Influence of a Niosomal Formulation on the Oral Bioavailability of Acyclovir in Rabbits

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ABSTRACT

The purpose of this research was to prepare acyclovir niosomes in a trial to improve its poor and variable oral bioavailability. The nonionic surfactant vesicles were prepared by the conventional thin film hydration method. The lipid mixture consisted of cholesterol, span 60, and dicetyl phosphate in the molar ratio of 65:60:5, respectively. The percentage entrapment was ~11% of acyclovir used in the hydration process. The vesicles have an average size of 0.95 μm, a most probable size of 0.8 μm, and a size range of 0.4 to 2.2 μm. Most of the niosomes have unilamellar spherical shape. In vitro drug release profile was found to follow Higuchi's equation for free and niosomal drug. The niosomal formulation exhibited significantly retarded release compared with free drug. The in vivo study revealed that the niosomal dispersion significantly improved the oral bioavailability of acyclovir in rabbits after a single oral dose of 40 mg kg^{-1}. The average relative bioavailability of the drug from the niosomal dispersion in relation to the free solution was 2.55 indicating more than 2-fold increase in drug bioavailability. The niosomal dispersion showed significant increase in the mean residence time (MRT) of acyclovir reflecting sustained release characteristics. In conclusion, the niosomal formulation could be a promising delivery system for acyclovir with improved oral bioavailability and prolonged drug release profiles.

KEYWORDS: Acyclovir niosomes, oral acyclovir, bioavailability of acyclovir.

INTRODUCTION

Acyclovir is a synthetic acyclic purine nucleoside analog that is currently used for the prevention and treatment of herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections.[1] The oral bioavailability of acyclovir is low, variable, and species dependent.[2] The pharmacokinetic properties of acyclovir are well established.[3] The effects of dosage size on the extent of oral absorption are not well understood. Some reports suggest that absorption from the gastrointestinal tract may be a saturable, dose-dependent process.[4] In contrast, another study reported a relative constancy in the urinary recovery of unchanged drug and in the bioavailability calculated from urinary excretion data, concluding that the net absorption of acyclovir is nearly proportional to the dose.[2] Acyclovir is categorized as a class III drug according to the Biopharmaceutical Classification System (BSC) because of its high solubility and low permeability.[5] The US Food and Drug Administration (FDA) guidelines[6] reported that a drug substance is considered highly soluble when the highest dose strength is soluble in 250 mL or less of the aqueous media over the pH range of 1 to 7.5. The intrinsic solubility of acyclovir was 1.2 mg/mL as measured by the acid-base titration method.[7] Acyclovir has dissociation constants (pk_a) of 2.34 and 9.23 and partition coefficient (*P-octanol*) of ~0.023.[8] This low partition coefficient may reflect the low membrane permeability of acyclovir. According to the FDA guidelines,[6] the drug is considered highly permeable when the extent of absorption in human is determined to be >90% of the administered dose in comparison to an intravenous reference dose. In humans, acyclovir showed poor and variable oral bioavailability (15%-30%), probably due to the relatively low lipophilicity of the drug.[2] Thus, the rate-limiting factor in acyclovir absorption is its membrane permeability. The inclusion of absorption-enhancing excipients in the formulations can enhance the drug bioavailability. Trials that have been made to improve the oral bioavailability of acyclovir concerned mainly with chemical modification of the drug.[9,10] Luengo et al[11] studied the pharmacokinetics of different preparations of acyclovir with β-cyclodextrin and found that β-cyclodextrin showed no significant effect on the oral drug bioavailability. This finding is because the effect of cyclodextrin was mainly on the solubility of the lipophilic drug not the permeability of the hydrophilic drug. Encapsulation of acyclovir in lipophilic vesicular structure may be expected to enhance the oral absorption and prolong the existence of the drug in the systemic circulation. Niosomes are nonionic surfactant vesicles that are well recognized as drug delivery vehicles. Niosomes can carry hydrophilic drugs by encapsulation, are quite stable, and require no special conditions for production or storage. Preliminary studies indicate that niosomes may increase the absorption of certain drugs from the gastrointestinal tract following oral ingestion.[12]

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In the present study, acyclovir-loaded niosomes were formulated and evaluated for their in vitro as well as in vivo characteristics in an attempt to improve the oral bioavailability of the drug. The in vivo evaluation of acyclovir niosomes in comparison with free drug solution was conducted in rabbits after a single oral dose.

