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Research Paper

Bioavailability enhancement of zaleplon via proliposomes: Role of surface charge

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ABSTRACT

The present systematic study focused to investigate the combined advantage of proliposomes and surface charge for improved oral delivery of zaleplon. The zaleplon loaded proliposomes were prepared using hydrogenated soyphosphatidylcholine (HSPC) and cholesterol (CHOL) in varying ratios, and the optimized formulation was tailored with dicetyl phosphate and stearylamine to obtain negative and positive charged vesicles, respectively. The formulations were characterized for micromeritics, size, zeta potential, and entrapment efficiency. Further, *in vitro* release and dissolution study carried out provide an insight on the stability and enhanced dissolution of zaleplon from proliposome formulations. The solid state characterization (SEM, DSC, and PXRD) studies unravel the transformation of zaleplon to amorphous or molecular state from the native crystalline form. To depict the conclusions, *in situ* single-pass perfusion and bioavailability studies were carried out in rats. The significant increase in effective permeability coefficient (Peff) and rate and extent of absorption from cationic vesicles indicate the importance of surface charge for effective uptake across the gastrointestinal tract. Overall a two- to fivefold enhancement in bioavailability in comparison with control confers the potential of proliposomes as suitable carriers for improved oral delivery of zaleplon.

1. Introduction

Oral drug delivery continues to be the preferred route of administration. Nevertheless many of the new chemical entities synthesized by combinatorial chemistry suffer from bioavailability problems due to inadequate solubility and stability in gastrointestinal (GI) fluids, poor permeation across the GI barrier, degradation in the enterocytes and liver while exiting of drug into systemic circulation via portal circulation. Several strategies have been adopted for enhancing the dissolution behavior of insoluble drugs by complexation, drug derivatization, solid state manipulation, inclusion of surfactants, increasing the surface area by micronization or nanonization, spray drying and microencapsulation [1,2]. Inspite of improving the dissolution behavior, the drug systemic exposure is poor and baffling the formulation scientists so as to obviate the barrier function of GI tract and avoidance of presystemic metabolism.

Earlier reports suggest that the oral bioavailability of poorly water soluble, lipophilic drugs can be enhanced when co-administered with a meal rich in fat [3,4]. The concept has made to develop the formulation of colloidal lipid carrier systems as a means to improve the drug solubilization and permeation across the GI barrier [5,6]. Among different colloidal particulate drug delivery systems, liposomes are very distinct when compared with conventional dosage forms, because the particles can act as the drug containing reservoirs and modify the particle composition or surface to adjust the drug release rate or the affinity for the target site. Liposomes are phospholipid vesicles and can entrap both amphiphilic and hydrophobic solutes. Despite the advantages, the limited success of liposomes in oral delivery can be explicit in terms of physicochemical stability issues such as sedimentation, aggregation, fusion, phospholipid hydrolysis, and/or oxidation. In addition, large scale production of liposome remains unresolved [7].

The proliposome tactic has provided a major breakthrough in resolving these stability issues by using dry, free-flowing product, which is more stable during sterilization and storage [8]. Ease of distribution, transfer, measuring and storage make proliposome a versatile delivery system. Proliposomes are the dry powder formulations containing water soluble carrier particles coated with phospholipids and can be reconstituted to form liposomal dispersion on brief agitation in aqueous media. The liposomes formed after the dispersion is similar to conventional liposomes and more uniform in size. Some drugs entrapped in liposomes could not be well absorbed, but they can augment the rate and extent of absorption in GI tract [9,10]. The improved delivery of liposome entrapped drug is mediated by vesicle adsorption onto the cell surface followed by endocytosis. Further, the liposomes are bioadhesive

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and biocompatible and can adhere to the surface of GI tract to make good absorption [11]. In this context, the surface charge is a key factor for the increased vesicle interaction with the cell surface. It can be speculated that the positive charge vesicles can interact more effectively due to stronger electrostatic interaction because of the negative surface charge on the epithelial cell surface. While many studies explored the utility of proliposomes for the improvement of solubility and bioavailability of poorly soluble drugs [12–14], limited focus was given to examine the contribution of surface charge in improving the oral delivery.

Zaleplon, a pyrazolopyrimidine hypnotic drug, is indicated in insomnia and also it is a potential anticonvulsant against phenylenetetrazole- and electroshock-induced convulsions [15]. After oral administration, zaleplon is rapidly absorbed, nonetheless its absorption is dissolution rate limited due to limited aqueous solubility and thus delay the onset of action [16]. In conjunction, the bioavailability is only 30% because of extensive first pass metabolism [17]. Earlier researchers have tried solid dispersions of zaleplon using hydrophilic carriers (Poloxamer, PVP K30 and PEG 6000) and have shown improvement in the dissolution rate of zaleplon [16]. Our previous studies on proniosomes [18] and proliposomes [19] demonstrate their potential to serve as carriers for improvement in the dissolution and permeation, respectively. Keeping this in view, the present systematic study was focused to combine the advantages of proliposomes and surface charge for improved oral delivery of zaleplon. The zaleplon loaded proliposomes (neutral, negative and positive) were prepared by film deposition method and characterized. Subsequently the solid state characterization was done to ascertain the morphology, physical state and possible interaction between the formulations ingredients if any. In order to depict the conclusion of the influence of surface charge on the bioavailability of zaleplon, *in situ* single-pass perfusion and *in vivo* pharmacokinetic studies were conducted in rats.

2. Materials and methods

2.1. Materials

Zaleplon was a kind gift sample from Symed laboratories, Hyderabad, India. Phospholipon 90H (highly purified hydrogenated soyphosphatidylcholine, 90% purity, HSPC) was generously donated by Lipoid, Ludwigshafen, Germany. Cholesterol ($\geqslant$99%, CHOL), dicetyl phosphate (>98%, DCP) and stearylamine (99%, SA) were procured from Sigma, St. Louis, MO, USA. Centrisart I (20,000 MWCO) was purchased from Sartorius AG, Goettingen, Germany. Spray dried mannitol (pearlitol SD200) was a generous gift sample from Dr. Reddy's laboratories, Hyderabad, India. 2-Naphthol (G.R) used as internal standard was purchased from Merck, India. All other chemicals used were of analytical grade, and solvents were of HPLC grade. Freshly collected double distilled water was used all throughout the experiments.

2.2. Preparation of proliposome powders

The film deposition method was used for the preparation of proliposome powders [20], and the composition was represented in Table 1. In brief, accurately weighed amounts of lipid mixture (250 μM) comprising of HSPC and cholesterol at various molar ratios (1:0, 2:1, 1:1, and 1:2 respectively) and drug (10 mg) were dissolved in 20 mL of solvent mixture containing chloroform and methanol (9:1). The resultant solution was transferred into a 250 mL round bottomed flask, and spray dried mannitol (250 mg) was added to form slurry. The flask was attached to a rotary flash evaporator (Laborota 4000, Heidolph, Germany), and the organic solvent was evaporated under reduced pressure at a temperature of 45 ± 2 °C. After ensuring the complete removal of solvent, the resultant powders were further dried overnight in a vacuum oven at room temperature so as to obtain dry, free-flowing product. The obtained proliposome powders were sieved with a US 60 mesh screen (250 μm) and stored in a tightly closed container at 4 °C for further evaluation.

Table 1
Composition of zaleplon loaded proliposome powders.

Formulation	Molar ratio (HSPC:CHOL)	HSPC (mg)	CHOL (mg)	DCP (mg)	SA (mg)
ZPL-0	1:0	187.5	–	–	–
ZPL-1	3:1	140.5	24.1	–	–
ZPL-2	2:1	125.0	32.5	–	–
ZPL-3	1:1	94.0	49.0	–	–
ZPL-4	1:2	62.5	64.5	–	–
ZPL-DCP	1:1	94.0	49.0	13.7	–
ZPL-SA	1:1	94.0	49.0	–	6.7

HSPC, CHOL, DCP, and SA represent hydrogenated soyphosphatidylcholine, cholesterol, dicetyl phosphate, and stearylamine, respectively.
Total 250 μM lipid mixture was used in all the preparations.
Each formulation contains 10 mg and 250 mg of zaleplon and spray dried mannitol, respectively.

