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Characterization and *in vitro* assessment of paclitaxel loaded lipid nanoparticles formulated using modified solvent injection technique

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This study investigates the design and characterization of solid lipid nanoparticles (SLNs) containing paclitaxel fabricated by a modified solvent injection technique using stearic acid as lipid and stabilized by a mixture of surfactants, for future evaluation of this colloidal carrier system for the oral delivery of paclitaxel, devoid of the side effects of Cremophor® EL. SLN formulations of paclitaxel stabilized by mixture of surfactants i.e. lecithin/poloxamer 188 were developed with smaller size and narrow size distribution. The paclitaxel-loaded SLNs exhibited spherical shape with smooth surface as analyzed by transmission electron microscopy (TEM). The average particle size obtained through this method was found to be ~113 nm. The zeta potential was between −32 and −39 mV with poloxamer 188. Encapsulation efficiencies of about 72.18 ± 3.7 and 89.0 ± 2.4% were achieved using 0.05 and 0.25 mmol of paclitaxel, respectively. Paclitaxel showed a sustained *in vitro* release profile and was found to follow Higuchi kinetic equations. *In vitro* cytotoxicity assay confirmed that paclitxel entrapped in SLNs showed higher cytotoxicity against cultured hepatocelluler carcinoma cells than paclitaxel alone. The modified solvent injection technique used in this research proved to be a simple, easily available and effective method to produce SLNs and could be used for controlled delivery of different lipophilic drugs for cancer chemotherapy.

1. Introduction

Paclitaxel, a naturally occurring diterpenoid originally extracted from the Pacific Yew tree, is one of the best antineoplastic drugs, being used against a wide spectrum of cancers, including breast cancer, ovarian cancer, lung cancer, head and neck carcinomas, and acute leukemia. However, the success of its clinical application is limited by its low therapeutic index and low solubility in most pharmaceutical solvents. Currently, the only available dosage form of paclitaxel is Taxol® as intravenous (i.v.) infusion, which is a solution of paclitaxel in an adjuvant called Cremophor EL, which causes serious side effects such as hypersensitivity reactions, nephrotoxicity, neurotoxicity and cardiotoxicity (Spencer and Faulds 1994; Szebeni et al. 1998; Singla et al. 2002; Van-Tellingen et al. 1999; Ten-Tije et al. 2003). Taxol® infusion is cumbersome for the patients and limits the use of frequent dosing schedule for a prolonged systemic exposure to the drug. Thus, the development of successful paclitaxel delivery system devoid of Cremophor EL is essential for a better clinical administration. Moreover, it would be an ideal solution to achieve best therapeutic efficacy with least side effects and also greatly improve the quality of life of patients, if paclitaxel shows improved bioavailability, when administered orally.

More effective chemotherapy using paclitaxel is relying on development of its new dosage forms, among which solid lipid nanoparticles (SLNs) of biodegradable lipids and lipid bilayer vesicles (liposomes) seem the most prospective. Nanoparticles of biodegradable lipids could provide an ideal solution for intravenous or oral delivery of paclitaxel as well as of other anticancer drugs. Except for solving the poisonous vehicle problem, nanoparticles could also achieve controlled and targeted delivery of paclitaxel (Xu et al. 2005). Moreover, SLNs may have advantages in favor of adhesion to and adsorption into the cancer cells to cross various physiological barriers for drug delivery, which include the gastrointestinal (GI) barrier, the blood-brain barrier (BBB), the microcirculation barrier, etc. due to its extremely small size and appropriate surface modification (Wong et al. 2007). It has been found that the size and surface characteristics of the nanoparticles play a key role on their adhesion to and interaction with the biological cells. Also it has been shown that SLNs stabilized by surfactant mixtures, such as lecithin/Poloxamer 188 and lecithin/tyloxapol, resulted in more stable, smaller particle sizes than formulations of the same lipid and a single surfactant (Zur-Mühlen 1996). When using lecithin as the surfactant with taurodeoxycholate and monooctylphosphate as cosurfactants, Cavalli et al. produced stearic acid

SLNs having 70 ± 2 nm diameters (Cavalli 1998). Surfactant mixtures often reduce interfacial tension more than single surfactant formulations on a mole per mole basis, particularly if the cosurfactant head group is significantly smaller than the surfactant head group. This phenomenon is largely due to an increased surfactant concentration at the interface, or surface excess, made possible by the minimization of repulsion forces of closely packed, like surfactant molecules (Porter 1994).

