Effect of Acyclovir Solid Lipid Nanoparticles for the Treatment of Herpes Simplex Virus (HSV) Infection in an Animal Model of HSV-1 Infection

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Abstract: Background: Acyclovir use is limited by a high frequency of administration of five times a day and low bioavailability. This leads to poor patient compliance.

Objectives: To overcome the problem of frequent dosing, we used nanotechnology platform to evaluate the proof of concept of substituting multiple daily doses of acyclovir with a single dose.

Methods: Acyclovir was formulated as solid lipid nanoparticles (SLN). The nanoparticles were characterized for particle size, surface charge and morphology and in vitro drug release. The pharmacokinetic and pharmacodynamic of SLN acyclovir were compared with conventional acyclovir in a mouse model.

Results: SLN showed drug loading of 90.22% with 67.44% encapsulation efficiency. Particle size was found to be of 131 ± 41.41 nm. In vitro drug release showed 100% release in SIF in 7 days. AUC$_{0-\infty}$ (119.43 ± 28.74 µg/ml h), AUMC$_{0-\infty}$ (14469 ± 4261.16 µg/ml h) and MRT (120.10 ± 9.21 h) were significantly higher for ACV SLN as compared to ACV AUC$_{0-\infty}$ (12.22 ± 2.47 µg/ml h), AUMC$_{0-\infty}$ (28.78 ± 30.16 µg/ml h) and MRT (2.07 ± 1.77 h), respectively (p<0.05). In mouse model, a single dose of ACV SLN was found to be equivalent to ACV administered as 400mg TID for 5 days in respect to lesion score and time of healing.

Conclusion: The proof of concept of sustained-release acyclovir enabling administration as a single dose was thus demonstrated.

Keywords: Acyclovir, HSV-1, pharmacodynamic, pharmacokinetic, Solid Lipid Nanoparticles (SLN), sustained release.

1. INTRODUCTION

Infections due to herpes simplex virus (HSV) are among the most common viral infections [1, 2]. Acyclovir was the first specific antiviral drug to become widely used in the treatment of HSV and herpes zoster virus (HZV) infections [3, 4]. One of the major limitations of oral acyclovir is that it needs to be administered 5 times a day due to its short half-life and low oral bioavailability [5]. This is inconvenient and can lead to poor patient compliance.

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We and others have shown that nanotechnology-based drug delivery platforms are able to provide sustained release plasma profile for various drugs [6-9]. Acyclovir loaded PLGA microparticles have shown increased efficacy against HSV-1 in cell culture [10]. Majority platforms include polymer-based systems. However, polymer-based systems use organic solvent like dichloromethane (DCM) and acetone which can cause toxicity. Solid lipid nanoparticles (SLN) provide an alternative drug delivery platform. It comprises of solid lipids as matrix for drug delivery which has biodegradable lipids and omits the use of organic solvents [11]. Camptothecin [12], piribedil [13] and cyclosporine [14] have been encapsulated in SLN and have shown sustained release profile. Various antiviralis including those for HIV have been encapsulated in SLN and targeted in vivo [15, 16]. Acyclovir SLN has been developed in various studies, however, they have been developed for dermal and ocular delivery which is not the desired route for infections requiring systemic delivery [17-19].

Therefore, with this background, the current study was planned to prepare SLN based oral sustained release formulation of acyclovir, evaluate the pharmacokinetics of the formulation and its efficacy in an animal model of cutaneous herpes infection.

2. MATERIALS AND METHODS

2.1. Materials

Acyclovir was purchased from Sigma Aldrich, Inc. St Louis, MO, USA. Soy lecithin (Phospholipon 90H) was received as a gift sample from Lipoid GmbH, Germany. Compritol 888 ATO® was a gift sample from Colorcon Asia Pacific Pvt. Ltd., India. Stearic acid and Tween 80 (polysorbate 80) were purchased from Central Drug House, Mumbai. Chemicals and solvents used were analytical or HPLC grade. 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) was purchased from Sigma Aldrich, Inc., St Louis, MO, USA. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin (50U/ml) hepes buffer 1M, non-essential amino acids, L-glutamine, phosphate buffer saline pH 7.2 (PBS) were obtained from Gibco-BRL, Spain. Crystal violet were purchased from Hi-Media, India.

