IJPSR (2021), Volume 12, Issue 5 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 May 2020; received in revised form, 19 October 2020; accepted, 21 October 2020; published 01 May 2021

FORMULATION AND EVALUATION OF VALACYCLOVIR LIPOSOMES

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Keywords:

Liposomes, Valacyclovir, HSV, VZV, CMV, Phospholipid, Vesicles, Suspension

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ABSTRACT: The main aim of the present study was to formulate and evaluate valacyclovir-loaded liposomes. A drug and excipient compatibility study was performed by FT-IR, and the study revealed that there was no interaction between drug and excipients. Liposomes are prepared by using two different methods, *i.e.*, ether injection method and thin-film hydration method. Various formulations are prepared by varying the concentration of phospholipid and cholesterol. All formulations are evaluated for entrapment efficiency and *in-vitro* drug release studies. The optimized formulation showed the highest entrapment efficiency (96.51%) and highest drug release (84.21%). The optimized formulation was evaluated for FT-IR, SEM and particle size analysis, and zeta potential studies. The FT-IR study revealed that there is no interaction between drugs and excipients. The optimized formulation was found to be stable with zeta potential -40.9 mV and particle size of 67.4 nm with uniform distribution. The regression coefficient (R^2 value) 0.966 indicating release as zero-order, where "n" value 1.212 states the mechanism as a super case-II transport mechanism. Stability studies were carried out for the optimized formulation for 1 month by storing at three different temperatures. After 1 month, the samples were analyzed for their physical properties, drug entrapment, and *in-vitro* release profile. The formulation, which is stored at 4-8 ºC, was found to be stable without any significant changes in the drug entrapment and *in-vitro* drug release profile.

INTRODUCTION: The goal of any drug delivery system is to provide therapeutic action at the proper site in the body and then maintain the desired drug concentration range. Nano formulations cause a reduction in particle size, resulting in an improved ratio of surface area to volume and enhanced bioavailability [1, 2, 3]. To follow optimal drug action, a carrier might be used for transportation to the site of action and released to perform their task; thus carrier should be non-toxic, biodegradable, and of the appropriate size to accommodate a wide variety of substances.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(5).2913-19

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(5).2913-19

Liposomes discovered in 1965 by Bangham and his colleagues are spherical colloidal particles containning an aqueous core surrounded by a phospholipid bilayer, which replicates cell membrane [4]. Liposomes were a better model to investigate membrane structure and functionality as it is mainly composed of phospholipids [5].

Liposome's structural resemblance to the cellular membrane has allured scientists to explore the potency of using the liposomes as drug carriers to transport therapeutics with peculiar properties to targeted parts of the body since 1940's [6, 7, 8, 9]. Due to its similarity with cell membrane structure, liposomes can incorporate both lipophilic and hydrophilic drugs into phospholipid bilayer and aqueous core. Another advantage is it can simultaneously encapsulate other active ingredients into its complex formulation [10]. Valacycloviristhe L-valine ester of acyclovir.

It is a member of the purine (guanine) nucleo-side analog drug class. The first stage of drug phosphorylation for acyclovir requires activation by the virus-specific enzyme. In the case of HSV, VZV, and EBV this enzyme is the viral thymidine kinase (TK), which was found only in the virus-infected cells. The phosphorylation process is completed (conversion from mono- to triphosphate) by cellular kinases. Acyclovir triphosphate competitively inhibits the virus DNA polymerase and results in DNA chain termination, stopping virus DNA synthesis and blocking virus replication. The inhibitory capabilities of acyclovir are highly selective due to the drug's strong affinity for thymidine kinase (TK) [11, 12, 13].

Sudiptadas *et al.,* (2018) prepared Liposomes of isoniazid by thin-layer film hydration method. L-α-phosphatidylcholine and cholesterol were used to make multilamellar vesicles. Six batches of liposomes were prepared based on the different weight ratios of L-α-phosphatidylcholine and cholesterol. The percentage entrapment efficiency was found to be 8.99 ± 0.15 to 4.19 % ± 0.12 %, respectively. From the release profile, it was seen that F1 batch was the fastest and F6 was the slowest to release the drug. The satisfactory batch F1 was packed in an Eppendorf tube and stored at 4 °C temperature for one month. At the end of one month, the samples were analyzed for their physical properties, drug entrapment, and *in-vitro* release profile. The percentage release was found to be 96.5 ± 3.2 after 4 h[14].