The negative and positive charged proliposome powders were prepared by adding 25 μM of dicetyl phosphate and stearylamine (10 mol% of total lipid), respectively, to the proliposome formulation (ZPL-3) comprising of HSPC and cholesterol in 1:1 ratio and processed as described above. For comparison, the control formulation was also processed similarly excluding the phospholipids and cholesterol.

2.3. Physico-chemical characterization of proliposome powders

2.3.1. Formation of liposomes from proliposome powders and morphological evaluation

The formation and morphology of the liposomes was evaluated by optical microscopy. The proliposome powder was placed on a cavity glass slide, and few μL of water was added drop wise along the side of the cover slip. The formation of vesicles on the surface of solid particle due to hydration was monitored through an optical microscope (Coslabs micro, India) and photomicrograph was taken. For the morphological evaluation, the proliposome powder was hydrated with distilled water and agitated manually for 2 min, and the transmission electron microscope observations were performed to know the morphology of formed liposomes [21]. A thin film was made on a carbon-coated copper grid by placing a drop of liposome dispersion. Before the film dried on the grid, it was negatively stained with sodium phosphotungstate solution (0.2%w/v); any excess solution was drained off with a filter paper. The grid was allowed to air dry, and samples were viewed under a transmission electron microscope (JEOL-100CX-II, Tokyo, Japan).

2.3.2. Flow properties of proliposome powders

The content uniformity of the powder formulations is dictated by the flow properties of powder. The flow properties of proliposome powders were assessed through measuring the angle of repose, Carr's compressibility index and Hausner's ratio. The angle of repose was determined by using conventional fixed funnel method. The Carr's compressibility index and Hausner's ratio were calculated from the bulk and tapped density of the proliposome powders [22].

2.3.3. Number of vesicles per mm^3

The abundant formation of vesicles is one of the important prerequisite to optimize the composition of the proliposome formulation. The liposomes formed after hydration of proliposome powder

were counted by optical microscope using a hemocytometer, and the number of vesicles per cubic mm was calculated by using the following formula [19].

$$\text{Total no. of liposomes per mm}^3 = \frac{\text{Total no. of liposomes counted} \times \text{dilution factor} \times 4000}{\text{Total number squares counted}}$$

2.3.4. Measurement of vesicle size and zeta potential of liposomes

The proliposome powders were hydrated with distilled water and agitated manually for 2 min, and the resultant liposome dispersion was used for the determination of size, zeta potential and entrapment efficiency.

The mean size and size distribution of liposomes were determined by photon correlation spectroscopy using Zeta sizer NanoZS90 (Malvern Instruments, Malvern, UK). Each sample was diluted to a suitable concentration with distilled water, and analysis was performed at 25 °C with an angle of detection of 90 °C. Size and polydispersity index of liposomes were obtained from the instrument. The zeta potential values were also obtained from Zetasizer NanoZS90 (Malvern Instruments, Malvern, UK), and the measurement is based on the Smoluchowski equation [23].

$$\zeta = U_E \eta / \varepsilon$$

where 'ζ' is zeta potential, 'U_E' is electrophoretic mobility, 'η' is viscosity of the medium, and 'ε' is dielectric constant.

2.3.5. Assay and entrapment efficiency

The zaleplon content was determined by dissolving the proliposome powder (5 mg) in 50 mL of solvent mixture comprising of methanol:water (60:40 v/v). An aliquot of sample was taken in microcentrifuge tubes and followed by centrifugation at 10,000 rpm for 15 min. The supernatant was separated, suitably diluted with mobile phase, and 20 μL of the sample was injected onto HPLC and quantified.

The entrapment efficiency of the liposomal formulation was determined by measuring the concentration of free drug in the dispersion medium using ultra-filtration [24]. In brief, ultra-filtration was carried out using centrisart (Sartorius AG, Gottingen, Germany) at 3500 rpm for 15 min, which consist of filter membrane (Molecular weight cut off 20,000 D) at the base of the sample recovery chamber. The amount of the drug in the aqueous phase was quantified by HPLC. The experiment was performed in triplicate, and percentage entrapment of zaleplon in liposomes was calculated from the following equation:

$$\%\text{Drug entrapment} = (\text{Total amount of drug added} - \text{Unentrapped drug})/(\text{Total amount of drug added}) \times 100$$

2.3.6. In vitro drug release study

The liposome dispersion formed after hydration of proliposome powders was subjected to release study in order to understand the release behavior of zaleplon from liposomes using the dialysis membrane (DM-70; MW cut off 12,000–14,000). After soaking the dialysis membrane in the release medium (20%w/v propylene glycol in 0.1 N HCl was used to maintain sink condition) for 24 h, control (drug solution in 20% w/v propylene glycol) or liposome dispersions (2 mL) equivalent to 2 mg of drug were placed in the dialysis bag and kept in 50 mL of release medium which was stirred continuously at 200 rpm and maintained at 37 °C. At preset time intervals, 1 mL of sample was withdrawn and replenished with equal volume of fresh medium to maintain constant volume. The samples were analyzed by HPLC, and the obtained data were fitted into mathematical equations (zero order, first order, Higuchi and Korsmeyer Peppas models) [25] and regression analysis was carried out to describe the kinetics and mechanism of drug release from the liposome formulations.

2.3.7. In vitro dissolution study

In vitro dissolution study of proliposome powders and control formulation was performed using USP type II (paddle) apparatus (Electrolab, TD L8, Mumbai, India) in simulated gastric fluid (pH 1.2) without enzyme. The volume of dissolution medium used was 500 mL, and the temperature was maintained at 37 ± 0.5 °C with paddle speed set at 50 rpm throughout the experiment. At predetermined time intervals, an aliquot of 5 mL was withdrawn and replenished with fresh dissolution medium to maintain constant volume. The samples were filtered by passing through 0.45 μm membrane filter (Millipore, USA) and analyzed by HPLC.

Dissolution efficiency (DE) was calculated from the area under the dissolution curve (measured using the trapezoidal rule) and expressed as a percentage of the area of the rectangle described by 100% dissolution in the same time. It is calculated by using the following equation [26].

$$\text{DE} = \frac{\int_0^t y dt}{y_{100} t} \times 100\%$$

The relative dissolution rate (RDR) was determined by dividing the dissolution efficiency of proliposome powder formulations with control formulations [27].

$$\text{RDR} = \frac{\text{DE of proliposome powder formulation}}{\text{DE of control formulation}}$$

2.4. Solid state characterization

2.4.1. Scanning electron microscopy (SEM)

The surface morphology of the pure drug, mannitol, and proliposome powders was investigated by scanning electron microscope (SEM) (S-4100, Hitachi, Japan). Samples were fixed on a brass stub using double-sided adhesive tape and were made electrically conductive by coating with a thin layer of gold, and SEM images were recorded at 15 keV accelerating voltage.

2.4.2. Differential scanning calorimetry (DSC)

The molecular state of the drug in optimized proliposome formulation was evaluated by performing DSC analysis of pure drug, mannitol, and proliposome powder. The DSC curves of the samples were obtained by a differential scanning calorimeter (Mettler DSC 823e, Mettler-Toledo, Germany). Average sample weight of 5 ± 2 mg was heated in hermitically sealed aluminum pan over a temperature range of 20–300 °C under a constant nitrogen gas flow of 30 mL/min at a heating rate of 10 °C/min. The instrument was calibrated with indium (calibration standard, purity >99.9%) for melting point and heat of fusion.

