Design and Development of Multivesicular Liposomal Depot Delivery System for Controlled Systemic Delivery of Acyclovir Sodium

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ABSTRACT

The aim of the present study was to design a depot delivery system of acyclovir sodium using multivesicular liposomes (MVLs) to overcome the limitations of conventional therapies and to investigate its in vivo effectiveness for sustained delivery. MVLs of acyclovir were prepared by the reverse phase evaporation method. The loading efficiency of the MVLs (45%-82%) was found to be 3 to 6 times higher than conventional multilamellar vesicles (MLVs). The in vitro release of acyclovir from MVL formulations was found to be in a sustained manner and only 70% of drug was released in 96 hours, whereas conventional MLVs released 80% of drug in 16 hours. Following intradermal administration to Wistar rats, the MVL formulations showed effective plasma concentration for 48 hours compared with MLVs and free drug solution (12-16 hours). C_{max} values of MVL formulations were significantly less (8.6-11.4 μg/mL) than MLV and free drug solution (12.5 μg/mL). The AUC_{0-48} of the MVL formulations was 1.5- and 3-fold higher compared with conventional liposomes and free drug solution, respectively. Overall, formulations containing phosphatidyl glycerol as negatively charged lipid showed better results. The MVL delivery system as an intradermal depot offers the advantage of a very high loading and controlled release of acyclovir for an extended period of time. The increase in AUC and decrease in C_{max} reflects that the MVL formulations could reduce the toxic complications and limitations of conventional IV and oral therapies.

KEYWORDS: multivesicular liposomes, acyclovir sodium, herpes simplex virus, sustained delivery, intradermal depot systems

INTRODUCTION

Herpes simplex virus (HSV) causes a variety of infections including orofacial and cutaneous herpes, genital herpes, chicken pox, varicella zoster infections, and herpes keratitis. Acyclovir is being used in the treatment of most of these infections but currently available therapies have a number of limitations. The oral absorption is dose dependent and highly variable with a bioavailability ranging from 10% to 30%.[1,2] Nevertheless, the oral route is preferred to parenteral administration because of the risk of local toxicity at the injection site. The mean plasma half life ($t_{1/2}$) of acyclovir is ∽2.5 hours. Hence, repeated administration of high doses is required (200 mg 5 times daily for 10 days) for the effective management of HSV infections.[3] On topical application the absorption of drug is very slow and needs a permeation enhancer, whereas bolus rapid injection causes renal precipitation of the drug. None of these regimens reproducibly reduces the risk of recurrent genital lesions. Frequently recurring genital herpes can be suppressed effectively with chronic acyclovir dosage regimens.[4] The dosage requirements are very high for immunocompromised patients and recurrence is common after discontinuation of therapy.[5] Topical acyclovir ointment is not associated with clinical benefits in recurrent herpes labialis. Furthermore, oral acyclovir has been associated infrequently with nausea, diarrhea, rash, or headache. Looking at these problems, an attempt was made to develop a depot intradermal acyclovir sodium formulation for prolonged systemic delivery.

Liposomes have shown great potential as versatile drug delivery systems to deliver numerous bioactives. These include proteins, peptides, antineoplastic agents, antibiotics, and antiviral drugs.[6,7] Liposome formulations of interferons, peptides, and hormones (calcitonin) have successfully been used as intramuscular depots.[8]

However, the conventional liposomes (unilamellar and multilamellar liposomes) have certain limitations viz low entrapment efficiency for water-soluble drugs, stability problems, and release of drugs after a single breach in the external membrane.[9] Very low aqueous entrapment (2-4 μL/μmol of lipid) precludes effective use of these carriers for hydrophilic drugs.[10] This challenge has been successfully met by the use of a new carrier, ie, a multivesicular liposomal (MVL) drug delivery system.[11-13] The MVL systems are characterized by their sui-generis structure of multiple nonconcentric aqueous chambers surrounded by

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a network of lipoidal membranes.[9] These systems can be differentiated from conventional liposomes (Unilamellar Vesicles [ULVs] and multilamellar vesicles [MLVs]) in 2 major aspects: size and composition. MVL carriers have a size range of 5 to 30 μm compared with ULVs (1 μm) and MLVs (1-5 μm). In addition to the general chemical composition of conventional liposomes, MVLs contain a neutral lipid (triolein, tricaprylene, trilaureine, tributyrine, etc) as an integral component, which is responsible for its unique multivesicular structure.[14,15] As MVLs are composed of multiple nonconcentric aqueous chambers, they contain 95% water, therefore providing a unique carrier system for hydrophilic drugs including a variety of therapeutic proteins.[16] This MVL technology has successfully been used to deliver several small molecules, analgesics, antitumor drugs, and antiviral drugs for prolonged therapeutic concentrations.[17-22]

