Original Research Article

Development and In Vitro-In Vivo Evaluation of Oral Drug Delivery System of Acyclovir Loaded PLGA nanoparticles.

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A b s t r a c t

Acyclovir (ACV) is an antiviral drug, used for treatment of herpes simplex virus infections with an oral bioavailability of only 10 to 20 % (limiting absorption in GIT to duodenum and jejunum),half-life about 3 hrs, soluble at acidic pH (pKa 2.27) and distilled water at 37ºC. Polymeric nano drug delivery systems of ACV have been designed and optimized. Poly (lactic-co-glycolic acid) (PLGA) (50:50) was used as polymer and Pluronic F68 was stabilizer. *In vitro* evaluation of prepared formulations showed drug entrapment up to 90.06 % and particle size from 395nm. Drug: Polymer ratio and concentration of stabilizer were found to influence the particle size and entrapment efficiency of ACV loaded PLGA nanoparticles (NPs). *In vitro* drug release studies indicated controlled and sustained drug release of drug for a period of 32 hours. *In vivo* evaluation was carried out for selected formulations in comparison with marketed tablet (Zovirax®) in rabbits. The AUC values for developed formulations clearly indicated two to three fold improvement in bioavailability of ACV when compared to Zovirax® tablets. These preliminary results indicate ACV NPs are superior to marketed tablet Zovirax® as particle size and release rate of entrapped drug is controlled, which results in enhanced bioavailability and probable decrease in dose and dosing frequency. Ultimately increasing adherence to drug therapy and patient comfort.

Keywords: Acyclovir, PLGA, nanoparticles, 3 factorial design, sustained release, in vitro-in vivo evaluation..

Introduction

Herpes simplex virus (HSV) has affected more than one third of the world's population, also it is believed that the prevalence of HSV in the general population in Asian countries is about 30% to 70% and is responsible for a wide array of human diseases, with effects ranging from discomfort to death.[1] In India, it is believed that 60% of sexually active adults carry Herpes viruses. Currently world-wide, approximately 86 million people are infected with genital herpes. Genital herpes is the very common infection in Human Immuno deficiency Virus (HIV) positive persons.[2] In fact, from 60% to 85% of persons with HIV have HSV-2 antibodies. People who have genital herpes are more likely to acquire HIV than those who don't have the virus. Both Genital herpes (GH) and HIV can be sexually transmitted.[3]

Currently, the only treatments available for genital herpes are conventional tablets and topical gel for application on outbreaks. The drugs that are commonly used for genital herpes are ACV, Valaclovir and Famciclovir. In spite of the addition of new antiherpes drugs (Famciclovir and Valacyclovir), ACV has been regarded as the original gold standard of therapy, a widely prescribed and reliable drug. Conventional dosage forms for the treatment of genital herpes infection has a dosage regimen which

is difficult and very rigorous. The adult dose of ACV, whose oral bio availability is only 10 to 30% and the half life is about 3 hrs, is 200 mg five times daily, for the treatment of primary genital herpes infections.[4] Treatment and control of Genital Herpes infections can also be extremely beneficial in reducing the incidence of HIV infections as well. In view of the difficulties like high dose, frequent dosing regimen and side effects, associated with adhering to therapy with the presently available conventional dosage forms, a new treatment modality for genital herpes infections is the need of the day.

NPs are solid colloidal polymeric carrier systems having particle size between 1 to 1000 nm. The polymeric NP have been developed as a promising tool for controlled drug delivery systems wherein the active drug is encapsulated, entrapped, adsorbed or conjugated with the polymer. The application of biodegradable polymeric NP in controlling drug delivery has generated immense interest in recent times. Biodegradable NP can be successfully used for modulating the drug release profile by controlling the polymer degradation. One of the best known biodegradable carriers for controlled and sustained release is poly (lactide-co-glycolide) (PLGA). Features such as biocompatibility, prediction of biodegradation kinetics, ease of fabrication and regulatory approval has attracted its attention for a variety of biomedical applications.[5] Moreover, both hydrophilic and lipophilic drugs can be successfully

encapsulated in the PLGA matrix. PLGA has been used for delivery of drugs for both oral and parenteral routes.[5] PLGA degrades *in vivo* to lactic and glycolic acids, which are subsequently eliminated as carbon dioxide and water via the Krebs cycle.[5] The release of drug from the NPs depends on polymer degradation, which is governed by the nature of copolymer composition and its molecular weight. For this study, we used PLGA 50:50, which is known to hydrolyze at a faster rate than those containing a higher proportion of polylactic acid.

Materials and Methods

Materials

ACV was a gift sample from M/S Ajanta Pharmaceuticals Ltd, Research and Development Division, Mumbai. poly DL-lactide-co-glycolide (50/50 DL-PLGA) inherent viscosity: 0.58 dL/g MW75,000–80,000 Da was purchased from Sigma Aldrich, India. Poloxamer 188 (F-68) was obtained as gift samples from M/S Glenmark Pharmaceutical Ltd. Nashik, India. Acetone, cellophane membrane etc. were purchased from S.D. Fine Chem. Ltd.,

Mumbai, India. All other reagents and chemicals used in this study were of analytical grade.

Formulation of poly (lactic-co-glycolic acid) (PLGA) NPs

PLGA NPs were prepared by the solvent deposition method.[6] ACV was dissolved in neutral water (pH =7) kept at 35-40 C containing a hydrophilic stabilizer (Pluronic F68) at various concentrations. PLGA (50:50) was solubilized in acetone (40ml) at various concentrations. The organic phase was poured into the aqueous solution drop wise, under stirring (RPM 5000) for 2 h, thus forming a milky colloidal suspension. The organic solvent was then evaporated by using a rota evaporator. The resultant dispersion was dried using a freeze drying method.

Experimental Design

The formulations were fabricated according to a 3 full factorial design, allowing the simultaneous evaluation of two formulation variables and their interaction. The experimental designs with corresponding formulations are outlined in table 1. The dependent variables that were selected for study were particle size (Y_1), and % drug entrapment (Y_2).

Table 1. Experimental design and particle properties of 3 Full Factorial Design Formulations

Formulation Code	X_1*	X_2#	% drug entrapment±SD**	Particle size (nm) ±SD**	Poly dispersity index±SD**
F1	+1	+1	64.06±5.3	1230.3±67.4	0.65±0.01
F2	+1	0	81.32±7.3	1310.4±87.1	0.50±0.03
F3	+1	-1	90.06±8.9	1500.2±97.2	0.77±0.01
F4	0	+1	66.24±5.9	1030.5±88.7	0.66±0.05
F5	0	0	82.36±7.6	1074.6±85.1	0.32±0.02
F6	0	-1	94.5±8.4	1130.7±63.8	0.31±0.05
F7	-1	+1	54.36±5.3	395.9±33.1	0.74±0.07
F8	-1	0	74.36±6.8	580.7±41.9	0.44±0.04
F9	-1	-1	84.67±6.4	925.2±76.3	0.59±0.05

**All values are represented as mean standard deviation
* For PLGA (50:50) (X_1) transformed levels in drug: polymer ratios are: –1= 1:1; '0'=1:1.5, +1=1:2,
# For surfactant (Pluronic F68) (X_2) transformed levels in % are: –1=0.25; '0'=0.5, +1= 1