In the present study we investigated the possibility and potential of incorporating paclitaxel in SLNs made up of bioacceptable and biodegradable lipid; stearic acid, using a modified solvent injection method and stabilized by mixture of surfactants; soya lecitihin/poloxamer 188 to produce stable and smaller sized nanoparticles with a possible future aim to evaluate them as carrier for oral administration of paclitaxel. The effect of various concentrations of poloxamer 188 was analyzed for its influence on particle properties such as SLNs diameter, zeta potential, encapsulation efficiency, and drug release profile. The system developed can also be applied to the controlled delivery of other lipophilic anti-cancer drugs.

2. Investigations, results and discussion

Paclitaxel loaded SLNs synthesized with the bioacceptable and biodegradable lipid, stearic acid, using a mixture of surfactants; soya lecithin/poloxamer 188 were manufactured by a modified solvent injection method in view of developing small and stable SLNs.

2.1. Preparation of SLNs using a modified solvent injection method

Size and size distribution play an important role in determining drug release behaviour of the paclitaxel loaded nanoparticles as well as their fate after administration. A simple, economical and reproducible method was developed for the preparation of small sized SLNs. Diethyl ether was selected as the injected solvent of choice due to its low boiling point. The entire process for manufacturing SLNs was carried out at 40 ± 2 °C. The same method was previously used for investigating SLNs preparation using acetone, ethanol, isopropanol, methanol and ethylacetate (Schubert and Müller-Goymann 2003).

The surfactant type played a critical role in the formation of small and stable nanospheres. Moreover, when the target applications of the nanoparticles were in the biomedical area, the presence of toxic surfactant residues over the surface of the nanospheres was of concern. To address this concern, the triblock copolymer poloxamer 188 which was biodegradable and biocompatible, and produced a protective hydrophilic coating was selected. Poloxamer was expected to attach to the surface of the particles via its hydrophobic part (poly(propylene) and the poly(ethylene oxide) chains protuding into the surrounding medium generating a hydrophilic surface. Increase in its concentration from 0.1 to 1.5% w/v caused a distinct particle size decrease from more than 217 nm with no emulsifier to about 95 nm for stearic acid SLNs as shown in Table 1. The difference between the two values was due to the presence of small amounts of aggregates in the former sample as lack of stabilizing surfactant caused the particles to aggregate. The data depicted in Table 1 revealed that the zeta potential of SLNs decreased with increase in poloxamer concentration, this decrease in zeta potential confirms the formation of a sterically stabilized adsorbed polymer layer. Increasing the emulsifier concentration further to 2.0% w/v led to an increase of the particle size and particle size distribution, this result suggested that a reduced diffusion rate of the solute molecules caused by an increased viscosity of the outer phase might be responsible for the particle size shift. Also, at the increased concentration, poloxamer was observed to show flocculation that may be attributed to the dehydration of the poloxamer chains and reduced steric stabilization efficiency. The mean particle size obtained by PCS remained lower than 250 nm and the polydispersity index remained lower than 0.3. It was concluded that the high zeta potential values obtained would provide increased stability by electrostatic repulsion. Thus depending on the size, polydispersity and zeta potential obtained for all the batches, SLN-1.5 formulations were selected as optimal, which would result in the most stable formulation.