Materials and Methods

Materials

Acyclovir was obtained from Glaxo Welcom (Cairo, Egypt). Acyclovir sodium (freeze-dried acyclovir as the sodium salt) was purchased from Welcom (London, UK). Dicetyl phosphate (DCP), sorbitan monostearate (span 60), and sephadex G-25 were purchased from Sigma Chemical Co (St Louis, MO). Cholesterol (CHOL) was from BDH (Pool, UK). Triton X-100 was obtained from PARK (Northampton, UK). All other chemicals used were of analytical grade.

Preparation of Acyclovir Niosomes

The nonionic surfactant vesicles were prepared by the conventional thin film hydration method. Cholesterol, span 60, and dicetyl phosphate (47.5 mg CHOL, 47.5 mg span 60, and 5 mg DCP) in a molar ratio of 65:60:5 were dissolved in 2.5-mL chloroform-methanol mixture (1:1 vol/vol). The lipid mixture was added to a 100-mL rounded bottom flask, and the solvent was evaporated under reduced pressure at a temperature of 60ºC by a rotary evaporator (BÜCHI, HB-140, Flawil, Germany) until a thin lipid film was deposited on the wall of the flask. The excess organic solvent was removed by leaving the flask in a desiccator under vacuum overnight. The lipid film was hydrated with 2.5 mL of the aqueous phase containing acyclovir sodium in a concentration equivalent to 6 mg mL^{-1} acyclovir in normal saline. The use of drug in its sodium salt was to increase the drug concentration in the hydration fluids in a trial to increase the weight of the entrapped drug in the prepared niosomes.

The hydration was continued for 1 hour, while the flask was kept rotating at 60°C. It was essential to prepare the vesicles at a temperature above the gel-liquid transition temperature of the nonionic surfactant; span 60 has the highest phase transition temperature of ~50°C. The niosomal suspension was further hydrated at room temperature for 2 hours in order to complete the swelling process. The hydrated niosomes were sonicated for 20 minutes in a bath type sonicator (Ultrasons, Selecta, Barcelona, Spain). This niosomal dispersion containing both free and entrapped drug was used for in vivo study. Niosomes were separated from unentrapped drug by gel permeation chromatography. A 2.0-mL aliquot of the niosomal dispersions was eluted with normal saline on a 2×30-cm column of sephadex G-25. The niosomal fraction was diluted with the eluent to obtain a total lipid concentration of 5 mg mL^{-1}. This purified niosomal dispersion was used for in vitro study.

Particle Size Determination

The freshly purified niosomal dispersion was scanned and imaged using an optical microscope (Biomed, Carl Zeiss, Germany) attached to video camera (Panasonic, Japan) with a magnification power of ×40. Full measurement of the size and size distribution of the examined niosomes was performed using computer software that is locally designed and calibrated at the National Institute of Laser Enhanced Science (Cairo, Egypt).

Determination of Entrapment Efficiency

An aliquot of the freshly purified niosomal dispersion (5 mg lipid mL^{-1}) was diluted with 10% Triton X-100 in a ratio of 1:99 vol/vol. The detergent dissolved the niosomes and yielded a clear solution. The resultant solution was analyzed for acyclovir concentration using the described high-performance liquid chromatography (HPLC) method to calculate the amount of entrapped acyclovir. The percentage of entrapped acyclovir was calculated by applying the following equation:

$$\% \text{ Entrapment} = (A_E \times 100)/(A_I) \qquad (1)$$

where, A_E is the amount of entrapped drug, and A_I is the initial amount of drug in the aqueous phase.