The structural characteristics of MVLs make them ideal vehicles for locoregional drug delivery. The MVL particles are manufactured with lipids, which are a membrane tissue component of the human body, hence they are highly biocompatible and nonimmunogenic.[15] Their unique particle size is large enough to preclude their entry into the capillaries and thus remain at the site of administration. Since most of the volume of MVLs is occupied by water, lipid remnants are minimal once the contents are released.

Acyclovir is being used to treat herpes simplex virus infections in both immunocompetent and immunocompromised patients.[5] Currently available dosage regimens of acyclovir have a number of limitations including the following: (1) variable bioavailability by oral administration, (2) poor percutaneous absorption, and (3) thrombophlebitis on IV bolus injection.[1,23] Most earlier studies to improve the physico-chemical characteristics of acyclovir are based on chemical modification viz amino acid ester prodrug,[24] highly water soluble alkylamines and benzylcarbamate prodrugs,[25] aliphatic prodrugs,[26] redox-based chemical targeting systems to enhance nasal transport,[27] 6-deoxyacyclovir prodrug to enhance intradermal delivery,[28] and 1-valyl ester prodrug, valacyclovir with greater oral bioavailability. Very limited work has been reported on dosage form modification, which includes transbuccal delivery[29] and ocular delivery. Positively charged acyclovir liposomes were developed for ocular delivery[30] for their possible use in the treatment of herpes keratitis. High loading efficiency and the sustained release pattern of MVLs for hydrophilic drugs prompted us to investigate the usefulness of this carrier system as a depot for systemic delivery of acyclovir sodium. MVL depot delivery through the topical route overcomes the first pass effect of oral therapy and possibly reduces the side effects of IV therapy.

The objectives of the present study were to design a depot delivery system of acyclovir sodium using MVL to overcome the problems associated with conventional therapies and to study in vivo effectiveness for sustained action. Also, the efficacy of MVLs was compared with plain liposomes in vivo.

MATERIALS AND METHODS

Materials

Acyclovir sodium was a gift from Cipla Pharmaceutical Ltd (Mumbai, India). Amphipathic lipids (phosphatidyl choline [PC], dipalmitoyl phosphatidyl choline [DPPC], dimyristoyl phosphatidyl choline [DMPC], and distearoyl phosphatidyl choline [DSPC]), negatively charged lipids (phosphatidyl serine [PS], phosphatidyl glycerol [PG], and phosphatidic acid [PA]), cholesterol, and tributyrine were purchased from Sigma Chemical Co (St Louis, MO). Glucose, glycine, and free-base lysine were purchased from Himedia (Mumbai, India). All other solvents and reagents were of analytical grade.

Preparation of Liposomes

The MVLs containing acyclovir were prepared by the reverse phase evaporation (REV) technique using a double emulsion (w/o/w) method, described in previous reports.[15,16,31] Briefly, an aqueous solution of acyclovir sodium (10 mg/mL) containing varying amounts of glucose (2.5%-7.5%) was emulsified (to get a w/o primary emulsion) with an equal volume of chloroform solution containing 13.2 mM amphipathic lipid (PC/DMPC/DPPC/DSPC), 19.88 mM cholesterol, 2.44 mM tributyrine, and 2.80 mM negatively charged lipids (PS/PA/PG) at ambient temperature (23-28°C) for 9 minutes at 9000 rpm. Then, this w/o emulsion was subsequently emulsified with a second aqueous solution containing 1.5% glycine/40 mM lysine at 6000 rpm for 1 minute to get the w/o/w double emulsion. The chloroform was removed by flushing nitrogen at 37°C over the surface of the emulsion (w/o/w), with gentle stirring on a magnetic stirrer. Similarly, the conventional liposomes were prepared by the same method using optimized concentration of PC and cholesterol. The resulting liposomes were washed to remove unentrapped drug and harvested by centrifugation for 10 minutes at 600*g* and then resuspended in phosphate buffered saline (PBS) with pH 7.4.

