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FORMULATION AND EVALUATION OF OCULAR *IN-SITU* GEL OF ANTIVIRAL AGENT: ACYCLOVIR

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ABSTRACT

The key objective of the present study was to formulate and evaluate *in-situ* gelling system based on sol-to-gel transition for ophthalmic delivery of an antiviral agent acyclovir, to overcome the problems of poor bioavailability and therapeutic response exhibited by conventional formulations. Acyclovir is an antiviral agent preferentially used in the treatment of infections caused by herpes simplex virus and highly useful to treat herpes simplex keratitis. The significant problem in the ocular drug delivery is the achievement of optimal drug concentration at the site of action. Development of therapeutic agents which need a repeated long-term administration is a carter for the sustained release drug delivery systems, to result in less frequent dosing and less invasive techniques. Therefore, *in- situ* gel a novel drug delivery system has been developed to overcome the anatomical barriers and ocular bioavailability constrains. Gellan gum natural polymer was used as the gelling agent and also acted as a viscosity enhancing agent. The prepared formulations were evaluated for pH, viscosity, clarity, drug content, gelling capacity, *invitro* drug release. The developed *in-situ* gel provided sustained drug release over a 6 hours period of time. The optimized formulation was found to be nonirritating with no ocular damage to the cornea and conjunctiva. Hence the developed formulation can be used as an in-situ gelling vehicle by virtue of its increased corneal residence time and prolonged drug release could be considered a viable alternative to achieving enhanced bioavailability and helped in the reduction in the frequency of instillation there by resulting in better patient compliance.

KEYWORDS: *In-situ* gel, Acyclovir, Natural polymer, Gellen gum.

INTRODUCTION

Opthalmic Drug Delivery System

Ophthalmic drug delivery is one of the challenging endeavors which are being faced by the pharmaceutical scientist, owing to the anatomy, physiology and biochemistry of the eye that renders it impervious to foreign substances. Topical administration of ophthalmic medications is the most common method for treating conditions that affect the exterior parts of the eye. The unique anatomy and physiology of the eye makes it difficult to achieve an effective drug concentration at the target site. Therefore, the major challenge remains to efficiently deliver a drug past the protective ocular barriers accompanied with a minimization of its systemic side effects.

It does formulate in-situ gelling system of **"HERPES SIMPLEX KERATITIS"** disease using antiviral drug **(ACYCLOVIR).** Develop as insitu gel formulation using a suitable phase transition polymer to effectively deliver the drug with sustained & prolonged release & enhance the drug bioavailability .Marketed eye drops and suspensions would not remain in the eye due to the lacrimal fluid turn over and so, the control over the intraocular pressure would not be maintained in anti viral drugs.

Herpes simplex virus (HSV) keratitis is the most frequent cause of corneal blindness in the United States and the most common source of infectious blindness in the Western world. The prognosis in HSV keratitis, however, is generally favorable with aggressive treatment.

HSV Keratitis is caused by the herpes simplex virus, a double stranded DNA virus made up of an icosahedral shaped capsid surrounding a core of DNA and phosphoproteins of viral chromatin. HSV I and HSV II are differentiated by virusspecific antigens. HSV I typically affects the oropharynx region while HSV II usually involves the genital area, though studies have shown that both viruses may affect either location.

The prime symptom of HSV keratitis is the presence of multiple small branching epithelial dendrites on the surface of the cornea, although often times it first presents as a coarse, punctuate epithelial keratitis (and may be mistaken for a viral keratitis).

Plan of work

Materials and methods

Acyclovir was obtained as a gift sample from Yarrow chem. pvt. Ltd.Mumbai, India. All other chemicals/reagents used were of analytical grade, available commercially and used as such without further processing. Gallen gum was obtained from Yarrow chem. pvt. Ltd., Mumbai, India..Boric acid, phenyl mercuric nitrate, Disodium edentate & PEG 400 were used as for drug analysis from Analytical grade.

Preformulation studies

Physical appearance

The sample drug (acyclovir) was found to be in powdered form, white in color which is in accordance with standard.

