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Design and Characterization of Acyclovir Loaded Poly- Lactic-Co-Glycolic Acid (Plga) Nanoemulsion for Ophthalmic Application

Subashini Rajaram[1]*, Srilatha T[1], Rajendran N.N[1]
1. Department of Pharmaceutics, Swamy Vivekananda College of Pharmacy, Elayampalayam-637205, Tamilnadu, India.

ABSTRACT

The present study attempted to evaluate acyclovir loaded PLGA nanoemulsions for ocular delivery. The acyclovir loaded PLGA nanoemulsions were prepared by spontaneous emulsification method. Five batches were prepared and labeled as NE-1, NE-2, NE-3, NE-4 and NE-5 by changing the concentration of PLGA polymer. The prepared nanoemulsions were subjected for its physico-chemical characterization, *in-vitro* diffusion, release kinetics and stability studies. FT-IR and DSC shown the drug and polymer were compatible with each other and no change in their chemical nature. The morphology of nanoemulsion shows spherical in shape with smooth surfaces. The particle size and zeta potential and Poly dispersity index were determined by malvern instrument and the results shown that the prepared nanoemulsion has significant ranges of particle size (164.67 - 244.43nm), zeta potential (-33.20 to -37.60) and poly dispersity index (0.256-0.499). Drug entrapment efficiency and % practical yield ranges between (54.97-79.67) and (46.83-58.01) respectively. The *in-vitro* % drug release of acyclovir indicates formulation NE-4 has significant sustained release compared with other formulations. The release kinetic data of all formulations are fitted with Higuchi's model and non-fickian diffusion mechanism. The stability study indicates 5°C±3°C and 25°C±2°C/60%±5% RH is ideal storage condition for nanoemulsion for longer period. Thus it can be conclusively stated that the acyclovir loaded PLGA nanoemulsions may be considered as an improved ophthalmic drug delivery system for the treatment of ocular viral infections.

Keywords: Acyclovir, PLGA polymer, Nanoemulsion, Ocular delivery, Spontaneous emulsification method.

*Corresponding Author Email: Subababu.r@gmail.com
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INTRODUCTION

Acyclovir is an anti viral drug with a significant and highly specific activity against herpes viruses and is widely used in the treatment of various ocular viral diseases. The topical application of acyclovir as eye ointment is limited by poor ocular drug bioavailability, pulse drug entry, systemic exposure due to the nasolacrimal duct drainage and poor entrance to the posterior segments of the eye due to the lens-iris diaphragm. Many attempts have been made to improve the ocular bioavailability and the therapeutic effectiveness of acyclovir. One of the most promising technology is the nanoemulsion drug delivery system, which is being applied to enhance the solubility and bioavailability of lipophilic drugs that is acyclovir. Nanoemulsions are defined as the oil-in-water (o/w) emulsions with mean droplet diameters ranging from 50 to 1000nm[1]. Usually, the average droplet diameters ranging from 100 to 500 nm. The particles can exist as water-in-oil and oil-in-water forms, where the core of the particle is either water or oil respectively. The nanosized droplets leading to an enormous increase in interfacial areas associated with nanoemulsion would influence the transport properties of the drug. Nano particulate drug delivery systems from biodegradable and biocompatible polymer are interesting option for controlled drug delivery and drug targeting. Poly (lactide-co- glycolide) has gained attention for preparation of wide variety of delivery systems containing several drugs due to their biodegradable and biocompatible properties and low toxicity. Because of their biodegradability and biocompatibility poly lactic acid and its copolymers with glycolic acid (PLGA) are widely employed for the preparation of sustained release preparations[2,3]. Among the various polymers used in drug delivery research, PLGA (poly –d, l-lactide- co- glycolide) is one of the most successfully used biodegradable polymer for the development of nanomedicines. Several methods are employed for the preparation of nanoemulsion such as high pressure homogenization, microfluidization, spontaneous emulsification and solvent displacement method[4] and selection of suitable method depends on the drug and polymer used. Considering the above factors, the present study was aimed to prepare acyclovir loaded PLGA nanoemulsion by spontaneous emulsification method for ocular infection and this study could project the importance of nanotechnology to improve the bioavailability of acyclovir as nanoemulsion for ocular delivery.

MATERIALS AND METHOD

Acyclovir was obtained as a gift sample from Micro labs, Hosur, Bangalore (India). PLGA polymer was obtained as gift sample from Purac biomaterials (Germany).Soyabean oil, Miglyol oil and Tween 80 were procured from Loba Chemie Pvt Ltd, Mumbai(India). Benzyl benzoate was

purchased from S.D. Fine Chemicals Ltd, Mumbai(India). All other reagents and solvents used were of analytical grade.