In Vitro Characterization

Acyclovir-entrapped MVL formulations were characterized for the following attributes:

Vesicle Size and Size Distribution

The vesicle size and size distribution of various formulations were determined using a particle size analyzer (model

Table 1. Formulation Composition and Physicochemical Properties of Various MVLs

Formulation Code	Lipid Combination	Unit in mM	Average Vesicle Diameter (μm)	Vesicle Count $mm^3 \times 1000$	Percent Encapsulation Efficiency
Acy-MVL-1	PC: CH:TB:PG	13.2:19.88:2.44:2.8	8.2 ± 0.3	32.4 ± 1.4	44.3 ± 2.1
Acy-MVL-2	PC: CH:TB:PS	13.2:19.88:2.44:2.8	7.5 ± 0.3	32.7 ± 1.3	43.2 ± 1.8
Acy-MVL-3	PC: CH:TB:PA	13.2:19.88:2.44:2.8	7.2 ± 0.0.3	29.7 ± 1.1	30.7 ± 1.3
Acy-MVL-4	DMPC: CH:TB:PG	13.2:19.88:2.44:2.8	9.2 ± 0.4	29.1 ± 1.2	72.2 ± 3.4
Acy-MVL-5	DMPC: CH:TB:PS	13.2:19.88:2.44:2.8	8.1 ± 0.3	32.8 ± 1.8	58.1 ± 2.5
Acy-MVL-6	DMPC: CH:TB:PA	13.2:19.88:2.44:2.8	7.6 ± 0.3	35.1 ± 1.7	53.8 ± 2.5
Acy-MVL-7	DPPC: CH:TB:PG	13.2:19.88:2.44:2.8	12.4 ± 0.5	29.4 ± 0.1	76.6 ± 3.3
Acy-MVL-8	DPPC: CH:TB:PS	13.2:19.88:2.44:2.8	13.3 ± 0.4	20.0 ± 0.8	64.2 ± 3.1
Acy-MVL-9	DPPC: CH:TB:PA	13.2:19.88:2.44:2.8	12.6 ± 0.5	21.0 ± 0.9	62.3 ± 2.9
Acy-MVL-10	DSPC: CH:TB:PG	13.2:19.88:2.44:2.8	16.0 ± 0.7	16.6 ± 0.6	82.0 ± 4.1
Acy-MVL-11	DSPC: CH:TB:PS	13.2:19.88:2.44:2.8	16.8 ± 0.6	16.9 ± 0.7	74.2 ± 3.6
Acy-MVL-12	DSPC: CH:TB:PA	13.2:19.88:2.44:2.8	14.8 ± 0.7	17.9 ± 0.7	76.3 ± 3.3
Acy-Lip	PC:CH (Plain liposome)	15:22	1.8 ± 0.07	144.1 ± 6.5	12.8 ± 0.6

1604 L, Cilas, Marcoussis, France) and the data are presented in the Table 1.

Vesicle Count

The MVL formulations were diluted 10 times with 0.9% sodium chloride solution and the number of vesicles/mm^3 was counted by optical microscopy using a hemocytometer (Feinoptik, Blakenburg, Germany). The liposomes in 80 small squares were counted and the total numbers of vesicles/mm^3 were calculated using the following formula:

$$\text{Total number of vesicles}/mm^3 = \frac{\text{Total number of vesicles} \times \text{dilution} \times 4000}{\text{Total number of squares counted}} \quad (1)$$

Encapsulation Efficiency

The HPLC (high pressure liquid chromatography) method described by Boulieu et al[32] was used for the estimation of acyclovir entrapped in MVLs with slight modifications. The HPLC system (Shimadzu LC10 Ai, Chiyoda-ku, Tokyo, Japan) was operated in a binary mode with a photodiode array detector and a communication bus module. The analysis was performed at 254 nm with a shimpack, reverse phase C18, 250 mm × 4.5 mm, 5-μm column maintained at 25°C (column oven) using a mobile phase of 0.02 M potassium dihydrogenphosphate, pH 3.5 (99%) and acetonitrile (1%) pumped at a flow rate of 1.5 mL/min. The retention time of the drug was 7.8 ± 0.1 minutes and the calibration curve was rectilinear in the concentration range of 0.1 μg/mL to 50 μg/mL with a correlation coefficient of 0.999.

MVL suspension was lysed by vortexing with an equal volume of Triton X-100 and estimated for acyclovir content by HPLC.