In Vitro Release Study

The release of acyclovir from niosomes was studied by employing the dialysis method. The dialysis sacks (cellulose tubing, 35/100 mm flat width/length, Sigma Diagnostics (St Louis, MO) were washed several times with distilled water and left to soak in normal saline for 24 hours before use. A 3-mL sample, either of the freshly purified niosomal dispersion or of free acyclovir solution in normal saline, was transferred to the dialysis sacks. The concentration of acyclovir in each of the 2 samples was ~88 μg mL^{-1} (determined according to the calculated entrapment efficiency of the niosomal dispersion). The sack was placed in 200 mL magnetically stirred normal saline at 37°C. Two milliliter samples were withdrawn at specified time intervals of 0.5, 1, 2, 3, 4, 5, and 6 hours and replaced by fresh medium, and drug content was determined according to the described HPLC method.

Kinetic Analysis of Release Data

To investigate the possible mechanisms of acyclovir release from the prepared niosomes, the release data were analyzed mathematically according to the following models:

$$Q = kt \quad (2)$$

$$LogQ = kt/2.303 \quad (3)$$

$$Q = k\sqrt{t} \quad (4)$$

where, Q is the amount of drug released at a time (t) and k is the rate constant.

In-Vivo Study

Animals

Twelve male New Zealand white rabbits weighing 1.5 to 2.4 kg (average weight of 1.858 kg) were selected for study. The animal study adhered to the principles of Institutional Animal Care and Use Committee Guidebook (2nd Ed., 2002). The study protocol was approved by the institutional ethical committee for the use of animals in research. The rabbits were fasted overnight for 12 hours with free access to water. On study days, rabbits were placed in metal restrainers at 8:00 am, and a 22 gauge × 12-in catheter (Charter Med Inc., Winston-Salem, NC) was inserted without anesthesia into the anterior vena cava via the marginal ear vein. Doses were administered orally by gavage at 9:00 am as a 15-mL bolus of the respective formulation. After collection of the final blood sample at 24 hours after dosing, catheters were removed and rabbits were returned to their cages.

Experimental Design

Each animal received 2 formulations as a single oral dose of 40 mg kg^{-1} in a complete cross-over design with a 2-week washout period. The studied formulations were free acyclovir solution in normal saline and the freshly prepared unpurified niosomal dispersion containing both the free (89%) and the entrapped drug (11%). The use of unpurified niosomal dispersion was the possible practical solution to attain the high dose of acyclovir (40 mg kg GA,$^{-1}$) in a volume (15 mL) suitable for oral administration.

Collection of Blood Samples

Blood samples (1 mL) were collected through the marginal ear vein catheter by insulin plastic syringe at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 hours after administration of free acyclovir solution and at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 9.0, 12.0, 15.0, and 24.0 hours after administration of the niosomal acyclovir dispersion. Blood samples were collected in Eppendorf tubes and centrifuged at 1800g for 15 minutes. The serum was collected in Eppendorf tubes and was frozen at −40ºC until assayed.

HPLC Analysis of Acyclovir

The concentrations of acyclovir were measured in serum samples using the HPLC technique described by Peh and Yuen[13] with the following changes. An aliquot (250 μL) of serum was mixed with 100 μL of 30% trichloroacetic acid and the mixture was vortexed (Thermolyne Corporation, Maxi 11, Dubuque, IA) for 30 seconds and centrifuged at 1800g for 25 minutes. A 50-μL sample of the clear supernatant was injected onto the HPLC system. The HPLC system consisted of a Waters auto-sampler (model 717 plus), solvent delivery system (model 600), scanning fluorescence detector (model 474), and Millennium 2010 chromatography manager (Waters, Milford, MA). The separation was achieved using a reversed phase column (Waters, Bondapak C_{18}, 15×0.39 cm, 125A°, 10 μm). The column effluent was monitored at an excitation wavelength of 270 nm and an emission wavelength of 380 nm, and the eluent flow rate was 1.2 mL min^{-1}. The mobile phase consisted of 1% acetonitrile in 0.02 M disodium hydrogen orthophosphate adjusted to pH 2.5 with 60% perchloric acid. Calibration curves were constructed in rabbit serum by spiking the blank samples with the standard amounts of acyclovir. Peak areas were used in the determination of drug concentrations in the analyzed samples. The data were acquired and processed using Waters Millennium 2010 chromatography manager. The obtained chromatograms showed no interfering peaks, and the retention time of acyclovir was 4.085 minutes. The calibration curves were linear over the range of 100 to 6000 ng mL^{-1}. The sensitivity of the assay under these conditions was 50 ng mL^{-1} in rabbit serum. The intraday precision was determined by assaying 3 samples; the coefficients of variation were 14.8% and 3.5% at concentrations of 100 ng mL^{-1} and 6000 ng mL^{-1}, respectively. Interday precision was determined by assaying 5 samples on separate days; the coefficients of variation were 19.5% and 5.1% at concentrations of 100 ng mL^{-1} and 6000 ng mL^{-1}, respectively.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated from the individual serum concentration-time curves for acyclovir after the oral administration of its free solution or niosomal dispersion. The values of peak height (C_{max}) and peak time (T_{max}) were obtained directly from the individual serum