Preparation of acyclovir loaded PLGA nanoemulsions

The formula of acyclovir nanoemulsion was given in Table 1. The nanoemulsions were prepared by using spontaneous emulsification process. In the first step different concentrations of polymer (100,200,300,400,500mg) and soya phospholipid were prepared separately dissolved in 25 ml of acetone at 40°c with continuous shaking. The soya bean oil or miglyol 812 and egg yolk lecithin was added to the phospholipid organic solution and then acyclovir previously dissolved in 5 ml of methanol was added to this solution so as to constitute the organic phase. The aqueous phase was prepared by adding 250 mg of tween 80 with 50ml of phosphate buffer solution (pH 7.4). The nanoemulsion was formed through the slow injection of the organic phase into the aqueous phase, under magnetic stirring at 150 rpm during 30 min at 25°C. The solvent was then evaporated under reduced pressure at 40°C, and the volume of nanoemulsion was concentrated to the initial volume of the aqueous phase[5]. The prepared nanoemulsion was subjected to its physicochemical characteristics, *in-vitro* diffusion study and stability studies.

Table 1: Formula for Acyclovir Nanoemulsion

Constituents	Formulations				
	NE-1	NE-2	NE-3	NE-4	NE-5
Organic Phase					
PLGA polymer(mg)	100	200	300	400	500
Acyclovir drug(mg)	100	100	100	100	100
Egg yolk lecithin(mg)	250	250	250	250	250
Methanol (ml)	5	5	5	5	5
Acetone (ml)	20	20	20	20	20
Benzyl benzoate(ml)	0.5	0.5	0.5	0.5	0.5
Soyabean oil (mg)	250	250	250	250	250
Miglyol oil 812 (mg)	250	250	250	250	250
Aqueous Phase					
p^H7.4 phosphate buffer(ml)	50	50	50	50	50
Tween80(mg)	250	250	250	250	250

Drug and carrier interaction by Fourier Transform Infra Red Spectroscopy(FT-IR)

FT-IR Spectroscopy was performed on pure drug, polymer and nanoemulsion (Perkin Elmer RX1). The pellets were prepared by gently mixing of 1mg sample with 200mg potassium bromide at high compaction pressure. The scanning range was 4000to400cm^{-1} and the revolution was 4 cm^{-1}. The pellets thus prepared were examined and the spectra of all the samples were compared.

Thermal Analysis by differential scanning colorimetry (DSC)

Differential scanning calorimetric measurement of acyclovir loaded PLGA nanoemulsion was carried out by using a thermal analysis instrument (DSC CA 60 Shimadzu. Japan) equipped with al liquid nitrogen sub ambient accessory. 2-6mg samples were accurately weighed in 64 aluminum pans thematically sealed and heated at a rate of 10ºC min^{-1} in a 30 to 300ºC temperature under nitrogen flow of 40 ml / min.

Morphology by scanning electron microscopy (SEM)

The size of the nanoemulsion was analyzed by scanning electron microscope (JEOL MODEL JSM 6400). The nanoemulsions were mounted directly on the SEM stub, using double –sided, sticking tape and coated with platinum and scanned in a high vacuum chamber with a focused electron beam. Secondary electrons, emitted from the samples were detected and the images formed[6].

Surface Characteristics by Zeta Potential

The Zeta potential of nanoemulsions was measured on a zeta potential analyzer (Zetasizer 3000 HS Malvern instrument U.K). The samples were diluted with pH 7.4 and placed in eletrophoretic cell and measured in the automatic mode[7].

Particle size and polydispersity index (PDI)

The particle size of nanoparticles was measured with a Malvern zetasizer (Zetasizer 3000 HS Malvern instrument U.K).The particle size distribution is reported as poly dispersity index. The sample were placed in the analyzer chamber and readings were performed at 25°c with a detected angle of 90^{o}[8,9].

Percentage entrapment efficiency

To determine the entrapment of acyclovir in nanoemulsions, 1 ml of freshly prepared nanoemulsion was taken and diluted with 100 ml of simulated tear fluid STF (p^{H} 7.4). Aliquots 10 ml were taken and diluted to 100 ml, further subjected to cold centrifuge at 4^{0}c and 15000 rpm using Sigma 3k30 centrifuge for 30mins. From the supernatant 1 ml was taken and diluted appropriately. The resulting solutions were analyzed for acyclovir content using single beam spectrophotometer and % entrapment efficiency (%EE) was calculated using following equation[10].

Entrapment efficiency = Total amount of acyclovir – Free acyclovir in supernatant/ Total amount of acyclovir x 100

Percentage yield

The lyophilized nanoparticles from each formulation were weighed and the respective percentage yield was calculated using the following formula.