MATERIALS AND METHODS: Valacyclovir hydrochloride was obtained as a gift sample from Yarrow chem products, Mumbai. Soya lecithin (HI Media Laboratories private limited, Mumbai.), Cholesterol (Sd. fine. Chemicals limited, Mumbai). All other chemicals and solvents were of analytical or pharmacopoeial grade. Phosphate buffer was prepared freshly with distilled water whenever required. General procedure for preparation of calibration curve by UV spectrophotometric method [15].

A standard stock solution was prepared later; suitable dilutions were made with phosphate buffer of pH 7.4. To a series of 10ml volumetric flasks aliquots standard solutions were taken, and the volume was made up using a phosphate buffer pH 7.4. The absorbance of these solutions was measured at a respective wavelength in a UV-Visible spectrophotometer. Absorbance values were plotted against respective concentrations to obtain a standard calibration curve.

Preparation of Liposomes [16, 17, 18]: Valacyclovir liposomes were prepared by using two different methods *i.e.,* ether injection method and thin film hydration method. The effect of various process variables such as speed of rotation, vacuum, temperature and hydration time was altered and the effect on the formation of uniform thin lipid film was evaluated. Here Drug: Soyalecithin: Cholestrol ratios were altered and drug entrapment efficiency was studied. In the ether injection method Drug, soya lecithin and cholesterol were taken in the prescribed ratio (5:1:1, 5:2:1, 5:3:1, 5:4:1) in a 250 ml beaker. The mixture was dissolved in diethyl ether and chloroform in 1:1 ratio and slowly injected through 14 gauge needle into a beaker containing Valacyclovir in 10 ml phosphate buffer pH 7.4 the temperature maintained during the injection was 40-60 ºC. The difference in temperature between phases causes rapid vaporization of ether, chloro-form resulting in spontaneous vesiculation.

In thin film hydration method Drug, Lecithin and cholesterol were taken in various ratios (5:1:1, 5:2:1, 5:3:1, 5:4:1) and transferred into a clean round bottom flask, then diethyl ether and chloroform (1:1) was added, and the flask was fixed to the rotary evaporator. A solvent is evaporated by maintaining the temperature at 50 ºC under vacuum at 50 rpm. It forms a dry, thin film along the sides of the round-bottomed flask. Valacyclovir is dissolved in phosphate buffer pH 7.4 and was added to the thin film and vortexed at room temperature for 20 min, which forms a milky white suspension.

Characterization of Liposomes:

Physical Appearance of Liposomal Suspension: Liposomal suspension was viewed by optical microscope at 40 X magnification to observe crystal characteristics of suspension by spreading a thin layer of liposomal suspension on a slide and placing the coverslip on it. The appearance for each formula was checked, such as color, consistency, fluidity, and comparison of each one with the other.

Vesicle Size Analysis by Optical Microscopy [19]: The prepared liposomes were viewed for observing the vesicle formation and discreteness of dispersed vesicles. Particle size determination of liposomes was done by using an Optical microscope. Determination of particle size as mean diameter is based on direct observation under the microscope. The procedure includes 2 steps: Calibration of an eye-piece micrometer. Measurement of globule size

Vesicle Morphology [19]: Shape and surface morphology of liposomes was studied using scanning electron microscopy (SEM). The liposomes formed were mounted on an aluminum stub with double-sided adhesive carbon tape. The vesicles were then sputter-coated with gold/palladium using a vacuum evaporator and examined with the scanning electron microscope equipped with a digital camera at 25 kV accelerating voltages.

Zeta Potential Determination [20]: Charge on empty and drug-loaded vesicles surface was determined using Zetasizer 300 HSA (Malvern Instruments, Malvern, UK). Analysis time was kept for 60 s and average zeta potential and charge on the liposome was determined.

