Indian Journal of Novel Drug Delivery

IJNDD

An Official Publication of Karnataka Education and Scientific Society

Research Article

Preparation and Evaluation of Liposomes of an Antiviral Drug

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ARTICLE DETAILS

Article history:
Received on 25 August 2009
Modified on 31 August 2009
Accepted on 9 September 2009

Keywords:
Liposomes, Acyclovir,
Phosphatidyl choline,
Cholesterol,
Cephalin
Reverse phase evaporation

ABSTRACT

Liposomes are well known to alter the biodistribution of entrapped substances by protecting the enclosed material. They are widely used as vehicles to target the specific molecule to specific organ especially in conditions of viral infections. A development of system which controls the release and enhances the bioavailability of Acyclovir an antiviral agent with low oral bioavailability (10-30%) is in demand. Thus present work is focused on design and development of acyclovir liposomes by reverse phase evaporation method using various ratios of phosphatidyl choline with cholesterol and Cephalin (phosphatidyl ethanolamine) with cholesterol. Based on evaluation of entrapment efficiency, the best formulations were subjected to physicochemical studies i.e., photo microscopy, *in vitro* drug release and stability studies. The % entrapped drug in soya lecithin liposomes of batch F17 and F21 are 60.51 and 59.69 and cephalin liposomes of F41 and F45 are 58.23 and 57.38 respectively. The formulations F17, F21, F41 and F45 sustained the release and at the end of 12 hr the % drug release was 73.89, 79.48, 76.78 and 79.77 % respectively. Short-term stability studies were carried out for the selected formulations (F17, F21, F41 and F45) for a period of two months at 4°C, 25±2°C/ 60±5% RH and 30±2°C/ 65±5% RH. The liposomes stored at 4°C were found to be stable for duration of two months compared to other storage conditions. Hence it can be concluded that four formulations can be used for controlled release of acyclovir.

INTRODUCTION

Liposomes are microscopic spheres with an aqueous core surrounded by one or more outer shell(s) consisting of lipids arranged in a bilayer configuration [1]. A liposome can be used as a carrier for both hydrophobic and hydrophilic drug by dissolving or encapsulating in a lipid bilayer respectively [2]. The schematic diagram of a typical liposome and its bilayer membrane is given in Fig 1a and 1b respectively [3]. Reasons to use liposomes [4] as drug carriers are briefly summarized in Table 1 and the targeting potential of various types of liposomes [4] are listed in Table 2.

The incorporation of drugs into liposomes have several advantages as they protect their contents from interaction with plasma components, while favorably altering the pharmacokinetics and biodistribution of free compound since liposomes do not readily penetrate biologic membrane. They can be used for controlled release of drugs within body cavities such as pleural, peritoneal or intrathecal spaces [5, 6].

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Drug loading can be achieved either passively (i.e., the drug is encapsulated during liposome formulation) or actively (i.e., after liposome formation). Hydrophobic drugs, such as amphotericin B, taxol can be directly incorporated into liposomes during vesicle formation, and the extent of uptake and retention is governed by drug-lipid interactions. Trapping efficiencies of 100% are often achievable, but this is dependent on the solubility of the drug in the liposome membrane [7]. Drugs can be incorporated into liposomes using three primary mechanisms as Encapsulation, Partitioning and Reverse loading.

Acyclovir (ACV), a synthetic analogue of 20-deoxiguanosine, is one of the most effective and selective agent against viruses of the herpes group [8, 9]. This drug is particularly active against herpes labialis (lesions caused by herpes simplex type 1, HSV-1) and genital herpes (caused by herpes simplex type 2, HSV-2) [10], which remain as common viral infections in humans [11]. Unfortunately, its absolute oral bioavailability is considerably poor (about 15–30%) because of its low water-solubility (about 0.2%, 25°C) and short half-life (about 2.5 h) [12]. Therefore, ACV must be taken in an oral dose of 200mg five times daily, which cause compliance problems to patients. The fact to increase the effectiveness of this drug, high drug concentrations over a prolonged period of time in the basal epidermis

resulted in development of several polymeric particulate systems [13] like poly (*N*-2-hydroxyethyl)-dl-aspartamide conjugate [14], malonylchitosan microspheres [15], cyclodextrincomplex [16] that could pass oral absorption barrier and promote the sustained release of the drug in the target site. In the present work the liposomes which offer better compatibility to polymeric particles are designed to deliver the ACV and to prolong the release.