Percentage yield = Weight of nanoparticles obtained/ Weight of drug, polymer and oil x 100

***In-vitro* diffusion studies of acyclovir nanoemulsion**

The release of acyclovir from nanoemulsion was evaluated over 24 hrs by a dialysis bag system consisting of a membrane with molecular cuts off value of 12000-14000 KDa, loaded with 10ml of nanoemulsion was suspended in simulated tear fluid (STF) P^H 7.4 at 37±0.5^{0}c temperature and under slow magnetic stirring. At regular intervals aliquots of 1 ml of dissolving media were withdrawn and immediately restored with same volume of fresh medium. The amount of released drug was assessed by UV analysis at 253nm after suitable dilution[11,12].

Release kinetics

In order to understand the mechanism and kinetics of drug release, the results of the *in vitro* drug release study were fitted with various kinetics equations like zero order (% Cumulative drug release vs Time), first order (log % Cumulative drug remaining vs Time), Higuchi matrix (%Cumulative drug release vs Square root of time)[89,90,91]. In order to define a model which will represent a better fit for the formulation, drug release data were further analyzed by Peppa's equation, M_t/M_∞= ktn, where M_t is the amount of drug released at time t and M_∞ is the amount released at ∞, Mt/M_∞ is the fraction of drug released at time t, k is the kinetic constant and n is the diffusional exponent, a measure of the primary mechanism of drug release. r^2values were calculated for the linear curves obtained by regression analysis of the above plots.

Stability of nanoemulsion during storage

The effect of temperature and humidity on the optimum formulation of acyclovir nanoemulsion was evaluated for 3 months under different storage conditions. The nanoemulsion prepared was filled into an amber glass bottle and flushed with nitrogen gas prior to air-tight closure with a plastic cap. Nanoemulsions were then stored at 5°C±3°C and 25°C±2°C/60%±5% RH and 40°C±2°C /75%±5%RH (as per ICH guidelines) prior to droplet properties characterization on the 30،60and 90th days. The droplet properties of nanoemulsion evaluated in this study were size, zeta potential value and PDI. The percentage of drug release was assessed by UV analysis at 253nm (Shimadzu uv-1700, Japan) after suitable dilution[13].

Statistical analysis

The release data were subjected to ANOVA with Tukey-Kramer multiple comparision test. This test was used to compare different formulations, and a P value of 0.05 is considered to be significant.

RESULTS AND DISCUSSION

Compatibility study

FT-IR spectroscopy was carried out to study the compatibility of pure acyclovir and PLGA polymer used in the formulation of nanoemulsions. The pure acyclovir has characteristic IR peaks at 1610.45cm^{-1},1633.67cm^{-1}(C=Ostretchingvibration),3440.77cm^{-1}(OHstretching),1217.00 cm$^{-1,}$ 1307.65 cm$^{-1,}$ 1346.22 cm^{-}1(N-H stretching), 1388.65 cm^{-1} (C-N stretching).PLGA has the characteristic IR absorption frequency at 1750.70cm^{-1}(-C=O) stretch, 2926cm^{-1}(-C-H) stretching. The IR spectrum of the physical mixture and nanoemulsion were exhibited all the characteristic peaks of acyclovir and PLGA as depicted in Figure 1. It shown acyclovir was highly compatible with PLGA polymer.

Differential scanning colorimetry(DSC)

DSC of acyclovir shown the endothermic peaks at 120.61^{0}c (ΔH=124.43 mJ), 150.48^{0}c (ΔH=415.44 mJ) and 254.07^{0}c (ΔH=557.48 mJ). PLGA thermogram displays a thermal event at 50^{0}c corresponding to the polymer glass transition temperature (Tg).Two endothermic peaks were observed in physical mixture at 121.06^{0}c and 260.46^{0}c. it shows the disappearance of characteristic peak of acyclovir at 150.48^{0}c. The endothermic peaks of acyclovir at 120.61^{0}c were disappeared and a new peak was observed at 148.49^{0}c in the best formulation (NE-4). This result suggests that acyclovir has lost its crystalline character and partially transformed to amorphous state, it might be the inhibition of acyclovir crystallization by PLGA during nanoemulsion formation. The thermogram of acyclovir nanoemulsion (NE-4) was shown in Figure 2.