2.2. Preparation of Acyclovir (ACV) Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles of acyclovir were prepared by microemulsion method [20]. Briefly, the lipidic phase (8% lipid) and the aqueous phase (polysorbate 80, soy lecithin and water) were heated to ~10°C above the lipid melt temperature. The proportion of surfactant and the volumes of two phases were so adjusted that a microemulsion was formed spontaneously upon mixing the two phases. Hot microemulsion thus formed, was transferred into the same amount of cold water (~2°C) under constant stirring (Wise Tis HG-15 D, 10,000 rpm) for 20 minutes to obtain SLN. The prepared SLN was used as such for further studies. Table 1 describes the composition of acyclovir solid lipid nanoparticles having both lipid and aqueous phase.

Table 1. Composition of acyclovir solid lipid nanoparticles.

<table>
<thead>
<tr>
<th>Aqueous Phase</th>
<th>Lipid Phase (0.985 g) (4:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (4.75 ml)</td>
<td>Compritol (0.788 g)</td>
</tr>
<tr>
<td>Drug (40% lipid) (0.394g)</td>
<td>Stearic acid (0.197 g)</td>
</tr>
<tr>
<td>Tween 80 (6.64g)</td>
<td>-</td>
</tr>
<tr>
<td>Soya lecithin (0.4%)</td>
<td>-</td>
</tr>
</tbody>
</table>

2.3. Bioanalytical HPLC Method

Acyclovir was analyzed using HPLC by modification of the previously described method [21]. (Shimadzu Pump LC-20AD, Prominence liquid chromatography) with SPD-20A prominence UV-Vis detector. C-18 column (Hibar 250×4.6 Purospher STAR, RP-18e (5µm) was used. Serial dilutions within the range of 0.2-2.56 µg/ml were made from stock solution and standard plot was obtained. The mobile phase consisted of potassium di-hydrogen phosphate and acetonitrile (KH₂PO₄: ACN) (97:3) at pH=2.5 The mobile phase was delivered at a flow rate of 1.5 ml/ min and the detection of acyclovir was carried out at 254 nm. The injection volume was 20 µl and the analysis was performed at 30°C (sample 4°C).
2.4. Solid Lipid Nanoparticles (SLN) Characterization

SLN formulation was characterized for particle size, PDI and zeta potential using Delsa Nano C, Beckman Coulter, Inc. Double distilled water (DDW) was used as a dispersant medium for diluting the sample to 10^(-1) concentration. The sizes of nanoparticles are expressed in nanometer scale. The polydispersity index (PDI) is the dimensionless number which indicates the width of the distribution, having a value between 0 and 1. It is self-calculated by the software. Zeta potential measures the surface charge of the particles and it is also measured by DelsaNano C. It is extremely important parameter of colloidal stability and flocculation properties of the colloidal systems.

Transmission electron microscopy (TEM) analysis of the prepared SLNs was carried out to study sphericity and aggregation. For TEM, samples were examined by TEM (H 100, Hitachi Ltd., Japan). Samples were stained with phosphotungstic acid (PTA, 2%) for 5 minutes and excess PTA was removed. These were then spread on a gold grid and examined. Nanoparticles from five to seven different TEM fields were measured to determine the mean particles size of nanoparticles.

Fourier Transmission Infrared Spectroscopy (FTIR) was conducted on samples of free acyclovir, blank SLN and acyclovir SLN to assess the lipid-drug interaction obtained using a Perkin Elmer spectrometer (Perkin Elmer, Boston, MA). The FT-IR spectrums were obtained by mixing the samples with Potassium Bromide and pressed to obtain pellets which were then scanned in the IR range from 400 to 4000 cm^-1, with a resolution of 8 cm^-1.

The concentration of acyclovir in solid lipid nanoparticles was determined by HPLC as previously described. The total amount of drug per unit volume present in the formulation was determined by suitably disrupting 0.1 ml of the SLN dispersion in 5 ml chloroform: methanol (2:1) volumetrically. The total drug content (TDC) or percent drug loading was determined by using the equation given below:

\[
TDC = \frac{\text{Calculated amount of drug}}{\text{Total amount of drug added}} \times 100
\]

The drug entrapment, drug loading and the in vitro drug release were expressed as percentages.

Stability study of ACV SLNs was performed. ACV SLNs kept for 1 year at 4 – 8°C. After 1 year, particle size, PDI, zeta potential, percent drug loading and encapsulation efficiency were measured. Effect of temperature was observed on the ACV SLNs. As we have to perform the studies on cell cultures, therefore, autoclaving needs to be done. ACV SLNs were autoclaved at 121°C, 15 per square inch (psi) above atmospheric pressure for 15 minutes. After autoclaving, particle size, PDI, zeta potential, percent drug loading and encapsulation efficiency were measured.