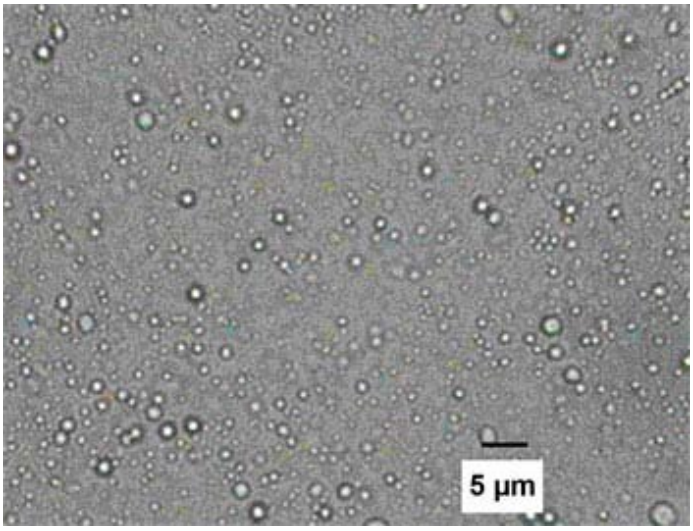

Figure 1. Photomicrograph of niosomes after sonication (original magnification ×40).

drug concentration time curves. The areas under the serum concentration time curves ($AUC_{0-\infty}$) were estimated by the linear trapezoidal rule. The ratio of individual $AUC_{0-\infty}$ values of niosomal acyclovir suspension to those of free acyclovir solution (relative bioavailability) was calculated to assess the extent of absorption from each formulation.

The terminal elimination rate constants (K) were calculated by applying linear regression on the log concentration vs time curve (3-4 points). The serum data were modeled using WinNonlin Version 2.0 pharmacokinetic software (Pharsight Corporation, 1994-1998, Palo Alto, CA) to calculate the absorption rate constants (K_a) of free and niosomal acyclovir according to the standard equation of 1-compartment open model with first-order absorption and elimination rates.

The mean residence time (MRT), corresponding to transit time of drug in the body, was calculated from Equation (5):

$$MRT = 1/K_a + 1/K \qquad (5)$$

Statistical Analysis

Analysis of variance (ANOVA, single factor) was employed in the statistical analysis of the determined parameters in this study. Statistical significance was defined at $P < .05$.

Results and Discussion

Size Distribution

Figure 1 shows a photomicrograph of acyclovir niosomes after sonication as recorded by the image analyzer system. Niosomes appeared as large unilamellar vesicles with spherical shape. Sonication may be responsible for the breakdown of the multilamellar vesicles to form unilamellar ones. Particle size analysis of the freshly prepared niosomes shows that the average size is ~0.95 µm and the most probable size is 0.8 µm (Figure 2). Azmin et al[12] assumed that the presence of dicetylphosphate in the formulation might be responsible for producing niosomes with diameter greater than 100 nm. The use of high cholesterol content in the formulation of acyclovir niosomes may lead to large vesicle size. McIntosh[14] found that cholesterol increases the width of lipid bilayers and consequently increases the vesicle size. Yoshioka et al[15] found that the mean size of the niosomes showed a regular increase with increasing the hydrophilic-lipophilic balance (HLB) of the surfactant because surface free energy decreases with increasing hydrophobicity. The authors found that the mean size of carboxyfluorescein niosomes prepared with span 60 (HLB 4.7) was 0.96 µm. This result is in good agreement with that obtained in the present study, where the average size of acyclovir niosomes is 0.95 µm. The particle size distribution of the prepared niosomes reflects a wide size range of 0.4 to 2.2 µm as shown in Figure 2. This finding may be owing to the influence of certain preparation conditions such as the hydration time and the degree of shaking.

