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RESEARCH ARTICLE

Development and evaluation of nanosized niosomal dispersion for oral delivery of Ganciclovir

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Abstract

Encapsulation of Ganciclovir in lipophilic vesicular structure may be expected to enhance the oral absorption and prolong the existence of the drug in the systemic circulation. So the purpose of the present study was to improve the oral bioavailability of Ganciclovir by preparing nanosized niosomal dispersion. Niosomes were prepared from Span40, Span60, and Cholesterol in the molar ratio of 1:1, 2:1, 3:1, and 3:2 using reverse evaporation method. The developed niosomal dispersions were characterized for entrapment efficiency, size, shape, *in vitro* drug release, release kinetic study, and *in vivo* performance. Optimized formulation (NG8; Span60:Cholesterol 3:2 molar ratio) has shown a significantly high encapsulation of Ganciclovir (89 ± 2.13%) with vesicle size of 144 ± 3.47 nm (polydispersity index [PDI] = 0.08). The *in vitro* release study signifies sustained release profile of niosomal dispersions. Release profile of prepared formulations have shown that more than 85.2 ± 0.015% drug was released in 24 h with zero-order release kinetics. The results obtained also revealed that the types of surfactant and Cholesterol content ratio altered the entrapment efficiency, size, and drug release rate from niosomes. *In vivo* study on rats reveals five-time increment in bioavailability of Ganciclovir after oral administration of optimized formulation (NG8) as compared with tablet. The effective drug concentration (>0.69 µg/mL in plasma) was also maintained for at least 8 h on administration of the niosomal formulation. In conclusion, niosomes can be proposed as a potential oral delivery system for the effective delivery of Ganciclovir.

Keywords: Ganciclovir, niosomes, entrapment efficiency, *in vivo* study

Introduction

Ganciclovir (a synthetic acyclic nucleoside analog of 2′-deoxyguanosine) is a potent inhibitor of human and animal herpes viruses[1–3] which shows excellent antiviral activity against varicella-zoster virus, cytomegalovirus, and Epstein-Barr virus. Ganciclovir is converted to Ganciclovir-5′-triphosphate *in vivo* which competitively inhibits viral DNA polymerase enzyme with respect to deoxyguanosine triphosphate[4–6]. Phosphorylated Ganciclovir shows greater affinity for DNA polymerase. This affinity is selective as inhibiting only the viral enzyme[7]. Ganciclovir is the first-line therapy for cytomegalovirus in immunocompromised patients, like organ transplant patients and patients with acquired immunodeficiency syndrome (AIDS)[3,8]. It is administrated through intravenous, oral, and intra-ocular route.

Ganciclovir is a BCS-III/IV drug having high solubility and low permeability[9]. Oral Ganciclovir has been shown to be effective in preventing CMV disease, but its poor oral bioavailability limits the degree of viral suppression and may predispose to the emergence of resistance[4,10–14]. Intravenous administration of Ganciclovir offers therapeutically effective plasma concentration levels, but the drawbacks associated due to long-term administration of i.v. Ganciclovir are patient inconvenience, higher cost (40% higher), and incidence of catheter-related

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infections and sepsis[15] as compared with orally administered Ganciclovir. Due to hydrophilic nature (log $P = -2.0$) and poor membrane permeation characteristic (BCS-III/IV), the bioavailability of Ganciclovir is very poor (5–7%) which require administration of large dose per day to reach effective plasma concentration. Furthermore, poor oral bioavailability of Ganciclovir is associated with greater intersubject variability of plasma concentrations and development of drug resistance[9].

So the purpose of present study was to improve the oral bioavailability of Ganciclovir by encapsulating the drug in lipophilic non-ionic surfactant-based nanosized niosomal colloidal dispersion.

Niosomes, the non-ionic surfactant vesicles are microscopic lamellar structures formed on admixture of different non-ionic surfactant classes such as alkyl or dialkyl polyglycerol ethers and Cholesterol, with subsequent hydration in aqueous media[16–19].

The *in vivo* behavior and fate of niosome is similar to the liposome like prolonging the drug release and circulation of entrapped drug and altering its organ distribution and metabolic stability[17]. Niosomes may overcome the problems associated with liposomes, most important is chemical instability of the phospholipids, cost, oxidation susceptibility, and variable purity.

Materials and methods

Materials

Ganciclovir was obtained from Ranbaxy laboratories (Gurgaon, India). Sorbitan monopalmitate (Span40) and Sorbitan monostearate (Span60) were obtained from S.D. Fine chemicals (Delhi, India). Cholesterol was obtained from BDH chemicals (Delhi, India) and ethanol and diethyl ether, dimethyl sulfoxide from Merck India Ltd. (Mumbai, India).