In Vitro Drug Release Profile

MVL suspensions (1 mL) were sealed in dialysis bags (Sigma, 12 000 MW cutoff) and immersed in PBS pH 7.4 (25 mL) at 37 ± 1°C and kept on magnetic stirring. The samples were withdrawn at scheduled intervals (replaced with equivalent amount of PBS pH 7.4) and analyzed for acyclovir by HPLC as described above.

In Vivo Performance Studies

On the basis of in vitro studies, 4 MVL formulations (Acy-MVL-1, Acy-MVL-4, Acy-MVL-7, and Acy-MVL-10) were selected for pharmacokinetic study to assess in vivo performance of these systems. Plain liposomes (Acy-Lip) and free drug solution were taken as control formulations to study the effectiveness of MVL formulations.

Thirty male Wistar rats (150-170 g) were divided into 6 groups of 5 animals each. All formulations were administered to respective groups intradermally at a dose of 7 mg/kg of acyclovir. Blood samples were collected from the tail vein into Eppendorf tubes containing EDTA sodium solution (0.2 M) at scheduled intervals up to 48 hours. The blood samples were centrifuged and the plasma was deproteinized by adding one tenth the volume of 35% perchloric acid, vigorously vortexed, and centrifuged. Supernatants were filtered through a 0.45-μm membrane filter (Pall-Pharmalab, Mumbai, India) and estimated for acyclovir content by HPLC. The extraction efficacy of acyclovir from spiked plasma samples was 96% ± 3% (n = 6) and the calibration

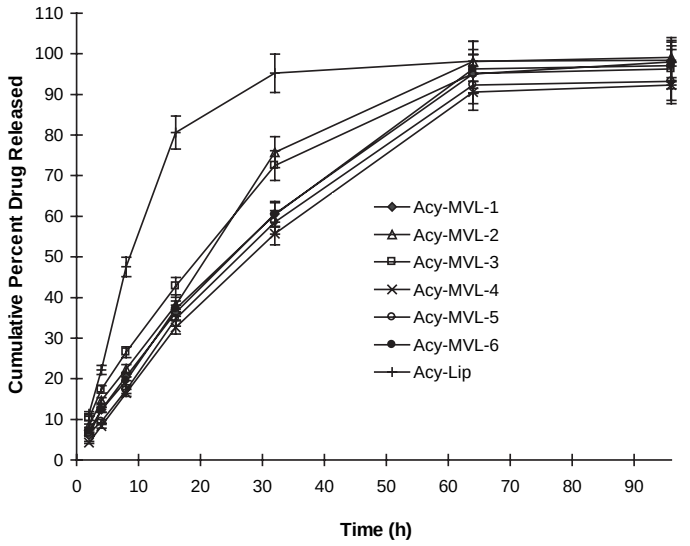

Figure 1. In vitro drug release profile of various MVL formulations (Acy-MVL-1 to Acy-MVL-6 and Acy-Lip) in PBS pH 7.4 (mean SD; n = 3).

curve was rectilinear in the concentration range of 0.1 μg/mL to 50 μg/mL ($r^2 < 0.99$). The interday and intraday accuracy and precision were within an rsd of ≤5%.

Statistical Analysis

The results were expressed as mean ± SD and the statistical analysis was done by ANOVA (analysis of variance). A probability level of $P < .05$ was considered to be significant.

RESULTS AND DISCUSSION

Preparation of Liposomes

The MVL formulations of acyclovir were prepared by a w/o/w double emulsification method using synthetic amphipathic lipids, neutral oil, and cholesterol. Lipid compositions were altered with negatively charged lipids and amphipathic lipids to optimize the formulation. Negatively charged lipids constitute an essential component of MVL structure because they increase the interlamellar distance between the successive bilayer of MVL structure, which leads to a greater overall captured volume. The presence of charged lipids also reduces the likelihood of aggregation following the formation of MVLs.[14] Neutral oil is an integral structural component and allows for the unique MVL structure. It becomes a part of the corner or edges where membranes meet each other and thus stabilizes membrane boundaries.[15]

Vesicle Size and Size Distribution

The particle size analysis reflects the monomodal size distribution of MVL formulations. Median size of the MVL formulations is in the range of 8 to 16 μm, and 95% of the MVL lies between 5 and 40 μm, whereas the size of MLVs is 1.8 μm (95% of particles sizing between 0.4 and 2.1 μm). It was observed that the size of the vesicle increased as on increasing the acyl chain of amphipathic lipids, and incorporation of negatively charged lipids has further increased the size. Maximum size was noted for Acy-MVL-11 containing PS compared with MVL containing PG (Acy-MVL-10) and PA (Acy-MVL-9). The amphipathic lipids with a longer acyl chain containing PG as a negatively charged lipid produced vesicles of larger size.

