg/ml (by pippeting out 0.2, 0.4, 0.6, 0.8, 1 ml of 100 µg/ml of BSA solution respectively). in all the above dilutions a constant volume of reagent, 8 ml was added and finally the solutions were made to 10ml with distilled water the absorbance of the solutions was measured at 590nm against blank using blank using UV-spectrophotometer.

Construction of calibration curve of lamivudine

The analytical method development employed in the present research work was carried out by UV spectroscopy. Analytical method for lamivudine was developed by UV spectrophotometry and shown absorption maxima (λ_{max}) at 271nm. Calibration curve of LV was constructed and the values obtained were used in the drug release data analysis.

Standard solution (0.1mg/ ml) LV solution was prepared using phosphate pH.7.4.This stock solution was suitably diluted to get series of concentrations (6, 8, 10, 12, 14 µg/ml) of LV. The absorption of these solutions was measured in UV Spectrophotometer at 271nm using phosphate buffer pH 7.4 as blank. The concentration was plotted against absorbance and the calibration graph was constructed.

Preparation of lamivudine loaded BSA nanoparticles

Bovine serum albumin nanoparticles were prepared by solvent desolvation process followed by cross linking with gluteraldehyde. lamivudine was incubated with required amount of protein solution (10%w/v) for 2 hrs at room temperature. The pH of the system was adjusted above the isoelectric point of BSA (4.7) and it was incubated for 30 min then ethanol was added to the solution until the appearance of bluish white colour under magnetic stirring the rate of ethanol addition was carefully controlled at 1ml/min. the desolvates so formed were hardened with 25% glutaraldehyde for overnight 5hrs to allow cross linking of protein Amount of GA addition was carried out at three different levels (low, medium, high) The residual organic solvents were then removed under reduced pressure by rotary vacuum evaporation at ambient temperature and the resulting nanoparticles were purified for further study Similarly

Albumin nanoparticles were prepared by the same procedure with 3% and 5%w/v protein solution.

Table no. 1: Preparative variables of LV-BSA-NP's.

Parameters	Formulation code					
	F1	F2	F3	F4	F5	F6
Drug in mg	100	100	100	100	100	100
BSA concentration mg/ ml	50	50	50	100	100	100
Gluteraldehyde in µl	115	115	115	115	115	115
Poloxamer concentration in mg/ml	-	1	5	15	25	50
Ethyl alcohol	q.s	q.s	q.s	q.s	q.s	q.s
HPLC grade water	10ml	10ml	10ml	10ml	10ml	10ml

Evaluation of lamivudine loaded BSA nanoparticles

Determination of drug entrapment efficiency (EE)[10]

The entrapment efficiency of LV loaded in BSA-NP'S were determined by separating the free drug from NP's by cooling centrifugation at 4°C for 30 min at 15000 RPM. During this process the free drug gets into supernatant while the NP'S will be obtained as pellet at the bottom. The amount of free drug present in the clear supernatant was estimated by UV-VIS spectrophotometer at 271nm using phosphate buffer Ph7.4 as blank.

The % Entrapment efficiency is given by:

%EE = (W-w)/W×100

W = Total amount of drug added during the solvent desolvation process.

W = Amount of free drug in the supernatant.

(W-w) = Amount of drug entrapped in the pellet.

Determination of the loading capacity (LC)[10]

The loading capacity of NP'S of different formulations were determined by ultracentrifugation of the formulation at 15000RPM at 4°C the amount of drug present in the supernatant was estimated by UV spectrophotometer at 271 nm phosphate buffer pH 7.4 as blank. the loading capacity of NP'S was calculated by using the followed formula:

LC =(W-w)/C×100

C = NP Weight

Determination of NP's size and zeta potential[11]

The size and the zeta potential of nanoparticles were determined by zetasizer(PALS-phase analysis light scattering) the samples were diluted with distilled water and measured at 25°C. Results were automatically calculated by the analyzer using the following equation.

$$m = \frac{ez}{h}$$

Where z is zeta potential, m is mobility, e is the dielectric constant and h is the viscosity of the electrolyte solution.