Melting Point

The Melting point was determined by the capillary method using Melting point apparatus. The capillary tube was filled by pressing the open end gently into Acyclovir (pure drug) sample by tapping the bottom of the capillary on a hard surface so that the drug pack down into the bottom of the tube. When the drug was packed into the bottom of the tube, the tube was placed into the slot behind the eye-piece on the Melt- temperature. Make sure the unit was plugged in and set to zero, and then turn it on. Temperature range was noted when sample started melting. Triplicate observations were recorded for melting range determination.

Determination Of Λ_{Max} By Uv Spectrophotometer

One hundred mg of Acyclovir drug powder was accurately weighed and transferred to a 100 ml volumetric flask. It was dissolved in an adequate amount of distilled water and the volume was made upto 100 ml with distilled water so as to obtain a stock solution of 1000 µg/ml. A dilution of 20 µg/ml concentration was made from the above stock solution with the distilled water and the resulting **solution was** scanned on a Double-beam UV-visible spectrophotometer (Shimadzu 1800) between wavelength ranges of 200 nm to 400 nm.

Preparation Of Calibration Curves

Preparation of calibration curve of Acyclovir in Distilled Water

Accurately weighed quantity of acyclovir (10 mg) was taken in 100 ml volumetric flask. It was dissolved in an adequate amount of distilled water and the volume was made upto 100 ml to obtain a stock solution of 100µg/ml. From the above stock solution appropriate dilutions were made in distilled water the concentration range of 4, 8, 12, 16, 20 and 24 µg/ml and absorbance was taken at λmax 252nm.

Preparation of calibration curve of Acyclovir in Simulated Lacrimal Fluid

Accurately weighed quantity of acyclovir (10 mg) was taken in100 ml volumetric flask. It was dissolved in an adequate amount of simulated lacrimal fluid and the volume was made upto 100 ml to obtain a stock solution of 100 µg/ml. From the above stock solution appropriate dilutions were made in simulated lacrimal fluid the concentration range of 4, 8, 12, 16, 20 and 24 µg/ml and were analyzed spectrophotometrically at 252 nm against a blank prepared in the same manner. The absorbance data for different concentrations were subjected to regression analysis.

Preparation of calibration curve of Acyclovir in Phosphate Buffer (pH 7.4)

Accurately weighed quantity of acyclovir (10 mg) was taken in 100 ml volumetric flask. It was dissolved in an adequate amount of PBS(pH 7.4) and the volume was made upto 100 ml to obtain a stock solution of 100 µg/ml. From the above stock solution appropriate dilutions were made in PBS(pH 7.4) the concentration range of 4, 8, 12, 16, 20 and 24 µg/ml in 10 ml volumetric flask and absorbance was taken at λmax 252nm.

Solubility Studies

Solubility may be defined as the spontaneous interaction of two or more substances to form a homogenous molecular dispersion. Excess of drug was suspended successively in 10 ml of each solvent at room temperature in a tightly closed 10 ml vial and shaked for few hours until the drug dissolve. Allow to sonicate in a Bath sonicatortan suspension was filtered and the filtrate was analyzed for soluble content using UV spectrophotometer.

Drug - Excipient Interaction Studies

The Drug were physically mixed with drug in 1:1 ratio and filled in amber colored vials which were then properly capped and sealed. The vials of each sample were kept at room temperature and in refrigerator for one month period. After every week for one month, the vials were withdrawn and changes in physical appearance and color of the contents were observed.

Selection of Excipients

Gellan gum shows ion activated phase transition behaviour. Gellan gum solutions of concentration range 0.1-1% were studied. It was observed that 0.3-0.7% solutions shows desired behaviour in presence of ions present in physiological fluid.

Gelation studies of Gellan Gum with Simulated Lacrimal Fluid

On the basis of physical characteristics of different concentration of gellan gum solution, concentration range of 0.1-0.7% was selected and integrity of gel formed with simulated lacrimal fluid was observed. For the estimation of gelling property cylindrical tube of 5

cm length and 1 cm diameter was taken and filled with the simulated lacrimal fluid. Gellan gum solution was added to it and assessed for gelling property**.**

\+ Gelation slowly and dissolve.
++ Gelation immediate and remains for few hours.
+++ Gelation immediate and remained for extended period of time.