Methods

Analysis of Ganciclovir by HPTLC in dissolution media and plasma

Ganciclovir was analyzed by using HPTLC. The samples were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60F-254 (20 cm × 10 cm) with 200 μm thickness (E. Merck, Germany) using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 150 nL s^{-1} was employed and space between two bands were 10 mm. The slit dimension was kept at 5mm × 0.45 mm and 20 mm s^{-1} scanning speed was employed. The mobile phase consisted of butanol:acetic acid:water (60:25:15). Linear ascending development was carried out in a twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2°C) at relative humidity of 55 ± 5%. The length of chromatogram run was 80 mm. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer. The source of radiation utilized was a deuterium lamp. A stock solution of Ganciclovir (100 μg mL^{-1}) was prepared in methanol. In case of plasma, the sample (1 μL) was transferred into Eppendorf tube and extracted with dichloromethane on a vortex-mixer for 2 min and centrifuged at 2100 rpm for 10 min. The supernatant was transferred to glass microvials and the solvent was evaporated at 45°C in water bath. The plasma was reconstituted with methanol (100 μL). Different volumes of stock solution 1, 2, 4, 6, 8 μL were spotted on the TLC plate to obtain concentrations of 100–800 ng $spot^{-1}$ of Ganciclovir. The plasma samples were also spotted in the similar fashion. The data of peak areas plotted against the corresponding concentrations were treated by least square regression analysis. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 254 nm. The R_f was found to be 0.36. Linear least squares regression analysis showed there was a good linear relationship ($r^2 > 0.997$) between peak area and concentration in the range 100–800 ng per zone. The intraday and interday precisions determined as relative standard deviation (%RSD) ranged between 0.4458–2.7873% and 0.711–1.8925%, respectively, whereas in case of plasma, it was found to be 0.4491–2.574% and 0.702–1.388%, respectively. Accuracy calculated as percent recovery was in the range of 97.2484–99.7772% (including plasma sample). The LOD, expressed as 3.3σ/(slope of the calibration plot), and the LOQ, expressed as 10σ/(slope of the calibration plot), were 2.511 ng/spot and 7.610 ng/spot, respectively. For plasma sample, the LOD and LOQ was found to be 2.893 ng/spot and 7.992 ng/spot, which indicates the sensitivity of the method is adequate.

Preparation of niosomal formulation

Noisomes were prepared by using the commonly used reverse evaporation method[20]. In this method, surfactant and Cholesterol (3:2 molar ratio) were dissolved in a mixture of 5 mL of ether and chloroform (1:1 v/v), called as organic phase. An aqueous phase (10 mL) containing drug (20 mg) is added to this phase and sonicated at 4–5°C for 30 min. Clear gel form appeared which was further sonicated after the addition of a small amount (2 mL) of phosphate buffered saline (PBS). The organic phase is removed at 40°C under low pressure. The resulting viscous niosome suspension is diluted with PBS and heated on a water bath at 60°C for 10 min. The composition of the developed niosomal dispersions are given in Table 1. For the further experiment, the developed niosomes were stored at 15 ± 0.5°C under ambient humidity.

In vitro characterization of niosomes

Particle size determination

Morphology of niosomal formulation was confirmed by optical microscopy (Motic, UK) and transmission electron microscopy (*TEM*). TEM (Morgagni 268D SEI, USA) was set at 200 KV and of a 0.18 nm capable of point-to-point resolution. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form and size of the Ganciclovir

Table 1. Composition of different developed niosomal formulations.

Formulation code	Surfactant	Cholesterol	Ratio of surfactant and Cholesterol (molar ratio)	Amount of drug (mg)
NG1	Span40	29 mg	1:1	20
NG2		19 mg	2:1	
NG3		14 mg	3:1	
NG4		23 mg	3:2	
NG5	Span60	29 mg	1:1	
NG6		19 mg	2:1	
NG7		14 mg	3:1	
NG8		23 mg	3:2	

niosomal formulation. In order to perform the TEM observations, the diluted niosomal formulation was deposited on the holey film grid and observed after drying.

Vesicle size, size distribution, and zeta potential

Vesicle size was determined by photon correlation spectroscopy that analyzes the fluctuations in light scattering due to Brownian motion of the particles using a Zetasizer (Nano-ZS, Malvern Instruments, UK). The formulation (0.1 mL) was dispersed in 50 mL of water in a volumetric flask, mixed thoroughly with vigorous shaking, and light scattering was monitored at 25°C at a 90°angle. In addition, zeta potential was also determined using Zetasizer (Nano-ZS, Malvern Instruments, UK).

Entrapment efficiency

Unentrapped drug was separated by *centrifugation and after complete vesicle disruption using Triton X-100, the* remaining amount of the drug in niosomes was determined.