In Vitro evaluation of the prepared NPs

% Free drug and % entrapped drug

i Estimation of Free Drug

The free drug (per 50mg of formulation) was estimated by taking said quantity of formulation in dialysis bag (cellophane membrane, molecular weight cut off 12000-14000 Da, Hi-Media, India) which was tied and placed into 100ml 0.1N HCL on magnetic stirrer. At pre-determined time intervals, 5ml of the sample was withdrawn by

means of a syringe. The volume withdrawn at each interval was replaced with the same quantity of fresh media. The samples were analyzed for free drug by measuring the absorbance at 252nm using UV-visible spectrophotometer (Shimadzu UV-1700) after suitable dilutions. Above described process of withdrawing sample and analysis was continued for each half minute till a constant absorbance was obtained for consecutive three readings.[7]

Estimation of encapsulated drug

Encapsulated drug (per 50mg of formulation) was estimated by taking the residue formulation remained in dialysis membrane after estimation of free drug content, as described above. Quantity remains in dialysis membrane was added to acetone (10ml) to dissolve PLGA and filtered. Residue remaining on filter paper was dissolved in 0.1N HCL and after removing supernatant, sample was analyzed for drug content by measuring the absorbance at 252nm using UV-visible spectrophotometer (Shimadzu UV-1700) after suitable dilution. The percentage of drug entrapped and the percentage of free drug are calculated by following Eq. Formulae to calculate % free drug and % drug entrapment,

$$\text{1) \% free drug} = \frac{\text{Amount of free drug present in 50mg of formulation}}{\text{Total amount of drug present in 50mg of formulation}} \times 100$$

$$\text{2) \% Drug entrapment} = \frac{\text{Amount of encapsulated drug present in 50mg of formulation}}{\text{Total amount of drug present in 50mg of formulation}} \times 100$$

Particle Size and polydispersity analysis

The particle size and size distribution of the ACV loaded PLGA NPs were characterized by laser light scattering method using Particle Size Analyzer (Zetasizer 1690S, Malvern UK).

Development of polynomial equations

The results from factorial design were evaluated using PCP Disso 2000 V3 software. Step-wise backward linear regression analysis was used to develop polynomial equations for dependent variables particle size (Y_1) and % drug entrapment (Y_2) which bear the form of equation-1:

$$Y=b_0+b_1X_1+b_2X_2+b_{12}X_1X_2+b_{11}X_1^2+b_{22}X_2^2 \quad ....1$$

Where Y is dependent variable, b_0 arithmetic mean response of nine batches, and b_1 estimated coefficient for factor X_1. The main effects (X_1 and X_2) represent average result of changing one factor at a time from its low to high value. The interaction term (X_1X_2) shows how the response changes when two factors are simultaneously changed. The polynomial terms (X_1^2 and X_2^2) are included to investigate non-linearity.

% Yield and Flow properties of NPs

Dried NPs were accurately weighed, and by considering the total amount of drug and polymers used for preparing the feed solution, the yield of production (*YP*) was calculated, as a percentage, using the following equation[8],

$$YP = \frac{NPs}{PLGA+ACV+SURFACTANT} \times 100$$

Where NPs stands for the amount of produced NPs and (PLGA+ACV+ SURFACTANT) for the sum of the amounts of Polymer (PLGA), ACV and surfactant for the preparation of NPs. Flow properties were assessed by measuring the angle of repose by fixed funnel method and compressibility index (Carr's index). The bulk density and tapped density were measured and the compressibility index was calculated using the formula[8], Compressibility index or Carr's index (I)

$$I = \frac{\text{Tapped density - Bulk density}}{\text{Tapped density}} \times 100$$

Surface Morphology / (Scanning Electron Microscopy)

The microscopic appearance of the coated and uncoated NPs, was observed under optical and scanning electron microscopes (Jeol JSM-5410 LV, Tokyo, Japan). The scanning electron microscope photomicrographs were taken at 15 kV in various magnifications appropriate to each formulation.

Thermal studies (DSC)

DSC is very useful tool in the investigation of thermal properties of prepared dosage form (NPs) and can provide both qualitative and quantitative information about the physicochemical state of the drug inside the NPs. The absence of endothermic peak indicated the amorphous nature of drug in the NPs. Differential scanning calorimetry (DSC) was conducted using Mettler Toledo Star System, Diya labs, Mumbai, India. Samples were weighed (1.00-4.00 mg) and placed in sealed aluminium pans. The coolant was liquid nitrogen. The samples were scanned at 10ºC/ min from 10ºC to 300ºC were carried out. The thermograms are shown in Figure 4.

X-Ray Diffraction studies (XRD)

X-ray diffraction patterns of the ACV loaded NPs were determined using a diffractometer equipped with a rotating target X-ray tube and a wide-angle goniometer in Diya Labs., Mumbai, India. The X-ray source was Ká radiation from a copper target with graphite monochromater. The X-ray tube was operated at a potential of 50 kV and a current of 150 mA. The range (2è) of scans was from 0 to 70º and the scan speed was 2º per minute at increments of 0.02º. The obtained results are shown in Figure 5.

In vitro drug release studies and release kinetics

NPs retained in dialysis bag after separation from free drug as described earlier, were taken for further drug release study. Separated NPs were transferred to a flask containing 50 ml phosphate buffer saline (pH 7.4). The entire system was then kept at 37±0.5 C with continuous magnetic stirring at 100 rpm. Required quantity (5ml) of the medium was withdrawn at specific time periods (1, 2, 3, 4, 6 , 8,10, 12, 24, 32, 48 hours) and same volume of dissolution medium was replaced in the flask to maintain a constant medium volume.[9] The withdrawn samples were filtered

and then 5 ml of the filtrate was made up to volume with 100 ml media. The samples were analyzed for drug release by measuring the absorbance at 252nm using UV-visible spectrophotometer (Shimadzu UV-1700).

To study the underlying mechanism of drug release, drug release data was computed by the use of following mathematical models; zero-order kinetics, first-order kinetics and Higuchi kinetics;

$$Q_t = k_0 t$$

$$\ln(Q_0 - Q_t) = \ln Q_0 - k_1 t$$

$$Q_t = K_h . t_{1/2}$$

The following plots were made; Q_t vs t (Zero-order kinetic model), $\ln(Q_0 - Q_t)$ vs t (First-order kinetic model) and Q_t vs $t_{1/2}$ (Higuchi model). Where Q_0 is the initial amount of drug present in the microspheres, Q_t is the amount of drug released at time t and k_0, k_1, and k_h are the constants of the above-mentioned equations. In order to define a model, which would represent a better fit for the formulation, dissolution data was further analyzed by Korsmeyer-peppas equation,

$$M_t / M = k_t^n$$

Where M_t is the amount of drug released at time t and M is the amount of drug released at time , thus the M_t/M is the fraction of drug released at time t. k is the kinetic constant and n is the diffusion exponent, a measure of the primary mechanism of drug release. r^2 values were calculated for the linear curves obtained by regression analysis of the above plots.