***In-vitro* drug release study of Acyclovir In-situ Gel formulation (Batch 1,2,3,4,5,6)**

Since the drug from the formulation will be released on the surface of eye, simulated lacrimal fluid was selected as the medium for drug release study.

Experimental Conditions
Release Medium - Simulated lacrimal fluid (SLF)
Volume - 25 ml
Temperature - 37˚C
Speed - 30 rpm

Procedure

The *in-vitro* release of acyclovir from the prepared formulations was 100µl of developed Acyclovir in-situ gel formulation (Batch 1) was applied over the egg membrane fitted at one end of cylinder having a length 7 cm and diameter 1 cm. The cylinders were then suspended into 25 ml of SLF separately maintained at 37˚C and adjusted to rotation at 30 rpm in a 100 ml beaker. Aliquots of fluid were withdrawn at fixed time intervals over 8 hrs and were replaced with an equal volume of fresh SLF maintained at same temperature. Aliquots of withdrawn samples were analyzed for drug content at 252 nm using a double beam UV- visible spectrophotometer (Shimadzu1800) the same procedure was repeated for formulation (Batch 2,3,4,5,6)

Formulation of *In-Situ* Gelling System

Boric acid was dissolved in 100 ml of distilled water. Gellan gum was dispersed in a part of above solution and stirred for 20 min at 90◦C temperature. After cooling to room temperature, drug and preservative were added and stirred until drug dissolved. Remaining amount of boric acid solution was added into it. The resulting solution was stored in a refrigerator for further use. Crystallization of the drug was observed after 48 hour. To avoid crystallization of drug in freeze thaw conditions, 0.05% of disodium edetate was incorporated in the formulation. It is also reported in literature, that disodium edetate prevents crystallization in freeze thaw conditions. Formulation was terminally sterilized by autoclaving at 121◦C temperature and 15 psi pressure for 15 min. Composition of the formulation with and without disodium edetate is shown in table.1

Table 1: Composition of Acyclovir In-Situ Gel Formulations.

S. No.	Ingredient	Quantity (gm)					
		Formulation Code					
		F1	F2	F3	F4	F5	F6
1.	Acyclovir	0.3	0.3	0.3	0.3	0.3	0.3
2.	Gellan gum	0.6	0.6	0.6	0.6	0.6	0.6
3.	Boric acid	1.68	1.68	1.68	1.6	1.68	1.68
4.	Phenyl mercuric nitrate	0.002	0.002	0.002	0.002	0.002	0.002
5.	Disodium edentate	-	0.01	0.05	0.05	0.05	0.05
6.	PEG 400	-	-	-	0.1	0.5	1
7.	Distilled water	100	100	100	100	100	100

Evaluation Studies

Transcorneal Permeation Study

Permeation study of developed Acyclovir in-situ gel formulation (Batch 05) was carried out using excised cornea of goat's eye and was compared to that of marketed formulation of Acyclovir (Acivir) in order to determine the permeation profile of Acyclovir from gel across the corneal membrane. Whole eyeballs of goat were procured from slaughter house and safely transported to laboratory in cold condition. The corneas were carefully removed along with 5-6 mm of surrounding sclera and washed with cold saline solution. The washed corneas were stored in cold freshly prepared solution simulated lacrimal fluid.

Corneal Membrane Integrity Test

Phenol red is generally used as a non absorbable marker in in-vitro, in-situ and in- vivo absorption experiments and has been also proposed for assessing the integrity of biological membranes. The purpose of this test was to ensure that barrier properties of the membrane were maintained not only in the beginning but also at the end of the experiment.

Phenol red was added to the cylindrical tube to which cornea was tied after permeation study of 7 hr. It was observed for 30 min and also estimated spectrophotometrically at 546 nm. It was then compared with the positive control i.e. the cornea which was pierced and then tied to the cylindrical tube of same dimensions. Within few seconds, solution in receptor compartment became red which was estimated by double beam UV-visible spectrophotometer at 546 nm.

Isotonicity Evaluation

Isotonicity is an important characteristic of the ophthalmic formulations. Isotonicity has to be maintained to prevent tissue damage and irritation to the

eye. The final optimized in-situ gel formulation (Batch 5) was subjected to the isotonicity testing.

