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Original Article

Polymeric Ocular Nanosuspension for Controlled Release of Acyclovir: In Vitro Release and Ocular Distribution

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Abstract

The aim of this study is to formulate a novel ophthalmic nanosuspension (ONS), an alternative carrier system to traditional colloidal carriers for controlled release (CR) of acyclovir (ACV). In the present study, ONS is employed to avoid some of major disadvantages of colloidal carriers systems such as instability in cul de sac and short half life by increasing efficiency of drug encapsulation as well as by CR. A quassi-emulsion solvent evaporation method was used to prepare ACV loaded Eudragit RS 100 ONS with the aim of improved ocular bioavailability and distribution. Five different formulations were prepared and evaluated for pH of ONS, particle size, entrapment efficiency, differential scanning calorimetry (DSC), in vitro release profile, in vivo release studies and stability studies. An average size range of 100 to 300 nm in diameter was obtained and encapsulation efficiency up to 95.0% was observed for all the formulations. Cumulative percent drug released for all formulations after 24 h was between 79.28 to 95% indicating effective CR property of ONS. The release profile revealed from best formulation followed Non-Fickian diffusion mechanism. In vivo studies showed that ACV concentration in aqueous humor at 8 h was 82.83, 77.49 and 34.15 mg/ml. Stability studies showed a maximum drug content and almost similar in vitro release compared to the initial data found for the sample stored at 4°C. Overall, the study also revealed that ONS was capable of releasing the drug for a prolonged period of time and increased bioavailability.

Keywords: Acyclovir (ACV); Nanosuspension; Ophthalmic delivery; In vitro release; Paracentesis.

Introduction

Local application of drugs in the forms of drops, colloidal carrier system, gel, etc., to eye is the most popular and well-accepted route of administration for the treatment of various eye disorders. Bioavailability of ophthalmic drug is however very poor due to efficient protective mechanisms of the eye. Blinking, baseline and reflex lachrymation and drainage remove rapidly foreign substances including drugs from the surface of the eye. Moreover, the anatomy, physiology and barrier function of the cornea compromise the rapid absorption of drugs. Frequent instillations of eye drops are necessary to maintain a therapeutic drug level in the tear film or at the site of action. But the frequent use of highly concentrated solutions may induce toxic side effects and cellular damage at the ocular surface (1).

Ophthalmic nanosuspension (ONS) can be

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defined as colloidal dispersions on nano-sized drug particles that are produced by a suitable method and stabilized by a suitable stabilizer; these can prove to be a boon for drugs that exhibit poor solubility in lachrymal fluids (2, 3). Eye diseases can cause therapeutic discomfort and anxiety in patients, with the ultimate fear of loss of vision or even facial disfigurement. Many regions of the eye are relatively inaccessible to systematically administered drugs and as a result, topical delivery remains the preferred route of delivery in most cases (4).

Currently in the management of Herpes Simplex infections, ACV is used as a 3% ointment preparation. The drug bears 10-30% of bioavailability with mean 2.5 h plasma half-life. These conventional preparations are ill-accepted on account of their short pre-corneal retention time, greasiness, vision-blurring effects, etc. (5). Considering the drawbacks associated with the current formulation, the present investigation has been dedicated to prolonging the retention time of medication on the eye surface and to the improvement of transcorneal penetration as well as solubility of poorly soluble drug ACV by formulating nanoparticles.

Experimental

Materials

Acyclovir was a gift sample from Glaxosmithkline Pharmaceuticals Mumbai, India. Pharmaceutical grade Eudragit RS 100 was a gift sample from Rohm Pharma, Degussa, Germany. Tween 80 was purchased from Himedia Laboratories Pvt., Ltd., Mumbai. Benzalkonium Chloride was purchased from Ranbaxy Fine-Chem. Pvt., Ltd., Mumbai.

Methods

Compatibility studies

Compatibility of the ACV with Eudragit RS 100 used to formulate nanosuspension was established by Fourier Transform Infra Red spectral analysis, Thermo Nicolet Corporation, Madison, WI. FT-IR spectral analysis of ACV, Eudragit RS 100 and combination of the ACV with Eudragit RS 100 was carried out to investigate any changes in chemical composition of the drug after combining it with the excipients.