2.4.3. Fourier transform infrared (FT-IR) spectroscopy

Infrared spectra of zaleplon, mannitol, and optimized proliposome powder formulation were obtained using FT-IR spectrophotometer (Paragon 1000, Perkin Elmer, USA) by the conventional KBr pellet method. The scanning range was 4000–500 cm^{-1} and the resolution was 4 cm^{-1}.

2.4.4. Powder X-ray diffractometry (PXRD)

The PXRD patterns of zaleplon, mannitol, and optimized proliposome powder formulation were obtained using X-ray diffractometer (X' Pert PRO PANalytical, the Netherlands). The measuring conditions were as follows: Cu Kα radiation, nickel filtered; graphite monochromator; 45 kV voltage; and 40 mA current

with X'celerator detector. All samples were run at 1° (2θ) min^{-1} from 3° to 45° (2θ).

2.5. In situ intestinal absorption study

The *in situ* single-pass perfusion studies were performed using established methods reported elsewhere [28]. The study was conducted with the prior approval of Institutional Animal Ethical Committee, St. Peter's Institute of pharmaceutical sciences. Euthanasia and disposal of carcass were in accordance with the guidelines. Male wistar rats weighing between 180 and 200 g used in the study were obtained from Mahaveera Enterprises (146-CPCSEA no: 199; Hyderabad, India). The animals were housed in separate cages in a clean room and maintained under controlled condition of temperature and the rats had free access to food and water.

Before perfusion experiments, the rats fasted overnight with free access to water were anesthetized by an intraperitoneal injection of thiopental sodium (60 mg/kg body weight) and placed on a thermostatic surface to maintain body temperature. Under anesthesia, an incision was made through a midline to expose the abdominal content. The lower part of the small intestine segment used for perfusion was exposed, and semi-circular incisions were made on both ends and cannulated with PE tubing followed by ligation with silk suture. After cannulation, the surgical area was covered with cotton soaked in physiological saline (37 °C). The intestine segment was flushed with phosphate buffered saline (PBS) (pH 7.4 at 37 °C) and stabilized by perfusing the blank PBS for 15 min. The perfusates prepared by dispersing different proliposome powders (ZPL-3, ZPL-DCP and ZPL-SA) equivalent to 3 mg of zaleplon containing phenol red (7.5 μg/mL) in PBS were passed at a steady flow rate of 0.2 mL/min (NE-1600, New Era Syringe Pumps, USA) for 90 min. The perfusate was collected for every 15 min and at the end of the perfusion, the circumference and length of the perfused intestine were measured. The samples were stored at −20 °C until further analysis by HPLC. Control formulation processed without phospholipids and cholesterol containing the same amount of the drug was included in the study for comparison. Each experiment was performed in triplicate. Prior to analysis, the perfusate samples were allowed to thaw, deproteinized with methanol, centrifuged, and the drug content in the supernatant was quantified for zaleplon by HPLC.

2.5.1. Data analysis

The absorption rate constant (Ka) was calculated from the slope of the remaining amount of drug vs. time plot. The effective permeability coefficient was determined using the following equation

$$P_{\mathrm{eff(rat)}} = -[Q_{\mathrm{in}} \cdot \ln(C_{\mathrm{in}}/C_{\mathrm{out(corrected)}})]/A$$

where Q_{in} is the rate of entering intestinal perfusate (0.2 mL/min), A is the surface area within the intestinal segment that is assumed to be the area of a cylinder ($2\pi rL$) with the length (L) (measured at the end of the experiment) and radius (r) of 0.18 cm. C_{in} and C_{out} are the inlet and fluid-transport-corrected outlet solution concentrations, respectively. The $C_{\mathrm{out(corrected)}}$ was calculated by using the formula given below [29].

$$C_{\mathrm{out(corrected)}} = C_{\mathrm{out}} \cdot (Q_{\mathrm{out}}/Q_{\mathrm{in}})$$

where Q'_{out} is the measured flow (mL/min) of exiting intestinal perfusate for the specified time interval.

The enhancement ratio (ER) was calculated by using the following equation: ER = Peff of proliposome formulation/Peff of control [30].

2.6. Pharmacokinetic study

2.6.1. Study protocol

The study was conducted with the prior approval of Institutional Animal Ethical Committee, St. Peter's Institute of Pharmaceutical Sciences, Warangal, India. Male albino wistar rats (180–200 g) used in the study had free access to food and water. The animals were kept for overnight fasting prior to dosing and were divided into four groups containing six in each and were randomly administered with each treatment. Control group received an oral suspension of zaleplon (control formulation processed excluding phospholipids and cholesterol) and the test groups were treated with selected proliposome dispersions (ZPL-3, ZPL-DCP and ZPL-SA) at an equivalent dose of 2 mg of zaleplon. At predetermined time intervals, blood samples (500 μL) were collected from retro orbital plexus into microcentrifuge tubes. The blood was allowed to clot, and the serum was separated by centrifugation at 10,000 rpm for 10 min in a microcentrifuge (Remi equipments, India) and stored at −20 °C until analysis.

2.6.2. Sample analysis

Zaleplon was quantitatively determined in serum by HPLC using 55:45 (v/v) acetonitrile and water, respectively, as mobile phase at a flow rate of 1.0 mL/min equipped with LC-10 AT solvent delivery unit (Shimadzu, Japan). An octadecylsilane (C_{18}) reverse phase stainless steel analytical column (250 × 4.6 mm) with 5 μm particle size was employed for chromatographic separation (Lichrospher, Merck, Germany). The column eluent was monitored at a wavelength of 232 nm using an SPD-10 AVP ultraviolet detector, and the sensitivity was set at 0.005 AUFS at ambient temperature. The serum samples were processed as described earlier in reports [31]. Briefly, 200 μL of serum sample was treated with 100 μL of methanol, 100 μL of internal standard (1 μg/mL of 2-naphthol in methanol) and 100 μL of 2.0 M sodium hydroxide solution and vortexed for 3 min. The mixture was extracted with 3 mL of ethyl acetate followed by centrifugation, and the separated organic layer was dried under vacuum. The residue was reconstituted with 100 μL of mobile phase and an aliquot of 20 μL was injected onto the HPLC. The limit of detection and quantification were 10 and 25 ng/mL, respectively. The concentration vs. peak area ratio plot was linear ($r^2 > 0.9961$) over the concentration range of interest, and the zaleplon content in samples was quantified using this plot.

2.6.3. Pharmacokinetic parameters

The peak concentration (C_{max}) and its time (T_{max}) were obtained directly from the serum concentration vs. time profile. The area under the curve (AUC_{0-t}) was calculated by using trapezoidal rule method. The $\mathrm{AUC}_{t-\infty}$ was determined by dividing the serum concentration at last time point with elimination rate constant (K). The relative bioavailability (RA) was estimated by dividing the $\mathrm{AUC}_{0-\infty}$ of proliposome formulation with control oral suspension.

2.7. Statistical analysis

The data obtained were subjected to student's 't' test and one-way analysis of variance (ANOVA), and the significance of difference between formulations was calculated by student-Newman–Keuls (compare all pairs) with Instat Graphpad prism software (version 4.00; GraphPad Software, San Diego California). The level of statistical significance was chosen as $p < 0.05$.

2.8. Stability studies

The formulations stored in glass vials were covered with aluminum foil and kept at room temperature and in refrigerator

(4 ± 2 °C) for a period of 90 days. At definite time intervals (0, 30, 60, and 90 days), samples were withdrawn and hydrated with distilled water and observed for any sign of drug crystallization under optical microscope. Further, the samples were evaluated for vesicle size and % retention of zaleplon as described in Sections 2.3.4 and 2.3.5, respectively.