2.2. Effect of amount of drug incorporated on the nanoparticle characteristics

Drug entrapment is another important factor to be considered, especially for expensive drugs like paclitaxel (Dong and Feng 2004). Since loading of drug may negatively or positively affect the physicochemical characteristics of SLNs, we studied the effect of loading of paclitaxel on the mean particle diameter and zeta potential of SLNs dispersion, and the observed diameters ranged from 95 to 160 nm (Table 2). If unstable SLNs were obtained, the free drug typically precipitated out of the suspension in the form of long acicular crystals. The paclitaxel loaded SLNs (F2) with a drug load of 0.25 mmol, were of 113 ± 6.6 nm with a polydispersity of 0.156 ± 0.01. Drug incorporation did not change the size distribution. When homogeneous particles were observed without precipitation of free drug, it was assumed that the drug was entrapped inside the lipid matrix.

The loading level and encapsulation efficiency of drug-loaded lipid systems are affected by lipid concentration and initial drug loading. Higher lipid concentrations yield greater encapsulation efficiency and drug loading level

Table 1: Effect of poloxamer 188 concentrations on the particle size, polydispersity index and zeta potential of SLNs (F1) (mean ± S.D., n = 3)

Lipid	Sample code	Poloxamer (% w/v)	Mean particle size (nm)	PI	ZP (mV)
Stearic acid	SLN-0.1	0.1	217 ± 9.7	0.145 ± 0.02	−38.7 ± 0.7
	SLN-0.5	0.5	195 ± 2.0	0.152 ± 0.05	−37.9 ± 0.8
	SLN-1.0	1.0	148 ± 6.7	0.161 ± 0.03	−36.3 ± 0.3
	SLN-1.5	1.5	95 ± 9.5	0.166 ± 0.01	−35.9 ± 0.5
	SLN-2.0	2.0	150 ± 11.0	0.229 ± 0.04	−31.9 ± 0.7

PI: Polydispersity index, ZP: Zeta potential

Table 2: Effect of paclitaxel content on properties of paclitaxel loaded SLNs

Formulation	Paclitaxel content (mmol)	Mean particle size (nm)	PI	ZP (mV)	% EE	% Loading
F1	0.0	95 ± 9.5	0.166 ± 0.01	−35.9 ± 0.5	–	–
F2	0.05	99 ± 3.9	0.158 ± 0.06	−35.2 ± 0.3	72.18 ± 3.7	16.3 ± 4.1
F3	0.25	113 ± 6.6	0.156 ± 0.01	−34.8 ± 0.6	89.0 ± 2.4	25.0 ± 3.2
F4	0.5	157 ± 13.4	0.161 ± 0.05	−35.7 ± 0.7	66.47 ± 1.2	23.6 ± 4.8

PI: Polydispersity index, ZP: Zeta potential, EE: Entrapment efficiency

but, the particle size also increases (Fonseca et al. 2002). Thus in this study, lipid was fixed at the optimal concentration of 0.23 mmol so as to maintain a small size. As shown in Table 2, when the initial paclitaxel load increased from 0.05 to 0.25 mmol, the encapsulation efficiency and loading of paclitaxel increased. Further increasing the initial drug loading to 0.5 mmol did not lead to greater drug loading level and paclitaxel precipitated during the fabrication process, possibly because the initial paclitaxel loading reached the maximum loading capacity of the system. The encapsulation efficiency and loading level obtained for F3 formulation was 89.0% and 25.0%, respectively. This study showed the significant impact of paclitaxel incorporation on particle size but the particle size distribution remained unaffected. Incorporation of paclitaxel did not alter the zeta potential values of the nanoparticles as the formulations prepared without paclitaxel had similar zeta potential values. This can be explained by the lack of ionizable functional group in the paclitaxel molecule.