Preparation of nanosuspension

Nanosuspensions were prepared by the quassi-emulsion solvent diffusion technique. The drug and polymer were co-dissolved at room temperature in ethanol (5 ml) and sonicated for 10 minutes. The solution was slowly injected with a syringe into 50 ml water containing Tween 80 (0.02% w/v) and benzalkonium chloride (0.1% w/v) and kept at a low temperature in an iced water bath. During injection, the mixture was mixed by mechanical stirring (propeller 4000 rpm) for 1 h.

The solution immediately turned into a pseudo-emulsion of the drug and polymer-ethanol solution in the external aqueous phase. The counter-diffusion of ethanol and water out of and into the micro-droplets, respectively. After completion of stirring, the solution dispersion was subjected to ultra sonication for a period of 10 min. The gradual evaporation of the organic solvent determined the in situ preparation of the polymer and the drug, with the formation of matrix-type nanoparticles. Ethanol residues were left to evaporate off under slow magnetic stirring of the nanosuspensions at the room temperature for 8-12 h (6).

Using this above method, 5 formulations of nanosuspensions FM-1, FM-2, FM-3, FM-4 and FM-5 were prepared by varying polymer ratio as shown in Table 1.

Evaluation of pH

pH is one of the most important factors involved in the formulation process. Two areas of critical importance are the effects of pH on solubility and stability. The pH of ophthalmic formulation should be such that the formulation will be stable at that pH and at the same time there would be no irritation to the patient upon administration of the formulation. The pH of the prepared formulations was checked by using pH meter.

Particle size and surface morphology

Particle size analysis was done by Scanning Electron Microscopy (SEM). SEM is the most commonly used method for characterizing drug delivery systems, due to simplicity in sample

Table 1. Formulation plan for acyclovir loaded nanosuspensions.

Ingredients	FM-1	FM-2	FM-3	FM-4	FM-5
Acyclovir (% w/w), (mg)	350	350	350	350	350
Eudragit RS 100 (% w/w), (mg)	550	450	350	250	150
Tween 80 (% w/v)	0.02	0.02	0.02	0.02	0.02
Benzalkonium chloride (% w/v)	0.1	0.1	0.1	0.1	0.1
Ethanol (ml)	5.0	5.0	5.0	5.0	5.0
Water 50 (ml) qs to	50	50	50	50	50

preparation and ease of operation. Particle size analyses were done by SEM using JEOL JSM-T330A scanning microscope. Cleaned brass specimen studs were used for mounting the samples. Wet solvent paint was applied on these studs and while the paint was wet, the pellets were placed on each stud and allowed to dry. Then the sample was observed in scanning electron microscope and photographs were taken (6-8).

Determination of drug entrapment efficiency

The percentage of incorporated ACV (entrapment efficiency) was determined spectrophotometrically at 254 nm. After centrifugation of the aqueous suspension, amount of the free drug was detected in the supernatant and the amount of incorporated drug was determined as the result of the initial drug minus the free drug (6, 10).

The entrapment efficiency (EE %) could be achieved by the following equation:

$$\text{Entrapment efficiency (\%)} = \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \times 100$$

Differential scanning calorimetry (DSC)

Differential scanning calorimetric analysis was performed using Shimadzu DSC-60 system. Sample of ACV drug (10 mg) was placed in a sealed aluminium pan and formulations i.e., FM-1, FM-2, FM-3, FM-4, FM-5 were taken (50 µl) in an aluminium pan and sealed with lid so that liquid should not spill outside. The prepared samples were heated at a rate of 10°C/min in 50-300°C range, using an empty sealed pan as a reference. Enthalpy changes (ΔH) were calculated from peak areas of samples and to study the polymorphic changes in the formulations (11, 12).

In Vitro drug release studies

The in vitro release of ACV from the formulation was studied through Dialysis membrane-110 (cut-off: 3500 Da) using modified apparatus. The dissolution medium used was freshly prepared 0.14 M phosphate buffer solution (pH 7.4). Dialysis membrane-110, previously soaked overnight in the dissolution medium was tied to one end of a specifically designed glass cylinder (open at both ends). Five ml of formulation was accurately placed into this assembly. The cylinder was attached to a stand and suspended in 50 ml of dissolution medium maintained at 37±1°C so that the membrane just touched the receptor medium surface. The dissolution medium was stirred at low speed using magnetic stirrer. Aliquots, each of 1 ml volume were withdrawn at hourly intervals and replaced by an equal volume of the receptor medium. The aliquots were suitably diluted with the receptor medium and analyzed by UV-Vis spectrophotometry at 254 nm.