Determination of surface morphology of NP

The surface morphology, size and shape of the nanoparticles were examined by scanning electron microscope.

***In vitro* study of nanoparticles[12]**

In-vitro release kinetics study across diffusion membrane (cut of 2-12KDa) precluding Albumin was performed in a specially designed diffusion chamber consisting of two compartments separated by a diffusion membrane. 10 mg of the drug loaded nanoparticles suspended in phosphate buffer pH 7.4 was placed in the donor compartment and the other compartments was filled with 30 ml of phosphate buffer pH7.4 to determine the amount if LV diffused through the diffusion membrane. At regular prefixed time intervals 1ml of the sample was withdrawn and replaced with fresh buffer solution in the receptor compartment. The amount of drug present in the sample was estimated by UV-spectrophotometer at 271nm.

Drug release kinetic analysis by using different release models[13]

Drug release kinetics of different formulations was studied by various mathematical models.

Zero order equation

Drug dissolution from pharmaceutical dosage forms that do not disaggregate and release the drug slowly (assuming that area does not change and no equilibrium conditions are obtained) can be represented by the following equation:

$$C_t = C_0 + K_0$$

Where,

C_t = Amount drug released at time t.

C_0 = Initial amount of drug.

K_0 = Zero order release rate constant.

First order equation

This model has been also used to describe absorption and /elimination of some drugs it is difficult to conceptualize this mechanism in a theoretical basis.

$$InC_t = InC_0 + k_1t$$

Where,

C_t = Amount of drug released at time t

C_0 = Initial amount of drugs.

k_1 = First order release rate constant.

Higuchi equation[13]

Higuchi developed several theoretical models to study the release of water soluble and low soluble drugs

incorporated in semi solid and /solid matrixes. Mathematical expressions were obtained for drug particles in a uniform matrix behaving as the diffusion media. In a general way it is possible to assume the Higuchi model to the following expression. This relation can be used to describe the drug dissolution from different types of modified released pharmaceutical dosage forms, as in the case of some transdermal systems and matrix tablets with water soluble drugs.

$$Q_H = KH\ t^{1/2}$$

Q_H = Amount of drug released at time t.
K_H = Higuchi diffusion rate constant.

Korsmeyer –peppas equation[13]

Korsmeyer et.al developed a simple, semi-empirical model, exponentially the drug release to the elapsed time (t).

Peppas used n value in order to characterize, different release mechanisms, concluding for a slab, of n = 0.5 for Fickian diffusion and higher values of n, between 0.5 and 1.0 or n = 1.0, for mass transfer following a non-Fickian model

$$M_t \setminus M_\infty = k\ t^n$$

Where,
M_t = Amount of drug released at time t.
M_∞ = Amount of drug released after infinite time.
K = kinetic constant incorporating characteristics.
N = Diffusion exponent indicative of the drug release mechanism.

RESULTS AND DISCUSSION

Drug carrier interaction studies

The FI-IR spectra of lamivudine, bovine serum albumin, and their physical mixture and prepared formulations were taken. The FT-IR spectra of lamivudine and bovine serum albumin show intense peaks at their characteristic functional groups mentioned. The same intense peaks were observed even in the physical mixture of lamivudine and BSA and the prepared formulation indicating that there are no interactions between them and they are suitable to each other for the preparation of formulation.