Procedure

Blood was procured from the slaughter house and was stored in a refrigerator for further use. It was centrifuged using cooling centrifuge at 4°C applying centrifugal force of 14000 G for 10 min. From each centrifuge tube supernatant was discarded and 0.5 ml of RBCs was recovered. Saline solution (0.9% NaCl) was added to each tube and resuspended the RBCs on the vortex for 2 min. Smear of Acyclovir in-situ gel formulation (AN/ACYISG/05) was prepared with the resuspended RBCs and observed under the microscope at 45X magnification. Same procedure was followed for the marketed Acyclovir ointment (Acivir), isotonic solution (negative control) as well as hypertonic and hypotonic solution (positive controls). Shape of the blood cells with Acyclovir in-situ gel formulation was compared with that of blood cells with marketed Acyclovir ointments (Acivir) as well as with the positive and negative controls.

Viscosity Studies

Viscosity determinations of the developed Acyclovir in-situ gel formulation was done using Brookfield viscometer (LVT) model. Viscosity of the sample solution was measured over a range of 0.3 to 30 rpm speed. The hierarchy of speed was reversed from 30 to 0.3 rpm. The average of the two dial readings was used to calculate the viscosity. To evaluate viscosity change after instillation and mixing with lacrimal fluid, rheological measurements were taken after diluting the formulation with the simulated lacrimal fluid.

Het-Cam (Hen's Egg Test or Hulner Embryogen) Test

This test is used for the detection of ocular corrosives and irritants. The potential ocular irritancy of a test substance is measured by its ability to induce toxicity in the chorioallantoic membrane of a chicken. The effects are measured by the onset of hemorrhage, coagulation, and vessel lysis. These assessments are considered individually and then combined to derive a score; irritation score (IS) which is used to classify the irritancy level of the test substance.

$$\left(\left(\frac{(301-\textit{Hemorrhage time})}{300}\right)\times 5\right)+\left(\left(\frac{(301-\textit{Lysis time})}{300}\right)\times 7\right)+\left(\left(\frac{(301-\textit{Coagulation time})}{300}\right)\times 9\right)$$

Hemorrhage time = observations in seconds of hemorrhage reactions on CAM

Lysis time = observations in seconds of vessel lysis on CAM

Coagulation time = observations in seconds of coagulation formation on CAM

A test is considered acceptable if the negative and positive controls each induce a response, which falls within the classification of nonirritating and severely irritating, respectively.

Cam Preparation

In this method, fertilized hen's eggs weighing between 50-60 gm were obtained from poultry farm. The eggs were candled to identify and discard the defective ones. These eggs were incubated in a humidified incubator at a temperature 37°C and 75% RH. The trays containing eggs were rotated manually in a gentle manner every hour. On 10th day, a window (2x2 cm) was made on pointed end of eggs through which 0.2 ml of developed Acyclovir in-situ gel formulation (AN/ACYISG/05) was instilled. A 0.9% NaCl solution was used as negative control and 1% NaOH as positive control in present study. After instillation of the test samples, the chorioallantoic membrane was observed for a period of 5 min for hemorrhage, coagulation and vessel lysis.

S. No.	Effect	Score	Inference
1.	No visible haemorrhage	0-0.9	Non- irritant
2.	Just visible membrane discoloration	1-4.9	Slight irritant
3.	Structures are covered partially due to membrane discoloration	5-8.9	Moderate irritant
4.	Structures are covered totally due to membrane discoloration/haemorrhage	9-21	Severe irritant

Stability Studies

The physical stability, including appearance, colour, and pH of the formulation were studied under various storage conditions. None of the samples showed any change in colour or appearance under all storage conditions for one month period. The drug content of the formulation was estimated initially and then after one month using double beam UV-visible spectrophotometer (Shimadzu1800).