Entrapment efficiency was calculated by using the following formula:

$$\text{Entrapment efficiency (EF)} = \left(\frac{\text{Amount entrapped}}{\text{Total amount}}\right) \times 100$$

Transition temperature analysis of niosomes

Transition temperature analysis was studied by DSC (Perkien Elmer, USA). A small amount of freeze-dried non-ionic surfactant vesicles and pure semisolid surfactant (Span40, Span60) was sealed in a 40-µL aluminum crucible and empty aluminium crucible is taken as reference. The temperature of the pans was raised from 40°C to 400°C, at a rate of 10°C/min. The heat flow calibration was performed with indium. The reproducibility of the thermograms was determined by repeating the temperature cycle three times for each sample.

In vitro drug release performance

In vitro release study was performed by USP XXII method (Dissolution apparatus II at 100 rpm and 37 ± 0.5°C filled with 900 mL of phosphate buffer (pH 7.4). 2 mL of niosomal dispersion (containing 4 mg of Ganciclovir) was placed in activated dialysis bag dialysis membrane bag (molecular weight cut off 12,000 Da). 5 mL of samples were withdrawn at regular time intervals (0, 2, 4, 6, 8, 12, 16, 20, and 24 h) and replaced with same volume of fresh phosphate buffer (pH 7.4) to maintain the sink condition. Samples were analyzed for the drug content by HPTLC.

Release kinetic study

Kinetic analysis of *in vitro* release data of optimized formulation was done according to zero-order, first-order, and Higuchi model.

Zero-order model $Q = kt$

First-order model $\text{Log } Q = kt/2.303$

Higuchi model $Q = k\sqrt{t}$

where, Q = amount of drug released at time t; k = dissolution rate constant (with unit of µg/mL/h for zero-order model, 1/h for first-order model, and µg/mL/h for Higuchi model.

Estimation of Ganciclovir in plasma

Approval to carry out *in vivo* study was obtained from Jamia Hamdard, Institutional Animal Ethics Committee and their guidelines were followed for the studies. The optimized niosomal dispersion, which showed the highest drug release, was taken for *in vivo* studies. In *in vivo* study, the drug analysis was performed by developed HPTLC method. Two groups containing six rats in each were taken for the study. The animals were kept under standard laboratory conditions, temperature at 25 ± 2°C and relative humidity (55 ± 5%). The animals were housed in polypropylene cages, three per cage, with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water ad libitum. The formulations (niosomal dispersion and marketed tablet) were given orally using oral feeding sonde. The rats were anesthetized using diethyl ether and blood samples (0.5 mL) were withdrawn from the tail vein of rat at 0 (pre-dose), 0.2, 0.4, 0.6, 1, 2, 2.5, 3, 4, 5, 6, 8, 12, and 24 h in vacutainer tubes, mixed and centrifuged at 5000 rpm for 20 min. The collected plasma was extracted with dichloromethane on a vortex-mixer for 2 min and centrifuged at 2100 rpm for 10 min. The combined extract was evaporated to dryness at 45°C in a water bath. At the time of analysis, all residues were reconstituted with methanol and applied on TLC plate. Pharmacokinetic parameters (PK) were calculated by

non-compartmental analysis, also called as model independent analysis, using WinNonLin version 4.0 (Pharsight Corp., Mountain View, CA). Peak plasma concentration (C_{max}) and time of its occurrence (t_{max}) were read directly from the individual plasma concentration–time profiles. Area under concentration time curve ($AUC_{0\to t}$) was calculated according to linear trapezoidal method.

Pharmacokinetic and statistical analysis

Data of *in vivo* analysis was expressed as mean of six animals ± SD. All pharmacokinetic parameters (t_{max}, C_{max}, $AUC_{0\to t}$) were calculated individually for each subject in the group and the values were expressed as mean ± SD. The data were compared for statistical significance by the one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test using GraphPad Instat software (GraphPad Software Inc., CA).

Results and discussion

Photomicroscopy and transmisson electron microscopy

The photomicrograph of optimized niosomal formulation revealed the spherical shape of vesicles NG8 (Figure 1A). The electron micrograph of optimized formulation are shown in Figure 1B. They show the clear outline and the core of the well-identified vesicles displaying the vesicular structure. Similar structural feature were reported earlier[21]. Vesicles were presented in dispersed and unaggregated form with the average vesicular size of 144 ± 3.47 nm (Figure 1C).