To check the eventual limiting effects of the dialysis membrane on drug dissolution, separate experiments were run in duplicate with a solution of saline, freshly prepared 0.14 M phosphate buffer solution (pH 7.4) in pure ACV of the drug concentrations present in the nanosuspensions (3, 6, 10).

In Vivo studies

In vivo studies were performed on groups of six

male New Zealand albino rabbits weighing 1.8-2.2 kg and with no signs of ocular inflammation or gross abnormalities. The animal's procedure is followed as per CPCSEA guidelines.

The animals were divided into two groups, one for FM-2 formulation containing 2 rabbits and second group for FM-3 formulations that contains four rabbits. For FM-3 formulation, the left eyes of rabbits for control preparation and the right eyes of the rabbits for prepared formulation.

Out of 5 batches of formulations FM-2 and FM-3 were taken for in vivo study on the basis of in vitro drug release studies. The pure drug ACV was dissolved in saline, freshly prepared 0.14 M phosphate buffer solution (pH 7.4) as controlled preparation. The preparations were sterilized by using UV radiation before in vivo study. The 100 µl of preparations were instilled into the conjunctival sac (left eye control preparation and right eye nanosuspension) at 60, 120, 240 and 480 min durations.

To perform the paracentesis (the removal of fluid from a body cavity using a needle/ puncture of the wall of a fluid-filled cavity by means of a hollow needle to draw off the contents), animals were lightly anaesthetized with an i.v. injection of ketamine hydrochloride (20 mg kg^{-1}). One drop of local anesthetic (0.4% Topical Xylocaine) was instilled into the conjunctival sac. Aqueous humour samples from each animal were collected with a 26 gauge needle attached to tuberculin syringe. The needle was introduced into the anterior chamber through the cornea, tacking care not to damage the iris, the lens and the anterior uvea. Eye conditions were examined using a slit lamp every hour after paracentesis and Cyclopentolate hydrochloride (Cyclogik) eye drop were put to avoid inflammation. Fifty microlitres of aqueous humour were collected and analyzed by HPLC for drug concentration. Samples were denatured by the addition of an equivalent volume of 2% $ZnSO_4$. $7H_2O$ solution, centrifuged and the supernatants were filtered through a 0.2-µm Teflon membrane. Samples (20 µl) were analyzed by an HPLC at detection wavelength 254 nm (6, 7, 13, 14).

Mobile phase = ammonium acetate (10 mM; pH 6.8)-acetonitrile (99:1)

Flow rate = 1 ml/ min

Detection = 254 nm

Column = Chemisorb C-18 cartridge column (250 mm × 4.5 mm i:d.; 5 µm)

Concentration in µg/ml =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Standard weight}}{\text{Dilution factor}} \times \frac{\text{Dilution factor}}{\text{Sample. weight}} \times \text{Purity}$$

Short-term stability study

Information on the stability of drug substance is an integral part of the systematic approach to stability evaluation. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under influence of variety of environmental factors such as temperature, humidity and light, and to establish a re-test period for drug substance or a shelf life for the drug product and recommended storage conditions. Stability is defined as the extent to which a product remains within specified limits throughout its period of storage and use. A drug formulation is said to be stable if it fulfils the following requirements:

- It should contain at least 90% of the stated active ingredient
- It should contain effective concentration of the added preservatives, if any
- It should not exhibit discoloration or precipitation, nor develops foul odor
- It should not develop irritation or toxicity.

Procedure

From the 5 batches of ACV loaded nanosuspension, formulation FM-3 was tested for stability studies. Formulation was divided into 3 sample sets and stored at:

- 4°C in refrigerator
- 37°C±2°C/65% RH±5% RH in humidity control oven (GINKYA IM 3500 series).
- Ambient temperature and humidity.

After 30 days drug content of all the samples were determined by the method discussed previously in entrapment efficiency section. In vitro release study of formulation FM-3 was also carried out after 30-day storage (15, 16).

Table 2. Best – fit models for all formulations.