HPLC analysis

Standardization of HPLC method for the quantification of ACV in rabbit plasma

To quantify ACV in plasma, HPLC method was standardized. Precipitation technique was followed for the extraction of ACV from plasma.

Instrumentation

The method was developed using HPLC isocratic system with a UV–Detector. Chromatographic analysis was carried out at ambient temperature. The mobile phase for plasma samples consisted 0.03M phosphate buffer / methanol / acetonitrile (35:40:25) adjusted to pH 4 with phosphoric acid and C18 column as the stationary phase. The flow rate was maintained at 1.5 mL/min and detection wavelength was 252nm. The volume of injection was 50μL.

Sample Preparation and Analysis

Frozen rabbit plasma was thawed at room temperature, vortexed for 10 sec, and any residual clots removed. An aliquot (100 μL) of serum was mixed with 100 μL of acetonitrile and the mixture was vortexed (Thermolyne Corporation, Maxi 11, Dubuque IA) for 30 seconds and centrifuged at 8000 rpm for 10 minutes. A 50μL

sample of the clear supernatant was injected onto the HPLC system. Peak areas were used for the determination of drug concentrations in the analyzed samples.

In vivo evaluation of NPs

In vivo Study Design

The albino rabbit has been shown to be a good animal model for *in vivo* drug release studies when using ACV as the model drug. In this study, six albino rabbits weighing 1.5 and 2.4 kg (average weight of 1.858±0.12 kg) were selected. The study was conducted in accordance with standard institutional ethical committee guidelines. The rabbits were fasted overnight for 12 hours with free access to water. In order to eliminate inter-subject variability among animals, the study design was a balanced Latin-Square cross-over design with three experimental formulations.

Experimental Design

Each animal received one formulation or dose at each study period as a single oral dose of 10 mg/kg in a Latin-Square- cross-over design with a one week washout period to reuse the animals. The test formulation was given to each rabbit orally in suspension form with oral snode at a dose of 10 mg of ACV per kg of body weight.

Collection of blood samples

The blood samples (approximately 300-400 μL) were collected from the marginal ear vein at 0, 0.5, 1, 2, 3, 4, 6, 12, and 24 hours after oral administration of test formulations. A washout period of 7 days was allowed between the first and second trials in individual subjects.

Collection of Blood Plasma

The blood samples were collected into a heparinized Eppendorf tube. Then the samples were subjected to centrifugation on a laboratory centrifuge (Sigma, 3K30) at 10000 rpm for 15 minutes at 0 C, and supernatant plasma was collected into another Eppendorf tube and kept at –20 C or freeze it until analysis.

Pharmacokinetic Analysis

Data were generated assuming first-order absorption and non-compartment model with first-order elimination. The maximum plasma concentration (C_{max}) and time of its occurrence (T_{max}) were directly computed from the plasma concentration vs. time plot. The elimination rate constant (K_{el}) was determined from the terminal phase of plasma concentration vs. time profile by least squares regression analysis. From this, K_{el} was calculated as $K_{el} = 2.303$ x slope. The elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693 / K_{el}$. The absorption rate constant (K_a) was calculated using the Wagner-Nelson method equation. All other pharmacokinetic parameters like volume of distribution (Vd), Total body clearance (CL/F), mean residence time (MRT), area under

the first-moment curve (AUMC), were calculated using WinNonlin software (Standard Version 1.5).

Statistical analysis

All statistical analysis was undertaken using ANOVA test followed by using Tukey-Kramer Multiple Comparisons Test (Graph Pad Instat3 software) for multiple comparisons at p 0.0001.

Result and Discussion

Preparation of NPs

PLGA NPs were prepared by the solvent deposition method using acetone as organic phase and water containing drug and stabilizer as aqueous phase. The formulations were fabricated according to a 3 full factorial design, drug: polymer ratio and concentration of stabilizer were selected as independent variable whereas particle size and % drug entrapment as dependent variables.

% Free drug and % entrapped drug

From Table 1. it is revealed that as drug:polymer (ACV:PLGA) ratio increased from 1:1 to 1:2 drug entrapment increases but thereafter, further increase in drug : polymer ratio showed reduced or insignificant change in the drug entrapment efficiency (p 0.05).

This can be explained by observing drug entrapment efficiency of factorial formulations F3, F6, F9 where Drug:Polymer ratio increased from 1:1,1:1.5 and 1:2 respectively with constant concentration of stabilizer (Pluronic F68) i.e. 0.25%. Drug entrapment efficiency increased from 84.67% to 94.5% and then decreases it to 90.06 %. It also observed that as percentage of stabilizer increased from 0.25% to 1% entrapment efficiency decrease significantly (p 0.001), the same can be explained with respective to factorial formulation F1, F2, F3 where Drug: Polymer ratio is constant i.e.1:2 and concentration of stabilizer decreased from 1% to 0.25%, drug entrapment efficiency increased from 64.06% to 90.06%. Thus it can be stated that the amount of stabilizer and polymer has an effect on the % drug entrapment of NPs. If the concentration of stabilizer is too low, aggregation of the polymer will take place, whereas, if too much stabilizer is used, drug incorporation could be reduced as a result of the interaction between the drug and stabilizer.

Particle size and polydispersity analysis

The mean diameters of the ACV loaded PLGA NPs are presented in Table 1. All NPs prepared showed discrete spherical appearance with mean diameters in the range of 395.9 nm to 1500 nm. The polydispersity index (PI) varied from 0.31–0.77, indicating higher particle size distribution.

From Table 1. it is revealed that as drug : polymer (ACV : PLGA) ratio increased from 1:1 to 1:2 particle size increased significantly (p 0.001). It also observed that as percentage of stabilizer increased from 0.25% to 1% particle size decrease significantly (p 0.001). For factorial formulation F1, F2, F3 where Drug: Polymer ratio is constant i.e.1:2 and concentration of stabilizer decreased from 1% to 0.25%, particle size increased from 1230nm to 1500nm. From the observed particle size of factorial formulations it can be conclude that the effect of the concentration of the polymers tested is negative or positive. A positive effect would imply that increasing the concentration causes the emulsion to have larger droplets, hence leading to larger particles. A negative effect means that increasing the concentration causes the emulsion to be more stable, hence leading to smaller particles.[10]

Development of polynomial equations for dependent variables

From the data of Experimental design and Parameters (Table 1.) for factorial formulations F1 to F9, polynomial equations for two dependent variables (particle size and % drug entrapment**)** have been derived using PCP Disso 2000V3 software.
The equation derived for particle size is:
$$Y_1 = 1.0193 + 0.3567 X_1 - 0.1500 X_2$$
…2
The equation derived for % drug entrapment is:
$$Y_2 = 83.49 + 3.6750 X_1 - 14.095 X_2 - 6.2283 X_1^2 - 3.6983 X_2^2$$
…3
In equations (2) negative sign for coefficient of X_2 indicates that the particle size of NPs increases when concentration of stabilizer (Pluronic F 68) is decreased and positive sign for coefficient of X_1 indicate positive effect of polymer concentration (PLGA) on particle size .