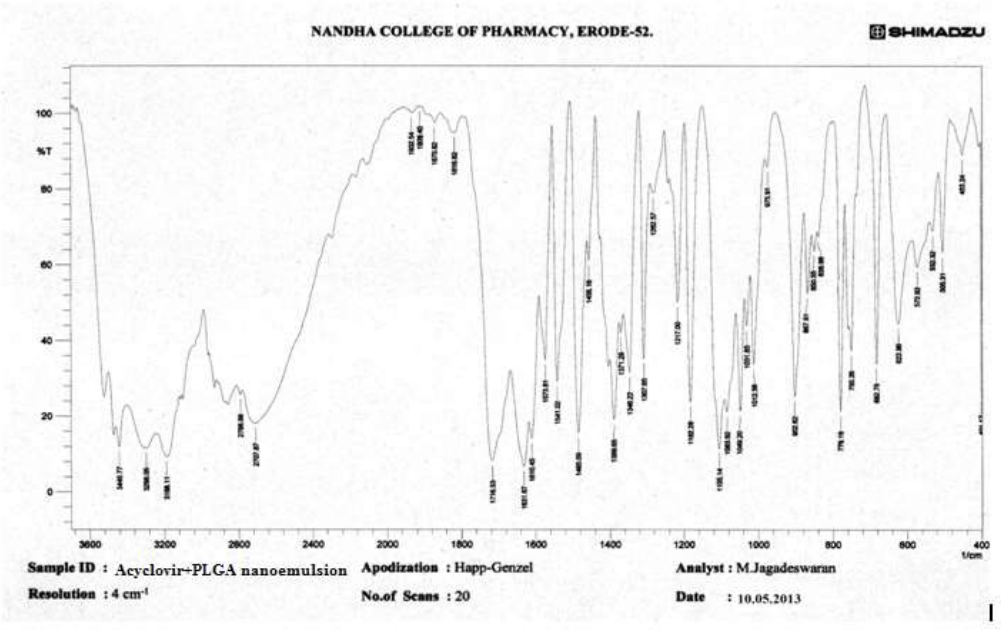

Figure 1: FT-IR spectra of acyclovir, PLGA nanoemulsion

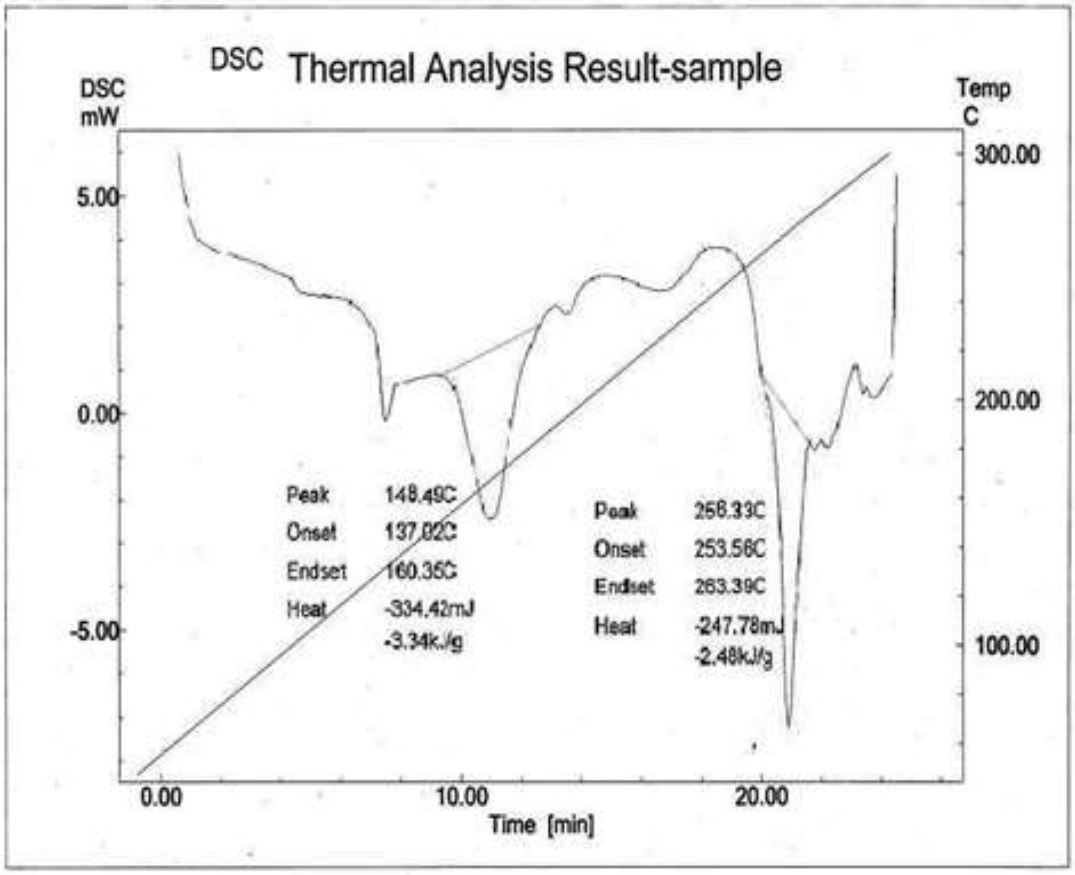

Figure 2: DSC thermogram of nanoemulsion (NE-4)

Surface morphology

Surface morphology of nanoemulsions was visualized by using SEM (JSM-T330A,JEOL) and it was shown in Figure 3. It was found that acyclovir loaded PLGA nanoemulsions were spherical in shape with smooth surface[6].

