Formulation and *In-vitro* Evaluation of Zidovudine-Lamivudine Nanoparticles

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ABSTRACT

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Human Immunodeficiency Virus infection and Acquired Immune Deficiency Syndrome commonly referred to as HIV/AIDS which have emerged as being the most serious and challenging public health problems in the world. Zidovudine-Lamivudine nanoparticles were prepared by emulsion polymerization in a continuous aqueous phase with different polymers poly(lactic-*co*-glycolic acid) PLGA (50:50), Poly(lactic acid) PLA, Poly (methyl methacrylate) PMMA, Methylmethacrylate-Sulfopropylmethacrylate (MMA-SPM).

The particle size and the surface morphology results revealed that PLGA nanoparticles (NPs) were smooth spherical with a size ranging from 58-224 nm. The drug content in lyophilized PLGA NPs was found to be 51.67% (Zidovudine) 58.33% (Lamivudine) and no drug loss was found after storage for 1 month at room temperature. *In vitro* release studies revealed that the rate of drug release from PLGA NPs was 95.38% in 10 h with zidovudine, and 97.37% in 10 h with lamivudine which was slower when compared to MMA-SPM, PLA and PMMA NPs. The rate of drug release from MMA-SPM NPs was 64.33% in 10 h with zidovudine and 95.43% in 10 h with lamivudine. Acute toxicity studies in mice revealed that the dose administered does not induce mortality in test animal.

Keywords: HIV, Nanoparticles, Emulsion Polymerization method, *In vitro* release, Stability study.

INTRODUCTION

The basic goal of novel drug delivery systems is to achieve a steady state of drug in blood or tissue level which is therapeutically effective and non toxic for an extended period of time. Carrier-mediated drug delivery has emerged as a powerful methodology for the treatment of various pathological disorders[1]. The therapeutic index of traditional and novel drugs is enhanced via the increase of specificity due to targeting of drugs to a particular tissue, cell or intracellular compartment, the control over release kinetics, the protection of the active agent or a combination of the above[2]. Targeting is the ability to direct the drug-loaded system to the site of interest[3]. Passive targeting is achieved by incorporating the therapeutic agent into a macromolecule or nanoparticle that passively reaches the target organ[4,5,6].

Nanotechnology is a rapidly emerging scientific field that is defined as the production of devices with atomic or molecular scale precision, but it also includes all devices with size less than 100 nm. One of the important areas of nanotechnology is nano medicine which refers to highly specific medical intervention at the molecular scale for diagnosis, prevention, and treatment of diseases.

Nanoparticles (NPs) were proposed as drug carriers over 30 years ago and have received growing attention since, mainly due to their stability, enhanced loading capabilities and control over physicochemical properties. In addition to systemic administration, localized drug release may be achieved using macroscopic drug depots close to the target site[7].

Monotherapy leads to development of *in vitro* and *in vivo* resistance with nucleoside reverse transcriptase inhibitor (NRTIs) zidovudine. Antiretroviral agents in combination has been shown to be superior to monotherapy for one or more of the following endpoints: delaying death, delaying development of AIDS, increasing CD4+ cell counts, and decreasing plasma HIV-1 RNA. Zidovudine has been shown to act additively or synergistically with other anti-HIV agents (indinavir, zalcitabine, didanosine, delaviridine, lamivudine, saquinavir, ritonavir, nevirapine, interferon-alpha) by inhibiting the replication of HIV in cell culture. The aim of the investigation is to formulate and characterize zidovudine and lamivudine nanoparticles for better product efficacy.

MATERIALS AND METHODS

Zidovudine and Lamivudine were the gift samples from Aurobindo Pharmaceuticals, Hyderabad. Poly (lactic – co – glycolic acid) (PLGA) 50:50 was the gift sample from Sigma-aldrich, USA. Methyl methacrylate, Poly methyl methacrylate and Poly lactide (PLA) were purchased from Loba chem. Pvt. Ltd., Mumbai. Dichloro methane, Ethanol and Pluronic F 68 were purchased from Loba chem. Pvt. Ltd., Mumbai.