In vitro release was determined in the following simulated fluids: simulated gastric fluid (SGF) pH=1.2; phosphate buffer saline (PBS) pH=6.8; simulated intestinal fluid (SIF) pH=7.8 by dialysis bag method using dialysis membrane (molecular weight cut-off of 12 kDa). The incubation took place in rotating vials at 37°C. 10 µl of samples were withdrawn at pre-set time intervals (0.25 hrs, 0.5 hrs, 1 hrs, 2 hrs, 3 hrs, 4 hrs, 5 hrs, 8 hrs, 12 hrs, 24 hrs, 48 hrs, 60 hrs, 72 hrs, 96 hrs, 120 hrs, 144 hrs, 168 hrs) and replaced by an equal volume of fresh dissolution medium. The aliquots were then diluted with mobile phase and filtered through 0.45 µm filters. The samples were analyzed by HPLC. The cumulative amount of acyclovir release from the nanoparticles was calculated using the following equation:

\[
\text{Encapsulation efficiency (EE)} = \frac{\text{TDC} - \text{Df}}{\text{TDC}} \times 100
\]

where Df = amount of drug in the clear supernatant fluid
calculated using the following equation:

\[ \% \text{Cumulative release} = \frac{W_t}{W_0} \times 100 \]

where \( W_t \) is the amount of drug released from the nanoparticles and present in the receiving phase at time \( t \) and \( W_0 \) is the initial amount of drug present in the nanoparticles. The cumulative percentage (%) of drug released was plotted against time. The samples were analysed by HPLC by the method described above to measure the amount of acyclovir in the samples.

2.5. Pharmacokinetics Studies

Swiss albino male mice weighing 20-40 g were used. The protocol was approved by the Institutional Animal Ethics Committee (IAEC), PGIMER, Chandigarh (Ref. No. 73/IAEC/444). Animals were divided into two groups (n=9), ACV and ACV SLN. They were dosed with 36 mg/kg of acyclovir, either in a solution form of acyclovir or as ACV SLN orally using an oral feeding canula. Blood samples were collected at pre-determined time points: 0, 15, 30 mins, 1, 2, 4, 8, 12 and 24 hrs after drug administration for ACV and for 0, 15, 30 mins, 1, 2, 4, 8, 12 and 24, 48, 72, 96, 120, 144 and 168 hrs after drug administration for ACV SLN. Plasma was separated out by centrifuging the blood samples at 4000 rpm for 10 min. Protein precipitation was carried out by adding 100 µl of 20% perchloric acid in 500 µl of sample, vortexing it for 1 min and then centrifuging the samples at 10,000 rpm for 15 min [22]. The supernatant was collected and then analyzed by standardized HPLC method. \( C_{\text{max}} \) (Peak plasma concentration) and \( T_{\text{max}} \) (time to reach peak plasma concentration) were obtained from actual plasma concentration versus time data. The area under the curve (AUC) was calculated using the trapezoidal rule, from the plasma vs. time plots. \( \text{AUC}_{0-\infty} \), total AUC and mean residence time (MRT) were calculated from the individual AUCs from the plasma-time plots.

2.6. Cell Culture and Viruses

Vero cells (African green monkey kidney cell line) were purchased from the National Institute of Virology, Pune, India. This cell line was grown in DMEM supplemented with 6% FBS, 1% penicillin-streptomycin, 1% Hepes buffer 1M, 1% non-essential amino acids and 1% l-glutamine. The maintenance medium for Vero cells was described above with 2% FBS. Herpes Simplex type 1 (HSV-1) (KOS strain) obtained from National Institute of Virology, Pune, India, was prepared in aliquots and stored at -80°C until use. Virus titer was determined by plaque assay in Vero cells and expressed as plaque-forming units (pfu) per ml.

2.7. Cell Infection

The titer of the virus stocks, determined by plaque reduction assay, was 3.3 x 10^6 plaques/ml. Monolayers of Vero cells grown in 24-well tissue culture plates, at 80% confluence were infected by adsorption of HSV-1 (300 µL) supplemented with 2% FBS for 1 hr at 37°C. The inoculum with the non-adsorbed virus was removed and cells were washed with the medium. Finally, the infected cells were treated according to the assay protocol.