Drug Encapsulation Efficiency Determination [21]: Entrapment efficiency of liposomes was determined by centrifugation method. Aliquots of liposomal dispersion were subjected to centrifugation on a laboratory centrifuge (Remi R4C) at 3500 rpm for a period of 90 min. The clear supernatants were removed carefully to separate non-entrapped drugs, and absorbance was recorded at 252 nm. The sediment in the centrifugation tube was diluted with phosphate buffer pH 7.4 (100 ml), and absorbance of this solution was noted at 252 nm. The additive quantity of drug in supernatant and sediment gave a total amount of drug in the dispersion. % entrapment of drug was calculated by the following formula:

Entrapment efficiency (%) = ((Total amount of the drug - amount of the free drug) / Total drug) × 100.

***In-vitro* Drug Release Studies [22]:** *In-vitro* release studies were performed using modified Franz diffusion cells. Dialysis membrane (HiMedia molecular weight 5000) was placed between receptor and donor compartments. Valacyclovir liposomal suspension was placed in the donor compartment, and the receptor compartment was filled with phosphate buffer, pH 7.4 (18 ml). The diffusion cells were maintained at 37 ± 0.5 ºC with stirring at 300 rpm throughout the experiment.

At fixed time intervals, 2 ml of aliquots were withdrawn and analyzed by UV Spectrophotometer at 252 nm. Data obtained from *in-vitro* release studies were fitted to various kinetic equations to find out the mechanism of valacyclovir release from liposomal suspension.

Stability Studies: Stability studies were conducted for best formulation *i.e.* F8 of valacyclovir liposomal suspension. The ability of vesicles to retain the drug (Drug Retention Behaviour) was assessed by keeping the liposome dispersion at different temperatures *i.e.*, 4-8 °C (refrigerator), 27 ± 2 °C (room temperature), 38 ± 2 °C for a specific period of 30 days.

The liposomal dispersion was kept in sealed aluminium foil-glass vials and analyzed for particle size, *in-vitro* drug release &percent drug entrapment at 252 nm.

RESULTS AND DISCUSSION:

Standard Calibration Curve of Valacyclovir Hydrochloride: Standard plot of valacyclovir hydrochloride was plotted, and its linearity was shown in **Fig. 1**. The standard graph of valacyclovir hydrochloride shows good linearity with R^2 value of 0.997, which indicates that it obeys beer-lamberts law in the concentration range 10-30 µg/ml.

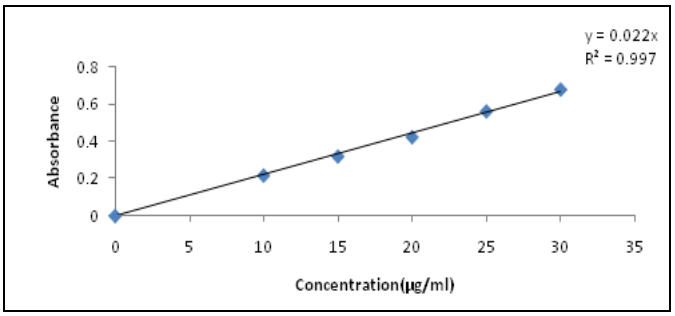

FIG. 1: CALIBRATION CURVE OF VALACYCLOVIR HYDROCHLORIDE

Characterization of Liposomes:

Vesicle Morphology: The morphology of liposomes was studied using scanning electron

microscopy. SEM images of valacyclovir revealed that liposomes were spherical in shape and discrete with sharp boundaries having large internal aqueous space.

SEM images of liposomes produced from optimized formulation F8 are shown in **Fig. 3**. The optical microscopy images of the liposomes are shown in **Fig. 2.**