3. Results and discussion

3.1. Preparation and physico-chemical evaluation of proliposomes

The proliposomes proved to be the efficient carriers for improved oral delivery of lipophilic and amphiphilic drugs [11,13,32]. In this study, the proliposomes have been prepared and evaluated their potential in improving the oral delivery of zaleplon. Several methods have been reported for the formulation of proliposomes that include crystal-film method [33], film deposition on carrier method [34], freezing and drying method [35], powder bed grinding method [36], fluided-bed method [37], and spray drying method [38]. According to the feasibility in our laboratory, we have employed film deposition on carrier technique for the preparation of zaleplon containing proliposomes.

The formation of liposomes after reconstitution depends on the ease of dispersibility of the carrier in aqueous fluids. Among the different carriers that include maltodextrin, sorbitol, microcrystalline cellulose, magnesium aluminum silicates, we preferred to use spray dried mannitol because it possess high porosity and surface area that enables the formulator for the easy adjustment of amount of carrier required to support the lipid and also to prepare proliposomes with high surfactant-to-carrier mass ratios [39]. The spray dried form allows for controlled particle size and distribution. Furthermore, its high compression characteristics and negative heat of solution allows transforming the resultant powder into a tablet with ease and also help to improve the palatability of the formulation, respectively. In addition, it is non-hygroscopic and has an excellent compatibility and safety with drugs [40].

The selection of phospholipid is important because it dictate the stability of the liposomes formed. Since the risk of oxidation is high in phosphatidylcholine due to the presence of unsaturated bonds in the fatty acid tails [41], hydrogenated soyphosphatidylcholine which is in powder form was used in the formulations. The high phase transition temperature and solid state render more stability in GI fluids and augment the flow characteristics of the proliposomes, respectively, which is an important prerequisite for solid dosage forms. Apart from this, lipid to carrier load can be increased so that lipophilic drugs with high dose can be incorporated without any hindrance to the flow properties [42]. The proliposome concept has resolved many stability issues pertaining to the aqueous liposome dispersions. The maximum benefit of proliposomes can be achieved when it forms stable vesicles with high entrapment efficiency after hydration in the gastric fluids. In this perspective, the structural lipid, cholesterol, was used which is known to increase the stability of the bilayer with high amounts of drug entrapment. However, the formation and stability of the formed liposomes are by and large dependent on the composition of phospholipid-to-cholesterol ratio because any alteration in their composition results in leakage of drug before the drug diffusion and fusion of vesicles with the GI membrane [43]. Therefore, the effect of cholesterol was investigated by varying the HSPC-to-cholesterol ratio keeping the total lipid constant (250 μM).

The proliposomes upon hydration derive the formation of liposomes and was spontaneous suggesting a rapid conversion to liposomes on contact with physiological fluids in the body (Fig. 1A and B). It is evident from the figure that in initial stages upon contact with water, the lipids tend to form tubular structures and upon manual agitation they have deformed into small multilamellar vesicles acquiring spherical shape. Further, the TEM analysis confirms the shape of vesicles formed after hydration of proliposomes (Fig. 1C).

The micromeritics of the proliposome powders is vital in handling and processing operations because the dose uniformity and ease of filling into container are dictated by the powder flow properties. In general, three types of flow measurements can be used to evaluate the nature of powder flow that is angle of repose; Carr's index and Hausner's ratio and the results were depicted in Table 2. The smaller the value of angle of repose, lesser the internal friction or cohesion between the particles and greater the flow characteristics and vice-versa. It is apparent from the results that small angle of repose (<30°) assure good flow properties for proliposome powder formulations. In addition to angle of repose, Carr's index and Hausner's ratio were also less than 21 and 1.25, respectively, ensuring acceptable flow for proliposome powder formulations (Table 2) [44]. The distinctive advantage of proliposome formulations can be speculated only when abundant numbers of vesicles are derived from hydration of proliposome powders in the gastrointestinal tract. Among all the formulations, the proliposome formulation (ZPL-3) containing equimolar ratio of HSPC and cholesterol (1:1) demonstrate good number of vesicles (Table 3). This is in correlation with our earlier reports [45].

One of the important parameter for the vesicular systems is vesicle size and size distribution [46]. The mean size of the vesicles was in the range of 180 to 290 nm (Table 3). The size of the vesicles seems to be dependent on the cholesterol concentration. The PI used as a measure of a unimodal size distribution was within the acceptable limits for all the proliposomal formulations (Table 3). The zeta potential of the proliposome formulations (ZPL-0 to

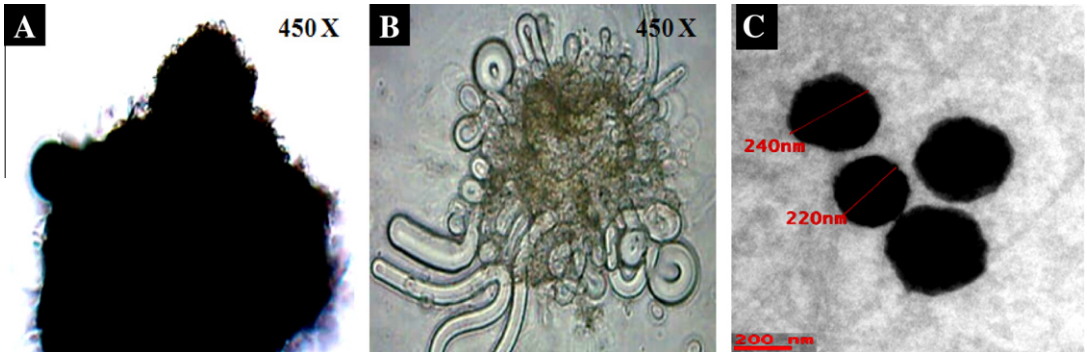

Fig. 1. Microphotographs showing (A) proliposome powder (B) formation of vesicles on pearlitol SD200 powder after hydration with distilled water (C) TEM image of liposome dispersion from reconstituted prolipsome powder (ZPL-3) upon manual agitation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Flow properties of zaleplon loaded proliposome powder formulations.

Formulation	Angle of repose (θ)	Compressibility index	Hausner's ratio
ZPL-0	16.1 ± 0.16	11.9 ± 0.04	1.03 ± 0.15
ZPL-1	18.0 ± 0.28	12.4 ± 0.12	1.14 ± 0.19
ZPL-2	21.6 ± 0.34	17.5 ± 0.45	1.12 ± 0.13
ZPL-3	21.1 ± 0.49	16.8 ± 0.67	1.11 ± 0.21
ZPL-4	24.0 ± 0.13	15.5 ± 0.33	1.32 ± 0.12
ZPL-DCP	22.3 ± 0.27	17.6 ± 0.36	1.22 ± 0.14
ZPL-SA	20.1 ± 0.12	16.1 ± 0.44	1.18 ± 0.09

Average of three determinations ± SD.
ZPL represents zaleplon loaded proliposomes.
ZPL-DCP and ZPL-SA represent zaleplon loaded proliposomes containing dicetyl phosphate and stearylamine, respectively.

ZPL-4) was between +3.1 and +5.4 mV (Table 3). Earlier reports suggest that the surface charge has a significant influence on the drug uptake by biological membrane due to electrostatic attraction or improved vesicle enterocyte interactions [47]. The classical liposomes carry a feeble charge and considered to be neutral; henceforth dicetyl phosphate and stearylamine were included at a concentration of 10 mol% of total lipid in the optimized formulation (ZPL-3) to yield negative and positive charged liposomes (ZPL-DCP and ZPL-SA), respectively. The zeta potentials of liposomes formed from ZPL-3, ZPL-DCP, and ZPL-SA were +4.3 ± 2.0 mV, −19.1 ± 2.4, and +26.0 ± 2.2 mV, respectively.