2.8. MTT Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is a yellow water-soluble tetrazolium dye that is reduced by live cells, but not by dead ones, to a purple formazan product that is insoluble in aqueous solutions. Monolayers of 1x10^5 Vero cells were placed in 96 well plates. Treatment was done on these plates with ACV and ACV SLN over a concentration range of 2.5-250 µg/ml and the plates were incubated for overnight at 37°C in 5% CO_2. MTT (20 µl of 5 mg/ml) was added to each well plate and incubated for 4 hrs. Media was discarded and DMSO (200 µl) was added. Absorbance in each well plate was measured at 490 nm.

2.9. Plaque Reduction Assay

ACV and ACV SLN were tested for antiviral activity against HSV-1 by plaque reduction assay over a concentration range of 0.25µM-8 µM. 1x10^5 Vero cells were seeded in 24-well plates and incubated at 37°C and 5% CO_2. When the cells reached 70% confluence, they were infected with 300 µl of 3.3x10^6 pfu/ml of HSV-1. After incubation for 1 hr at 37°C to allow viral adsorption, the plates were washed, and the medium replaced with maintenance medium containing different concentrations of ACV and ACV SLN. After 96 hrs incubation, the medium
was removed, and the monolayers were fixed with 10% formaldehyde (200 µl) for 1 hr and then stained with 300 µl of 1% solution of crystal violet in 30% methanol for 1 hr. Percent plaque reduction from control was calculated for both the treatment groups.

2.10. Evaluation of Antiviral Activity in Cutaneous Model of HSV-1 Infection

Female BALB/c mice (6 weeks old) were used. The right mid flank of each mouse was depilated. One day later, the skin was scratched using a 27-gauge needle and 40 µL of HSV-1 suspension of 3.3×10^6 pfu /ml was applied to the scarified area [23-25]. ACV and ACV SLNs were orally administered as an oral gavage 24 h after HSV-1 infection. ACV (400 mg) was given thrice a day for 5 days whereas ACV SLN was a single dose administration (400mg). Beginning 1 day after infection, the abraded site was evaluated and scored as follows: 0, no visible change in the abrasion or surrounding tissue; 0.5, the earliest lesion characterized by slight swelling near the abrasion and mild erythema; 1, papule(s) at or around the abrasion; 2, ulcerated papules at or around the abrasion site with eschar formation; 3, fusion of ulcerated lesions into large eschar; 4, large open ulcerated lesion; 5, death or sacrifice. The animals were evaluated daily for 11 days beginning 1 day after infection.

2.11. Statistics

PK data were expressed as mean ± standard deviation (SD). The difference in PK parameters and results of plaque reduction assay between the two groups were analysed by unpaired t-test. For lesion scoring in animal model, among the control, ACV and ACV SLN group. Data were analysed by ANOVA and post-hoc Bonferroni test was used for between-group comparisons. p value of <0.05 was considered significant.

3. RESULTS

3.1. HPLC Estimation

ACV was standardized for estimation by HPLC. A standard curve was obtained with 0.2-25.6 µg/ml concentration range. The correlation coefficient value of 0.99 was obtained. The standard curve was found to be eligible for the PK-PD studies as acyclovir is active against HSV-1 in concentration range of 0.02-0.9 µg/ml (Fig. 1). Recoveries of acyclovir from plasma samples were found to be 88.11 ± 0.27% which confirms less sample loss. Inter-day and intra-day variability were also found to be within range (15%) [26].

3.2. Characterization of ACV Solid Lipid Nanoparticles

ACV SLN were prepared by microemulsion technique by optimizing the ratio of the lipid phase and aqueous phase. ACV SLN had a size of 131 ± 41.41nm with a polydispersity index (PDI) of 0.30 ± 0.014. Zeta potential was found out to be -16.00 ± 1.90 mV (Table 2).

TEM micrographs showed smooth spherical surface morphology and non-aggregated nanopar-
Table 2. Results of parameters used for characterization of nanoparticles PDI, particle size, zeta potential results are of 3 sets of experiments.

<table>
<thead>
<tr>
<th>Particle Size (nm) (Mean ± S.D)</th>
<th>Zeta Potential (mV) (Mean ± S.D)</th>
<th>PDI (Mean ± S.D)</th>
<th>Percent Drug Loading (Mean ± S.D)</th>
<th>Percent Encapsulation Efficiency (Mean ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>131 ± 41.44</td>
<td>-16 ± 1.90</td>
<td>0.30 ± 0.014</td>
<td>90.22 ± 1.18%</td>
<td>67.44 ± 6.21%</td>
</tr>
</tbody>
</table>