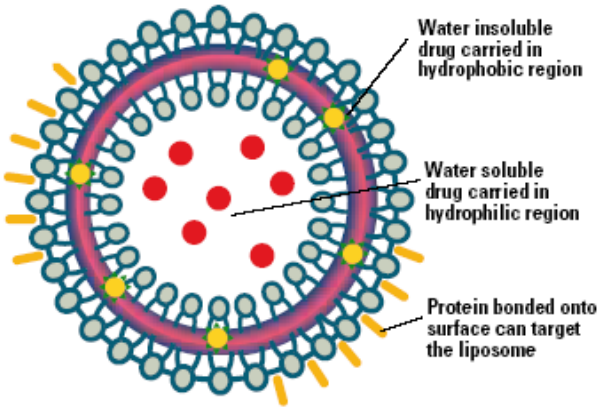


Figure 1 a: A Typical Liposome

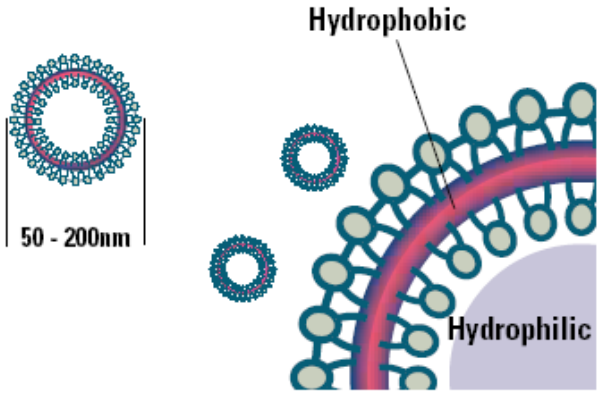


Figure 1 b: Bilayer Membrane

MATERIALS AND METHODS

Materials

Acyclovir (Complimentary sample from Cadila Pharma, Ahemdabad), Phosphatidyl choline (PC) was purchased from Hi media, Mumbai, Cholesterol (Chol) was obtained from Spectrochem, Mumbai, Cephalin was procured from Fluka), Potassium di hydrogen phosphate, chloroform and methanol was supplied by by S.D Fine Chem Ltd, Mumbai. Sodium hydroxide was purchased from E. Merck (India) Ltd, Mumbai**.** All the chemicals were of analytical grade.

Preformulation Studies

Compatibility Studies

The thermal analysis was carried by subjecting the samples to DSC to study the compatibility between the drug and the polymers selected.

Table 1: Reasons to use liposomes as drug carrier

Solubilization	Liposomes may solubilize lipophilic drugs that would otherwise be difficult to administer intravenously
Protection	Liposomes encapsulated drugs are inaccessible to metabolizing enzymes, conversely, body components (such as erythrocytes or tissues at the injection site) are not directly exposed to the full dose of the drug
Duration of action	Liposomes can prolong drug action by slowly releasing the drug in the body
Directing potential internalization	Targeting options change the distribution of the drug over the body. Liposomes are endocytosed or phagocytosed by cells, opening up opportunities to use 'liposome-dependent drugs'. Lipid-based structures (not necessarily liposomes) are also able to bring plasmid material into the cell through the same mechanism (non-viral transfection systems)
Amplification	Liposomes can be used as adjuvants in vaccine formulations

Table 2: Targeting potential of various types of liposomes

Liposome type	Targeting potential
Conventional liposomes	Macrophage targeting
Longcirculating liposomes	Selective targeting to pathological areas, acting as circulating micro reservoir of drug molecule.
Immunoliposomes	Specific targeting

Standard curve for ACV

57.8 mg of ACV was accurately weighed and dissolved in 50 ml of PBS to prepare stock solution. The Aliquot of stock solution was further diluted with PBS pH 7.4 to get 2.312 µg, 4.624 µg, 6.936 µg and 9.248 µg of drug per ml, the absorbance was measured in a UV spectrophotometer at 252 nm against blank PBS pH 7.4 as blank. The absorbance's so obtained were tabulated as in Table-3. Calibration curve was constructed and is shown in Fig 2.