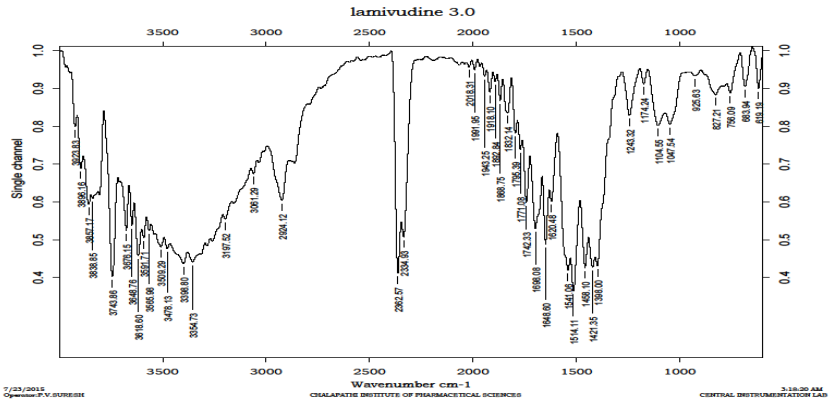


Fig no 1: FT-IR spectrum of lamivudine.

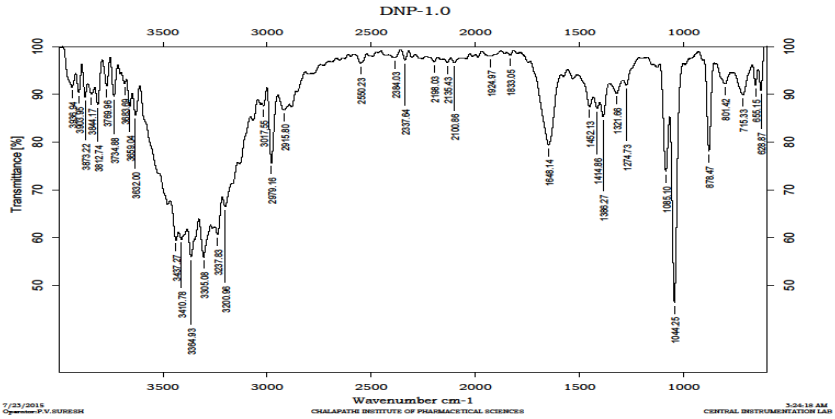


Fig no 2: IR-Spectrum of formulation F4.

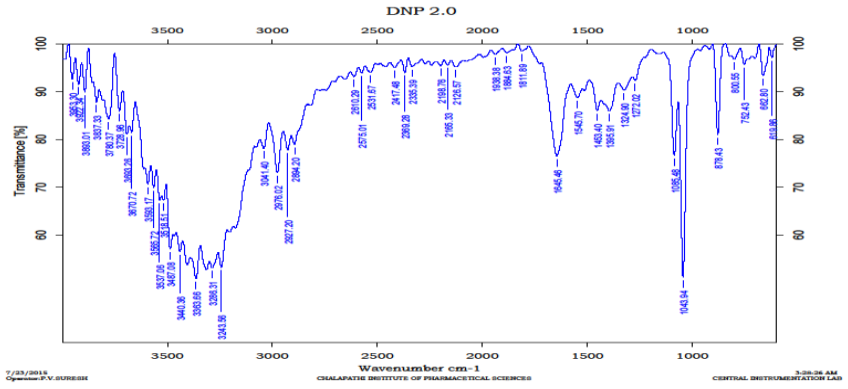


Fig no 3: IR Spectrum of formulation F5.

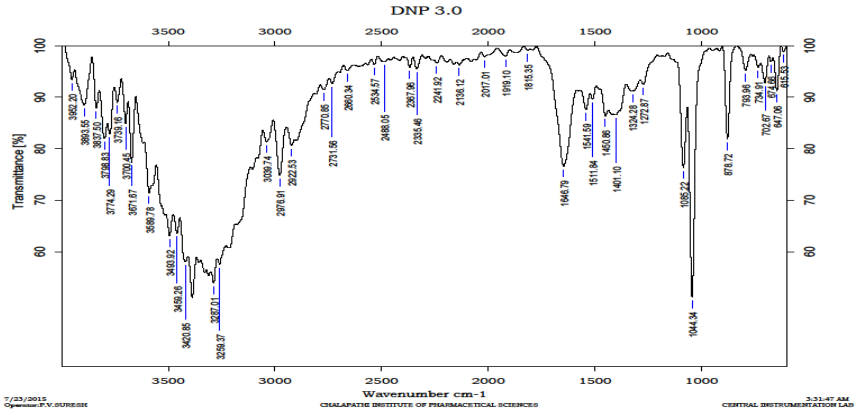


Fig no 4. IR-Spectrum of formulation F6.