Preparation of nanoparticles by emulsion polymerization method

Fixed amounts of drugs Zidovudine (30 mg) – Lamivudine (15 mg), with various polymers poly(lactic-*co*-glycolic acid)

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PLGA (50:50) (or) Poly(lactic acid) PLA (125 mg), was dissolved in dichloromethane. Poly (methyl methacrylate) PMMA (125 mg) (or) Methylmethacrylate-Sulfopropylmethacrylate (MMA-SPM) (125 mg) was dissolved in ethanol. To this mixture oil (1 ml) was added and sonicated at 50 Hz in a bath-sonicator for 10 m. The organic phase was added slowly through a needle into a well stirred aqueous phase pH 7.4 (25 ml) containing surfactant (0.5% Pluronic F- 68), and then magnetically stirred at 1200 rpm at room temperature for 4 h to evaporate the organic solvent. The resultant colloidal suspension is concentrated by evaporation under vacuum. The dichloromethane was evaporated at 45°C under reduced pressure using a rotary flash evaporator (Super fit, India). After evaporation the beaker was kept under vacuum overnight in a nitrogen atmosphere to remove residual solvent[8].

Preparation of MMA-SPM Co-Polymer

10 mg of sulfopropyl methacrylate (SPM) was mixed with 0.99ml of methyl methacrylate (MMA) in 20ml of ultrapure deionized water. 6 mg of Ammonium persulfate was added as an initiator, into the above solution under constant magnetic stirring at 78°C in 400 rpm over a period of 24 h to form methyl methacrylate sulfopropyl methacrylate[9].

Particle Size Determination

The particle size of sonicated particles was determined by Blue wave micro track particle size analyzer, (Blue wave model S 4521). About 5 ml of the colloidal suspension were poured in a chamber with an average of three run time 30 sec for each sample. By means of laser scattering mechanism particles size was evaluated.

Scanning Electron Microscopy Analysis

The morphology of Zidovudine-Lamivudine lyophilized PLGA NPs was examined by scanning electron microscopy (SEM) JEOL JSM-6360. The powder sample for SEM analysis was coated with a thin layer of platinum using the physical vapor deposition (PVD) process at 30 mA current from the distance of 50 mm for 180 s.

Determination of drug content

Zidovudine-Lamivudine lyophilized PLGA NPs is suspended in Acetonitrile:0.05M Phosphate buffer pH 7 (15:85). The amount of drug present in formulation was estimated using HPLC at 285 nm. The drug content was estimated by correlating the peak area obtained from the lyophilized product with the standard plot peak area containing pure drugs.

DSC study

The thermal behavior of the polymer, drug, and formulated nanoparticles were analyzed using a DSC (Shimadzu DSC-

60) instrument. About 5 mg of polymer, drug (Zidovudine & Lamivudine), nanoparticle formulation containing polymer drug mixture was weighed, crimped into an aluminium pan and analyzed at a scanning temperature range from 50 to 600°C at the heating rate of 10°C/m. Baseline optimization was performed before each run. Indium was used as the standard reference material to calibrate the temperature and energy scale of the apparatus[10, 11].

In vitro release studies

In vitro release was carried out using Himedia dialysis membrane with the molecular weight cut-off ranges from 12000 – 14000 Daltons which has the capacity of holding 1.61 ml/cm. Dialysis bag which acts as a donor compartment was soaked in warm water for 10 m and Himedia closure clips were used to close dialysis bag on both the sides to prevent the leakage of formulation during the release study.

Nanoparticle suspension (1 ml) enclosed in dialysis bags were placed in 50 ml phosphate buffer pH 7.4 at 37°C under mild agitation in a beaker. At pre-determined time intervals, entire buffer medium was removed and replaced with same amount of fresh buffer. Collected samples were analysed by HPLC at 285 nm.

Determination of drug release kinetics

The mechanism of release from two best formulations PLGA NPs and MMA-SPM NPs were determined using the following mathematical models: zero-order kinetics, first-order kinetic, Higuchi kinetics, and the Korsmeyer-Peppas. The regression coefficient and slope values were determined.