Preparation of Liposomes

Liposomes were prepared using rotary flash evaporator. The 100 ml solution of each polymer (PC and Chol) and ACV were prepared in different ratio using chloroform and methanol as solvents in 2:1 ratio respectively. The above three solutions were transferred in to round bottom flask which was attached to rotary flask evaporator which was rotated at 150 rpm at 40^0C to get a thin residual film. The process was continued for another 30 min to remove the residual solvent. The vacuum was released and the film was hydrated immediately by adding PBS pH 7.4 and the flask was

rotated under similar conditions for another 30 min till all the lipid film comes to the aqueous buffer resulting in the formation of liposomes which was identified by the conversion of the buffer solution to a milky white colloidal solution. The flask was removed and liposomes were transferred to a container subjected to mechanical shaking for 30 min to improve the hydration efficiency. Liposomes were allowed to swell overnight under refrigeration [17, 18].

Estimation of Entrapped Drug

Drug entrapped within the liposomes was estimated after removing the unentrapped drug, which was separated by collecting the supernatant after subjecting the dispersion to centrifugation in a cooling centrifuge at 10,000 rpm at a temperature of 4°C for 30min, where upon the pellets of liposomes were washed again with buffer to remove any unentrapped drug and the washing was combined with supernatant and was analyzed for drug content at 252nm [19].

$$\% \text{ Drug entrapment} = \frac{\text{Total drug} - \text{unentrapped drug}}{\text{Total drug}} \times 100$$

Particle size determination

The particle size analysis was carried by laser diffraction technique using Malvern mastersizer 2000s by wet analysis method.

Photomicroscopy

All batches of liposomes prepared were observed under trinocular microscope (Olympus, labomed)

***In vitro* diffusion studies**

The diffusion studies were carried using franz diffusion cells. The donar compartment containing 2ml of liposomal suspension was separated from receptor compartment containing 100ml of PBS pH 7.4 by a treated cellophane membrane. The receptor compartment was agitated throughout the process by magnetic stirrer. The samples were withdrawn at scheduled intervals (replaced with equivalent amount of PBS pH 7.4) and analyzed at 252nm [20].

Stability studies

The best formulations were subjected to accelerated stability studies by storing at 25°C / 60% RH, 30°C/65% RH and 4°C for 60 days and were analyzed for its drug content at an interval of 15 days.

RESULTS

Compatibility studies

The physical mixtures of the drug and polymers produced their own characteristic peaks without any disposition or disappearance of the peak observed for pure drug.

Standard curve for ACV

The obtained absorbance of the solutions is tabulated in Table 3 and the calibration curve was constructed by plotting absorbance versus concentration (Fig 2).

Table 3: Spectrophotometric estimation of acyclovir at 252 nm

Sl. No	Concentration (µg/ml)	Absorbance
1.	2.312	0.165
2.	4.624	0.305
3.	6.936	0.455
4.	9.248	0.620

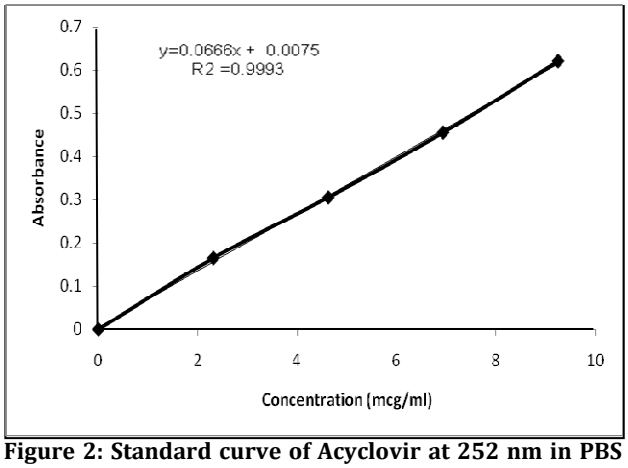


Figure 2: Standard curve of Acyclovir at 252 nm in PBS pH 7.4

Preparation of Liposomes

The various batches of liposomes of F1 to F48 were prepared by varying the ratio of PC and Chol and Cephalin and Chol with different weight ratio of the drug as shown in Table 4 for optimization of ideal batch for delivery of ACV.