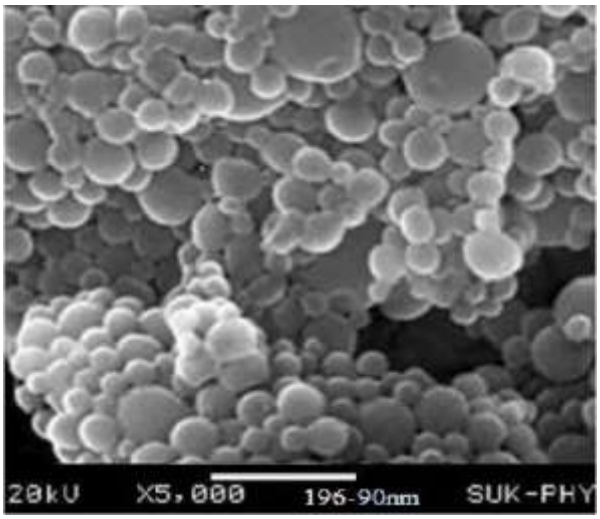

Figure 3: SEM image of nanoemulsion(NE-4)

Particle size

The particle size of acyclovir loaded PLGA nanoemulsions (NE-1 to NE-5) are shown in Table2. Particle size is one of the important parameter for ocular delivery in order to avoid the irritation to ocular surface. Large particle size will induce the rapid tear production which lead to the rapid drainage of the instilled dose and therefore reduce bioavailability. Generally the mean hydrodynamic diameter of the particle increased with increasing the polymer concentration. The particle size of nanoemulsion was increased with increasing concentration of PLGA polymer. The prepared nanoemulsion (NE-1 to NE-5) shown particle size below 250nm which may reduce rapid drainage of the instilled dose and increase bioavailability of the drug[8].

Table 2: Particle size, zeta potential and PDI of nanoemulsion

Formulation code	Drug : Polymer(mg)	Mean particle size (nm)	Poly dispersity index	Zeta potential(mV)
NE1	1:1	164.67± 3.32	0.256± 0.03	-37.60
NE2	1:2	175.33±3.76	0.375± 0.006	-35.86
NE3	1:3	182.87±5.35	0.438± 0.005	-34.60
NE4	1:4	196.90±7.51	0.226± 0.0075	-33.78
NE5	1:5	244.43±13.83	0.499± 0.030	-33.20

(Mean ± SD, n=3)

Zeta potential

Table 2 indicates the zeta potential of prepared nano emulsion. Zeta potential is an index of the stability of the nano formulations. Under most conditions, the higher the absolute value of the zeta potential of the nano emulsion, the larger the charge on their surface, leading to stronger repulsive interaction between the dispersed nano particles and higher stability and more uniform size. A high potential value of above ±25mV, ensure a high energy barrier that stabilizes the nano emulsion. The zeta potential values of all formulations are shown in Table 2. The obtained values remains in the ranges of negative values for all the formulations (-33.20 to -37.60mV).It shows the particle stability of nano emulsion because of the repulsive forces prevent aggregation with aging. The negative charge on the PLGA nano particles is due to the ionization of the carboxylic end groups on the surface of the polymer[7].

Poly dispersity index (PDI)

PDI of all formulations were given in Table 2. Poly dispersity index is another factor that represents the dispersion homogeneity. The range for the poly dispersity index is from 0 to 1. Values close to 0 indicates the homogenous dispersion and those greater than 0.5 indicate high heterogeneity. The PDI for all formulations was between 0.256 to 0.499 it indicates a relative homogenous dispersion[9].

Entrapment efficiency and percentage yield

The results of entrapment efficiency and percentage yield are shown in Table 3.The entrapment efficiency and % yield depends upon the drug and polymer interaction. The observed result shows that the entrapment efficiency was enhanced from 54.97%(NE-1) to 79.67%(NE-4), when PLGA concentration was elevated from 3 to 15 mg/ml. PLGA concentration above 15mg/ml resulted in a decrease in entrapment efficiency. It is consistent with the earlier report that a high concentration of polymer in the organic phase resulted in the increase in particle size and decrease the surface charge of nano particles that would affect the amount of acyclovir adsorbed on the surface of nanoparticles[10]. Percentage yield was increased with increase the concentration of polymer. Formulation NE-5 had high % yield in comparison with other formulations.