Among different methods used for determination of entrapment efficiency, we have employed ultra-filtration technique because no dilution step is involved unlike dialysis and column chromatography. The entrapment efficiency of proliposome formulations was between 80% and 95% (Table 3). Our results envisage that the entrapment efficiency of zaleplon is dependent on the composition of liposomes. The entrapment efficiency has been increased with an increase in the concentration of cholesterol (ZPL-0 to ZPL-3). This can be owed to the decreased leakage of the drug because of high compactness and hydrophobic interactions making the bilayer more stable. Further, the effective intercalation of hydrophobic drug, zaleplon within the hydrophobic core of the bilayer, may enhance drug pay load [48]. Interestingly, the same findings we could not extrapolate with proliposome formulation (ZPL-4) and in converse the entrapment value reduced (Table 3). The perturbation of the linear structure of bilayer and decreased packing space available for drug molecules might have resulted in expulsion of drug molecules [19].

3.2. In vitro release and dissolution study

In order to ascertain the effect of composition of proliposomes on the drug release and stability of liposomes, *in vitro* release study was conducted for reconstituted liposomes across dialysis membrane. Drug solution shows a very rapid drug diffusion indicating permeability of the membrane and prevalence of sink condition for the drug. A typical biphasic pattern was observed for liposomes with an initial rapid phase followed by a slow sustained phase for a period of 24 h (Fig. 2A). The initial rapid rise in the release as expected could be due to the burst release of drug because of the presence of unentrapped drug in the outer region of liposomes.

Table 3
Physico-chemical characterization of zaleplon loaded proliposome formulations.

Formulation	Size (nm)	PI	Zeta potential (mV)	Entrapment efficiency (%)	No. of vesicles per $mm^3 \times 10^3$
ZPL-0	188 ± 14	0.187	3.1 ± 2.6	81.2 ± 1.5	3.38
ZPL-1	215 ± 16	0.197	3.8 ± 1.1	84.2 ± 1.5	3.78
ZPL-2	237 ± 11	0.267	7.4 ± 2.2	86.5 ± 1.8	3.44
ZPL-3	252 ± 13	0.123	4.3 ± 2.2	94.6 ± 2.3	3.92
ZPL-4	285 ± 09	0.285	5.4 ± 1.3	79.4 ± 1.5	3.03
ZPL-DCP	266 ± 12	0.231	−19.1 ± 2.4	92.5 ± 3.1	3.72
ZPL-SA	248 ± 19	0.145	+26.0 ± 2.2	93.8 ± 4.8	3.62

PI: Polydispersity index.
ZPL represents zaleplon loaded proliposomes; ZPL-DCP and ZPL-SA represent zaleplon loaded proliposomes containing dicetyl phosphate and stearylamine, respectively.

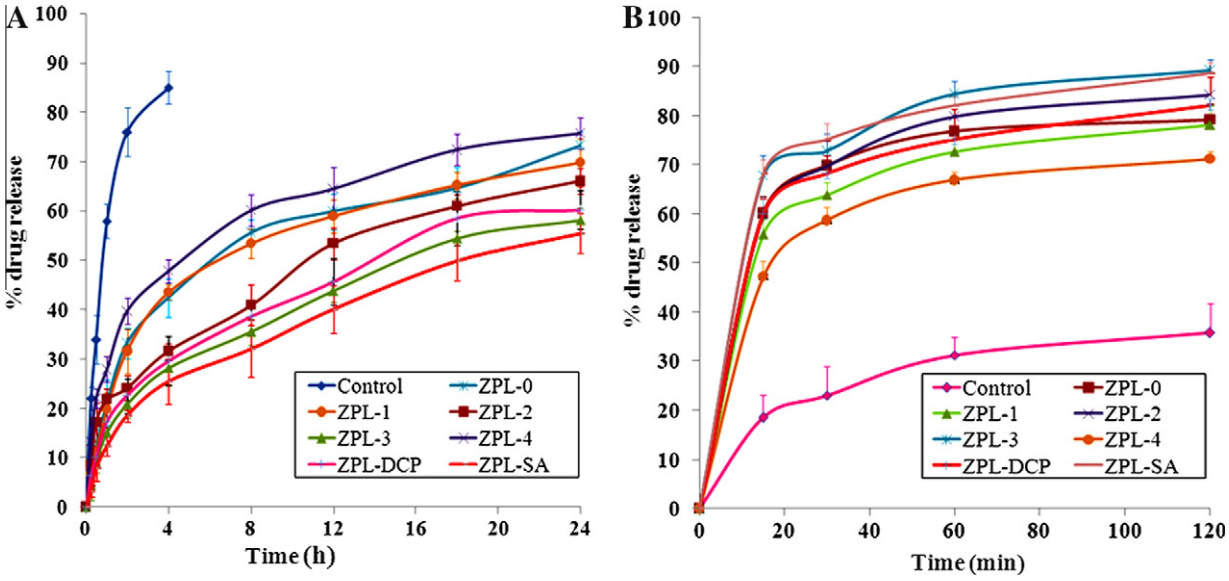

Fig. 2. In vitro release (A) and dissolution (B) profiles of zaleplon from proliposome formulations (mean ± SD; $n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
In vitro release kinetics of zaleplon from liposome dispersions.

Formulation code	Regression coefficient (R^2)				Release exponent 'n'
	Zero order	First order	Higuchi	Hixson crowell	
Control	0.773	0.686	0.950	–	0.467
ZPL-0	0.798	0.602	0.952	0.486	0.367
ZPL-1	0.740	0.558	0.921	0.465	0.310
ZPL-2	0.876	0.728	0.982	0.496	0.419
ZPL-3	0.892	0.667	0.990	0.554	0.426
ZPL-4	0.764	0.641	0.937	0.415	0.304
ZPL-DCP	0.884	0.702	0.987	0.534	0.404
ZPL-SA	0.910	0.654	0.994	0.579	0.462

ZPL represents zaleplon loaded proliposomes; ZPL-DCP and ZPL-SA represent zaleplon loaded proliposomes containing dicetyl phosphate and stearylamine, respectively.

Table 5
Dissolution parameters of zaleplon from proliposome formulations in simulated gastric fluid (pH 1.2). Each data expressed as mean ± SD; $n = 3$.

Formulation	Q15	Q60	DE	RDR
Control	18.5 ± 4.6	31.2 ± 6.1	27.3 ± 1.1	–
ZPL-0	60.5 ± 2.9	76.8 ± 3.3	69.2 ± 1.1	2.79 ± 0.22
ZPL-1	55.8 ± 3.1	72.7 ± 3.9	65.7 ± 0.8*	2.65 ± 0.25
ZPL-2	59.8 ± 3.8	79.8 ± 3.7	71.5 ± 1.2*	2.84 ± 0.29
ZPL-3	67.8 ± 4.1	84.5 ± 2.2	76.1 ± 1.8*	3.06 ± 0.31
ZPL-4	47.8 ± 3.2	66.8 ± 1.6	59.8 ± 1.6*	2.41 ± 0.21
ZPL-DCP	60.1 ± 3.5	75.2 ± 1.2	69.0 ± 1.4*	2.78 ± 0.25
ZPL-SA	68.2 ± 2.9	82.1 ± 2.3	75.6 ± 1.3*	3.08 ± 0.30

Q15 and Q60 indicate percent drug release in 15 and 60 min, respectively.
DE, RDR indicate dissolution efficiency and relative dissolution rate, respectively.
ZPL represents zaleplon loaded proliposomes; ZPL-DCP and ZPL-SA represent zaleplon loaded proliposomes containing dicetyl phosphate and stearylamine, respectively.
* Significant difference at $p < 0.01$ vs. control.