Estimation of Entrapped Drug

The percentage entrapment of ACV in preparations of PC and cephalin liposomes is reported in Table 5.

Photo microscopy

The photographs of the selected formulations (F17, F21, F41 and F45) of liposomes are shown in the Photograph No 1 to 4.

***In vitro* diffusion Studies**

The study was performed for selected formulations (F17, F21, F41 and F45) and the results are reported in Table 6. A graph of percentage drug diffused versus time was plotted as shown in Fig 3 to 6.

Stability studies

Short-term stability studies were carried out for a period of two months at 4°C, 25±2°C/ 60±5% RH and 30±2°C/ 65±5% RH. Four formulations (F17, F21, F41 and F45) were selected based on best entrapment efficiency. The drug content was estimated at an interval of 15 days, which is reported in Table 7.

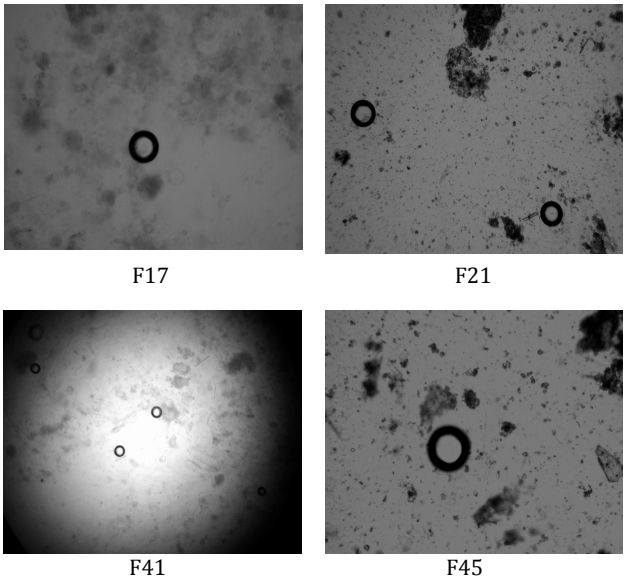
Table 4: Formulation of soya lecithin and cephalin liposomes

Sl No	Formulation No.	Polymer Ratio (PC:Chol)	Drug (mg)	Weight Taken (mg) (PC: Chol)	Formulation no.	Polymer Ratio (Cephalin: Chol)	Drug (mg)	Weight Taken (mg) (Cephalin: Chol)
1	F1	1.0:0.5	100	400:200	F25	1.0:0.5	100	400:200
2	F2	1.0:1.0	100	400:400	F26	1.0:1.0	100	400:400
3	F3	1.0:1.5	100	400:600	F27	1.0:1.5	100	400:600
4	F4	1.0:2.0	100	400:800	F28	1.0:2.0	100	400:800
5	F5	0.5:1.0	100	200:400	F29	0.5:1.0	100	200:400
6	F6	1.0:1.0	100	400:400	F30	1.0:1.0	100	400:400
7	F7	1.5:1.0	100	600:400	F31	1.5:1.0	100	600:400
8	F8	2.0:1.0	100	800:400	F32	2.0:1.0	100	800:400
9	F9	1.0:0.5	200	400:200	F33	1.0:0.5	200	400:200
10	F10	1.0:1.0	200	400:400	F34	1.0:1.0	200	400:400
11	F11	1.0:1.5	200	400:600	F35	1.0:1.5	200	400:600
12	F12	1.0:2.0	200	400:800	F36	1.0:2.0	200	400:800
13	F13	0.5:1.0	200	200:400	F37	0.5:1.0	200	200:400
14	F14	1.0:1.0	200	400:400	F38	1.0:1.0	200	400:400
15	F15	1.5:1.0	200	600:400	F39	1.5:1.0	200	600:400
16	F16	2.0:1.0	200	800:400	F40	2.0:1.0	200	800:400
17	F17	1.0:0.5	300	400:200	F41	1.0:0.5	300	400:200
18	F18	1.0:1.0	300	400:400	F42	1.0:1.0	300	400:400
19	F19	1.0:1.5	300	400:600	F43	1.0:1.5	300	400:600
20	F20	1.0:2.0	300	400:800	F44	1.0:2.0	300	400:800
21	F21	0.5:1.0	300	200:400	F45	0.5:1.0	300	200:400
22	F22	1.0:1.0	300	400:400	F46	1.0:1.0	300	400:400
23	F23	1.5:1.0	300	600:400	F47	1.5:1.0	300	600:400
24	F24	2.0:1.0	300	800:400	F48	2.0:1.0	300	800:400