In equation (3) positive sign for coefficient of X_1 indicates that the % drug entrapment increases when concentration of polymer (PLGA) increases and negative sign for coefficient of X_2 indicates that % drug entrapment of NPs increases when concentration of stabilizer (Pluronic F 68) decreases.

Graphical presentation of the data can help to show the relationship between response and independent variables. Graphs gave information similar to that of the mathematical equations obtained from statistical analysis. The response surface graphs for particle size and % drug entrapment are presented in Figure 1 and 2 respectively.

Based on highest % drug entrapment and lowest particle size (below 1100 nm) batches F5, F8, and F9 were chosen to carry out the further evaluation study.
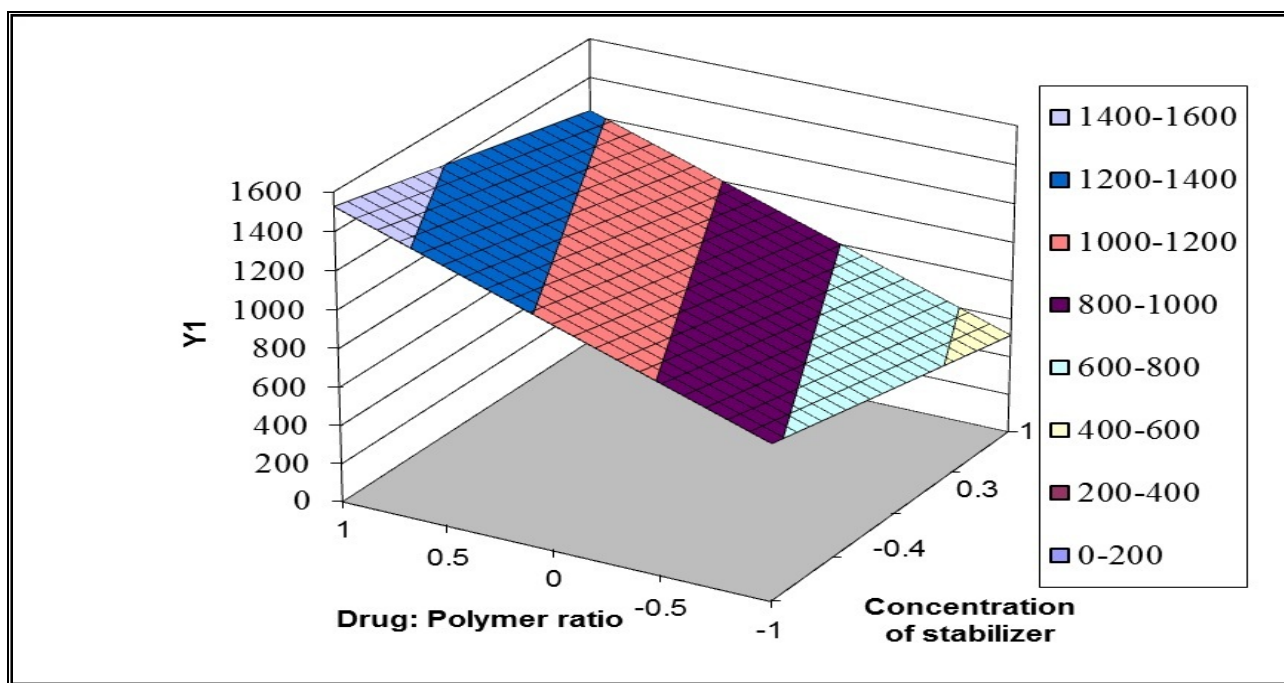
Figure 1.Response surface plot showing effect of factorial variables (Drug : Polymer ratio and concentration of stabilizer) on particle size (Y1) in nm

% Yield and Flow properties

The percentage practical yield varies between 70.51% to 81.78%. All the formulations were found to be free flowing i.e. non sticky and white powdery in apprearance.

The flow properties of all the formulations were found out by measuring the angle of repose by fixed funnel method and compressibility index (Carr's index). The results are shown in Table 2. The values of angle of repose were of the range 24 to 29,

which are within the normal acceptable range of 20 –40 . Drug loaded PLGA NPs thus showed reasonably good flow potential. This is further substantiated by the values of Compressibility index (I) which was in the range 11.43-13.77, indicating good flow characteristics of the powder. This also implies that prepared PLGA NPs are nonaggregated.

Table 2. % Yield and Flowability properties of PLGA NPs

Formulation Code	% Yield	Carr's Index±SD**	Angle of repose (º)±SD**
F1	81.78±3.24	12.77±1.5	25±1.3
F2	80.33±2.92	13.77±1.7	24±1.5
F3	79.43±2.78	13.11±1.3	26±2.0
F4	77.1±2.44	12.45±1.6	27±1.5
F5	76.17±2.86	12.45±1.7	24±1.9
F6	77.85±3.85	11.71±1.4	27±2.7
F7	75.42±3.01	12.77±1.6	26±1.9
F8	73.16±2.42	11.43±1.8	29±2.1
F9	70.51±2.10	12.54±1.2	28±2.5

Surface Morphology / (Scanning Electron Microscopy)

All the formulations were found to be free flowing i.e. non sticky, spherical and white powdery in appearance as shown in Figure 3.
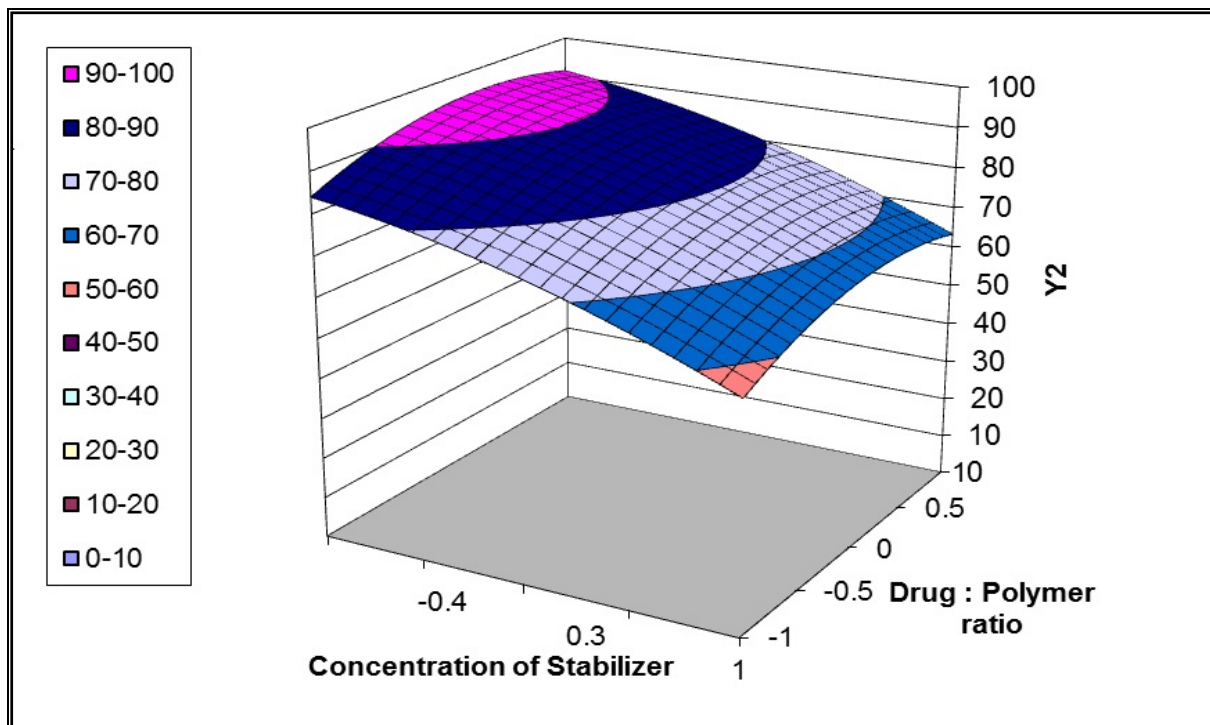