We could observe an inverse relation between the entrapment efficiency and drug release, higher the entrapment efficiency slower the drug release. From Fig. 2A, it is apparent that the drug was slower from the formulation containing equimolar ratio of HSPC and cholesterol (ZPL-3) compared to other formulations. The entrapment efficiency results also support the *in vitro* release data. The *in vitro* release data subjected to mathematical modeling reveal that the drug release from proliposome formulations is diffusion controlled following zero-order kinetics (higher R^2 values) (Table 4). The dissolution profiles of proliposomes were shown in Fig. 2B. The amount of zaleplon released from proliposomes was ranging between 65% and 84% in 60 min and was higher compared to control (31%) (Table 5). The dissolution efficiency of insoluble drug zaleplon has been significantly improved when encapsulated in proliposomes (Table 5) ($p < 0.01$). This might be due to the enhanced solubility of zaleplon by phospholipid molecules or transformation of the crystalline state of the drug to amorphous state [32]. Further, these results were consistent with the DSC and PXRD studies. However, we could not notice any remarkable change in the dissolution behavior with different proliposome formulations ($p > 0.05$).

3.3. Solid state characterization

The surface morphology of the pure drug, pearlitol SD200, and proliposome powders were examined by SEM, and the images are represented in Fig. 3. The absence of typical crystalline structures of zaleplon in proliposome formulation indicates the transformation of drug to amorphous or molecular state. Further, the porous structure of spray dried mannitol as evident in Fig. 3b was illegible in proliposome powder because of the deposition of phospholipids on the surface of pearlitol SD200.

The DSC, PXRD, and FT-IR analyses of zaleplon, pearlitol SD200, and proliposome formulation (ZPL-3) were represented in Figs. 4–6. The disappearance of peak in DSC thermogram of proliposome formulation over the melting range of zaleplon unravels the transformation of the physical state of the drug (crystalline to amorphous) which was further confirmed by PXRD analysis wherein the characteristic zaleplon peaks in proliposome formulation were reduced in intensity or absent. No additional peaks in FT-IR spectra of proliposome formulation indicate the absence of chemical interaction between the drug and formulation ingredients (Fig. 6).

3.4. Stability studies

The stability of the SLN formulation was ascertained by monitoring the physical appearance, particle size and % retention of zaleplon after storage at refrigerated temperature for a period of 3 months. At definite time intervals, the proliposome powder was reconstituted to form liposomes dispersion. The powder was free flowing without any conglomeration, and upon hydration, the formation of liposomes was rapid without aggregation problems and also we could not notice any signs of drug crystallization when observed under optical microscope. Further, no dramatic change in particle size and size distribution indicates the stability of the proliposome formulation upon storage at refrigerated

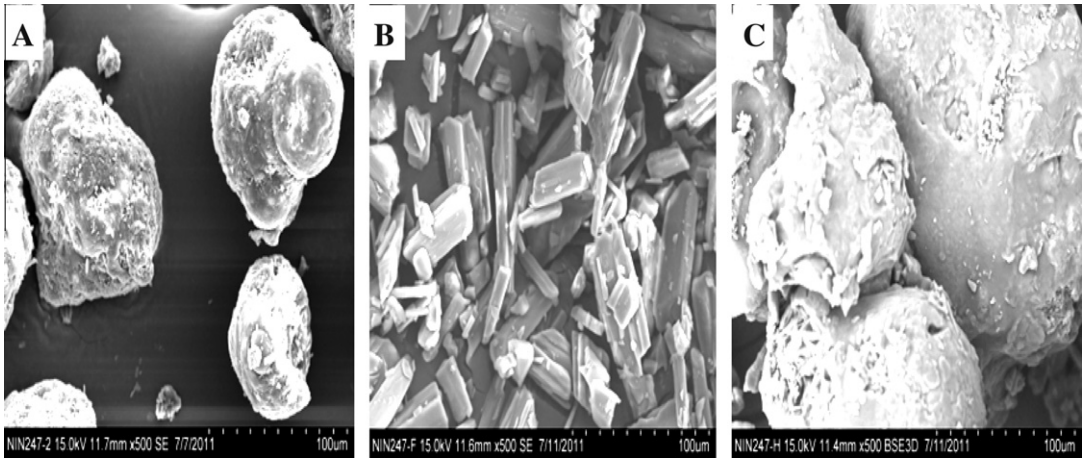

Fig. 3. Scanning electron microscope images of: (A) pearlitol SD200 (B) zaleplon, and (C) proliposome formulation (ZPL-3).

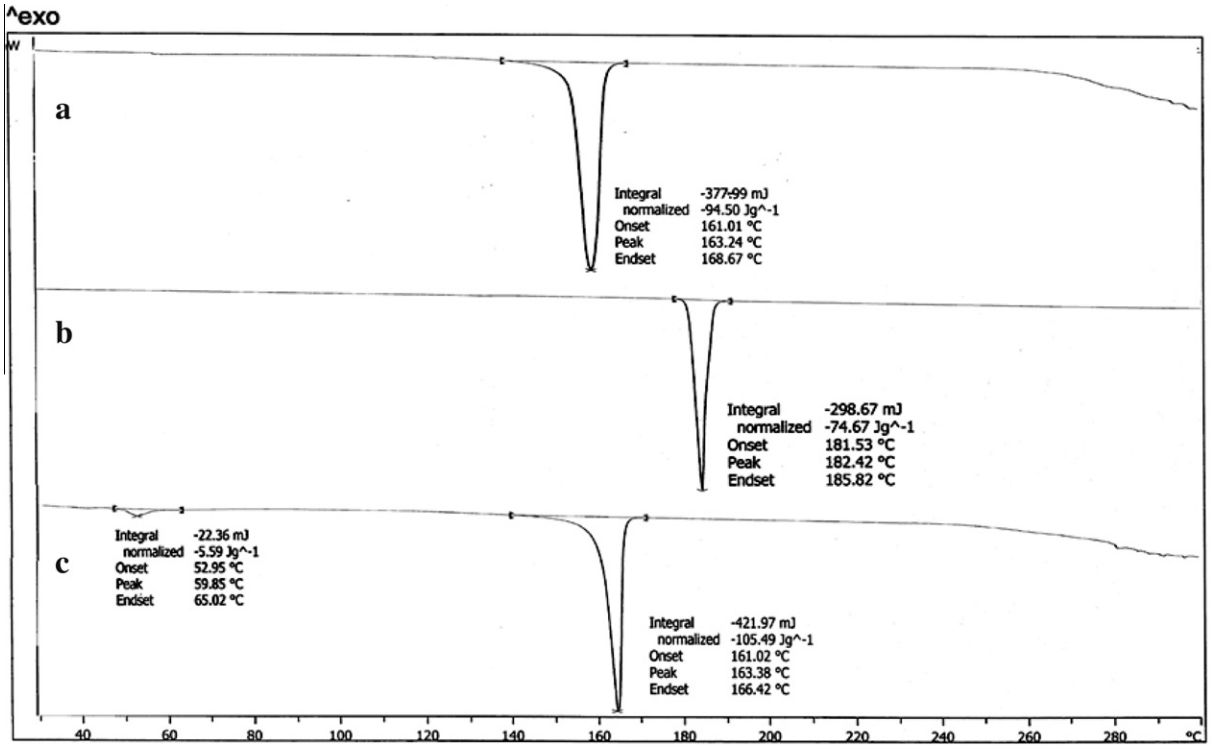

Fig. 4. DSC thermograms of: (a) pearlitol SD200, (b) zaleplon, and (c) proliposome formulation (ZPL-3).