Vesicle Count

The vesicle count (count/mm^3) of MLVs (144.14 ± 6.54 × 10^3) was greater than MVL formulations. The smallest vesicle count (16.60 ± 0.62 × 10^3) was observed with amphipathic lipids containing longer acyl chains (Acy-MVL-10) due to their larger size (Table 1).

Encapsulation Efficiency

Almost 82% encapsulation efficiency was observed in certain MVL formulations (Acy-MVL-10) (Table 1). This increase in percent encapsulation efficiency may be attributed to their unique structure. These MVL formulations contain numerous nonconcentric aqueous chambers and the aqueous-to-lipid ratio is much higher, which results in high encapsulation efficiency for water soluble drug. Among these formulations, PG containing MVL exhibited higher entrapment efficiency as it consists of amphipathic lipids with longer acyl chains.

In Vitro Drug Release Profile

The in vitro release profile of various formulations is shown in Figures 1 and 2. MLV formulation released more than 80.63% ± 3.84% drug within 16 hours while MVL formulation released drug in a sustained manner for 96 hours. Acy-MVL-10 formulation has shown only 70.34% ± 3.12% drug release in a 96-hour period. The slow release of drug from MVL formulations compared with the MLV formulation may be attributed to the fact that there are more barriers to the diffusion of the drug from MVL particles. In the case of MLV formulations, a single breach in the internal membrane results in the total release of the internal aqueous content. MVLs showed no rapid initial release of drug as they have multiple known concentric compartments, hence drug first would pass through these compartments and then would diffuse into the external surrounding medium. The in vitro release of acyclovir sodium from MVL formulations occurs in near first order manner. They are osmotically responsive and stored in isotonic

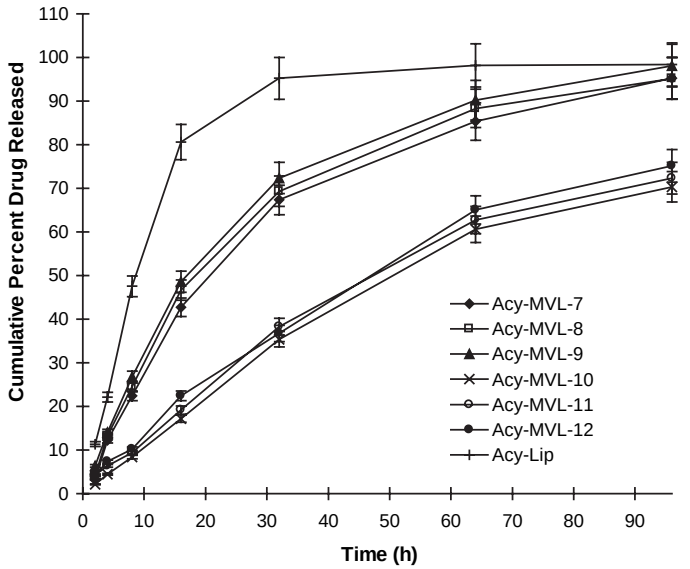

Figure 2. In vitro drug release profile of various MVL formulations (Acy-MVL-7 to Acy-MVL-12 and Acy-Lip) in PBS pH 7.4 (mean ± SD; n = 3).

medium. In vitro release indicates that MVL formulations exhibited a better-sustained and controlled acyclovir release compared with conventional liposomal formulations.

In Vivo Performance Studies

The in vivo behavior of the MVL formulations of acyclovir sodium was compared with control liposomal formulations (MLV) and free drug solution after intradermal administration to Wistar rats (Figure 3). Various pharmacokinetic parameters such as t_{max} (time required to achieved maximum plasma concentration), C_{max} (maximum plasma concentration), and AUC (area under the drug plasma concentration – time curve) were calculated and are presented in Table 2. MVL formulations containing PG as negatively charged lipid showed superiority over the other formulations as evident from significantly reduced C_{max} and very high AUC values. The C_{max} values of Acy-MVL-7 (8.61 μg/mL) and Acy-MVL-10 (9.81 μg/mL) were significantly less ($P < .05$) than MLV formulations (12.53 μg/mL) as well as free drug solution (12.34 μg/mL). MVL formulations showed a t_{max} of 16 hours compared with 4 and 8 hours, respectively, of free drug solution and MLV formulation. The $AUC_{0\text{-}48}$ of the MVL formulations (Acy-MVL-10; 389 μg.h/mL and Acy-MVL-7; 352 μg.h/mL) was 1.5- and 3-fold higher compared with MLV formulations (Acy-Lip; 217 μg.h/mL) and free drug solution (119 μg.h/mL), respectively. The increase in t_{max} and $AUC_{0\text{–}48}$ and decrease in C_{max} reflects that the MVL formulations could reduce toxic complications such as parivascular inflammation and renal precipitation of IV therapy. Also, increase in