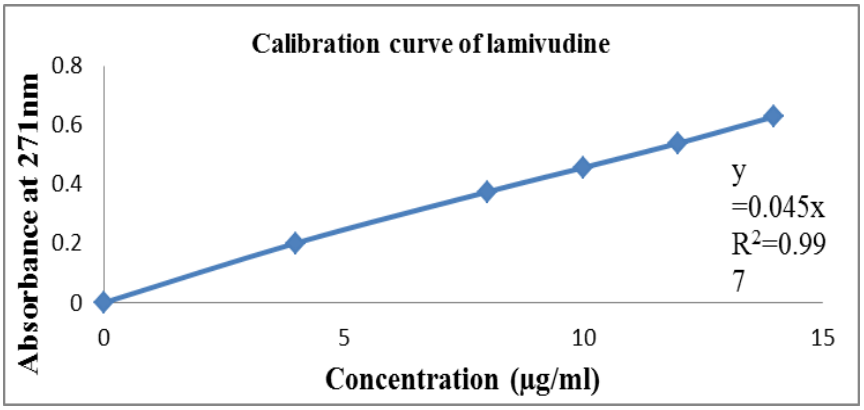


Table no 2: Calibration of lamivudine.

S. no.	Concentration (µg/ml)	Absorbance at 271nm				Relative standard deviation
		Trial 1	Trail II	Trail III	Average±S.D	
1	0	0	0	0	0	0
2	4	0.286	0.302	0.291	0.291±0.018	0.018
3	8	0.379	0.376	0.367	0.374±0.026	0.026
4	10	0.448	0.451	0.469	0.456±0.011	0.011
5	12	0.532	0.540	0.545	0.539±0.046	0.046
6	14	0.631	0.625	0.625	0.629±0.033	0.033

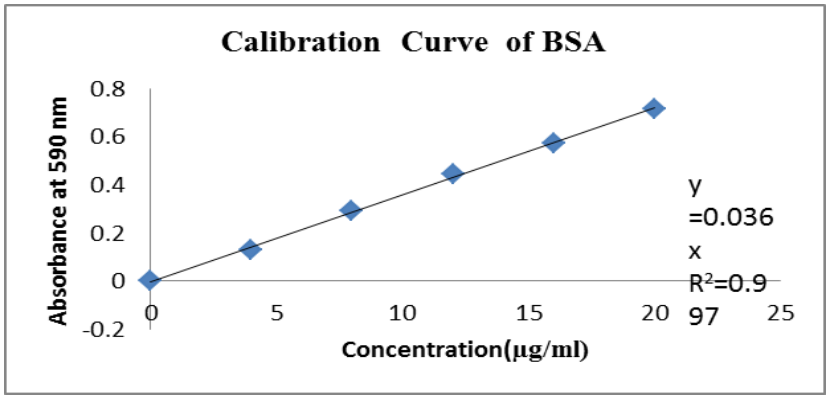


Fig no 6: Calibration curve of BSA.

Percentage yield, Entrapment efficiency and loading capacity of nanoparticles

The results of nanoparticles yield, entrapment efficiency and loading capacity of prepared nanoparticles were tabulated. As the volume of cross linking agent was increased more amount of carrier got cross linked thus stabilizing the nanoparticles, preparation entrapping the drug with it and finally the nanoparticles yield, entrapment efficiency and loading capacity was increased. in the similar way as the carrier ratio was increased more amount of carrier was available for the drug to be encapsulated and depending on the volume of cross linking agent in the particular formulations yield and entrapment efficiency was increased.