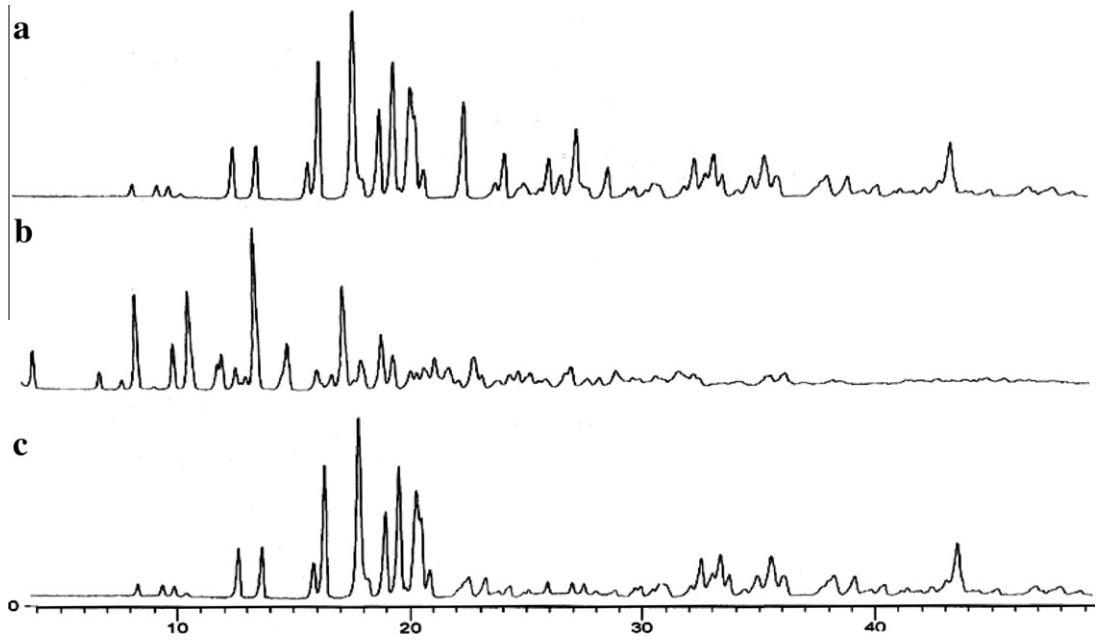

Fig. 5. Powder X-ray diffraction patterns of: (a) pearlitol SD200, (b) zaleplon, and (c) proliposome formulation (ZPL-3).

temperature for a period of 3 months (Fig. 7A). However a marginal reduction in the % retention of zaleplon was observed which is statistically insignificant (Fig. 7B) ($p > 0.05$).

3.5. In situ perfusion study

The *in situ* perfusion study facilitates to ascertain the potential of proliposomes for improved absorption of zaleplon across GI tract. The effective permeability coefficient (Peff), absorption rate constant (Ka), and enhancement ratio were calculated and represented in Table 6. The obtained Peff values for control, ZPL-3, ZPL-DCP, and ZPL-SA were 3.93 ± 0.23, 5.60 ± 0.31, 6.84 ± 0.30, and 7.72 ± 0.38 cm/s ($\times 10^{-4}$), respectively. The significant enhancement in Peff for zaleplon from ZPL-3 ($p < 0.01$), ZPL-DCP ($p < 0.001$), and ZPL-SA ($p < 0.001$) with respect to control reveals that proliposomes obviate the barrier properties of the

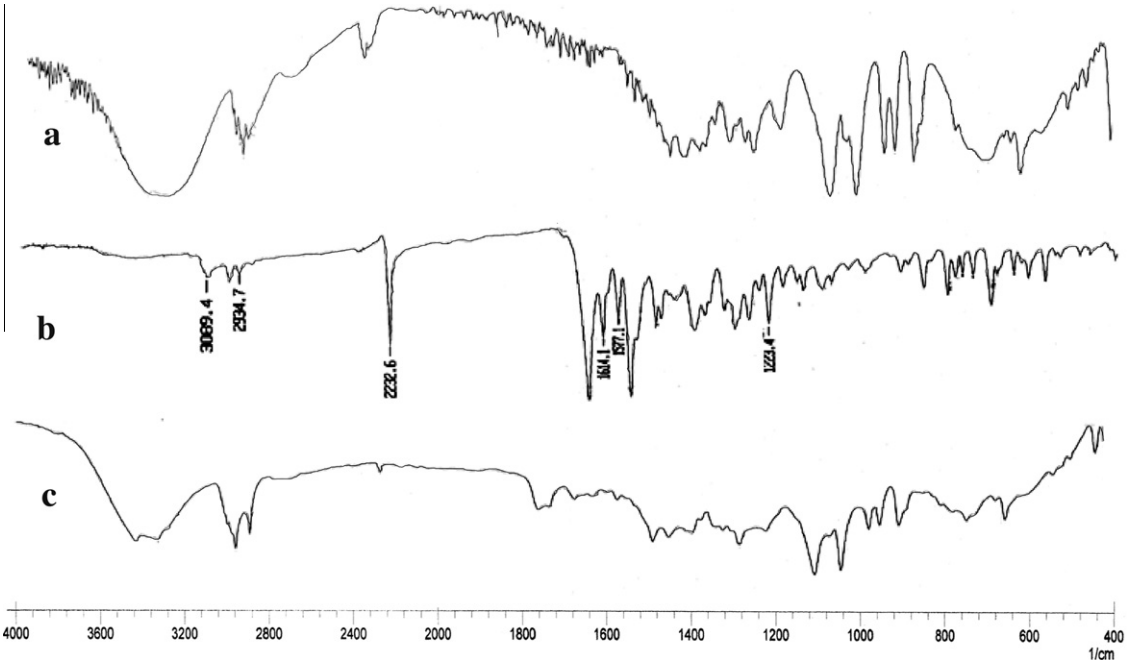

Fig. 6. FT-IR spectra of: (a) pearlitol SD200, (b) zaleplon, and (c) proliposome formulation (ZPL-3).

gastrointestinal tract thus favoring the absorption. The absorption rate constant (Ka) indicative of rate of absorption was also significantly higher for proliposomes compared to control. The enhancement ratio above 1 indicates an enhanced permeation, and in our case with all the proliposome formulations, we could observe an ER greater than 1 which suggests the potential of proliposomes for improved oral delivery.

In accordance with earlier reports, our results also envisage a preferential absorption with positively charged liposomes (ZPL-SA) when compared with negative (ZPL-DCP) and neutral liposomes (ZPL-3) [49]. This is obviously due to the effective interaction of cationic liposomes with the negative cell surface because of electrostatic attraction. Further, such an interaction might have led to an increased flux of zaleplon across the GI membrane.

Table 6
In situ parameters of zaleplon from proliposome formulations across rat intestine (mean ± SD; $n = 3$).

Formulation	Peff (cm/s) × 10^{-4}	Ka (h^{-1})	ER
Control	3.93 ± 0.23	0.018 ± 0.003	–
ZPL-3	5.60 ± 0.31^{b,1}	0.024 ± 0.002^{a,1}	1.43 ± 0.6
ZPL-DCP	6.84 ± 0.30^{c,1}	0.027 ± 0.004^{a,1}	1.75 ± 0.42
ZPL-SA	7.72 ± 0.38^{c,1,b,2,a,3}	0.037 ± 0.003^{c,1,b,2,3}	1.97 ± 0.48

Peff, Ka, and ER represents effective permeability coefficient in 2 h, absorption rate constant, and enhancement ratio, respectively.
ZPL represents zaleplon loaded proliposomes; ZPL-DCP and ZPL-SA represent zaleplon loaded proliposomes containing dicetyl phosphate and stearylamine, respectively.
1, 2, 3 indicates control, ZPL-3, and ZPL-DCP formulation, respectively.
a, b, c, indicates significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

3.6. Pharmacokinetic study

The objective of the present study is to check the feasibility of zaleplon loaded proliposomes to improve the oral bioavailability. It is speculated that the positive charge containing vesicles favor the drug absorption due to increased vesicle interaction with the cell surface because of electrostatic attraction. In this context, the