Table 5: Estimation of Entrapped Drug

Sl No	Formulation No.	% Drug Entrapped *	S. D	Formulation No.	% Drug Entrapped	S. D
1	F1	68.20	0.490	F25	63.0	0.283
2	F2	65.60	0.490	F26	58.13	0.340
3	F3	64.27	0.411	F27	55.86	0.525
4	F4	62.13	0.340	F28	52.26	0.249
5	F5	66.80	0.432	F29	61.26	0.411
6	F6	65.33	0.249	F30	57.93	0.411
7	F7	62.53	0.573	F31	54.13	0.188
8	F8	60.60	0.163	F32	50.53	0.340
9	F9	63.66	0.170	F33	61.73	0.170
10	F10	62.60	0.216	F34	59.87	0.188
11	F11	61.67	0.170	F35	57.73	0.205
12	F12	61.00	0.245	F36	56.07	0.205
13	F13	62.66	0.205	F37	60.17	0.170
14	F14	62.20	0.245	F38	59.33	0.340
15	F15	60.76	0.170	F39	56.23	0.170
16	F16	59.83	0.205	F40	55.8	0.141
17	F17	60.51	0.110	F41	58.23	0.114
18	F18	59.01	0.094	F42	56.95	0.136
19	F19	58.24	0.119	F43	56.13	0.094
20	F20	57.16	0.139	F44	55.50	0.114
21	F21	59.69	0.136	F45	57.38	0.033
22	F22	58.65	0.083	F46	56.75	0.033
23	F23	57.71	0.160	F47	55.07	0.094
24	F24	56.76	0.139	F48	54.38	0.083