Table 3: Entrapment efficiency and % yield of nanoemulsion

Formulation code	% Entrapment efficiency	% yield
NE1	54.97± 1.20	46.83
NE2	62.42± 0.79	48.15
NE3	69.35± 0.69	52.38
NE4	79.67± 1.01	53.70
NE5	70.72± 0.82	58.01

(Mean ± SD, n=3)

Diffusion studies

In-vitro acyclovir release from nanoemulsion (NE-1 to NE-5) was studied at various time intervals (0-24 hrs) at pH 7.4 phosphate buffer using dialysis bag diffusion technique[13,14].Comparative diffusion profile of all formulations was shown in Table 4 and Figure 4.The profiles are biphasic initial burst of drug release attributed to surface associated drug, followed by a phase of slower release as drug entrapped inside the particle diffuses out into the release medium.

Table 4: Comparative diffusion profile of nanoemulsion

S.NO.	Time(hrs)	% Drug Release				
		NE1	NE2	NE3	NE4	NE5
1	0	0	0	0	0	0
2	1	34.45	26.27	25.72	25.53	26.01
3	2	40.08	31.96	30.18	29.61	30.59
4	3	46.81	38.26	34.59	34.65	36.65
5	4	54.36	43.60	39.99	40.05	41.56
6	6	61.32	53.53	45.28	49.48	50.86
7	8	68.68	59.37	55.45	55.13	56.00
8	10	75.70	64.74	61.23	61.51	62.11
9	12	78.37	70.61	65.79	66.84	67.41
10	24	93.51	90.21	88.67	84.83	86.95

(Mean ± S.D, n= 3)

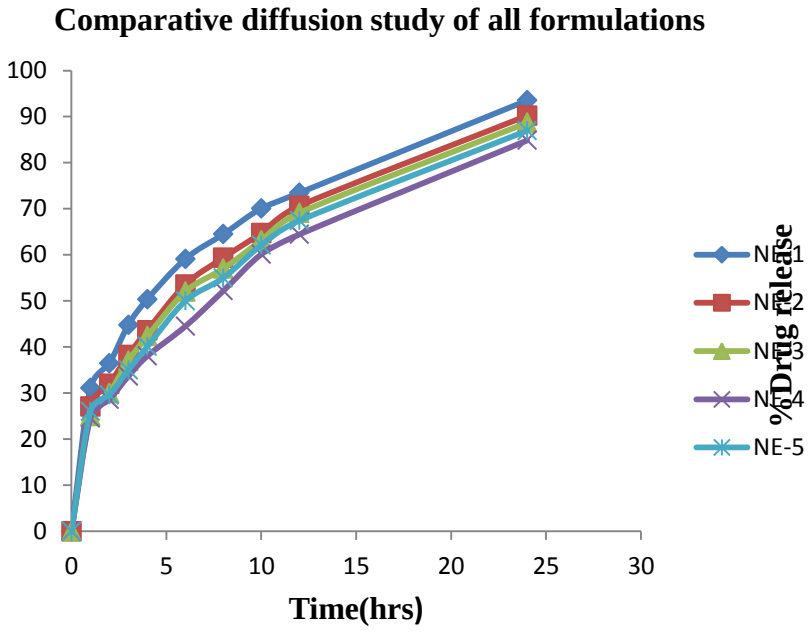

Figure 4: Comparative diffusion profile of all formulations

Formulation NE1 with smaller avg particle size 164.67 nm and large initial burst release of 34.45 %after 1hr and 93.51% drug release after 24 hrs. Formulation NE4 with large average particle size 196.90nm gave small initial burst release of 25.53 % after 1 hr and 84.83% drug release after 24 hrs. The results observed with formulation NE-2 (175.33nm) and NE-3 (182.87nm) which showed initial burst release of 26.27% and 25.72% after 1 hr while 90.21% and 88.67% drug release after 24 hrs respectively, whereas formulation NE-5 (particle size 244.43 nm) gave initial burst release of 26.01% after 1 hr and 86.95% drug release after 24 hrs. Smaller particles have a high surface area compared to their volume, so most of the drug will be at or near the particle surface and can be readily released. Furthermore diffusion distances encountered in particles are small which allows the drug entrapped in the core to rapidly diffuse out and also the release medium diffuse in. Whereas large particles have large cores which allow more drug to be encapsulated and slowly diffuse out[15]. Thus it can be inferred that large particles have a small initial burst release and a longer sustained release than smaller particles. In comparison with other formulations NE-4 has more sustained release character and it was statistically significant ($p<0.001$).

Release kinetics

The data release kinetics is shown in Table 5. Among the models tested, the drug release profiles of all formulations (NE1-NE5) were fitted with Higuchi matrix model based on the regression co-efficient (0.985, 0.9952, 0.9942, 0.992,0.996) respectively. The linearity of the plot indicated that the release was diffusion controlled .Thus the amount of drug released was dependent on the

matrix drug load. The diffusion exponent (n) values for all the formulations were less than 0.5 indicate non-fickian mechanism of drug release.