Formulation	Mathematical models					'n' Values	Best fit model
	First order	Zero order	Higuchi's matrix	Peppa's plot	Hixson Crowell		
FM-1	0.9825	0.8236	0.9931	0.9941	0.9498	0.5420	Peppas
FM-2	0.9429	0.8322	0.9946	0.9918	0.9868	0.5557	Higuchi matrix
FM-3	0.9880	0.8758	0.9983	0.9977	0.9889	0.5463	Higuchi matrix
FM-4	0.9973	0.8826	0.9982	0.9989	0.9848	0.5667	Peppas
FM-5	0.9678	0.8442	0.9922	0.9931	0.9472	0.5409	Peppas

Results and Discussion

Compatibility study

From the IR spectral analysis, it was found that IR spectrum of pure drug ACV and combination of pure drug with polymers like Eudragit RS 100 showed the all characteristic peaks of ACV confirming the compatibility of the pure drug and polymer.

pH

pH values for all the formulations are within acceptable range 5.8-6.5 and hence would not cause any irritation upon administration of the formulation. It was also observed that increase in Eudragit RS 100 polymer causes a slight increase in pH for formulations.

Particle size and surface morphology

Scanning electron photomicrographs of all the five formulations are shown in Plates No. 1 and 2. Magnifications of 15,000-20,000 X were used while taking these photomicrographs. Average particle size of nanoparticles of ACV was found for FM-1, FM-2, FM-3, FM-4 and FM-5 was 150-300 nm, 125-275 nm, 150-300 nm, 100-450 nm, 100-350 nm. Particles of all formulations except FM-4 were smooth, oval and discrete whereas particles of FM-4 were slightly rough surfaced and non discrete.

Drug entrapment efficiency

The drug content in five batches of ACV nanoparticles was studied. The amount of drug bound per 1 ml of nanosuspension was determined in each batch. It was observed that the entrapment efficiency increased with the increase in concentration of polymer in the formulations. The maximum entrapment was found in FM-2 (95.0%) and lowest entrapment in FM-5 (83.51%).

Differential scanning calorimetry (DSC)

The DSC thermograms for drug as well as formulations are represented in Figure 1 and 2. DSC analysis of ACV shows the endothermic peak at its melting point i.e. at 249.86°C (ΔH =

Scanning electronic micrographs of acyclovir loaded nanoparticles.

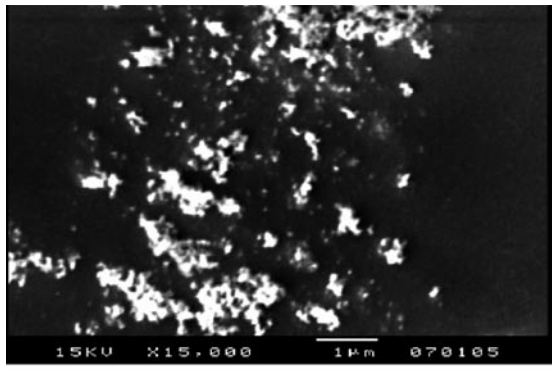

Plate 1: SEM of Formulation 2

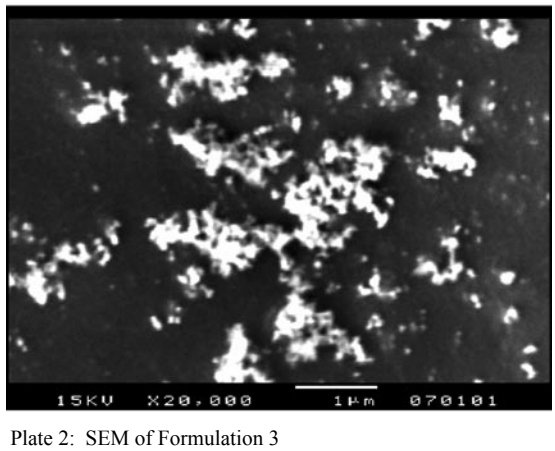

Plate 2: SEM of Formulation 3

Figure 1. SEM photographs of 1) Formulation-2 2) Formulation-3.

Table 3. In vivo release of acyclovir after topical administration of acyclovir loaded nanosuspensions.

Time (h)	Control C-1		Formulation FM-2		Formulation FM-3	
	AUC	Concentration (µg/ml)	AUC	Concentration (µg/ml)	AUC	Concentration (µg/ml)
1	138684±1.76	21.30±1.88	169075±2.09	25.97±2.04	178994±2.66	27.49±2.33
2	176457±2.40	27.10±2.20	225307±2.72	34.71±2.36	271575±2.33	41.71±3.16
4	222345±2.90	34.15±2.45	504527±2.30	77.49±2.15	539243±3.21	82.83±3.60
8	183462±1.51	28.18±1.75	346726±3.18	53.25±1.59	377126±2.02	57.92±2.01

132.16 J/g). DSC curves of the binary systems observed at 101.59°C, 100.49°C, 97.73°C, 123.7°C and 120.60°C, they showed complete disappearance of the melting endotherm of ACV, which could indicate the complete amorphization of the drug as well as loss of drug crystallinity, which indicates the change in melting point, release kinetics and bioavailability.