RESULTS AND DISCUSSION

1. Determination of Absorption Maxima (λmax)

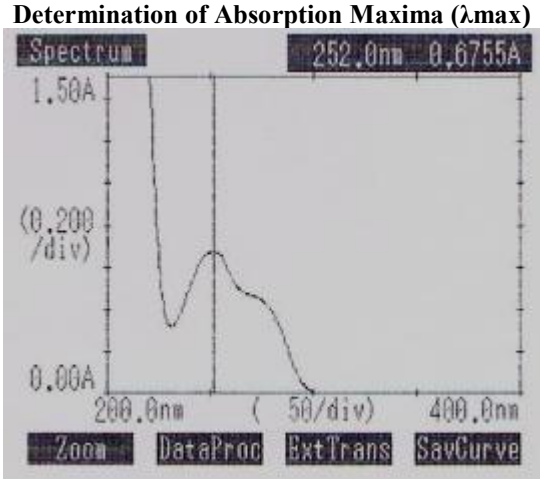
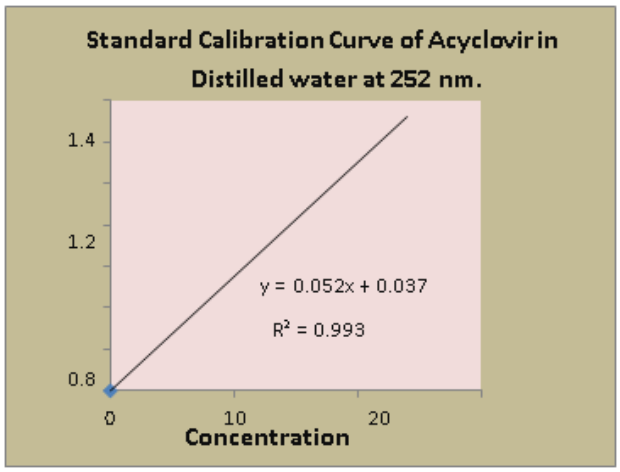
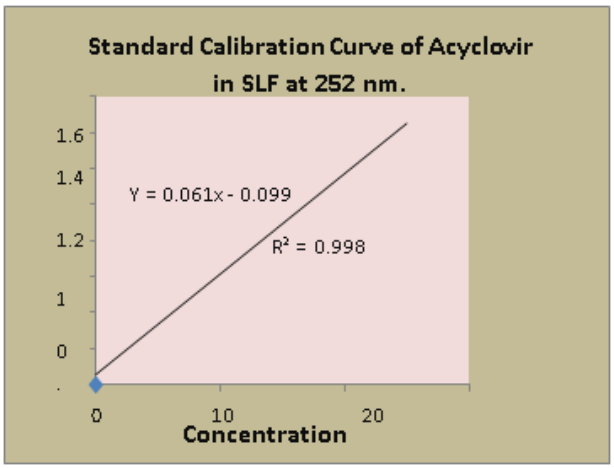


Fig. 1: UV Spectra of Acyclovir in Distilled Water.

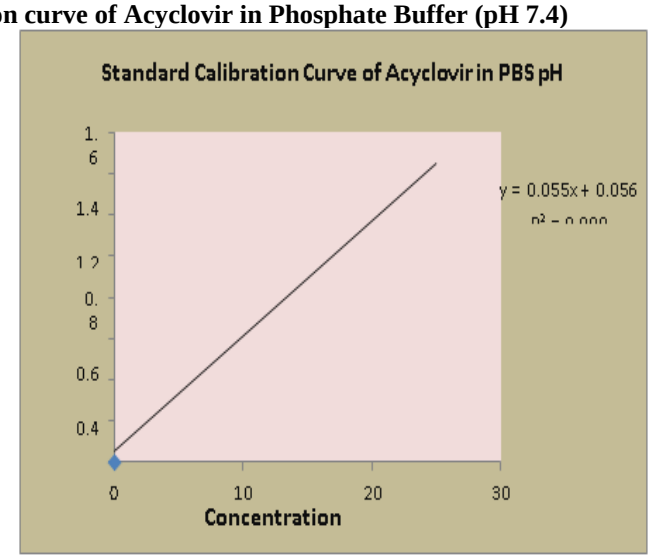
2. Preparation of calibration curve of Acyclovir in Distilled Water



3. Preparation of calibration curve of Acyclovir in Simulated Lacrimal Fluid



4. Preparation of calibration curve of Acyclovir in Phosphate Buffer (pH 7.4)



5. Solubility Studies of Acyclovir in Various Solvents.

S.No.	Solvent	Solubility (mg/ml)	Inference
1	Distilled water	6.80	Slightly soluble
2	Acetate Buffer	7.60	Slightly soluble
3	PBS (pH 7.4)	3.73	Slightly soluble
4	Simulated Lacrimal Fluid	3.81	Slightly soluble

Acyclovir was found to be slightly soluble in water, acetate buffer, simulated lacrimal fluid and PBS (pH 7.4).