Fig. (2). Transmission Electron Microscopy (TEM) micrograph of Acyclovir SLN showing spherical nanoparticles. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

ticles (Fig. 2). The drug loading and encapsulation efficiency were found out to be 90.22 ± 1.18% and 67.44 ± 6.21% respectively. In ACV SLNs, FT-IR spectra peak shifted from 3521 cm⁻¹ to 3404 cm⁻¹ in -NH stretch, and shift in 1485 cm⁻¹ to 1472 cm⁻¹ due to C-OH group was also noted (Fig. 3) (Table 3). *In vitro* drug release study demonstrated a biphasic release pattern where it was observed that there was an initial burst release of drug followed by a sustained release. An initial burst release of a drug was 51%, 45% and 52% in SIF, PBS and SGF respectively in the first 24 hours. 87% and 95% of entrapped drug were released until 7 days in PBS and SGF. 100% drug release was observed in SIF in 7 days (Fig. 4).

3.3. Stability of ACV SLN

Stability of ACV SLN was assessed for drug loading, particle size, polydispersity index and zeta potential. After storage for 1 year, percent drug loading was 85%. Particle size, PDI and zeta potential changed to 152 nm, -31.11 and 0.318,
Table 3. Major bands of FT-IR spectra of acyclovir, blank SLNs and acyclovir SLNs.

<table>
<thead>
<tr>
<th>Band</th>
<th>Acyclovir</th>
<th>Blank SLNs</th>
<th>Acyclovir SLNs</th>
<th>Functional Groups (Frequency Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3521.3</td>
<td>3437.56</td>
<td>3404.37, 3436.36</td>
<td>-NH stretch (3100-3500 cm⁻¹)</td>
</tr>
<tr>
<td>2</td>
<td>1485.2</td>
<td>-</td>
<td>1472.30</td>
<td>-C-OH group (1500-1065 cm⁻¹)</td>
</tr>
<tr>
<td>3</td>
<td>1715.0</td>
<td>1736.53</td>
<td>1736.53</td>
<td>-C=O group (1700-1800)</td>
</tr>
<tr>
<td>4</td>
<td>3309.1</td>
<td>-</td>
<td>3309.40</td>
<td>-OH stretch (3200-3500 cm⁻¹)</td>
</tr>
</tbody>
</table>
Table 4. Characterisation of formulation before and after 1 year of storage at 4-8°C and effect of sterilisation on formulation.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Initial</th>
<th>Final</th>
<th>After Sterilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Size (nm)</td>
<td>131.37 ± 41.44</td>
<td>151.9 ± 3.31</td>
<td>246.6 ± 16.2*</td>
</tr>
<tr>
<td>PDI</td>
<td>0.30 ± 0.014</td>
<td>0.315 ± 0.023</td>
<td>0.229 ± 0.079</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>-16.03 ± 1.63</td>
<td>-30.6 ± 1.89*</td>
<td>-12.0 ± 3.57</td>
</tr>
<tr>
<td>% Drug loading</td>
<td>90.3 ± 1.18</td>
<td>85 ± 2.44</td>
<td>85 ± 2.18</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D; *p<0.05.

Fig. (5). Plasma concentration-time profile of orally administered free acyclovir (36 mg/kg) and acyclovir solid lipid nanoparticles (equivalent 36 mg/kg of acyclovir in SLNs) in mice dosed orally. (n=3) mice were taken at each time point from 0 hour to 168 hours. Time points were shown as logarithmic scale. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

respectively (Table 4). Sterilisation did not have any effect on percent drug loading, though it did increase the particle size of SLN from 13 nm to 256.6 nm.

3.4. Comparative Assessment of Pharmacokinetics of Free and Nanoformulation of Acyclovir in Mice

ACV SLN demonstrated a prolonged release of drug for up to 7 days in comparison to ACV (Table 5). The drug levels observed in both the groups corresponds to a concentration which is needed for antiviral activity (0.02-0.9 µg/ ml) [5]. Plasma concentrations were maintained for up to 7 days in ACV SLN as compared to 2.5 hrs in the ACV group. The C\text{max} was significantly decreased (p<0.05) for ACV SLNs as compared to ACV (3.59 ± 3.00 vs. 10.54 ± 5.46 µg/ ml). The AUC\text{0-∞} (119.43 ± 28.74 µg/ ml h), AUMC\text{0-∞} (14469 ± 4261.16 µg/ ml h) and MRT (120.10 ± 9.21 hrs) were significantly higher for ACV SLN as compared to ACV AUC\text{0-∞} (12.22 ± 2.47 µg/ ml hrs), AUMC\text{0-∞} (28.78 ± 30.16 µg/ ml hrs) and MRT (2.07 ± 1.77 hrs), respectively (p<0.05, Table 5) (Fig. 5).