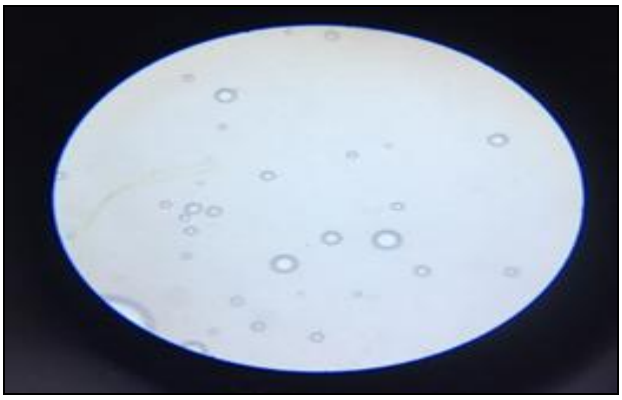

FIG. 2: OPTICAL MICROSCOPY IMAGE OF LIPOSOMES

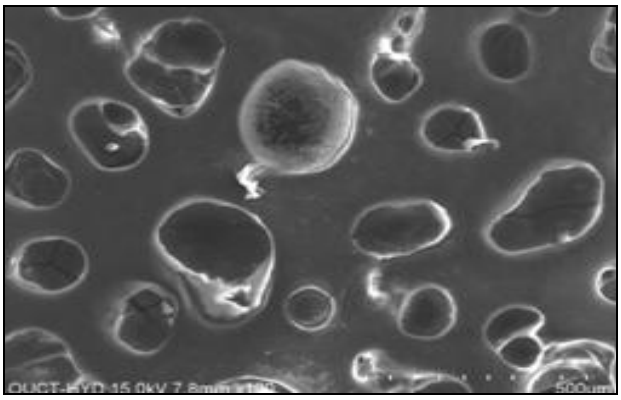

FIG. 3: SEM IMAGE OF OPTIMIZED FORMULATION

Vesicle size Analysis: Average vesicle size of Valacyclovir liposomes are presented in **Table 1**, which indicated that vesicles formed with lecithin and cholesterol in the concentration range of 4: 1 is smaller in size. Vesicles prepared by the thin-film hydration method were small in size compared to vesicles prepared with the ether injection method due to rotation applied during the thin-film hydration method. The vesicle size of optimized liposomal formulation F8 was found to be 175.2 nm (Zetasizer) which was shown in **Fig. 4.**

TABLE 1: AVERAGE VESICLE SIZE OF LIPOSOMAL FORMULATIONS

Formulation Code	Average Vesicle size in µm
F1	825.1±0.10
F2	642.8±0.05
F3	581.9±0.86
F4	451.3±0.12
F5	40.5±0.098
F6	321.7±0.04
F7	240.5±0.16
F8	175.2±0.03

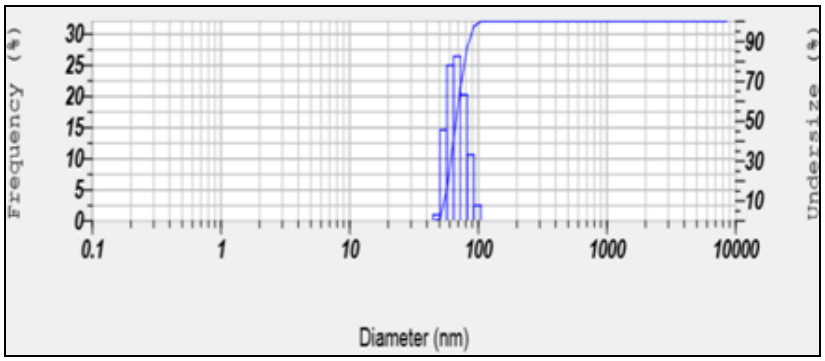

FIG 4: PARTICLE SIZE OF LIPOSOME FORMULATION (F8)

Determination of Zeta Potential: In the present study the zeta potential obtained for optimized liposomes are shown in **Fig. 5**. The values of zeta potential (-40 mV for vesicles) showed prepared liposomes have sufficient charge to avoid aggregation of vesicles.

Drug Encapsulation Efficiency: The encapsulation efficiency of liposomal formulations ranges from 74.78% to 96.51%. The drug encapsulation efficiency of all eight formulations of liposomes was shown in **Table 2**, **Fig. 6**. The liposome formulation containing drug, lecithin, and cholesterol in the ratio of 5:4:1 showed higher encapsulation efficiency than other formulations. Liposomes prepared by the thin-film hydration method showed higher encapsulation efficiency when compared with liposomes prepared by the ether injection method.