6. Drug-Excipients Interaction Studies

S. No.	Drug-excipient Blend	Initial Physical State	Observation at Different Storage Conditions											
			2-8 ˚C				25 ˚C				40 ˚C			
			Duration (weeks)											
			1	2	3	4	1	2	3	4	1	2	3	4
1.	AC	OWP	N	N	N	N	N	N	N	N	N	N	N	N
2.	AC + Gellan gum	OWP	N	N	N	N	N	N	N	N	N	N	N	N
3.	AC + Phenyl mercuric nitrate	OWP	N	N	N	N	N	N	N	N	N	N	N	N
4.	AC +Disodium Edentate	OWP	N	N	N	N	N	N	N	N	N	N	N	N
5.	AC + Boric acid	OWP	N	N	N	N	N	N	N	N	N	N	N	N
6.	AC+ PEG400	OWP	N	N	N	N	N	N	N	N	N	N	N	N

OWP = off white powder AC = Acyclovir N = No change in color and physical state

7. Gelation studies.

S. No.	Concentration of Gellan Gum Solution	Gelling Property in SLF
1	0.1%	-
2	0.2%	-
3	0.3%	+
4	0.4%	+
5	0.5%	+
6	0.6%	++
7	0.7%	++

- = no gelation + = immediate gelation ++ = immediate gelation which persisted for extended period of time.

8. Physico-chemical properties of Acyclovir In-situ Gel formulation.

S.NO.	Parameters	Observations					
		F1	F2	F3	F4	F5	F6
1.	Clarity	Clear	Clear	Clear	Clear	Clear	Clear
2.	pH	7.4	7.4	7.4	7.4	7.4	7.4
3.	Drug Content (%)	99.5 ±0.25	99.3 ± 0.45	99.1 ± 0.75	97.4 ± 0.15	97.8 ± 0.17	0.60

9. In-vitro drug release study of Acyclovir In-situ Gel formulation Batch (F1, 2, 3, 4, 5, 6).

Time	Cumulative % Drug Released (n=3)					
	Formulation Code					
	F1	F2	F3	F4	F5	F6
5 min	11.8	12.5	11.6	11.4	16.4	15.4
15 min	17.3	18.4	17.3	22.5	31.2	30.4
30 min	23.2	23.4	22.4	28.3	49.0	49.2
45 min	28.1	29.6	28.7	35.4	59.6	58.3
1 hr	32.5	32.8	32.6	43.1	65.1	62.3
2 hr	35.4	36.3	37.8	54.8	76.3	75.0
3 hr	40.9	40.5	40.8	66.3	79.5	78.2
4 hr	46.8	46.6	46.6	71.9	82.1	82.0
5 hr	58.8	58.0	59.0	77.4	87.2	83.9
6 hr	63.2	63.9	62.8	82.6	90.5	85.6
7 hr	66.5	66.7	66.3	84.7	92.2	86.6
8 hr	68.9	69.1	69.8	87.6	94.4	91.0