3.5. Pharmacokinetic/Pharmacodynamic Index of Acyclovir Solid Lipid Nanoparticles

EC\text{50} against HSV-1 isolates ranged from (0.02 to 13.5 µg/ mL) [5, 27, 28]. Time above EC\text{50} was found to be significantly higher for ACV SLN equivalent to (36 mg/kg) of acyclovir as compared to ACV (168 hours vs 4 hours) (Table 6).
Table 5. Pharmacokinetic parameters of acyclovir and acyclovir solid lipid nanoparticles (C<sub>max</sub>, T<sub>max</sub>, AUC, AUMC, MRT; n=3).

<table>
<thead>
<tr>
<th>Group</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml) (Mean ± S.D)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h) (Mean ± S.D)</th>
<th>AUC (µg/ml*h) (0-24) (Mean ± S.D)</th>
<th>AUC (µg/ml*h) (0-∞) (Mean ± S.D)</th>
<th>AUMC (µg/ml*h) (0-∞) (Mean ± S.D)</th>
<th>MRT (h) (Mean ± S.D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACV</td>
<td>10.54 ± 5.46</td>
<td>0.33 ± 0.144</td>
<td>15.11 ± 3.21</td>
<td>10.56 ± 1.30</td>
<td>28.78 ± 30.16</td>
<td>2.07 ± 1.77</td>
</tr>
<tr>
<td>ACV SLN</td>
<td>3.59 ± 3.00</td>
<td>1 ± 0</td>
<td>10.14 ± 1.21</td>
<td>119.43 ± 28.74</td>
<td>14469.73 ± 4261</td>
<td>120.10 ± 9.21</td>
</tr>
</tbody>
</table>

Fig. (6). Effect of Acyclovir and Acyclovir SLN on non-infected Vero cells viability at different drug concentration demonstrating cell toxicity. Each point represents the mean ± S.D (n=3). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Table 6. Time above EC<sub>50</sub> for free acyclovir and acyclovir solid lipid nanoparticles (Acyclovir SLNs) at an EC<sub>50</sub> (0.02-13.5 µg/ml).

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Time&gt;EC&lt;sub&gt;50&lt;/sub&gt; (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free acyclovir (36 mg/kg)</td>
<td>4</td>
</tr>
<tr>
<td>Acyclovir NPs (equivalent to 36 mg/kg)</td>
<td>168</td>
</tr>
</tbody>
</table>

3.6. Comparative Antiviral Efficacy of ACV and ACV SLN

3.6.1. Cellular Toxicity

To investigate cytotoxicity, uninfected Vero cells were treated with ACV and ACV SLN and then subjected to cell viability assay. The amount of surviving cells after incubation with an ACV and ACV SLN during a 24 hours incubation was estimated by MTT assay. The viability was expressed in percent by comparison with non-treated control. At higher concentration of ACV tested, 250 µg/ml, cell viability was 19.1% (Fig. 6). However, at 100 µg/ml, cell viability observed was 60.5% (Fig. 6). Similar results were obtained for ACV SLN and no significant difference was observed between the two treatment groups. Cytotoxicity was observed at concentration above ≤100 µg/ml for both the treatment groups.

3.6.2. Plaque Reduction Assay

The antiviral efficacy of ACV SLN against HSV-1 was assessed by plaque reduction assay in a monolayer of Vero cells. ACV showed a concentration-dependent response on plaque formation. As shown in Fig. (4), ACV decreased the percent (%) plaque count from 61% at the lowest (0.25 µM) to 2% at the highest concentration (8 µM). On the other hand, ACV SLN showed better antiviral efficacy at lowest concentration (0.25 µM) (Fig. 7) as no plaque formation occurred in wells having ACV SLN in concentration range of (0.5 µM - 8 µM) (p<0.05). The effective concent-
Fig. (7). Plaque reduction assay at different drug concentration of Acyclovir and Acyclovir SLN. Percentage plaque formation reduction from the control was shown as percentage HSV-1 plaques formed in both the treatment groups. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Fig. (8). Comparative pharmacodynamic activity of Acyclovir and Acyclovir SLN was shown. Treatment of HSV-1 infected mice was given after 24h infection. Control mice were given saline. ACV was given 3 times a day treatment for consecutive 5 days. ACV SLNs were given once administration during five days (n=6). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