Vesicle size, size distribution, and zeta potential

The particle size distribution and polydispersity index [PDI] of Span40- and Span60-based niosomes are given in Table 2. Average particle size was found to be 144 ± 3.47 nm with PDI = 0.08 for optimized formulation NG8 (Figure 1C). Regarding PDI, a value of zero indicates an entirely monodisperse population and a value of 1 indicates a completely polydisperse population[22]. So the PDI value of 0.08 for NG8 indicates the best uniformity of size among the formulation. During experiments, it was found that niosomes prepared using Span60 were slightly larger in size (133 ± 3.07 nm–144 ± 3.47 nm) than those prepared using Span40 (129 ± 3.35 nm–121 ± 3.12 nm). The result was in accordance with the previous finding[23,24]. Correlation between entrapment efficiency and particle size with the nature of surfactant and its ratio with Cholesterol are presented in Figure 2. The increased size of the developed niosomes may be due to the presence of Span60 which has a longer saturated alkyl chain as compared with Span40[25]. Furthermore, the larger vesicles are formed when the hydrophilic portion of the molecule is decreased relative to the hydrophobic portion[26], it may also be attributed to the fact that the increase in alkyl chain length as the series is ascended from the C12 to the C18 ester would result in an increase in the value of the critical packing parameter[27]. Furthermore, zeta potential of all the developed niosomal dispersions were

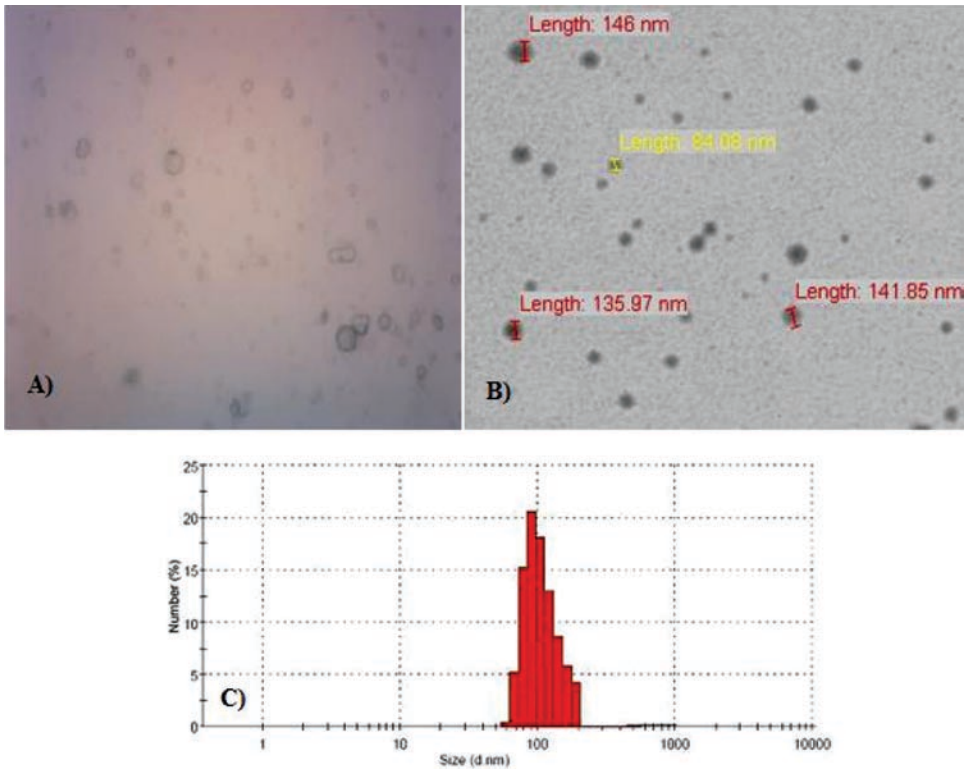

Figure 1. (A) Photomicrograph (400×), (B) Transmission electron microscopy (TEM) image, (C) Particle size distribution curve of Ganciclovir-loaded niosomes formulation-NG8.

determined which varied from –9.5 ± 0.9 mV to –27.9 ± 1.9 mV for NG1-NG8 (Table 2). The obtained zeta potential revealed that the developed formulations were stable and having uniformly dispersed particles.