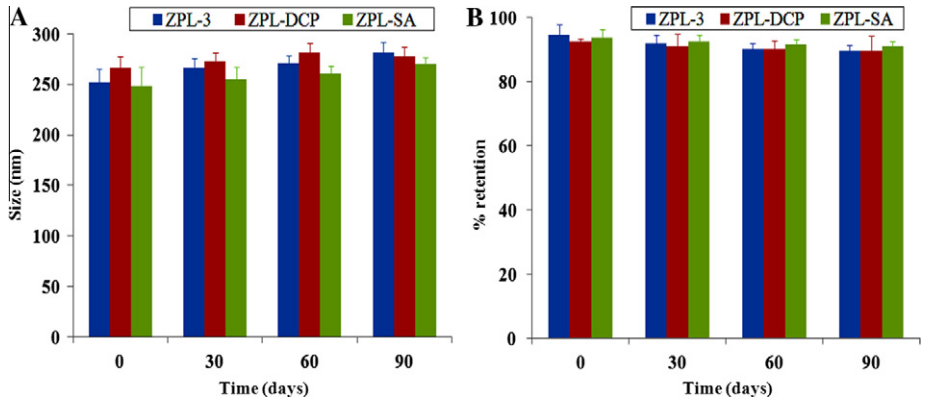

Fig. 7. Stability profiles of proliposome formulations with respect to size (A) and % retention of zaleplon (B) upon storage for 90 days at refrigerated temperature. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

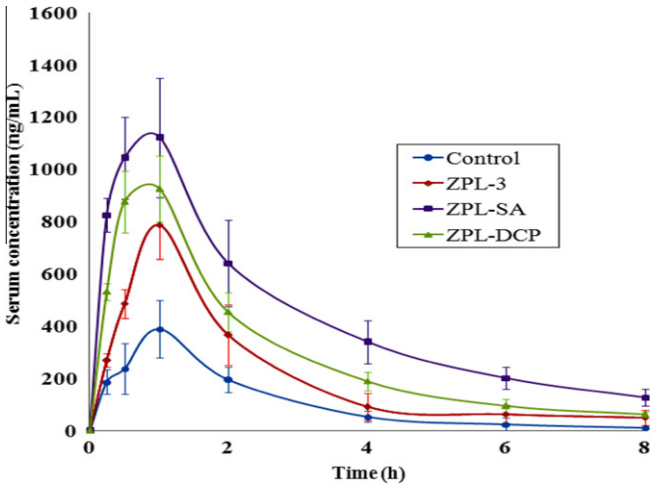

Fig. 8. Pharmacokinetic profiles of zaleplon in serum following oral administration of proliposome formulations (mean ± SD; $n = 6$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effect of surface charge was studied by intentional tailoring of proliposomes with negative and positive charge inducers that is dicetyl phosphate and stearylamine, respectively. The mean serum concentration vs. time profiles of zaleplon following peroral administration of different proliposomes in comparison with control is shown in Fig. 8. The pertinent pharmacokinetic parameters were derived and presented in Table 7. The obtained results reveal a higher C_{max} for proliposomes compared to control ($p < 0.001$). However, the time to reach the peak concentration (T_{max}) remained constant from all the formulations. The higher biological half-life and mean residence time of zaleplon from proliposomes with respect to control is obviously due to the slower elimination rate of zaleplon from these formulations (Table 7). The AUC values that indicate the extent of absorption were 2037 ± 214, 2970 ± 244, and 4646 ± 387 ng h mL^{-1} following oral administration of ZPL-3, ZPL-DCP, and ZPL-SA, respectively, and were significantly higher compared to control (1001 ± 106 ng h mL^{-1}) ($p < 0.001$). The relative bioavailability (RA) of zaleplon following oral administration of proliposomes was also significantly higher compared to control ($p < 0.001$). Overall, it is apparent from the results that the rate and extent of absorption of zaleplon have been markedly improved from proliposomes compared to control. Based on the AUC and RA values, the formulations can be ranked in the following descending order ZPL-SA > ZPL-DCP > ZPL-3 > Control. The improved bioavailability of zaleplon from proliposomes could be due to the contribution of several mechanisms either alone or in combination which include (i) by virtue of the surfactant property of the phospholipids can reduce the interfacial barrier and provide intimate contact with epithelial cell membrane thus favoring the partitioning of zaleplon into the hydrophobic domain of the cell membrane (ii) fusion of the liposomes with the epithelial cells by endocytosis also might be responsible for the augment in absorption across GI membrane (iii) direct transfer of liposomes formed at the vicinity of the GI tract may lead to an improved bioavailability due to avoidance of first pass metabolism.

Among the formulations tested, a significant improvement in the rate and extent of absorption was observed for the proliposome formulations tailored with negative and positive charge inducers (ZPL-DCP and ZPL-SA) compared to ZPL-3 formulations ($p < 0.001$). The permeability and potential uptake of slightly soluble drugs is increased, thus enhancing the bioavailability with the positively charged colloidal particles when compared with neutral or negatively charged particles [49]. Interestingly, our results also envisage a remarkable enhancement in AUC and (RA) with ZPL-SA in comparison with ZPL-DCP which indicates the importance of positive charge for the improved bioavailability of zaleplon. The epithelial cells of GI tract possess negative charge due to the presence of negatively charged proteins in the outer membrane of the cells [49]. Henceforth it is surmised that the uptake is selective toward the positive charged particles because of electrostatic attraction. Overall a two- to fivefold improvement in the relative bioavailability (RA) deduces the potential of proliposomes as a suitable carrier for improved oral delivery of zaleplon.

4. Conclusion

In this study, the combined advantage of proliposomes and surface charge has been explored for the improved oral delivery of zaleplon. The zaleplon proliposomes were prepared by film deposition method using spray dried mannitol as carrier at varying ratios of HSPC and cholesterol. The formulation containing equimolar ratio of HSPC and cholesterol was optimized based on the physicochemical characterization and *in vitro* drug release and dissolution studies. The *in situ* perfusion and *in vivo* pharmacokinetic studies reveal the importance of positive charge for enhanced absorption and oral bioavailability of zaleplon. In conclusion, the improved oral delivery of zaleplon proves the potential of proliposomes as suitable carriers for poorly soluble drugs. However, further *in vivo* studies in humans need to be warranted in order to derive the feasibility of these formulations for the improved oral delivery of zaleplon.

Table 7
Pharmacokinetic parameters of zaleplon in rats following oral administration of proliposome formulations (mean ± SD, $n = 6$).

Pharmacokinetic parameters	Formulations			
	Control	ZPL-3	ZPL-DCP	ZPL-SA
C_{max} (ng/mL)	389.2 ± 46.4	787.01 ± 63.2^{c,1}	925.15 ± 89.8^{c,1,2}	1122.4 ± 118.5^{c,1,2,3}
T_{max} (h)	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
$T_{1/2}$ (h)	1.35 ± 0.2	1.75 ± 0.36	1.80 ± 0.21	2.30 ± 0.42
K (h^{-1})	0.52 ± 0.16	0.38 ± 0.11	0.32 ± 0.10	0.30 ± 0.09
AUC_{0-t} (ng h mL^{-1})	885 ± 85	1743 ± 126	2463 ± 201	3612 ± 255
$AUC_{0-\infty}$ (ng h mL^{-1})	1001 ± 106	2037 ± 214^{c,1}	2970 ± 244^{c,1,2}	4646 ± 387^{c,1,2,3}
$MRT_{0-\infty}$ (h)	1.77 ± 0.2	1.95 ± 0.26	1.96 ± 0.24	2.68 ± 0.23
RA	1.0 ± 0.0	2.03 ± 0.26^{c,1}	2.96 ± 0.31^{c,1,2}	4.64 ± 0.66^{c,1,2,3}

K – elimination rate constant; MRT – mean residence time; AUC – area under the curve; RA – relative bioavailability.
ZPL represents zaleplon loaded proliposomes; ZPL-DCP and ZPL-SA represent zaleplon loaded proliposomes containing dicetyl phosphate and stearylamine, respectively.
1, 2, 3 indicates control, ZPL-3, and ZPL-DCP formulations, respectively.
a, b, c indicates significant difference at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

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