For this purpose, infected mice were divided into three groups and were treated with saline, ACV and ACV SLNs after 24 hours of infection. Lesion development in mice was assessed for 11 days. It was observed that as compared to the saline-treated mice, treatment with ACV and ACV SLNs both significantly reduced the lesion score in infected mice and also reduced the time of healing of lesions (p<0.005). At day 1, there was no significant difference observed in lesion scores between all the treatment groups. On the subsequent days, significant difference was observed in both the treatment groups when compared to the saline-treated group (p<0.005) (Fig. 8). Time of healing was also found to be significantly decreased in treatment groups as compared to control group (9 days vs. 4 days). When ACV and ACV SLNs groups were compared, no significant difference was observed in lesion scoring and time of healing (Fig. 9).

4. DISCUSSION

In the current study, we developed solid lipid nanoparticles (SLNs) of acyclovir containing
suitable lipids and surfactants which are biodegradable and biocompatible.

### 4.1. Preparation and Characterisation of Acyclovir Solid Lipid Nanoparticles

We were able to achieve a drug loading of 67.44%. Previously, Seyfoddin A 2013, have prepared acyclovir SLNs the microemulsion technique and have used Tween 80 as a surfactant and have reported encapsulation efficiency of 13.32% [29]. They have tried with different lipids and different drug lipid ratio to get the best encapsulation efficiency. We have used compritol 888 ATO as a lipid and Tween 80 as a surfactant to prepare microemulsion. The high encapsulation efficiency of 67.44% demonstrates that the lipid combination used have synergistic interaction with the drug molecule. By combining the two lipids, it resulted in better incorporation of acyclovir into the lipid mix melt into the empty spaces of the imperfect lipid matrix. Most of the other studies which have developed acyclovir nanoparticles have developed using different lipids and most of them have not prepared nanoparticles for oral delivery [30-33].

Acyclovir solid lipid nanoparticles had an average size of 131 ± 41.41 nm with total drug loading (TDC) of 90.22%. Particle size less than 200 nm is desirable for sustained release action as they remain inaccessible to the reticuloendothelial system (RES) and can show prolonged action [34]. A high total drug capacity holds promise in the drug development process as scaling up the formulation may not be a wasteful excess [34]. The suspension was also found to be stable over one-year showing non-significant change in particle size and total drug loading. The nanoparticles exhibited low negative zeta potential of -16 ± 1.90 mV which could be due to terminal carboxylic group of stearic acid. Under TEM, nanoparticles had smooth surface and were found to be spherical in shape. The particle size observed with TEM micrographs corresponds well with number distribution pattern. FTIR spectra of acyclovir showed an -NH stretch at 3521.3 cm⁻¹, -C=O stretch at 1715.0 cm⁻¹ and -C-OH stretch at 1485 cm⁻¹. The shift in the stretch of these groups indicates interaction with the lipids. In ACV SLNs groups a higher shift was observed at all these groups which suggest an interaction of acyclovir with compritol 888 ATO and stearic acid both which resulted in good encapsulation efficiency of 67.44%. In vitro drug release of ACV SLN showed an initial phase of burst release of drug in first 24 hours due to the presence of unentrapped drug onto the nanoparticles. Afterwards, it showed a sustained release of drug by diffusion from the lipid core, wherein the

Fig. (9). Representative images depicting HSV lesions in BalB/C mice at day 3. A) Control mice given saline; B) Free acyclovir group mice given 3 times a day administration for 5 days; C) Acyclovir SLNs group mice given once for five days; D, E, F focused images of above images above groups. (A higher resolution / colour version of this figure is available in the electronic copy of the article).
drug was homogeneously distributed. A slow release of drug is may be accounted by the high lipophilicity of the compritol 888 ATO. Higher carbon chain length of compritol AT0 888 contribute to higher sustained release [35]. Further, amorphous nature of formulation leads to high solubility [35].