S.D. = Standard Deviation
n=3



Photomicroscopy of selected formulations

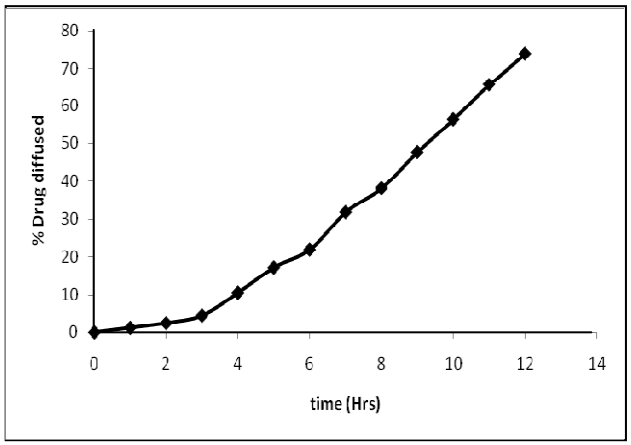


Figure 3: *In vitro* drug diffusion profile of F14 batch

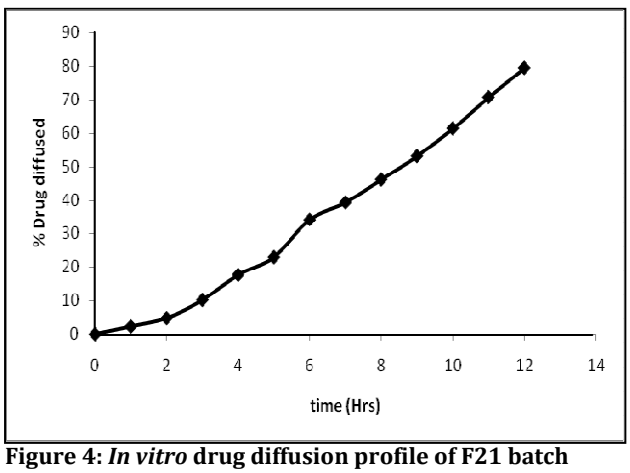


Figure 4: *In vitro* drug diffusion profile of F21 batch

DISSCUSION

In general viral infections are extremely difficult to treat and there are very few effective therapies. Antiviral therapy based on intracellular delivery of drugs may be enhanced using various techniques. Among the antiviral drugs, ACV is most widely used. However it's oral bioavailability is very low (10 to 30%) [20], thus development of a delivery system which can enhance the bioavailability and prolong effect of this drug, is in high demand.

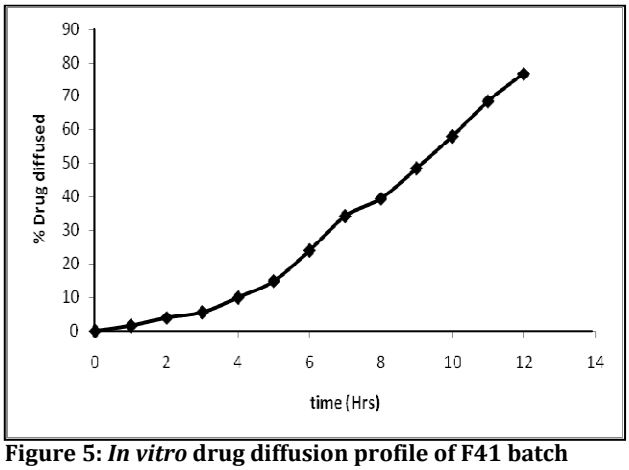


Figure 5: *In vitro* drug diffusion profile of F41 batch

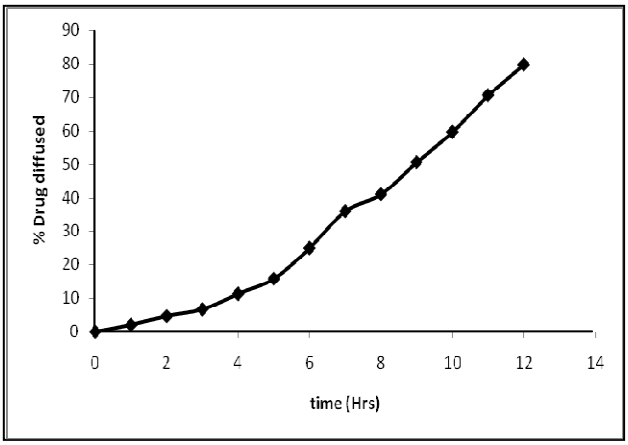


Figure 6: *In vitro* drug diffusion profile of F45 batch

ACV is a drug of choice for the treatment of the infection due to the herpes simplex or varicella zoster virus. The oral route is preferred one over parenteral administration due to the risk of local toxicity and topical application as the absorption of the drug is very slow and needs a permeation enhancer, where as bolus rapid injection causes renal precipitation of the drug. The mean plasma half life ($t_{1/2}$) of acyclovir is 2.5hrs. Thus repeated administration of high dose is required (200mg 5times daily for 10 days) to maintain the peak plasma concentration [20].

ACV is moderately (1 in 400) soluble in water and insoluble in most of organic solvents, it is thus reasonable to expect that trapping the drug with in aqueous compartment of liposomes or within large aqueous core area of liposomes will be more efficient than trapping this drug in small layers of interstitial water in multilamellar liposomes. Hence, liposomes of acyclovir were prepared by reverse phase evaporation method to obtain maximum percentage drug entrapment (PDE).

As the PC, Cephalin and Chol had no interaction with ACV they are considered for liposomal preparations by the above method. In order to obtain the maximum amount of drug entrapped in the lipid bilayer various ratios of them were tried. Drug entrapment in so formed formulations was calculated by correlating the absorbance values of them with the standard curve with a regression value of 0.9993.