Entrapment Efficiency

Entrapment efficiency was expressed as a percentage of the total amount of acyclovir used initially. The calculated average percentage entrapment efficiency of the niosomes was 11.003% ± 0.2% (n = 3). This means that ~16.5 µg of acyclovir was entrapped per 1.0 mg of the lipid phase. This result may be explained by the high cholesterol content (~50% of the total lipids) and the use of span 60 with its high phase transition temperature (T_c). Yoshioka et al[15] reported that entrapment efficiency was increased with increasing cholesterol content when niosomes were prepared by changing the molar ratio of nonionic surfactant to cholesterol. The authors found that vesicles prepared with span 60 (HLB 4.7) showed the

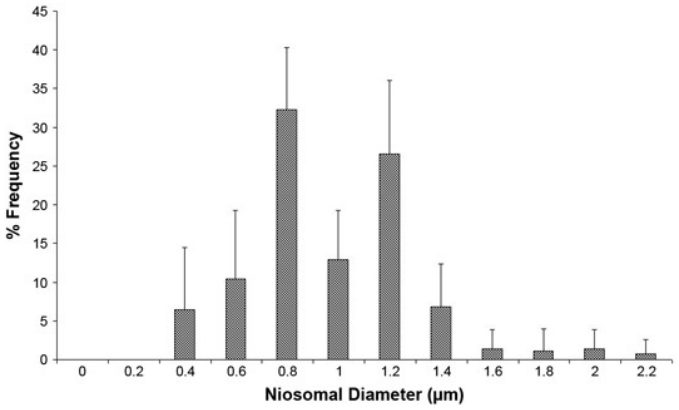

Figure 2. Particle size distribution of acyclovir niosomes (± SD).

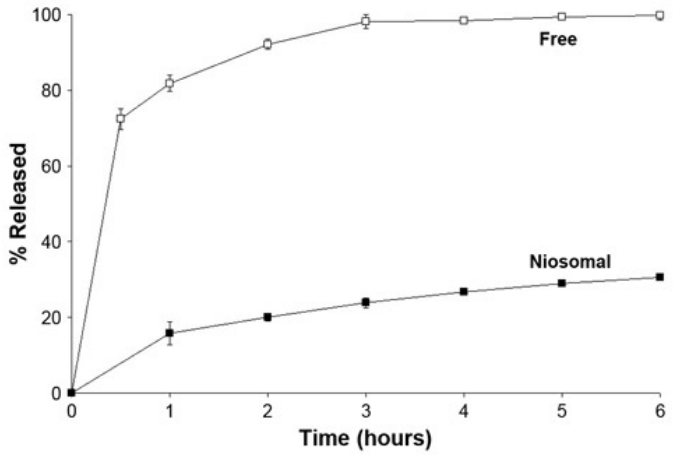

Figure 3. In vitro release (± SD) of free (■) and niosomal (□) acyclovir in normal saline at 37ºC.

most efficient entrapment compared with those prepared with other spans because of its highest phase transition temperature of ~50°C. The large size of the prepared unilamellar niosomes may also be responsible for the high drug entrapment. For water-soluble compounds, large or intermediate sized unilamellar vesicles are the most appropriate types to achieve as high a value as possible for entrapped volume: lipid ratio. This is because of the large core available for entrapping a large volume of drug solution. So, at a constant lipid concentration, the entrapment efficiency is higher for large vesicles.[16]