4.2. Pharmacokinetics of Acyclovir Solid Lipid Nanoparticles

Oral administration of acyclovir solid lipid nanoparticles led to a prolonged drug release with levels above EC$_{50}$ for 7 days in comparison to 4 hours in the free drug group. Several reasons may be given for improved oral bioavailability of acyclovir after administration of acyclovir solid lipid nanoparticles. Firstly, smaller particle size increases the surface area of particles itself which leads to increased absorption from the gastrointestinal tract [36]. Bioadhesion of SLN to the mucosa of the gastrointestinal tract due to lipid moieties may increase the residence time at the gut wall. This close contact with the epithelial membranes may enhance the drug absorption thus leading to increased oral bioavailability [36]. Secondly, absorption can also be facilitated by presence of fatty acids and mono or diacylglycerols being converted by triglycerides present in the nanoformulation by gastrointestinal lipase [37]. Thirdly, lipids present in SLN increase lipoprotein formation which stimulates lymphatic lipid flux. This increased lymphatic delivery of drug leading to enhanced bioavailability. Lastly, nanoparticles are preferentially taken by the M-cells present in the Peyer's patches of the gut-associated lymphoid tissue (GALT) followed by shuttling into the systemic circulation and release of drug in the blood. This specialised uptake mechanism can also improve absorption of drug [38]. Similar studies have shown increased bioavailability of drug encapsulated in solid lipid nanoparticles [37, 39, 40]. A significant increase in the MRT of acyclovir in case of acyclovir solid lipid nanoparticles as compared to free acyclovir was due to change in the acyclovir absorption and elimination after encapsulating into solid lipid nanoparticles. This may be also because of slow release of drug from the nanoparticles due to higher biodegradation time of acyclovir solid lipid nanoparticles [40].

4.3. Pharmacodynamics of Acyclovir Solid Lipid Nanoparticles

Efficacy studies were carried out both in cell culture by plaque reduction assay and in the animal model of cutaneous HSV-1 infection. 50% inhibition observed with the treatment groups corresponds to the value which is above the reported EC$_{50}$ value (0.02-13.5 µg/ml ≈ 0.08-60 µM). Approximately 100 times improvement in the efficacy was observed in the ACV SLNs group when compared to ACV alone. This improvement in antiviral efficacy could be due to the longer duration of contact of the drug-loaded SLNs with the infected cells [10]. Jalon et al., 2003 have also shown a significant decrease in IC$_{50}$ value (0.15 ± 0.03 µM), in cell plates treated with ACV microparticles as compared to drug solution alone (0.27 ± 0.02 µM). The difference in EC$_{50}$ value of the reported study, when compared to our study, maybe due to the reason that we are using nanoparticles unlike microparticles used in their study. Similar studies also showed increase efficacy of drugs incorporated in the SLN through increased cellular uptake in the cells [41-44].

Efficacy study done in an animal model of cutaneous herpes infection showed that acyclovir given three times a day for consecutive five days was comparable with a single dose of acyclovir solid lipid nanoparticles. The lesions observed in control animals are scored up to 3 which corresponds to large lesions fused into large eschar, whereas, for the treatment groups maximum lesion score of 2 was observed. The score of 2 corresponds to small ulcerated papules leading to eschar formation. Time of healing was also found to be decreased in treatment groups as compared to control group (9 days vs. 4 days). However, no significant difference was observed in lesion scoring and time of healing (p<1.000). The pharmacokinetic study observed significant increase in MRT in the ACV SLN group when compared to ACV alone (120.10 h vs. 2.07 h). The efficacy results observed are in accordance with the pharmacokinetics results which demonstrate that the drug levels were maintained above EC$_{50}$ for up to 7 days in ACV SLN group as compared to 4 hours in ACV alone group. This could be a valid reason for the comparable effect shown when free was given acyclovir given 3 times a day for five days.
and a single dose of ACV SLN. The animal model of cutaneous herpes used in our study is a well-validated model and has been used to evaluate efficacy in HSV-1 and HSV-2 infections models in various studies [45, 46].

There are limitations to our current study. We were not able to show the antiviral efficacy of our nanoparticles through a genotypic assessment like polymerase chain reaction (PCR) and western blotting. We have shown the efficacy in one animal model of herpes infections. Screening needs to be done on different models of herpes infections.

CONCLUSION

In conclusion, our study showed that the developed single dose acyclovir solid lipid nanoparticles have shown comparative efficacy to the multiple-dose regimen of conventional acyclovir. They found to be potential carriers for oral therapy in the treatment of HSV-1 infection. In future, we will plan for the ACV SLN toxicity studies and its efficacy studies in HSV-2 infection.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval from the Institutional Animal Ethics Committee, PGIMER, Chandigarh, India (Ref. No. 73/IAEC/444).

HUMAN AND ANIMAL RIGHTS

No humans were used. We have followed OECD guidelines for the PK-PD experiments and the protocol was approved by the Institute Animal Ethics committee.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The raw data that support the finding of the results of this study are available from the corresponding author, [Dr. NS], and the first author [Dr. RK] upon request.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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