Entrapment efficiency

Data in Table 2 shows entrapment efficiencies of various developed niosomal formulations based on different molar ratio of Span40:Cholesterol and Span60:Cholesterol. The highest entrapment efficiency (89 ± 2.13%) was found for NG8 which is Span60-based niosome having surfactant:Cholesterol ratio of 3:2. Effect of nature of surfactant and its ratio against the Cholesterol is established in Figure 2. Results showed that the incorporation of Cholesterol into niosomes significantly increased the drug entrapment efficiency up to an optimum ratio (3:2) of Span60:Cholesterol. Cholesterol alters the fluidity of chains by reducing the transition of gel to liquid phase of surfactant bilayer[27–29]. It also increased the micro-viscosity of niosomal membrane conferring more rigidity, resulting in a higher stability which leads to the greater drug retention[25]. However, after increasing the Span40:Cholesterol molar ratio, it starts reducing the entrapment efficiency as it starts disrupting the regular bilayered structure leading to lowering of drug entrapment. Table 2 clearly shows that the entrapment efficiencies for Span60-based niosomes were significantly higher than Span40-based niosomes with the same molar ratios of Span60 to Cholesterol. This might be due to the higher phase transition temperature of Span60[30], long hydrophobic alkyl chain surfactant produces high entrapment[23]. Hence, Span60 having longer saturated alkyl chain (C18) compared with Span40 (C16) produced niosomes with higher entrapment efficiency[29]. In addition, the length of the alkyl chain influences the HLB value of the surfactant mixture that directly affects the drug entrapment efficiency. The HLB values for Span40 and Span60 are 6.7 and 5, respectively, the lower the HLB of the surfactant, the higher will be the drug entrapment efficiency[24].

Transition temperature analysis (DSC)

DSC thermograms of Ganciclovir, surfactant, and drug-loaded optimized niosome composed of Span60:Cholesterol (3:2) is illustrated in Figure 3A, 3B,and 3C. DSC thermogram of Ganciclovir and Span60 showed an endothermic peak at 250.71°C (Figure 3A) and 62.165°C (Figure 3B), respectively. DSC thermogram of Ganciclovir-loaded niosomes showed disappearance of all characteristic melting endotherms (Figure 3C). This absence of all melting endotherms suggests the encapsulation of Ganciclovir in niosomal dispersion occurs. Similar results were found earlier as well with niosomal preparation[31,32].

Table 2. Mean particle size, PDI, zeta potential (mV), and entrapment efficiency (%) of niosomal dispersion.

Formulation code	Mean particle size ± SD (nm)	PDI	Zeta potential ± SD (mV)	Entrapment efficiency (%) ± SD
NG1	129 ± 3.35	0.21	–9.5 ± 0.9	49 ± 3.01
NG2	127 ± 3.12	0.19	–11.2 ± 0.7	43 ± 5.78
NG3	109 ± 2.29	0.32	–12.6 ± 1.0	39 ± 2.43
NG4	121 ± 3.12	0.11	–10.3 ± 0.4	53 ± 3.67
NG5	133 ± 3.07	0.12	–19.5 ± 1.1	81 ± 2.56
NG6	137 ± 2.17	0.23	–16.8 ± 0.7	76 ± 4.34
NG7	142 ± 2.12	0.40	–21.2 ± 1.4	71 ± 2.01
NG8	144 ± 3.47	0.08	–27.9 ± 1.5	89 ± 2.13%

PDI, polydispersity index.

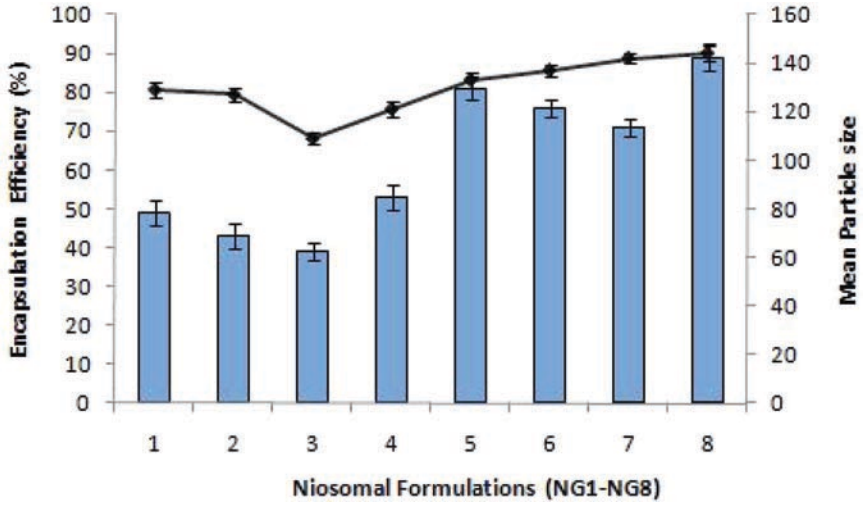

Figure 2. Effect of different surfactants and its ratio with Cholesterol on mean particle size and % encapsulation efficiency.

Dispersion study

Niosomes which gave the best dispersibility was Span60:Cholesterol-based NG8 formulations. In case of the other niosomal dispersions, higher sedimentation were seen that might be due to fusion of the niosomes. The sonication of the niosomal dispersion reduced the aggregation, making them more dispersive, thereby increasing dispersibility of the vesicular dispersion[24].