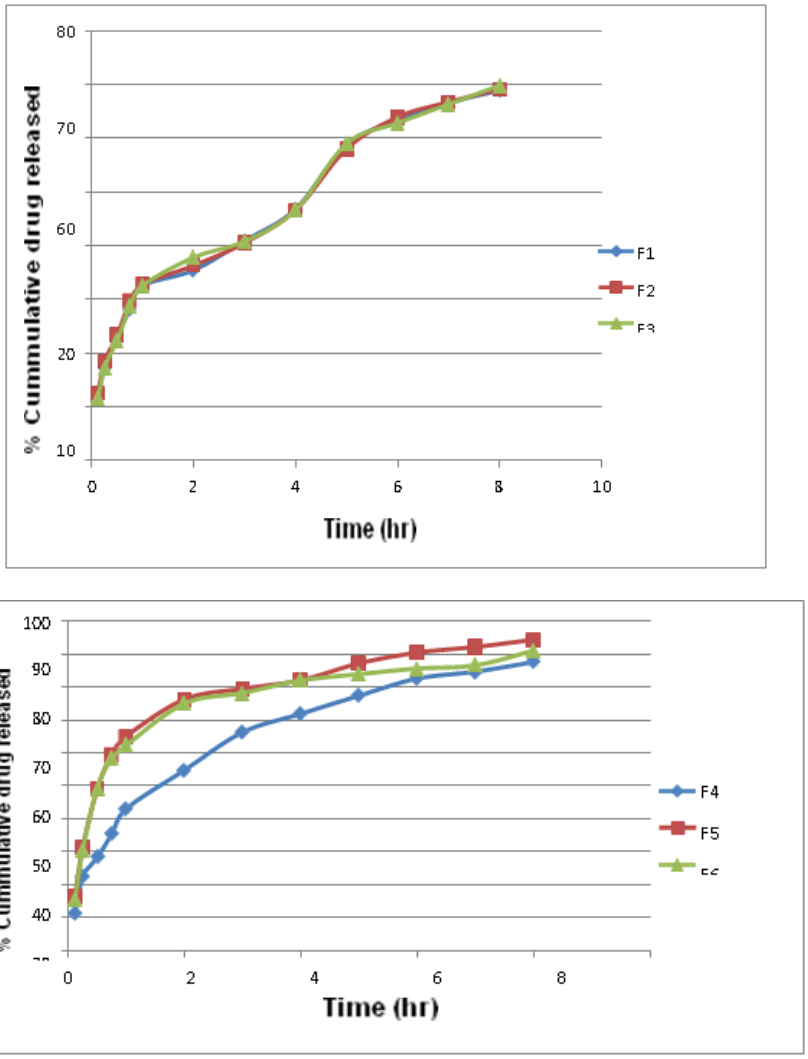


Fig. No. 1: In -Vitro Drug Release Profile of Acyclovir In-Situ Gel Formulations in Simulated Lacrimal Fluid (SLF) (Formulations F1, F2, F3, F4, F5, F6).

10. Transcorneal Drug Permeation Study.

Table: Transcorneal Drug Permeation Data of Developed Acyclovir In-situ Gel Formulation and Marketed eye ointment.

Time	Cumulative % Drug Permeated	
	Developed In-Situ Gel Formulation (Batch – no.5)	Marketed eye ointment (Acivir)
0	0	0
15 min	0	0
30 min	2.9	0
45 min	4.6	3.7
1 hr	9.6	7.0
2 hr	28.2	19.2
3 hr	44.9	33.4
4 hr	56.1	47.9
5 hr	65.8	57.4
6 hr	71.2	63.1
7 hr	79.3	65.8