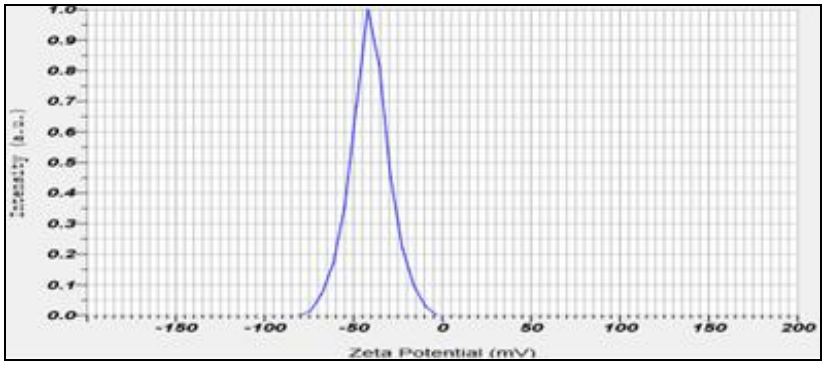

FIG. 5: ZETA POTENTIAL OF OPTIMIZED LIPOSOME FORMULATION

TABLE 2: CHARACTERIZATION OF LIPOSOMAL FORMULATIONS FOR ENCAPSULATION EFFICIENCY

Formulation Code	Percentage Entrapment Efficiency
F1	74.782±0.27
F2	77.790±0.33
F3	83.523±0.56
F4	88.129±0.78
F5	82.276±0.64
F6	86.708±0.36
F7	91.554±0.29
F8	96.512±0.97

All values represent mean ± standard deviation (SD), n=3

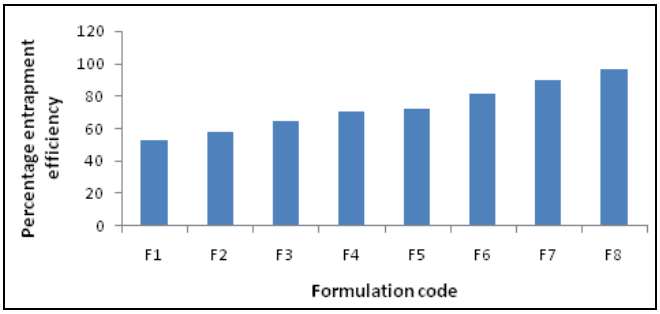

FIG. 6: COMPARISION OF ENTRAPMENT EFFICIENCY OF FORMULATIONS

***In-vitro* Drug Diffusion Profile of Liposomal Formulations:** The liposomal formulations of valacyclovir (F1-F8) were characterized for their drug permeation study using Franz diffusion cell through an artificial membrane. A drug diffusion study of all the formulations was carried out using a phosphate buffer of pH 7.4 for 12 h at 37 ± 0.5 ºC with 500 rpm speed.

Samples were withdrawn at regular intervals (0 1, 2, 3, 4, 6, 8, 10, 12 h). At every interval, 1 ml of sample was withdrawn; after appropriate dilution, the sample solutions were analyzed at 252 nm for valacyclovir by using a UV-Visible spectrophotometer. The cumulative percentage of valacyclovir released from liposome formulations containing different concentrations of lipids was shown in **Fig. 7** and **Fig. 8.** The results indicate that the liposomes with an increased ratio of lecithin have a high release rate. The percent cumulative release in 12 h was found to be 84.21%.

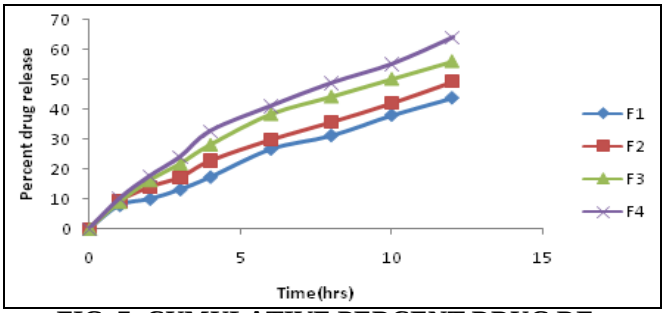

FIG. 7: CUMULATIVE PERCENT DRUG RELEASE PROFILES OF VALACYCLOVIR LIPOSOMAL FORMULATIONS (F1-F4)