In vitro drug release studies

On the basis of entrapment efficiency, we have selected only Span60-based niosomes for the *in vitro* drug release

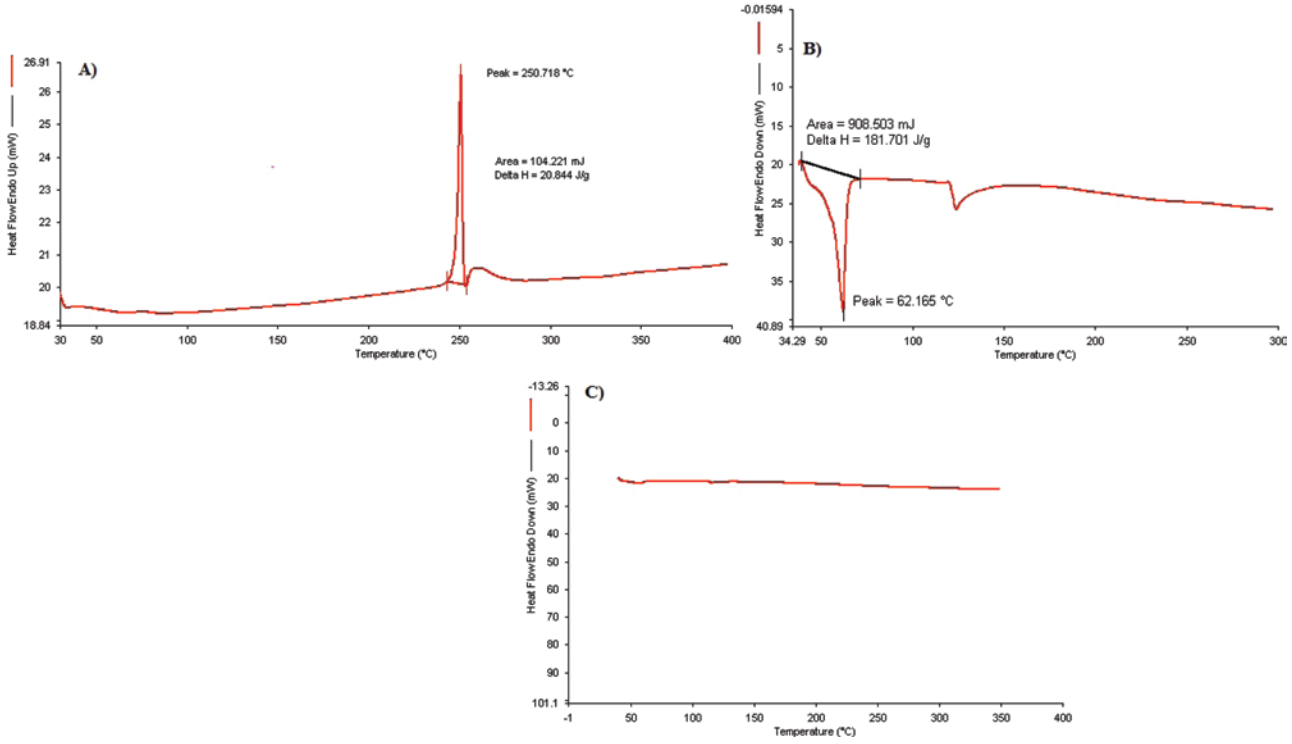

Figure 3. DSC thermogram of (A) Drug, (B) Span60, (C) Ganciclovir-loaded niosomes (NG8).