Stability studies

Lyophilized product was kept at room temperature $28 \pm 2^{\circ}$C for 1 month and the drug content in all the formulations were examined. During the study period relative humidity of atmosphere was noted each day and it was found to be $65 \pm 5\%$.

The change in drug content of the nanoparticles stored for 1 month at room temperature were evaluated periodically.

Acute toxicity studies

Acute toxicity studies was carried out as per as OECD Guideline for Testing of Chemicals 423. This study protocol was approved by the institutional ethical committee for the use of animals. .Toxicity studies were performed with male mice weighing 30 g. Starting dose selected was 2000 mg/kg body weight. After administration, animals were observed individually after dosing at least once during the first 30 m, periodically during the first 24 h, with special attention given during the first 4 h and daily thereafter, for a total of 14 days.

RESULTS AND DISCUSSION

Zidovudine-Lamivudine nanoparticles were prepared by emulsion polymerization in a continuous aqueous phase method with different polymers (Table1).

Particles size of PLGA NPs and MMA-SPM NPs were found using Blue wave microtrac particle size analyzer after probe sonication for 5 m and the particle size was found in the range of 58-224 nm and 91-823 nm respectively.

Scanning electron microscopy reveals that prepared PLGA nanoparticles had a homogeneous solid matrix structure. (Fig 1) The picture shows that the particles are spherical and nearly monodispersed.

The amount of drug present in formulation was estimated using HPLC at 285 nm. The drug content in different formulations was shown in (Table 2).

Drug Content from the lyophilized formulation containing PLGA was found to be higher when compared with PLA, PMMA. The amount of drug present in formulation containing MMA-SPM (co-polymer) was also found to be high when compared with PLA, PMMA (Table 2).

DSC studies were performed to assess interactions between various substituted polymers and drugs and to confirm the presence of drug in the NPs either in its crystalline or amorphous form. Melting endotherm of pure Lamivudine,

Fig.1: SEM Image of PLGA Nanoparticles
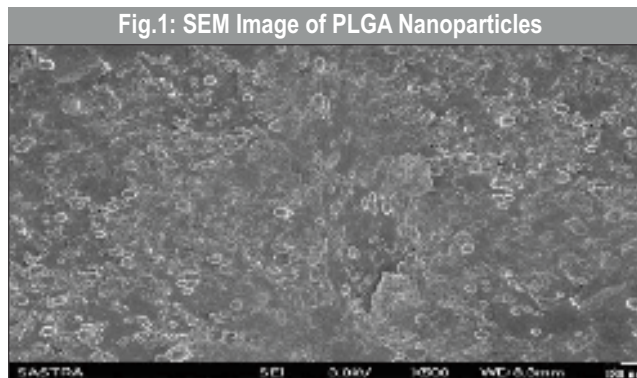
Zidovudine, and PLGA showed the presence of an endothermic peak at 124°C, 172°C and 45.12°C respectively (Fig. 2). The DSC profiles of drug polymer mixture show that after PLGA was conjugated, the drug mixture, showed a broad small hump (peak) at 122°C (Zidovudine), 184°C (Lamivudine) also the endothermic peak was shifted, indicating an altered polymer nanostructure (Fig 3). PLGA polymer is amorphous in nature. The presence of drug may either promote an amorphous or a crystalline state of the polymer. Polymer does not show any crystallization exotherm indicating it may be amorphous. The absence of a drug crystallization peak indicates that drug may be present in an amorphous form or molecularly dispersed state in the polymer matrix. The reduction of height and sharpness of the

Table 1: Formulation of PLGA NPs, PLA NPs, MMA-SPM NPs and PMMA NPs

Sl. No.	PLGA NPs		PLA NPs		MMA-SPM NPs		PMMA NPs	
	Ingredients	For 25 ml	Ingredients	For 25 ml	Ingredients	For 25 ml	Ingredients	For 25 ml
1	Zidovudine	30mg	Zidovudine	30mg	Zidovudine	30 mg	Zidovudine	30 mg
2	Lamivudine	15mg	Lamivudine	15mg	Lamivudine	15 mg	Lamivudine	15 mg
3	PLGA	125mg	PLA	125mg	MMA-SPM	125 mg	PMMA	125 mg
4	Dichloro methane	12.5ml	Dichloro methane	12.5 ml	Ethanol	12.5 ml	Ethanol	12.5 ml
5	Castor oil	1ml	Castor oil	1ml	Castor oil	1 ml	Castor oil	1 ml
6	Pluronic F- 68	125mg	Pluronic F- 68	125mg	Pluronic F- 68	125 mg	Pluronic F- 68	125 mg
7	Phosphate buffer(qs)	25 ml	Phosphate buffer(qs)	25 ml	Phosphate buffer(qs)	25 ml	Phosphate buffer(qs)	25ml