Table 2. Pharmacokinetic Parameters of Various Formulations*

Formulation Code	C_{max} (μg/mL)	t_{max} (h)	$(AUC)_{0\text{–}48}$ (ng.h/mL)
Free Acyclovir sodium solution	12.3 ± 0.6	4	119.2 ± 4.8
Acy-Lip	12.5 ± 0.5	8	216.7 ± 7.2
Acy-MVL-1	11.4 ± 0.5	8	205.8 ± 7.4
Acy-MVL-4	10.3 ± 0.4	8	258.3 ± 10.3
Acy-MVL-7	8.6 ± 0.3	16	351.5 ± 15.6
Acy-MVL-10	9.8 ± 0.4	16	389.4 ± 17.6

*t_{max} is time required to achieved maximum plasma concentration; C_{max} is maximum plasma concentration; AUC is area under the drug plasma concentration-time curve.

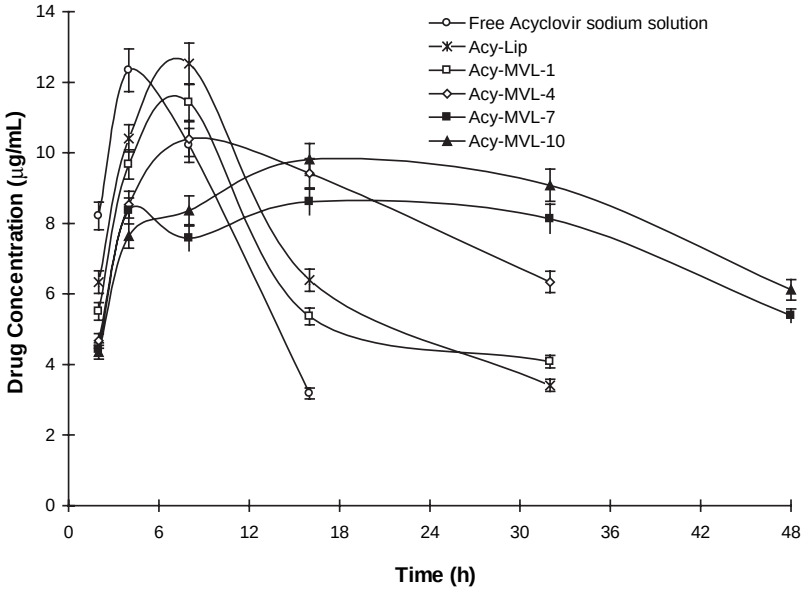

Figure 3. Acyclovir plasma concentration profile of various MVL formulations (mean ± SD; n = 3).

AUC_{0-48} value of this route (intradermal) is advantageous over the oral route, which suffers variable bioavailability.

Moreover, the drug is cleared rapidly from the blood on intradermal administration of free drug solution, but the liposomal formulation exhibited sustained action because of delayed release of drug from the formulation. MLVs, being smaller in size, are taken up by capillaries and the lymphatic system and reach systemic circulation where they are opsonized, thus releasing their contents. However, the effective drug levels were maintained up to 32 hours. On the other hand, MVL formulations, because of their larger vesicle size, cannot be taken up by the lymphatic/ capillary network. Hence, the MVL (12 μm) system remains at the site of administration and forms a depot. Therefore, acyclovir after diffusion from the vesicle reaches the systemic circulation slowly and gradually to maintain the drug concentration in the therapeutic window for an extended period of time.

CONCLUSIONS

This versatile delivery system offers the advantage of a very high loading and controlled release of acyclovir for an extended period of time compared with plain liposomes. Pharmacokinetic study reveals that MVL formulations showed reduced C_{max} and effective drug concentration for a prolonged period.

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