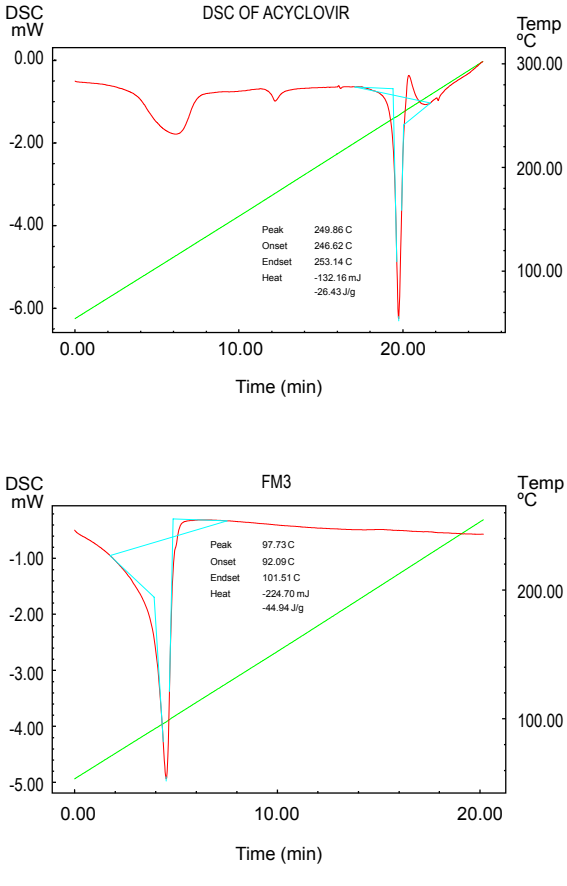

Figure 2. DSC Thermograms of 1: Acyclovir 2: Formulation-1 3: Formulation-2 4: Formulation-3 4: Formulation-4 5: Formulation-5.

In vitro release studies

Cumulative percent drug released for FM-2, FM-3 after 24 h was 92.14%, 95.0% and for FM-1, FM-4 and FM-5 after 24 h was 82.14%, 82.13% and 79.28%, respectively.

The in vitro release of all the five batches of nanosuspensions showed an interesting bi-phasic release with an initial burst effect. In the first hour, drug released was 15.0%, 15.71%, 16.42%, 15.36% and 12.85% for FM-1, FM-2, FM-3, FM-4 and FM-5, respectively. Afterwards the drug release followed a steady pattern approximating zero order release. The burst release in the first hour can be attributed to the drug loaded on the surface of nanoparticles.

The kinetic values obtained for different formulations are indicated in Table 2. The values of in vitro release were attempted to fit into various mathematical models. Plots of zero order, first order, Higuchi matrix, Peppas and Hixson Crowell are depicted in Figure 3, respectively.

The correlation coefficients for formulations FM-1 to FM-5 of zero order plots were found to be 0.8236, 0.8322, 0.8758, 0.8826 and 0.8442, respectively.

The correlation coefficients for formulations FM-1 to FM-5of first order plot were found to be 0.9825, 0.9429, 0.9880, 0.9973 and 0.9678, respectively. Based on the highest regression values (r), the best-fit model for FM-1, FM-3 and FM-4 followed first order FM-2 and FM-5 followed zero order release.

The correlation coefficients of formulations FM-1 to FM-5 of Higuchi matrix plot were found to be 0.9931, 0.9946, 0.9983, 0.9982 and 0.9922. It was observed that FM-2, FM-3 followed Higuchi matrix suggesting drug release by diffusion.

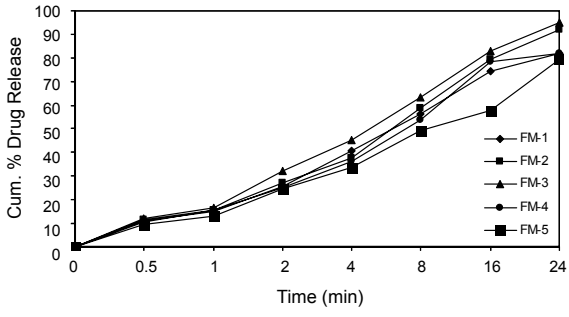

Figure 3. Comparative In vitro drug release profile for all formulations following zero order. FM-1, FM-2, FM-3, FM-4 & FM-5.