Figure 2. Response surface plot showing effect of factorial variables (Drug : Polymer ratio and concentration of stabilizer) on % drug entrapment (Y2)
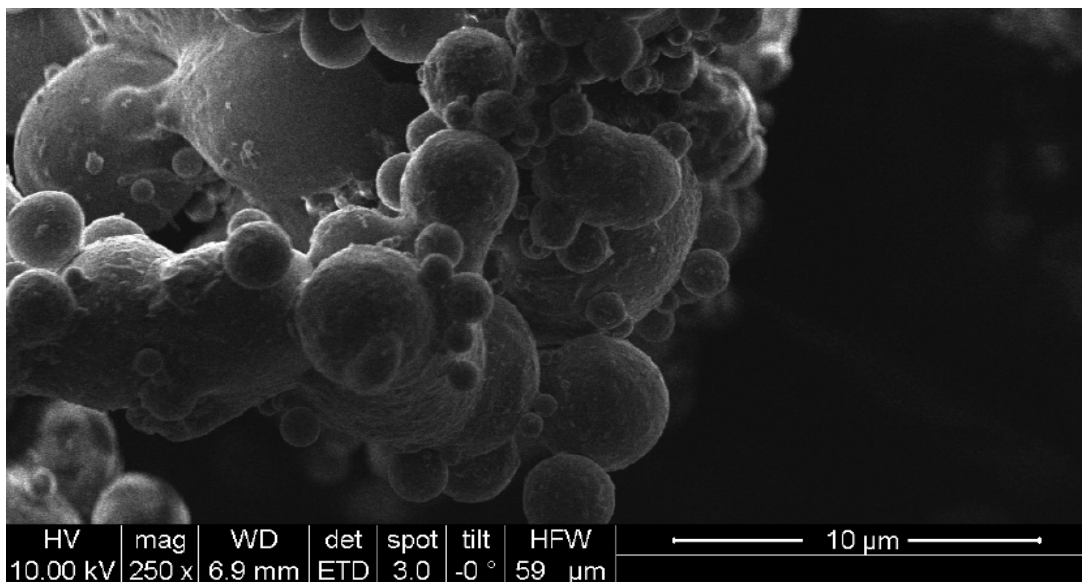

Figure 3. Scanning Electron Micrograph (SEM) of PLGA NPs (F8).

Thermal characterization of NPs

The DSC technique can provide qualitative and quantitative information about the physicochemical status of drug in NPs. The

related thermal transitions include melting, recrystallization, decomposition and out gassing or a change in heat capacity. DSC is useful to monitor different samples of same material to assess their similarities or differences or the effects of additives on the thermal properties of a material. Using the DSC analysis of drug, polymer materials and produced NPs, the nature of the drug inside the polymer matrix can be assessed, which may emerge as in solid solution, metastable molecular dispersion or crystallization. In order to identify the physical state of the drug within the selected nanoparticle formulations were subjected to DSC studies. A sharp and large onset/peak/endset peak of ACV at 246.16/252.15/258.14ºC as shown in Figure 4 was obtained which is corresponding to melting temperature of ACV (249ºC). However, the melting peak of the drug was absent in the DSC thermograms (Figure 4) of formulation F5, F8 and F9 indicating that the drug was dispersed in the NPs as an amorphous form.
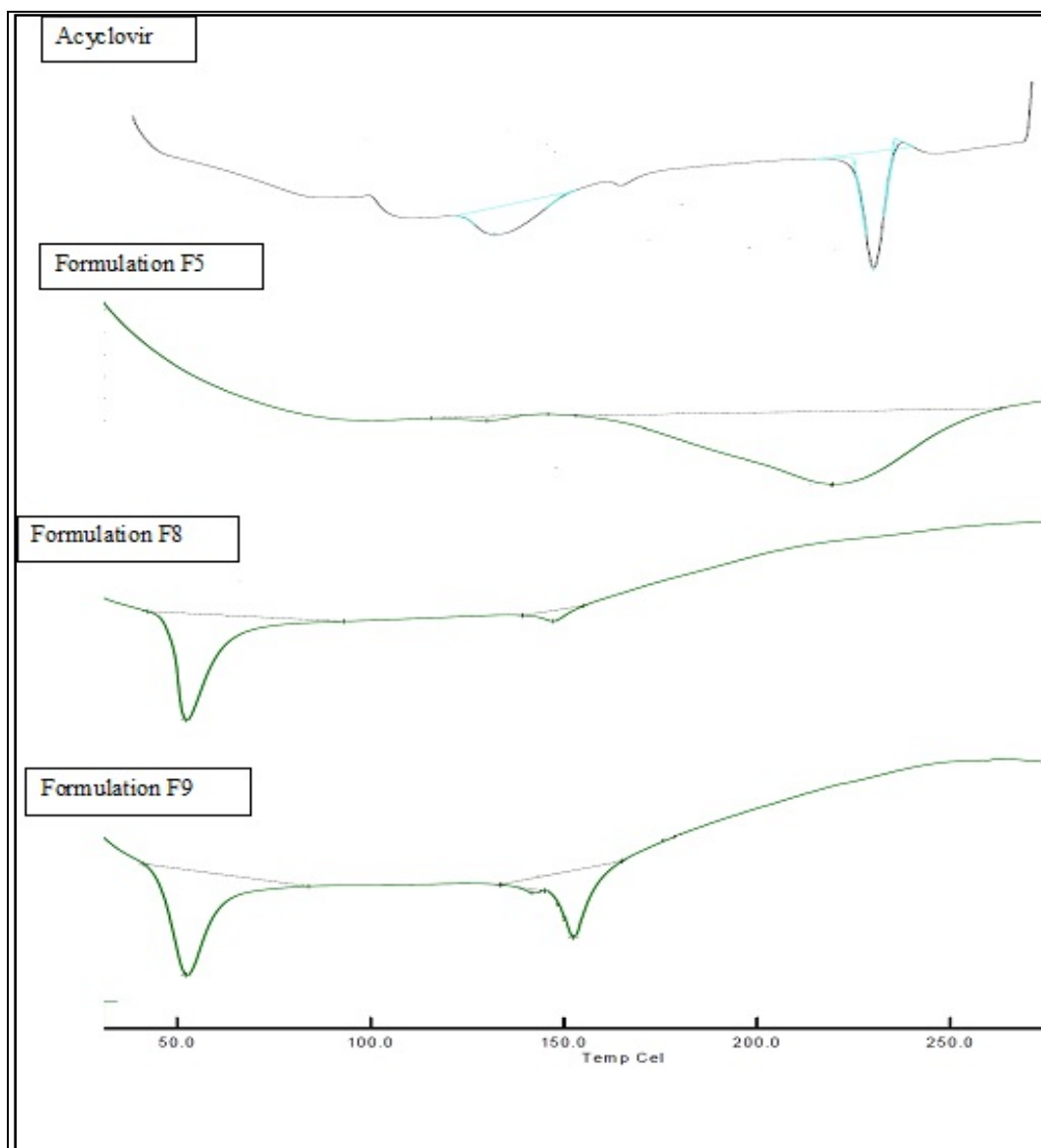

Figure 4. DSC thermograms of drug loaded PLGA NPs.