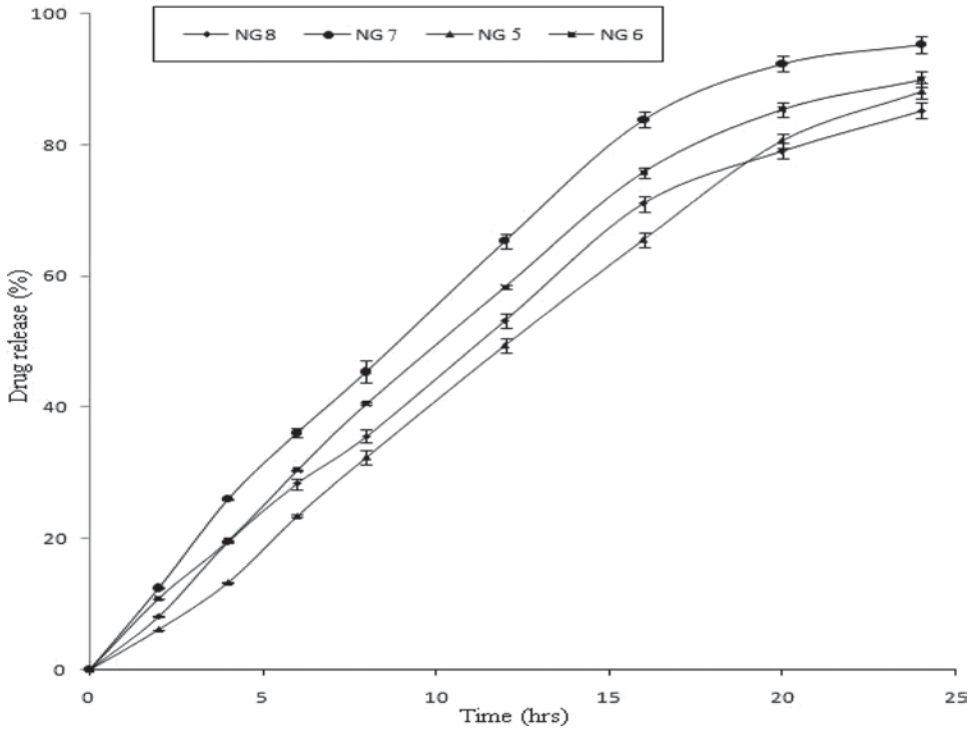

Figure 4. Percentage drug release profile of niosomal dispersion NG5, NG6, NG7, and NG8.

studies because they entrapped maximum amount of the drug used during niosomal preparation. Furthermore, maximum dose were available only when maximum entrapment occurs. A comparative *in vitro* release profile of four formulations [NG5, NG6, NG7, and NG8] is shown in Figure 4. Percentage release of Ganciclovir after 24 h was found to be 89.1 ± 0.016, 90.29 ± 0.17, 95.2 ± 0.014, and 87.34 ± 0.16 for NG5, NG6, NG7, and NG8, respectively. Result shows that the increase of Cholesterol molar ratio significantly reduced the efflux of Ganciclovir, showing Cholesterol membrane stabilizing ability and space-filling action[20,33]. Furthermore, Cholesterol is known to increase the rigidity of niosomes and abolish the gel-to-liquid phase transition of niosomal systems resulting in niosome formulations that are less leaky[34,35], thus decreasing the drug release from niosomes. Niosomal formulations prepared using Span60 showed a significantly slower rate of drug release compared with Span40 (data are not given). This can be explained by the fact that niosomes exhibit an alkyl chain length-dependent release. The higher the chain length, the lower the release rate[28]. Finally, NG8 was selected for *in vivo* study on the basis of entrapment efficiency and *in vitro* study.

Release kinetic study

Release data of optimized niosomal formulation were analyzed according to zero-order model (Figure 5A), first-order model (Figure 5B), and Higuchi model (Figure 5C). Release pattern was found to follow zero-order kinetics as average values of correlation coefficient were 0.9864 for zero-order model, 0.945 for first-order model, and 0.883 for Higuchi model.

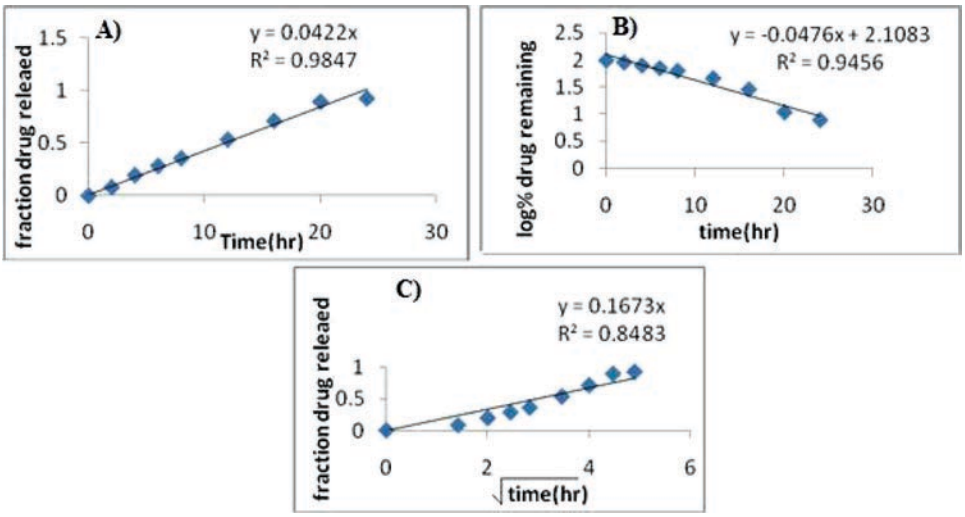

Figure 5. Figures reperesenting the *in vitro* release kinetic profile for (A) Zero-order model, (B) First-order model, (C) Higuchi model.