Table no 3: Entrapment Efficiency, %Yield and Drug loading of different lamivudine loaded BSA NP formulations.

S. no	Formulation code	%Yield $\overline{X}$ ±S.D	%EE $\overline{X}$ ±S.D	%LC $\overline{X}$ ±S.D
1	F1	52.2±0.013	28.5 ±.041	24.9±0.031
2	F2	57.6±0.012	31.4±0.040	26.2±0.045
3	F3	61.3±0.023	35.4±0.034	26.9±0.032
4	F4	64.4±0.012	39.6±0.013	16.2±0.012
5	F5	67.1±0.015	45.4±0.014	15.9±0.142
6	F6	69.7±0.013	52.3±0.012	15.4±0.010

Surface morphology and particles size

As submicron particles are not easily removed by the liver and spleen and have increased circulation time. These particles prolong the duration of drug activity and also increase the targeting efficiencies to specific sites. So the surface morphology and particles size are important parameters governing the efficiency of the formulation. The surface morphology and particles size of the prepared formulation was analyzed by scanning electron microscope (SEM). Aggregation of particles were observed by increased in the volume of the cross linking agent. The SEM images of the formulations. The particle size distribution of the formulation F5,F6 were found to be best compared to the other formulations.

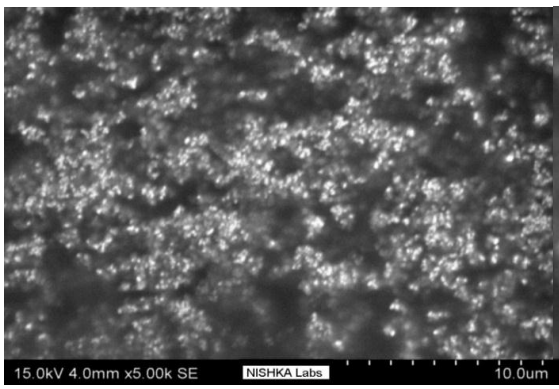


Fig no 7: SEM image of LV-BSA NP.

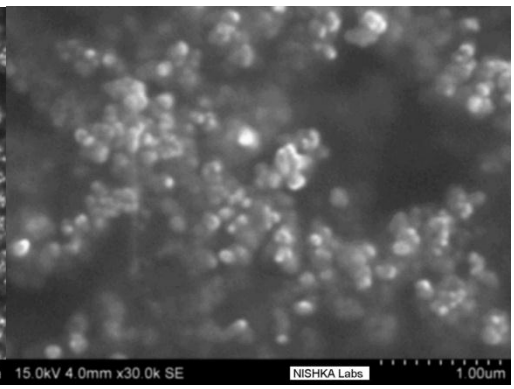


Fig no 8: SEM image of LV-BSA NP.

Zeta potential

Surface charge was the critical parameter for the stability of particulate drug delivery systems for which investigation of zeta potential is an important part of nanoparticles characterization. Zeta potential was significantly affected by volume of crosslinking agent,

amount of BSA and lamivudine in the desolvation process gluteraldehyde and lamivudine compete for the amino groups of BSA protein. After getting cross linking with Gluteraldehyde still few amino acids will remain on the BSA particle which contributes to the surface charge. This surface charge is responsible for the zeta potential (±30 mV) value will indicate the stability of colloidal systems. Increasing the volumes of GA reduced the number of amino group on the surface of BSA nanoparticles and also decreased the zeta potential of the carrier system. Zeta potential measurement of the prepared formulations indicated negative zeta potential value of F5-23.0, F6-21.4 (the zeta potential value were given in the table).

Table no 4: Zeta potential and size distribution data of nanoparticles formulations.