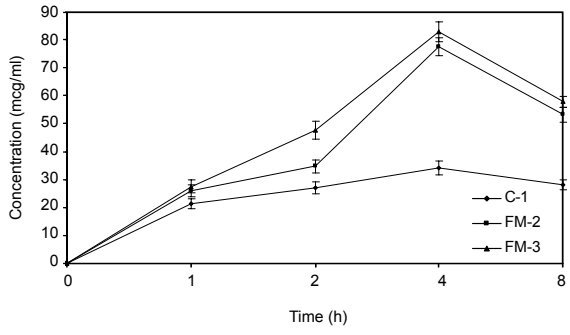

Figure 4. Plots of In vivo release of acyclovir after topical administration of acyclovir loaded nanosuspensions C-1, FM-2 & FM-3.

The 'n' values for FM-1 to FM-5 were -0.0791, -0.1036, -0.1130, -0.0968 and -0.0680 respectively. FM-1 and FM-5, which is less than 1 and greater than 0.5 follows Non Fickian diffusion mechanism

Hixson Crowell correlation coefficients of formulation FM-1 to FM-5 were found to be 0.9498, 0.9868, 0.9889, 0.9848 and 0.9472 respectively. These results indicate that FM-3 appears to fit in Hixson Crowell model. Here it can be assumed that the release rate was limited by the drug particles dissolution rate and erosion of the polymer matrix.

In vivo drug studies

Formulation, FM-2 and FM-3 with optimal particle size and satisfactory in vitro release was selected for in vivo drug studies. Drug concentration was determined by HPLC method. The drug concentration was determined in each formulation by calculating the peak areas of ACV pure drug and different formulations and control formulations as shown in Figure 4 with HPLC graphs. Table 3 results of concentrations in each of these formulations. The maximum concentration was found to be in FM-3 at fourth hour 82.83 µg/ml where as controlled preparation showing 34.15 µg/ml, and the lowest concentration was found to be at first hour by CF-1 is 21.30 µg/ml. In vitro release studies and in vivo studies were based on content of drug and polymer ratio present and their release at different time intervals. The concentrations of drug obtained from aqueous humors with formulations C-1, FM-2 and FM-3 were studied and pharmacokinetic parameters such as C_{max}, T_{max} and AUC values are shown in Table 3, significance was studied by one way ANOVA which showed the P value 0.0845.

Stability studies

Stability studies of the prepared nanosuspensions were carried out, by storing formulation FM-3 at 4°C in refrigerator, ambient temperature and humidity and 37±2°C, 65% ±5% RH in humidity control oven for thirty days. Two parameters namely residual percent drug content and in vitro release studies were carried out.

These studies revealed that there is a reduction in drug content after storage for thirty days at 4°C, ambient temperature and humidity and 37±2°C/65% ±5% RH. It was also revealed that FM-3, the one stored at 4°C showed maximum residual drug followed by that stored at ambient temperature and humidity and 37± 2°C/65% ± 5% RH.

In vitro release studies, which were carried out after storing a selected formulation (FM-3) at 4°C, ambient temperature and humidity and 37±2°C/65% ±5% RH for thirty days.

In vitro release studies proved that the formulation stored at 4°C showed 94.52 % release, the one which stored at ambient temperature and humidity showed 93.77 % and formulation stored at 37± 2°C/65% ±5% RH showed 92.15% release after 24 h.

These results indicate that the drug release from the formulation stored at 37±2°C/65% ±5%

RH was highest followed by formulation stored at ambient temperature and humidity and 4°C.

On comparing this data with the previous release data of FM-3, it was observed that there was an overall increase in the drug release.

These results may be attributed to erosion of nanoparticles to some extent during storage.

Conclusion

From the above findings it is evident that, polymeric system of ACV loaded nanoparticles have achieved the objectives of increased contact time, prolonged release and decreased frequency of administration. Drug release studies indicates that the no significant increase drug release with increase in drug to polymer ratio but also depends on the pH, particle size. In vivo release profile indicated that polymeric system of ACV has achieved the objectives of increased contact time, prolonged release, decreased frequency of administration, avoidance of eye irritation and redness of the rabbit eye

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