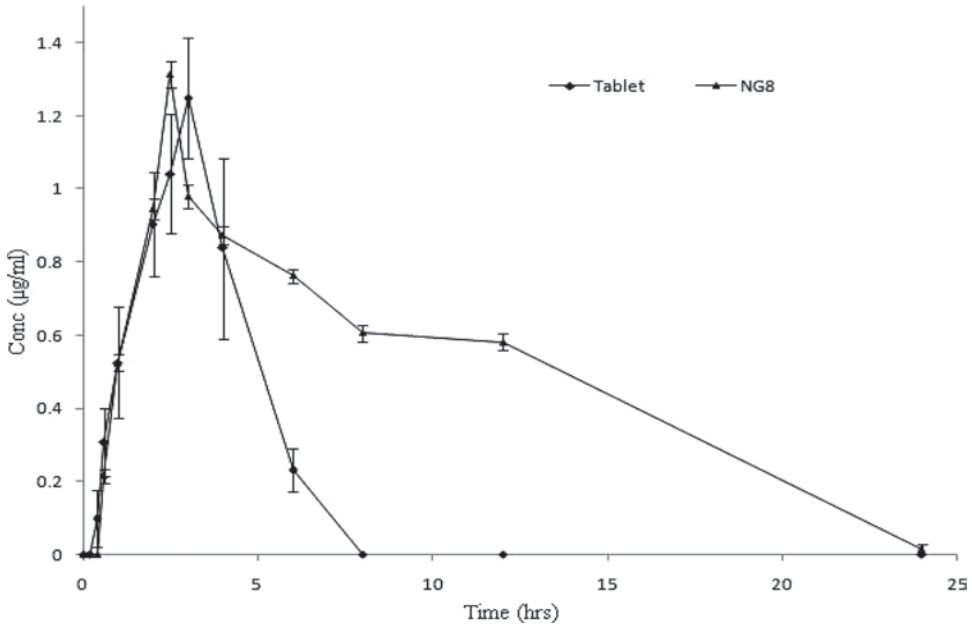

Figure 6. Pharmacokinetic profiles of Ganciclovir after oral administration of niosomal formulation (NG8) and marketed tablet.

Table 3. Pharmacokinetic parameters obtained following oral administration of drug-loaded niosomal dispersion and drug suspension to six rats (mean value ± SD; $n = 6$).

Formulation	t_{max} (h)	C_{max} (μg/mL)	AUC $_{0\rightarrow t}$ (μg.h/mL)
Tablet	3 ± 0.32	1.2101 ± 0.4	2120 ± 19.56
Niosomal dispersion	1.3512 ± 0.3	1.3512 ± 0.3*	11,200 ± 22.5†

†$p < 0.05$, compared with oral administration.
*$p < 0.01$, compared with oral administration.

In vivo study

Figure 6 shows the pharmacokinetic profiles of Ganciclovir after oral administration of niosomal formulation compared with oral suspension of drug. The calculated pharmacokinetic parameters are given in Table 3. For NG8 niosomal formulation, the $AUC_{0\rightarrow\infty}$ of ($AUC_{0\rightarrow\infty}$ of 11200 ± 22.5 μg/mL/h) was nearly 5-fold higher compared with orally administered drug suspension ($AUC_{0\rightarrow\infty}$ of 2120 ± 19.56 μg/mL/h), and the statistic calculation provided a significant difference between both values ($p < 0.05$). The effective drug concentration (>0.69 μg/mL in plasma) was maintained for at least 10 h through niosomal oral administration. The significant increase of C_{max} values and sustaining effect of the drug in plasma for prolonged period may be owing to enhanced absorption of the Ganciclovir with niosomal formulation. This finding may be a result of the influence of the vesicle size (250 nm) of niosomes, lipophilic nature of the niosomal formulation, improved partitioning of the lipophilic system to the mucosa, prolonged localization of the drug-loaded niosomes at the site of absorption, and its component (surfactants) as a penetration enhancer[36-38]. The *in vivo* data reveal that administration of Ganciclovir through niosomal dispersion had a sustained and enhanced absorption. Furthermore, Ganciclovir in niosomal form is effective at lower dose level with reduced dosing frequency. So the developed formulation can play a key role in reduction of dose and its associated side effects, which is observed with conventional dosage form.

Conclusion

The prepared Ganciclovir niosomes were homogenous in shape with an average size of 144 nm and maximum percentage drug entrapment was found to be 89 ± 2.13% with NG8. The niosomal formulation showed sustained release characteristics with zero-order drug release. *In vivo* study in rats discovered 5-fold increase in the oral bioavailability compared with tablet. Our studies provided evidence that niosomes were valuable as an oral delivery carrier to enhance the bioavailability of Ganciclovir.

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Declaration of interest

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