Formulation code	Average particle size (d.nm)	% area of peak indicating volume of particles formulation		PDI	Zeta potential (mv)
		Peak 1	Peak 2		
F5	163.1	184.4	000.0	0.100	-27.8
F6	465.4	495.4	000.0	0.112	-21.4

***In vitro*drug release**

The in vitro release study was carried out using 8 stage Franz diffusion cell apparatus, the release profiles of lamivudine from BSA nanoparticles formulation extended form 16 to 24 hrs depending on the drug to polymer ration. The drug release was faster for those particles with lower polymer concentration and as the concentration of polymer was increased, amount of drug release was also increased.

Table no 5: *Invitro* release data of lamivudine.

S.no	Time(Mins)	%Drug Released. $\overline{X}$ ±S.D
1.	0	0
2.	5	12.2±0.034
3.	10	22.2±0.041
4.	20	30.3±0.030
5.	30	42.8±0.024
6.	45	49.2±0.011
7.	60	56.4±0.027
8.	90	64.3±0.016
9.	120	71.8±0.013

Table no 6: *Invitro* release data of lamivudine loaded Bovine Serum Albumin nanoparticles.

S no	Time(hrs)	%Drug released		
		F1. $\overline{X}$ ±S.D	F2. $\overline{X}$ ±S.D	F3. $\overline{X}$ ±S.D
1	1	20.5±0.042	21.5±0.027	21.2±0.014
2	2	22.0±0.039	25.4±0.017	23.5±0.016
3	4	27.1±0.027	29.1±0.035	27.1±0.042
4	6	29.5±0.0141	31.2±0.025	32.2±0.031
5	8	33.2±0.016	35.6±0.021	39.2±0.014
6	10	37.9±0.012	40.4±0.015	42.8±0.021
7	12	41.6±0.013	43.8±0.017	48.5±0.022
8	16	45.5±0.020	49.1±0.021	53.2±0.018
9	20	49.9±0.014	54.8±0.019	58.5±0.020
10	24	56.2±0.015	59.5±0.016	63.4±0.014

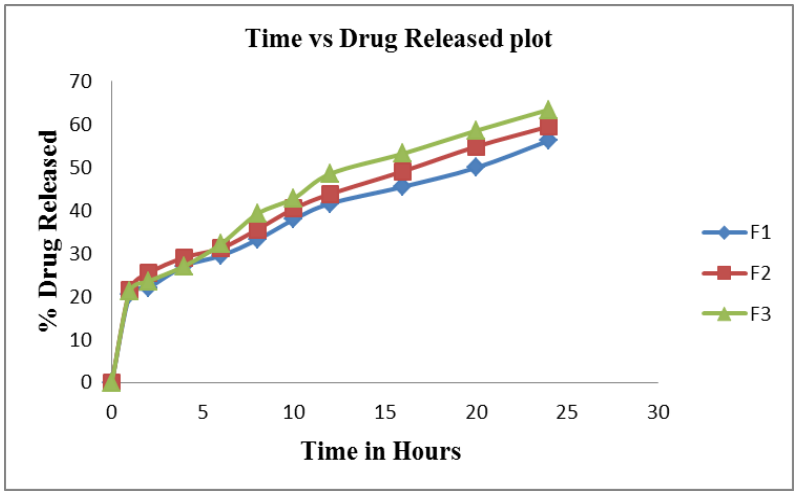
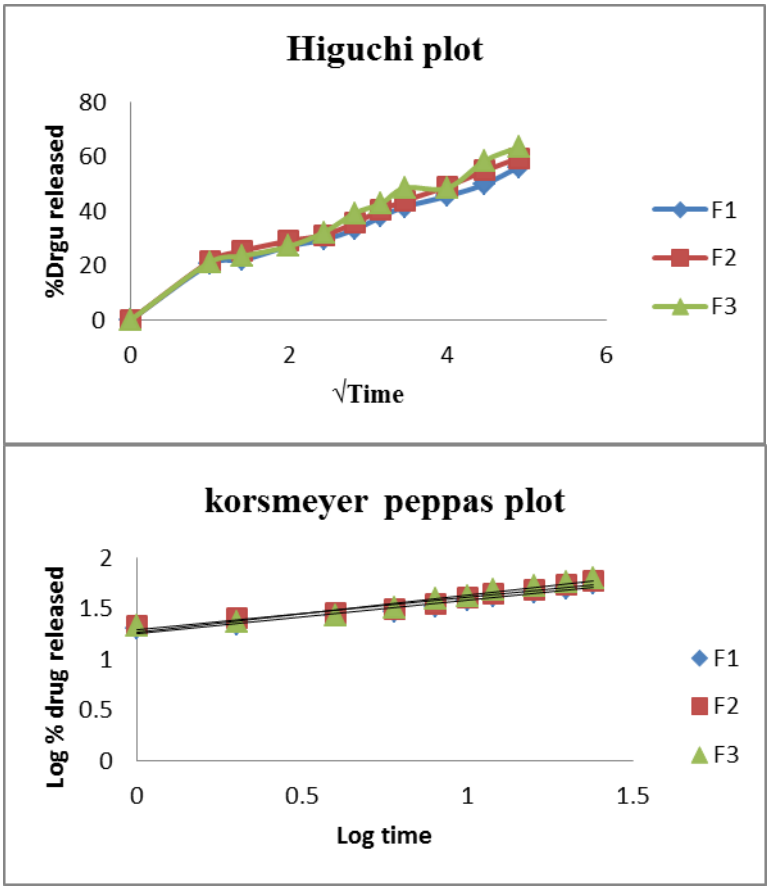