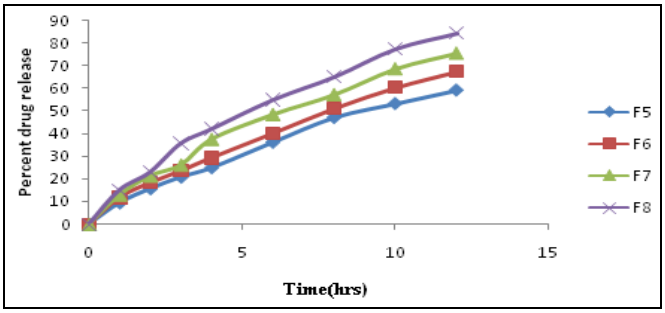

FIG. 8: CUMULATIVE PERCENT DRUG RELEASE PROFILES OF VALACYCLOVIRLIPIOSOMAL FORMULATIONS (F5-F8)

Stability Studies: The optimized liposome formulation was unstable at 37 ± 2 ºC and 40 ± ºC.

Stability studies revealed that physical appearance, particle size, *in-vitro* drug release & percent drug entrapment of F8 sample remained unchanged upon storage at 4-8 ºC.

Drug Release Kinetics: The kinetic and the release mechanisms were estimated by regression plots for

zero order, first order, Higuchi model, erosion model, and Korsmeyer Peppas model. When the R^2 values of regression plots for the first order and zero-order were considered, it is evident that the drug release from all valacyclovir formulations follows zero-order kinetics. By incorporating release data in Higuchi and erosion models, the R^2 values of all the formulations are greater for the Higuchi model. So, all the formulations in this study were best expressed by Higuchi's classical diffusion equation. The linearity of the plot indicated that the release process was diffusion controlled. To further confirm the exact mechanism of drug release, the data was incorporated into Korsmeyer Peppas model, and the mechanism of drug release was indicated according to the value of exponent 'n'. For all the liposomal formulations, the release exponent 'n' value was found to be >1, so it indicates all the liposomal formulations followed super case-II mechanism.

CONCLUSION: Liposomes were prepared by ether injection method and thin-film hydration method by varying the concentration of lecithin & cholesterol. F1-F4 is prepared by the ether injection method, and F5-F8 is prepared by the thin-film hydration method. All the formulations were compared for the evaluation parameters. Among all the formulations, the F8 formulation, which contains drug, lecithin, and cholesterol in the ratio 5:4:1, was found to be best with entrapment efficiency of 95.51% and percentage of drug release 84.21% in 12 h. In order to describe the release kinetics of all formulations, the dissolution data were fitted in various kinetic dissolution models like zero order, First order, Higuchi, Peppas, respectively. From R^2 values, it indicates that drug release from all formulations follows zero-order release, and the release mechanism was diffusion controlled. The Peppas model is used to confirm whether the release mechanism is Fickian diffusion, Non-fickian diffusion or zero order. The 'n' values for all formulations were found to be more than 0.89.This indicates that the drug release follows the super case-II mechanism.

ACKNOWLEDGEMENT: The authors are thankful for yarrow chem products, HI Media labs Pvt Ltd & Sd. Fine chemicals Ltd for providing valacyclovir, soya lecithin, and cholesterol as a Free gift sample.

Iam very grateful to Osmania University, Hyderabad for performing SEM studies and Zeta potential for my research work. The authors would like to thank the principal, Department of pharmaceutics and management of Hindu college of pharmacy, Guntur, for providing the necessary facilities during the conduction of this work.

CONFLICTS OF INTEREST: Authors do not have any conflicts of interest.

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How to cite this article:
Ramya K and Devi AS: Formulation and evaluation of valacyclovir liposomes. Int J Pharm Sci & Res 2021; 12(5): 2913-19. doi: 10.13040/IJPSR.0975-8232.12(5).2913-19.