X-Ray Diffraction (XRD) studies

The DSC studies revealed that the drug loaded in NPs was in an amorphous form. This phenomenon was further confirmed by X-ray diffraction patterns. The crystal peak (7.0º, 10.5º, 23.9º, 26.2º,29.2º) of ACV was clearly observed in the X-ray data as shown in Figure 5 However, the diffraction patterns of ACV loaded PLGA NPs (Figure 5) did not contain any peaks associated with crystals of the drug, suggesting that the drug was amorphous in the polymer matrix.
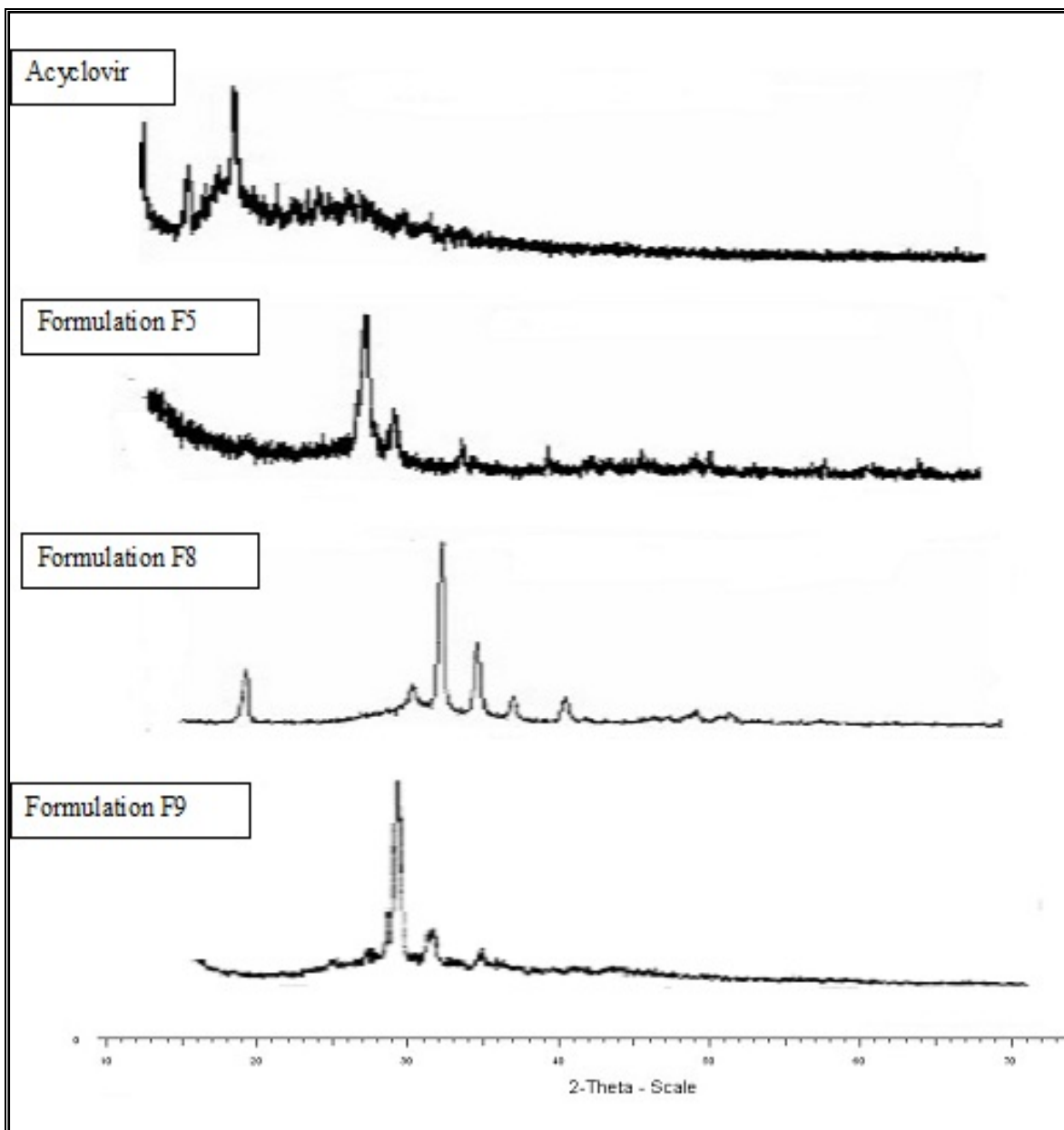

Figure 5. X-ray diffraction of drug loaded PLGA NPs.

In vitro drug release studies and release kinetics

Drug release from NPs and subsequent biodegradation are important for developing the successful formulations. The release rate of NPs depends upon i) desorption of the surface-bound/adsorbed drug; ii) diffusion through the nanopaticle matrix; iii)diffusion(in case of nanocapsules) through the polymer wall; iv)nanoparticle matrix erosion:and v) a combined erosion/diffusion process.[13] Thus, diffusion and biodegradation govern the process of drug release.

It is generally anticipated from a bulk eroding polymer such as 50:50 PLGA would give an initial burst release followed by a controlled release, in contrast to the release pattern observed in other controlled release systems for example sustain release tablets, pellets and beads.[13] In cases where there is an initial burst effect, the high initial release may be attributed to the presence of crystals of free and weakly bound drug on the surface of the particulate carriers.[13]

The cumulative percentage drug release at the end of 32 hours from selected factorial formulations F5, F8 and F9 was 58.48, 66.54 and 62.92 respectively.