Fig no 8: *Invitro* release data of lamivudine loaded bovine serum albumin nanoparticles of F1, F2, F3.



Release kinetics

The in vitro release kinetics of the formulation from F4 to F6 were fitted to various standard release equation viz., zero order, first order, Higuchi model and korsmeyer peppas equation. The results obtained. The resulted obtained were presented in the table. The model that best fits the release data was selected based on the highest coefficient of determination value (r) of various models. The results indicated that the release of the drug from nanoparticles was following zero order as indicated by higher 'r' values in the zero order models. The release data was also analyzed using the korsmeyer peppas equation; the n values indicated that mechanisms of drug release from the BSA nanoparticles were non-Fickian.

Table no 7: Mathematical model fitting *Invitro* release data of lamivudine loaded BSA Nanoparticles.

S. no	Formulation code	Zero order		First order		Higuchi model		Korsmeyerpeppas model	
		K_0	r	K_1	r	K_H	r	N	R
1	F1	1.422	0.975	0.226	0.935	10.65	0.962	0.684	0.923
2	F2	1.624	0.997	0.044	0.954	12.23	0.964	0.721	0.928
3	F3	1.666	0.968	0.052	0.959	12.74	0.966	0.705	0.963
4	F4	1.055	0.955	0.023	0.944	9.64	0.971	0.735	0.922
5	F5	1.216	0.986	0.036	0.941	11.45	0.945	0.692	0.917
6	F6	1.171	0.991	0.042	0.854	10.55	0.984	0.691	0.933

CONCLUSION

Nanoparticles drug delivery system of lamivudine was developed using bovine serum albumin as a carrier, gluteraldeyde as a cross linking agent and poloxamer as the stabilizer. Solvent desolvation technique was used to develop the nanoparticles. The aim of research work was to design and develop lamivudine loaded bovine serum albumin nanoparticles using solvent desolvation technique. This system was selected as effect can be increased with reduced side effects. In vitro release studies revealed that the release of drug from the Nanoparticulate formulations was retarded as the concentration of BSA was increased. The release from the nanoparticles varied from the 16 to 24. Hrs From the study it was concluded that the method selected was able to effectively load the drug. The in vitro release characteristics found to exhibit better release retardation and prolonging the drug therapy and minimization of multiple doses.

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