Table 6: In vitro drug release studies for the formulation F17, formulation F21, formulation F41 and formulation F45

Percentage drug diffused ± SD				
Time (h)	**Formulation F17**	**Formulation F21**	**Formulation F41**	**Formulation F45**
0	0	0	0	0
1	1.23±0.033	2.25±0.028	1.57±0.042	2.09±0.042
2	2.47±0.038	4.75±0.014	3.94±0.028	4.66±0.028
3	4.46±0.028	10.19±0.038	5.59±0.017	6.66±0.033
4	10.49±0.033	17.65±0.038	9.99±0.014	11.29±0.037
5	17.13±0.037	22.93±0.022	14.9±0.017	15.79±0.031
6	21.88±0.04	34.31±0.035	24.03±0.022	24.93±0.041
7	31.78±0.054	39.46±0.026	34.24±0.033	36.15±0.025
8	38.11±0.057	46.3±0.040	39.43±0.023	41.2±0.022
9	47.65±0.057	53.26±0.035	48.48±0.031	50.74±0.049
10	56.3±0.037	61.46±0.038	57.99±0.040	59.73±0.026
11	65.53±0.037	70.78±0.027	68.44±0.045	70.66±0.034
12	73.89±0.031	79.48±0.031	76.78±0.066	79.77±0.020

n=3

Table 7: Stability studies for the formulation F17, 21, 41 and 45

Batches	**Stability conditions**	**Drug Content (%)**				
		Day 0	**Day 15**	**Day 30**	**Day 45**	**Day 60**
F17	4°C	100	93.80	83.77	75.44	65.21
	25°C±2°C/60±5% RH	100	84.25	62.69	33.56	19.48
	30°C±2°C/65±5% RH	100	83.21	64.99	32.42	18.44
F21	4°C	100	91.40	82.61	74.26	65.64
	25°C±2°C/60±5% RH	100	82.69	65.59	34.11	19.99
	30°C±2°C/65±5% RH	100	82.12	64.97	33.25	19.40
F41	4°C	100	93.39	84.18	77.54	67.82
	25°C±2°C/60±5% RH	100	83.50	67.44	33.23	20.11
	30°C±2°C/65±5% RH	100	82.81	66.89	32.13	19.27
F45	4°C	100	89.04	79.78	70.60	64.67
	25°C±2°C/60±5% RH	100	79.09	62.37	32.83	19.13
	30°C±2°C/65±5% RH	100	78.04	61.57	32.03	18.54

All the formulations had more than 50% of the drug entrapped but F17, F21, F41 and F45 were selected for further studies considering the weight of drug entrapped for the same weight of the polymer. Formulations loaded with 300mg of drug were chosen as the optimum in both of the polymer concentration. The entrapment efficacy was reduced with increase in polymer concentration and the photomicroscopy shows that the liposomes are spherical in shape which may retard the diffusion of drug as the contact area with the membrane is least but theoretically as the size is less around 700nm the surface area gets reduced and the release and diffusion should enhance which is not seen practically. So the retard in release is not because of minimal contact area with the membrane rather it may be due to lipoidal bilayer which controls the release of hydrophilic drug ACV. These formulations were than subjected to stability studies. As the formulations are to be refrigerated during storage the study was carried at 4°C and also at room temperature to study the effect of temperature on the formulations. At the end of 2 months the drug content of all the formulations stored at 25 and 30°C reduced drastically which may be due to degradation of lipid. Whereas at 4°C the drug content was reduced but still around 60-70% was available for action.

CONCLUSION

The viral infections treated with extreme difficulty widely implies on Acyclovir but having the lower half life it is causing the patient incompliance. The present work was designed to provide patient compliance by sustaining the release of Acyclovir thereby enhancing the half life. The study showed that the successfully developed liposomes of PC and Chol, and Cephalin and Chol can prolong the release of drug for more than 12 h which in turn alters the elimination rate. The bioavailability of the drug is expected to improve as most of the research on liposomes had shown better bioavailability. Thus we conclude the administration of Acyclovir in form liposomal drug delivery will be the better therapy in treatment of viral infection.

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