The mechanism of drug release from NPs is determined by different physical–chemical phenomena. The exponent n has been proposed as indicative of the release mechanism. In this context, n 0.43 indicates Fickian release and n = 0.85 indicates a purely relaxation controlled delivery. Intermediate values 0.43 < n < 0.85 indicate an anomalous behavior (non-Fickian kinetics) corresponding to coupled diffusion/polymer relaxation.[14]

The average percentage release was fitted into different release models: zero order, first order and Higuchi's square root plot. The models giving a correlation coefficient close to unity were taken as the order of release. *In vitro* drug release profile of all selected factorial formulations (Figure 6) was subjected to goodness of fit test by linear regression analysis according to zero order and first order kinetic equations, Higuchi's and Korsmeyer-Peppas models to ascertain the mechanism of drug release.[14] From various parameters determined for drug release from NPs based on Peppas Model, Higuchi Model and Diffusion profile (Table 3), it is evident that values of 'R^2' for Higuchi plots of factorial formulations F5, F8 and F9 are 0.8477, 0.9343 and 0.8322 respectively, for first order plots 0.9125,0.8952 and 0.8834 respectively and those of 'n' (Diffusion exponent) values of Peppas equation are 0.2547, 0.2512 and 0.3637 respectively. This data reveals that drug release follows first order release kinetics with fickian diffusion mechanism. Finally, it can be concluded that the different drug release rates may be attributed to different sizes of the NPs. It is expected as the particle size of PLGA nanoparticle is smaller, their surface area will be more and the drug release is faster.
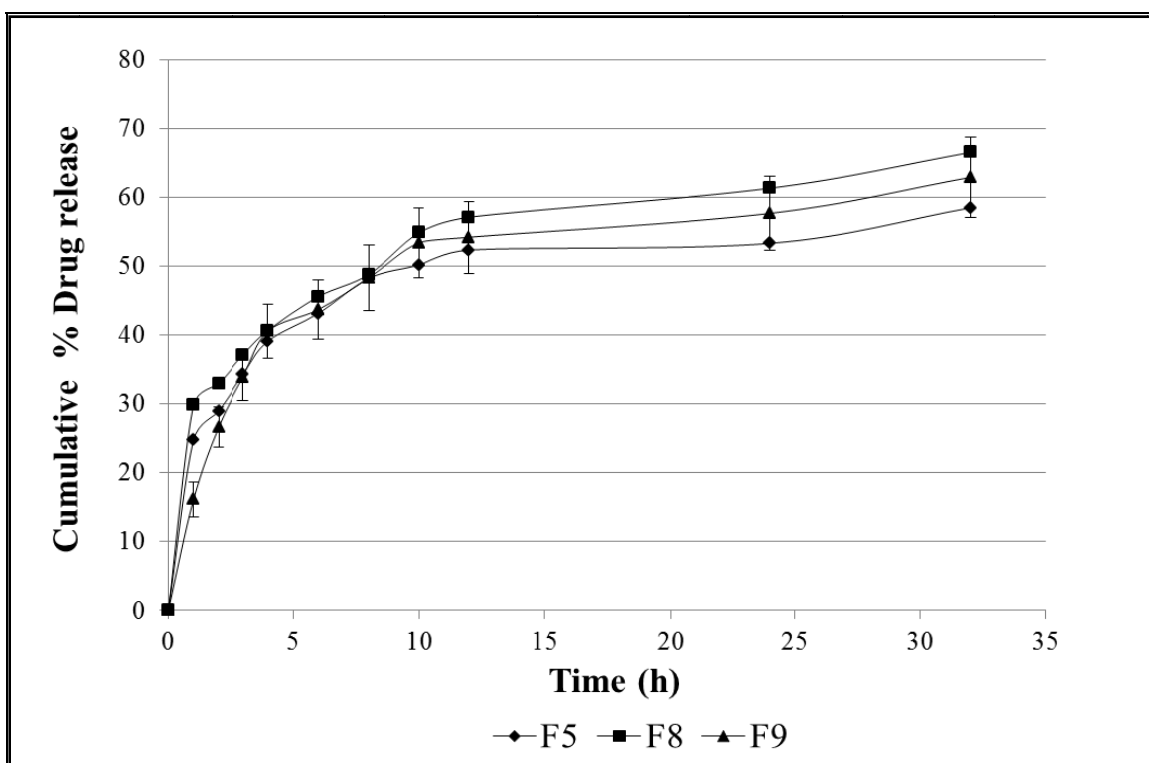

n=3

Figure 6. *In vitro* drug release profile of PLGA NPs.

In vivo pharmacokinetic studies

Animal study was carried out to determine the absorption pattern of ACV from the formulation F5, F8 and F9, which were administered orally to rabbits. Figure 7. Shows that the plasma drug concentrations as a function of time after oral administration of these preparations along with commercial tablet Zovirax®. The pharmacokinetic parameters such as C_{max}, T_{max}, $t_{1/2}$, K_a, K_e, AUC, AUC $_{(0-)}$ AUMC, AUMC $_{(0-)}$, Vd, MRT, Cl/F, F and Fr are summarized in Table 3. for oral administration of selected test formulations as well as i. v. administration of pure drug.
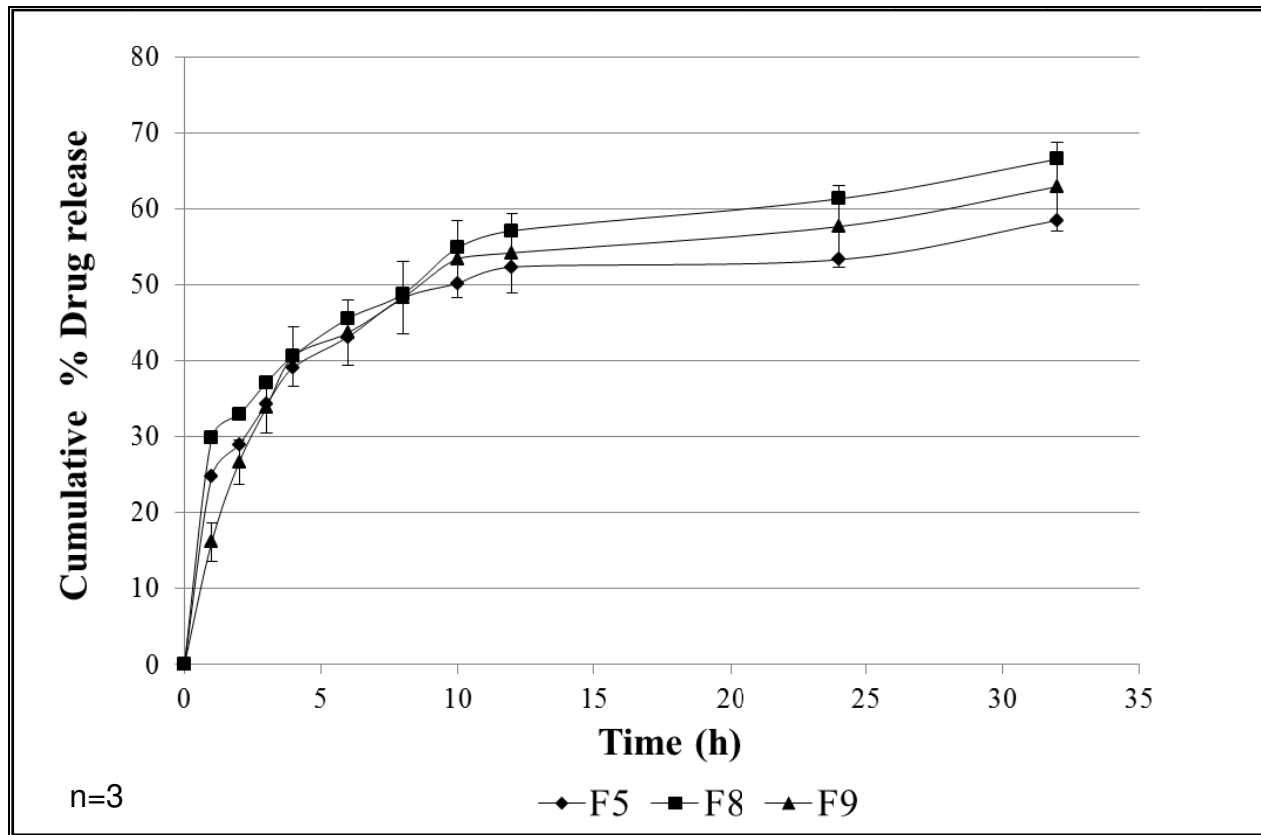
Figure 6. *In vitro* drug release profile of PLGA NPs.