Table 2: Drug content determination in formulations

S. No.	Formulations	Peak area		Drug content/ml	
		*Zidovudine	*Lamivudine	Zidovudine (%)	Lamivudine (%)
1	PLGA	10614372	6743665	51.67 ± 0.031	58.33 ± 0.031
2	PLA	8282678	3378206	40.25 ± 0.03	30.5 ± 0.03
3	MMA-SPM	9254478	5988389	45 ± 0.027	52.17 ± 0.027
4	PMMA	8028258	4057184	39.17 ± 0.034	35 ± 0.034
		* Average of 3 determinations			

Fig. 2: DSC of polymer PLGA and DSC of Lamuvidine and Zidovudine
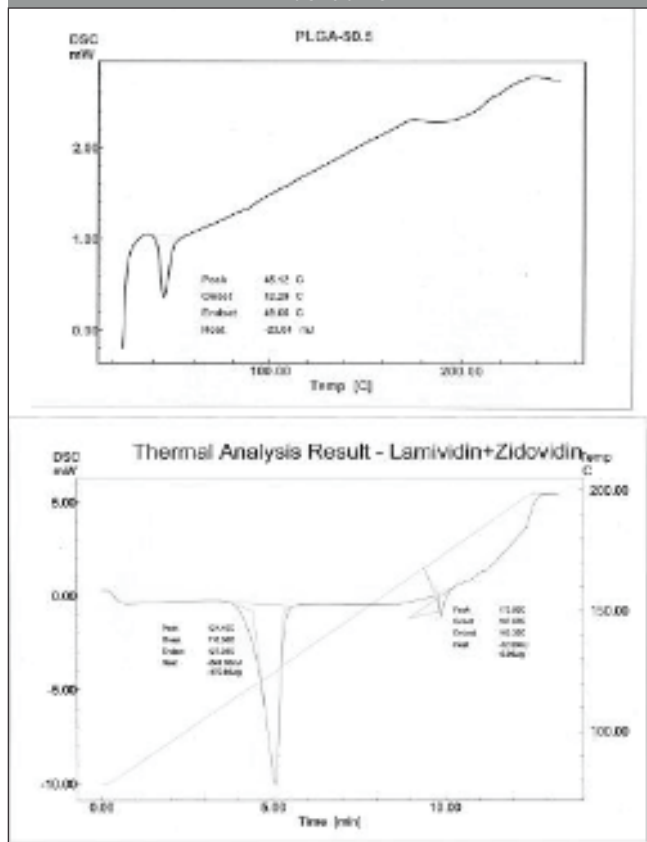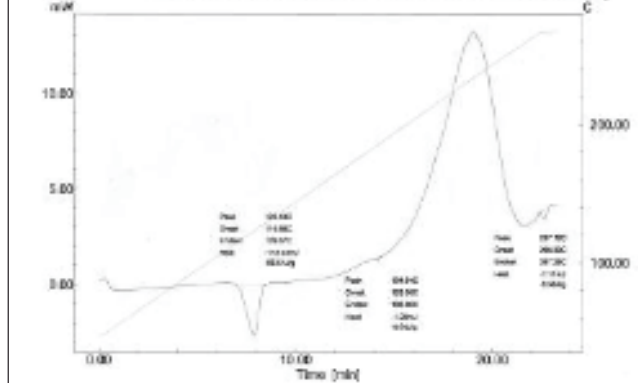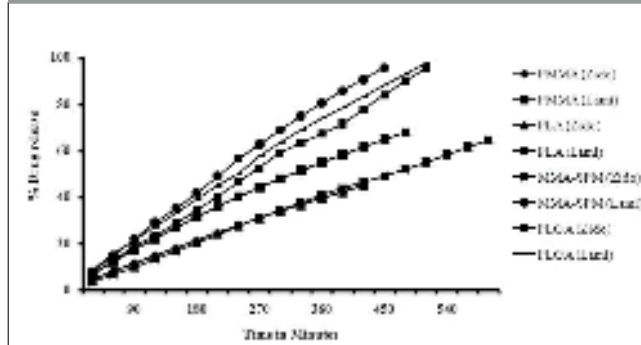