In Vitro Release

Figure 3 illustrates the drug release profiles from its free solution and niosomal dispersion. The release data were analyzed mathematically according to zero-order, first-order, and Higuchi's equations. The data were best fitted to Higuchi's equation for both free and niosomal drug with average R^2 values of 0.9864 and 0.9869, respectively. The release rate constants (K) and the half-life values ($T_{1/2}$) were calculated from the corresponding release profiles beyond 0.5 hour and 1.0 hour from the beginning of the release tests for free and niosomal drug, respectively (Table 1). As a result, K values do not reflect the initial amounts of drug released that differ significantly ($P < .05$) according to the tested form. Free drug solution gave a high initial percentage drug release of ~72% after 0.5 hour, whereas the niosomal drug dispersion showed only 16% drug release after 1.0 hour. The release rate of acyclovir from the niosomal dispersion (K = 0.0293 mg $h^{-1/2}$) was significantly lower ($P < .001$) than that from the free solution (K = 0.0654 mg $h^{-1/2}$). The drug release from the free solution began to plateau after 3 hours, whereas, the release from the niosomal dispersion was continued for 6 hours without reaching plateau. These results pointed to sustained release characteristics with a Higuchi

Table 1. Release Rate Constants (K) and Half-lives ($T_{1/2}$) for Release of Acyclovir From Its Free Solution and Niosomal Dispersion in Normal Saline at 37ºC*

Forms	K (mg $h^{-1/2}$)	$T_{1/2}$ (h)
Free drug solution	0.0654 (±0.0041)	0.819 (±0.059)
Niosomal dispersion	0.0293 (±0.0040)†	19.193 (±4.357)†

*Data are means ± SD (n = 3).

†Significantly different from free drug solution ($P < .05$).

pattern of drug release, where niosomes act as reservoir system for continuous delivery of drug. This slow release pattern of entrapped drug may indicate the high stability of the niosomal formulation. Presence of cholesterol in a high percentage and the use of span 60 in the niosomal formulation may explain this high stability of the niosomal membrane. Yoshioka et al[15] found that the release rate of carboxyfluorescein, a water-soluble compound, from niosomes prepared with span 60 was slower than the release rate from other span formulations (span 20, 80, and 85). This result is because at 25ºC, the molecules of span 60 are in the ordered gel state, but those of other spans are in the disordered liquid crystalline state.

Bioavailability and Pharmacokinetics

The average serum drug concentration time curves in rabbits after a single oral dose of acyclovir (40 mg/kg) as free solution and niosomal dispersion (containing both free and entrapped drug) are shown in Figure 4. The pharmacokinetic parameters of acyclovir were calculated from the individual curves and the mean values are presented in Table 2. The niosomal drug dispersion showed significantly ($P < .005$) higher values for C_{max}, $T_{1/2}$, $AUC_{0\rightarrow\infty}$, and MRT; and significantly ($P < .005$) lower values for absorption (K_a) and

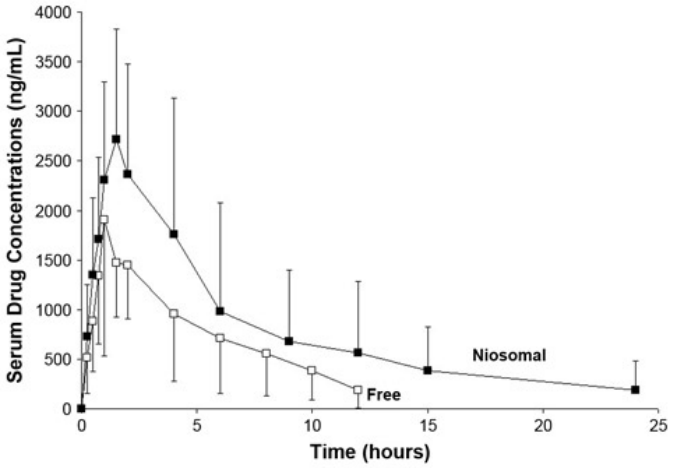

Figure 4. Mean serum acyclovir concentration time profiles (± SD) in rabbits after oral administration of free solutions (□) and niosomal dispersions (■) as a single dose of 40 mg/kg (n = 12).