Table 3. Various parameters determined for drug release from NPs based on Peppas Model, Higuchi Model and Diffusion profile

Formulation	Zero-Order		First Order		Higuchi Model		Korsemmeyer-Peppas Model		P value
	R^2	K_0	R^2	K_1	R^2	K_h	R^2	n	
F5	0.5395	22.17	0.912	-0.08	0.8477	5.074	0.631	0.2547	0.001
F8	0.609	25.87	0.895	-0.07	0.9343	6.331	0.552	0.2512	0.001
F9	0.599	24.44	0.883	-0.06	0.8322	7.412	0.640	0.3637	0.001

R^2 is the coefficient of correlation; K_0, K_1 and K_h are the release constant for zero-order, first model and Higuchi model respectively and n is the release exponent of Korsemmeyer-Peppas Model

Peak plasma concentration (C_{max}) values of 1.39, 1.01, 1.22, 1.06 and 26.37 µg/mL was observed after oral administration of Commercial Tablet Zovirax®, Formulation F5, F8, F9 and intravenous (i.v.) administration of pure drug solution respectively. These results revealed that the ACV i.v. solution gave the highest C_{max} value, followed by oral administration of commercial Tablet Zovirax®. However, there was no significant difference between C_{max} values of Formulation F5, F8 and F9. Peak plasma concentration of ACV was obtained at 3.00 hours i.e. T_{max} for all the formulations, as shown in Table 4. There was no significant difference between T_{max} values for all the formulations. The $t_{1/2}$ values for ACV were found to be 3.12, 4.96, 6.1 and 5.2 hours after oral administration of Commercial Tablet Zovirax®, Formulation F5, Formulation F8 and Formulation F9 respectively. Higher $t_{1/2}$ values for test formulations as compared to commercial Tabel Zovirax shows that more bioavailability can be achieve for the drug with oral administration NPs. The Ka values (Table 4) were found to be 1.46, 0.568, 1.66 and 0.732 hr^{-1} for oral administration of Commercial Tablet Zovirax®, Formulation F5, Formulatiuon F8 and Formulation F9 respectively. These results indicated that the rate of absorption of ACV from Formulation F8 was faster compared other F5 and F9, due to difference in particle size and % drug entrapment.

Table 4. Pharmacokinetic Parameters following oral administration of test formulations, conventional tablet and intravenous administration of pure drug.

Pharmacokinetic Parameters	F5	F8	F9	Zovirax	Pure Drug (I.V.)
$AUC_{(0-24)}$ (µg.h/mL)	15.22±0.2160	17.96±0.1760	16.81±0.363	6.53±0.515	42.72±3.12
$AUC_{(0-)}$ (µg.h/mL)	45.55±3.99	64.77±2.68	51.54±5.41	6.90±0.681	47.16±3.84
AUMC(µg.h/mL)	152.65±4.12	193.96±2.57	178.65±5.43	35.48±5.46	62.71±5.14
$AUMC_{(0-)}$ (µg.h/mL)	2145±435.978	5002.94±390.290	3317±757.978	47.29±11.21	80.31±7.59
C_{max}(µg/mL)	1.01±0.0082	1.22±0.0082	1.06±0.013	1.39±0.025	26.37±1.95
T_{max} (h)	3.00±0.00	3.00±0.00	3.00±0.00	1.00±0.00	0.0083±0.00
% Relative bioavailability	201.44±92	276.38±22.89	259.09±26.9	-	-
% Absolute bioavailability	4.297±0.457	4.92±0.299	4.610±0.278	1.78±0.16	-
$Ka(h^{-1})$	0.568±0.043	1.666±0.0438	0.732±0.022	1.466±0.0438	-
$t_{1/2}$ (h)	4.96±1.43	6.1±0.156	5.2±0.230	3.12±0.55	1.2±0.9
$Kel(h^{-1})$	0.018±0.0011	0.012±0.005	0.015±0.001	0.140±0.016	0.561±0.00
MRT (h)	10.48±0.064	10.79±0.040	10.61±0.097	5.4±0.43	1.49±0.09

The AUC values for oral administration of Commercial Tablet Zovirax®, Formulation F5, Formulation F8 and Formulation F9 found to be 6.53, 15.22, 17.96 and 16.81 µg.hr/mL respectively. These values clearly indicated the improvement in bioavailability of ACV from prepared formulation when compared to conventional commercial tablet.

The relative bioavailability (Fr) of ACV was found to be 201.44%, 276.38% and 259.09% for Formulation F5, F8 and F9 respectively. It was observed that, % absolute bioavailability of test formulations was significantly higher than commercial tablet Zovirax® i.e 4.29%, 4.92% and 4.61% respectively for formulation F5, F8 and F9, whereas Zovirax® has only 1.78% absolute bioavailability. These results indicate that the bioavailability of ACV was improved significantly when administered as a PLGA NPs.

Other pharmacokinetic parameters, like volume of distribution (Vd), total body clearance (Cl/F), has been calculated with the help of WinNonlin software (Standard version1.5), shows test formulations (F5, F8 and F9) have higher volume of distribution than conventional tablets.

Conclusion

ACV loaded NPs were prepared by the solvent deposition method. The application of factorial design gave a statistically systematic approach for the formulation of NPs with desired particle size and high entrapment efficiency. Drug: polymer ratio and concentration of stabilizer were found to influence the particle size and entrapment efficiency of ACV loaded PLGA NPs but the concentration of stabilizer had greater influence on both dependent variables (Particle size and Drug entrapment) as compared to Drug:Polymer rato. *In vitro* drug release study of selected factorial formulations (F5, F8, F9) showed 58.48 %, 66.54%, and 62.92% release respectively in 32 hrs. The release was found to follow first order release kinetics with fickian diffusion mechanism for all batches. The *in vivo* studies of selected formulations followed by comparison with pharmacokinetic parameters like AUC, % relative bioabaliability, % absolute bioavailability, $t_{1/2}$, MRT of conventional tablet (Zovirax®), revealed that 2.6 folds improvement in bioavailability which results in probable decrease in dose and dosing frequency due to controlled particle size, entrapment efficiency and sustain drug release pattern.

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Author's contribution

UVB prepared and characterized the physicochemical properties of ACV-loaded PLGA nanoparticles along with interpretation of all the results for final manuscript. KVD helped determine the entrapment

of ACV-loaded PLGA nanoparticles and draft the manuscript. SC conducted the *in vivo* study of ACV-loaded PLGA nanoparticles.

Declaration of interest

The authors report no conflicts of interest.

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