Table 5: Data of kinetic release rate, correlation co-efficient and diffusion exponent value

Formulation code	Zero order "R^2" value	First order "R^2" value	Higuchi plot"R^2" value	Peppa's plot "R^2" value	n value	Best fit model
NE1	0.7203	0.98	0.985	0.9716	0.3091	Higuchi matrix
NE2	0.8157	0.9892	0.9952	0.9926	0.4021	Higuchi matrix
NE3	0.8501	0.9883	0.9942	0.99	0.452	Higuchi matrix
NE4	0.8222	0.9809	0.992	0.9908	0.4181	Higuchi matrix
NE5	0.8224	0.985	0.996	0.9947	0.4098	Higuchi matrix

Stability study of acyclovir nanoemulsion as per ICH guidelines

The emulsion was kept under international conference on harmonization of technical requirements for registration of pharmaceuticals for human use conditions (ICH guidelines): 5°C±3°C, 25°C±2°C/60%±5% relative humidity (RH) and 40°C±2°C/75%±5% RH for 3 months[13]. The results show (Table 6) that there is no significant changes on their physico- chemical characteristics for 3 months at 5°C±3°C, 25°C±2°C/60%±5% relative humidity (RH) and 40°C±2°C/75%±5%RH. When exposed with high temperature and humidity (40°C±2°C/75%±5% RH) acyclovir content was reduced from 84.83% to 67.09% (Table 7, Figure 5, 6, 7). This might be to the heat sensitive effect of acyclovir when exposed to high temperature for longer time. It indicate 5°C±3°C, 25°C±2°C/60%±5% RH is the suitable condition for storage of nanoemulsion for longer period.

Table 6: Effect of temperature and duration of storage on particle size, PDI and zetapotential of the acyclovir nanoemulsion (NE-4)

Temp (°C/%RH)	Duration (days)	Size(nm)	PDI	Zeta potential
5 ±3	30	196.43±0.098	0.543±0.009	-33.41±0.0036
	60	197.8±0.182	0.551±0.0036	-34.12±0.0971
	90	199.5±0.225	0.559±0.0096	-35.54±0.0529
25± 2/60 ±5	30	196.75±0.064	0.498±0.0043	-33.98±0.0793
	60	198.4±0.1	0.543±0.0045	-35.89±0.1044
	90	199.8±0.185	0.561±0.0036	-36.87±0.0871
40± 2/75± 5	30	197.4±0.1	0.554±0.0062	-35.76±0.0871
	60	198.67±0.0624	0.571±0.0036	-38.78±0.0793
	90	199.98±0.441	0.561±0.0036	-39.97±0.0624

(Mean ±S.D, n = 3)

Table 7: *In-vitro* release profile of formulation (NE-4) at various stability conditions

Time (hrs)	Cumulative% drug release at 5°C±3°C			Cumulative % drug release at 25°C±2°C/60%±5%RH			Cumulative % drug release at 40°C±2°C/75%±5% RH		
	30 days	60 days	90 days	30 days	60 days	90 days	30 days	60 Days	90 days
1	26.91	27.89	29.98	26.76	26.01	28.12	20.07	20.92	22.49
2	31.89	31.56	33.76	30.21	30.12	32.01	22.66	23.67	25.32
3	36.91	36.79	38.97	35.71	35.15	37.78	26.79	27.6	29.23
4	42.99	42.53	45.86	41.12	40.12	44.11	30.84	31.93	34.4
6	52.54	51.58	54.45	50.98	50.01	53.32	38.24	38.78	40.84
8	57.78	58.64	61.45	56.01	57.11	60.16	42.01	44.04	46.09
10	63.98	63.24	65.78	62.12	61.99	63.21	46.59	47.43	49.34
12	68.91	68.45	69.87	67.01	67.31	68.23	50.26	51.34	52.41
24	86.71	87.34	89.45	85.78	86.01	88.1	64.34	65.51	67.09