Fig. 3: DSC of formulation

Fig. 4: Comparative release profiles of combined zidovudine–lamivudine in all formulations

endothermic peak is due to the presence of polymers in the nanoparticles.

Drug release from MMA-SPM nanoparticles showed a 27.26% of drug release at 240 m with zidovudine, and prolonged upto 600 m. Comparatively lamivudine at the end of 240 m 56.28% of drug release was observed and the release extended upto 450 m (Fig 4). MMA – SPM is a non biodegradable polymer and the slow release from this formulation may be because the drug is getting released only by diffusion.

Drug release from PLGA nanoparticles shows 46.16% of release at 240 m with zidovudine, and prolonged upto 600 mins. Comparatively in lamivudine at the end of 240 m 50.03% of drug release was observed and the release extended upto 480 m (Fig 4). The drug release from PLGA, a biodegradable polymer follows diffusion and bioerosion.

The drug release from PLGA nanoparticles often had a bi-phasic pattern *in vitro*. A rapid initial release phase (6.1% in 30 m in zidovudine), (8.3% in 30 m in lamivudine) followed by a second slow release phase.

The rapid initial release is attributed to drug localized on the surface of particles. Thereafter, a diffusion-controlled slower release phase follows.

The rate of drug release from PLGA formulation was slower and the entire amount of loaded drug was released when compared with PLA, PMMA, MMA-SPM (Fig 4).

Drug release kinetics was derived for best formulation from the *in vitro* profile. Result from Higuchi plot showed that PLGA NPs follows diffusion mechanism where the regression value was above 0.9. The release profile from PLGA had a correlation coefficient of 0.9932 for lamivudine and 0.9998 for zidovudine respectively. Slope value for

Table 3: Drug content of lyophilized formulations stored at room temperature				
		*Initial drug content/ml		*After 30 days drug content/ml	
S. No.	Formulations	Zidovudine (%)	Lamivudine (%)	Zidovudine (%)	Lamivudine (%)
1	PLGA	51.67 ± 0.031	58.33 ± 0.031	51.67 ± 0.016	58.33 ± 0.016
2	MMA-SPM	45 ± 0.027	52.17 ± 0.027	44.42 ± 0.013	51 ± 0.013
* Average of 3 determinations					

formulation (PLGA) from Peppas plot was found to be more than 0.5 (Slope 0.63) for lamivudine and (1.59) for zidovudine, which confirms that formulation follows non-Fickain's diffusion mechanism.

PLGA formulation followed first order kinetics and the regression value was found to be (0.993).

From stability studies minimal loss in the drug content was found in the PLGA NPs and MMA-SPM NPs when stored at room temperature $28 \pm 2^{\circ}C$ (RH65 ± 5%). (Table 3) Freeze-dried nanoparticle formulations overcome the advantage of exposure of the product to room temperature remains stable and the product remained solid.

After 14 days of observation, the dose of 2000mg/kg administered in male mice does not induce mortality in test animal. This indicates that administered dose of PLGA NPs is non-toxic.

CONCLUSION

Zidovudine and Lamivudine loaded nanoparticles were prepared with lower dose to improve the availability of the drug at the site and to minimize the drug related side effects. From the results we conclude that combined Zidovudine–Lamivudine PLGA nanoparticles gives promising results with respect to particle size, drug content, *in vitro* release studies and toxicity studies. We hope this nanoparticle will get easily phagocytosed by monocytes and macrophages which is responsible for the distribution of virus to various tissues.

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