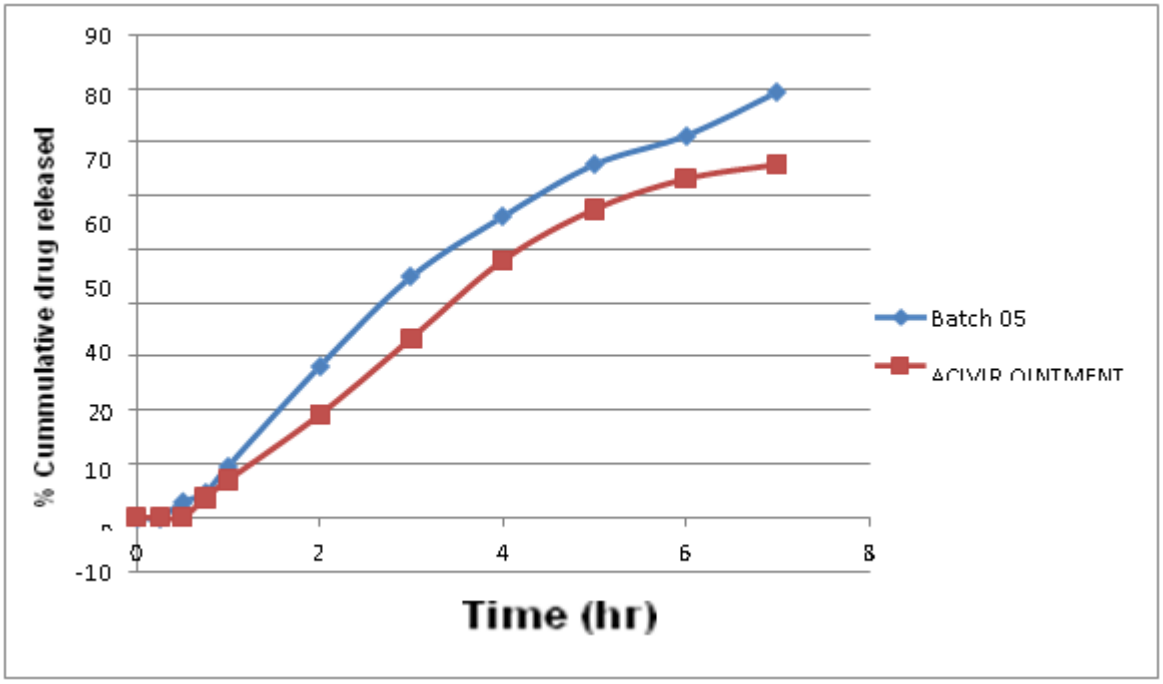


Fig. No. 2: Transcorneal Drug Permeation Profile of Developed Acyclovir In-situ Gel Formulation (Batch - 05) and Marketed Ointment.

11. HET-CAM (Hen's Egg Test or Hulner Embryogen) Test.

S. No.	Test Substance	Score	Inference
1.	0.9% NaCl (Negative control)	0	Non-irritant
2.	Developed Acyclovir in-situ gel formulation(AN/ACYISG/05)	0	Non-irritant
3.	1% NaOH (Positive control)	11.57	Severe irritant

The developed Acyclovir in-situ gel formulation (Batch - 05) was found to be non-irritant to the chorioallantoic membrane (CAM) of chicken. Since the immune response generated by chorioallantoic membrane of chicken simulates the ocular immune response of human eye, the developed formulation can be presumed to be non-irritant to the eyes.

12. Stability Data for Optimized In-Situ Gel of Acyclovir.

S. No.	Time	Storage Condition	Colour	Clarity	pH	Drug Content
1.	Initial	NA	Slight yellow	Clear	6.3	98.75%
2.	One week	2-8˚C	Slight yellow	Clear	6.3	98.53%
		RT	Slight yellow	Clear	6.3	97.46%
		40˚C	Slight yellow	Clear	6.28	97.15%

CONCLUSION

Physical appearance of the formulations was found to be white in color and clear. The pH value of all the prepared formulations ranged from 7.0-7.4, which is considered acceptable to avoid the risk of irritation upon application to the eye. The two main fundamentals of gelling system are viscosity and gelling capacity. The viscosity of the different formulations was compared as shown in Table 4. The *in-situ* formed gel should preserve its integrity without dissolving or eroding for prolonged period to facilitate sustained release of drugs locally. On the basis of physicochemical properties (viscosity and gelation capacity) six formulations (F1, F2, F3,F4, F5,and F6) were selected and evaluated for drug content, and *in-vitro* dissolution . The drug content of all the formulations was in range (96-99%). The results of the ocular irritation studies indicate that the developed formulation was non-irritant. Excellent ocular tolerance was noted. No ocular or abnormal clinical signs to the cornea, iris or conjunctiva were visible. The formulated insitu gelling system F1, F2, F3, F4, F5, F6. Therefore it can be concluded that drug is stable in final formulation at room temperature and at 40ºC for one month. Acyclovir is an antiviral drug used in the treatment of herpes infections of the eye was successfully formulated as an ion activated in situ gel forming ophthalmic solution using gellan gum as a viscosity enhancer. Acyclovir entrapped in an in situ gel forming systems was formulated in a solution form such that the acyclovir drops when instilled into the eye undergo a solution-gel transition in cul- de -sac.the loss of drug is overcome due

to the immediate gel formation. Buy considering the results of all the evaluation parameters Batch F5 were considered as ideal formulations.

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