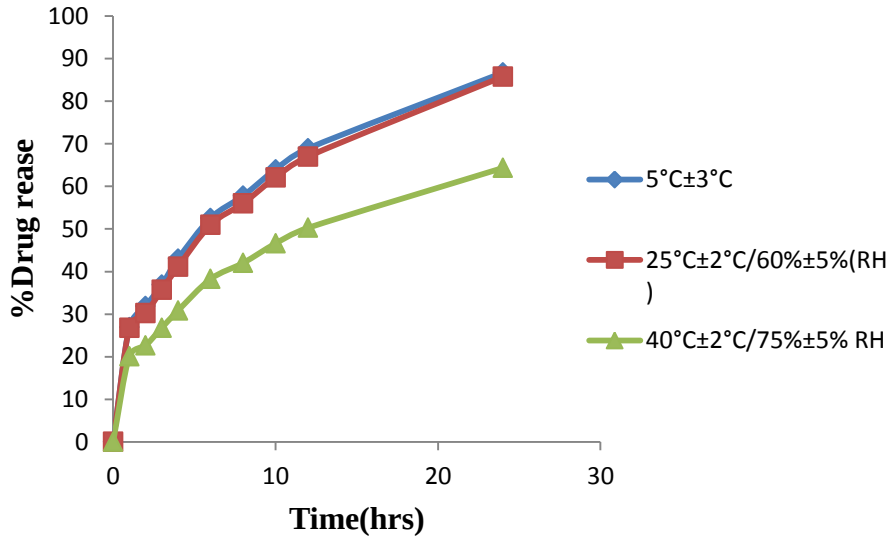

Figure 5*: In -vitro* drug release profile of formulation NE-4 after 30 days

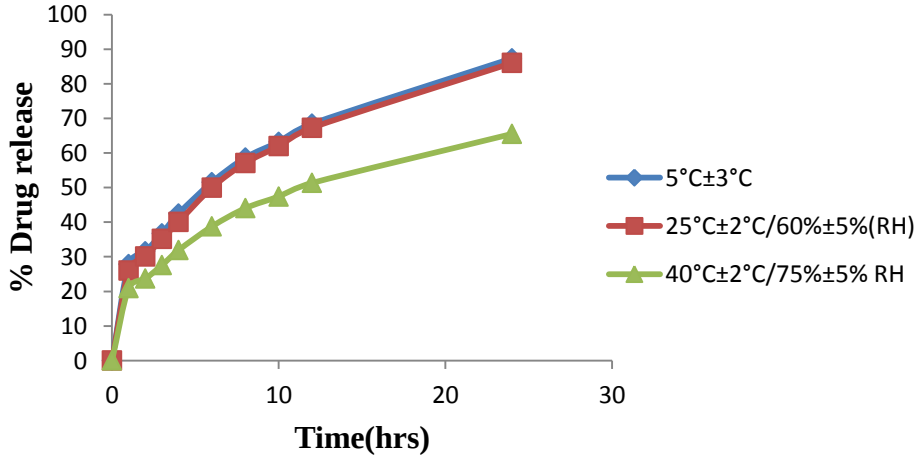

Figure 6: *In -vitro* drug release profile of formulation NE-4 after 60 days

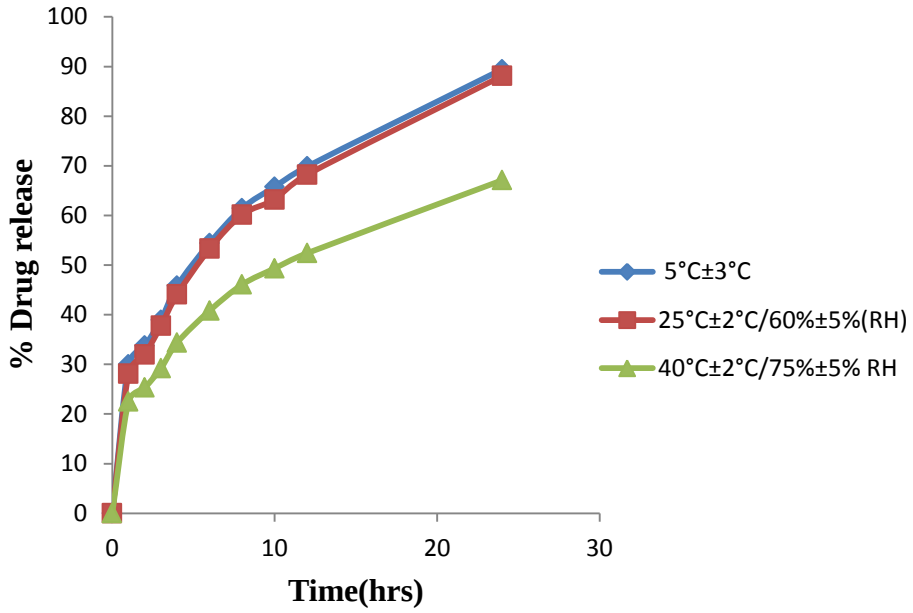

Figure 7: *In -vitro* drug release profile of formulation NE-4 after 90 days

CONCLUSION

The findings of this study demonstrate that acyclovir loaded PLGA nanoemulsion improves the ocular retention time of drug and thus improves the bioavailability of drug. Future work to be taken up with respect to the in-vivo pharmacokinetic and pharmacodynamic studies performed in order to characterize the delivery system for clinical use. It could be beneficial to improve the patient's compliance.

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