Table 2. Pharmacokinetic Parameters of Acyclovir in Rabbits After Administration of a Single Oral Dose of 40 mg/kg as Free Solution and Niosomal Dispersion*

Parameters	Free Solution	Niosomal Dispersion
C_{max} (ng.mL^{-1})	1857 ± 534	3386 ± 969†
T_{max} (h)	1.6 ± 0.9	1.9 ± 1.3
K_a (h^{-1})	1.16 ± 0.73	0.65 ± 0.29‡
K (h^{-1})	0.22 ± 0.1	0.08 ± 0.04‡
$T_{1/2}$ (h)	3.84 ± 1.95	10.36 ± 3.79†
AUC $_{0-\infty}$ (ng.h.mL^{-1})	10810 ± 5597	22393 ± 16710 †
MRT (h)	5.4 ± 2.8	14.3 ± 5.5 †

*Data are means ± SD (n = 12).

†Significantly higher than free solution ($P < .05$).

‡Significantly lower than free solution ($P < .05$).

elimination (K) rate constants compared with free drug solution. There was no significant difference between the values of T_{max} for the free and niosomal drug. The increase in the MRT and $AUC_{0\rightarrow\infty}$ values and the decrease in the K_a value may reflect the sustained release effect of the niosomal formulation. This sustained release effect was also investigated by the in vitro release study. A possible explanation for this sustained release effect is that niosomes act as a carrier and a slow release vehicle. The drug is carried by the niosomes through the epithelium into deeper layers of the mucosa, where the encapsulated drug is slowly released. This sustained release effect can improve the bioavailability of drugs with slow and limited absorption and narrow absorption windows. The significant increase of C_{max} values may be owing to enhanced absorption of the free drug included in the tested unpurified niosomal formulation (containing both the free and niosomal drug). This finding may be a result of the influence of span 60 as a penetration enhancer on the permeability of gastrointestinal membrane. The individual $AUC_{0\rightarrow\infty}$ values for the niosomal dispersions were compared with those for the free drug solutions to determine the relative bioavailability and the mean ratio was found to be 2.55 (±1.82). This result indicated that more than 2-fold increase in the oral bioavailability of acyclovir was achieved by the niosomal formulation. The oral bioavailability of acyclovir is low (15% to 30%), highly variable in humans, and is species dependent.[2] Studies of the mechanisms of oral absorption of acyclovir have produced conflicting results. The existence of a saturable process in the oral absorption of acyclovir by mice, rats, and dogs has been proposed based on a decline in the fraction of dose absorbed with rising dosage levels.[2] The same suggestion has been made for humans based on a decline in percentage acyclovir urinary recovery with increasing dosage.[17] The high degree of species variation and intestinal variability, along with the fact that some investigators have reported active transport for other purines, also seems to suggest that carrier-mediated transport may be involved in the intestinal uptake of acyclovir. On the other hand, the low oral bioavailability may simply be a function of poor membrane permeability owing to low partition coefficient of acyclovir (P = .023). Meadows and Dressman[18] studied the intestinal uptake mechanism of acyclovir in rate jejunum using in vitro and in situ methods. The authors found that the uptake mechanism of acyclovir in the rate jejunum is predominantly via passive diffusion with no evidence of a carrier-mediated transport system.

In the present study, the niosomal dispersion enhanced the bioavailability up to 2.5-fold despite the low content of entrapped acyclovir (11%) in comparison with free acyclovir (89%). This effect may be explicable in view of the poor oral bioavailability of acyclovir in its conventional forms (15%-30%). The improved oral bioavailability may be owing to the lipophilic nature of the niosomal formulation and the effect of the nonionic surface-active agent on the permeability of the gastrointestinal membrane. Improved partioning of the lipophilic system to the mucosa, a direct effect of the surface active agent (span 60) on the barrier function of the mucosa, and prolonged localization of the drug-loaded niosomes at the site of absorption may be possible reasons for the improved bioavailability. The present in vivo results support previously published in vitro findings indicating that passive diffusion is the main mechanism underpinning the intestinal absorption of acyclovir.

Conclusion

The prepared acyclovir niosomes have unilamellar spherical shape with an average size of 0.95 μm and percentage drug entrapment of 11%. The niosomal formulation showed sustained release characteristics with Higuchi pattern of drug release. In vivo study in rabbits revealed that more than 2-fold increase in the oral bioavailability and MRT was achieved by the niosomal formulation. So, the prepared niosomes could be promising delivery systems for acyclovir with sustained drug release profiles.

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