Through the use of modified solvent injection method, paclitaxel loading was further investigated with respect to entrapment efficiency and loading using different poloxamer concentrations (Fig. 1). SLNs fabricated without poloxamer showed low entrapment efficiency because of their porous surface, which resulted in drug loss from nanoparticles as a result of diffusion into the dissolution medium during the fabrication process. The incorporation of paclitaxel in SLNs using the modified solvent injection method and system stabilization by a mixture of surfactants; lecithin/poloxamer 188 resulted in smaller sized nanoparticles when compared to using a single surfactant (Chen et al. 2001). As poloxamer gave nanoparticles a smooth surface with very few or no pores to minimize the drug loss during the fabrication process and the incorporation of lecithin added to high entrapment because hydrophobic surfactant contributed to further incorporation of the highly hydrophobic paclitaxel into the formulation system. 1.5% w/v poloxamer resulted in small sized SLNs with high loading and entrapment efficiency when compared to 1% w/v poloxamer, which was also supported by the TEM image Fig. 2. Further increase of poloxamer (2% w/v) resulted in flocculation and formation of aggregates, which resulted in loss of drug, thus low entrapment and loading. In addition, some drug molecules may bind to the excessive surfactant molecules and thus drug may be lost during the preparation process. The disadvantage with the use of surfactants in drug delivery systems is their potential toxicity at higher concentrations. Surfactants are capable of causing disruption in biological membranes and display significant interaction with certain proteins and a high concentration of surfactants over a long period of time may disturb some bodily processes (Buckton 1995). Therefore depending on the above results, the optimal concentration of poloxamer chosen for further studies was 1.5% w/v which was in accordance with the optimal concentration obtained for blank SLNs.

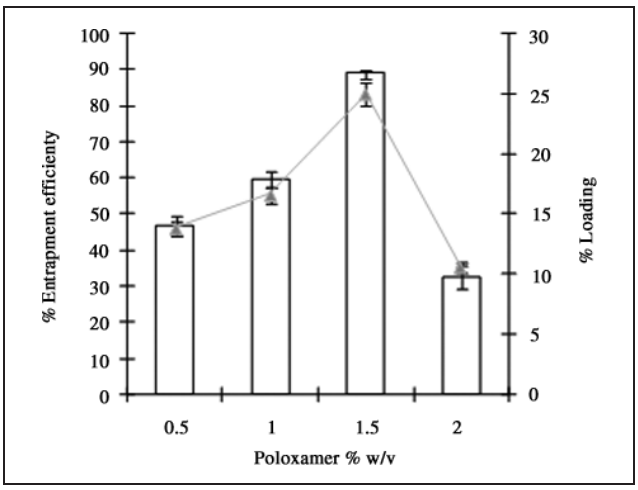

Fig. 1: Effect of different surfactant concentrations on % entrapment efficiency and % loading (n = 3)
%EE (SA)
% loading (SA)

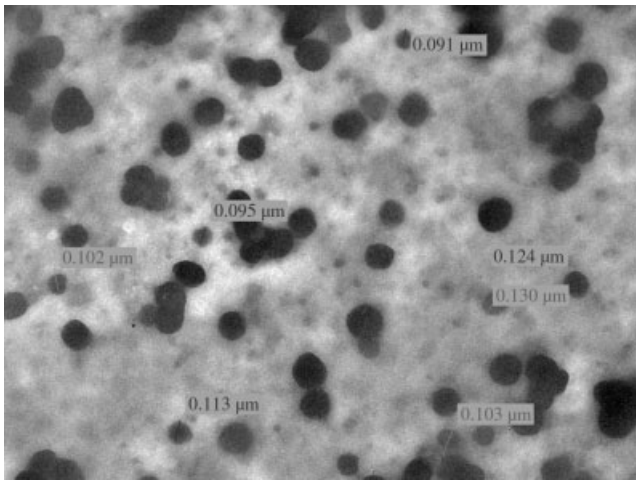

Fig. 2: TEM image of the F3 SLNs

2.3. Surface morphology characterization of drug-loaded SLNs

The morphology of drug loaded SLNs was analyzed by TEM. SLNs had a spherical shape with a relatively narrow size distribution, which is consistent with the size as determined by PCS (Table 2). Furthermore, no large aggregates (e.g. larger than 170 nm) were observed, indicating that SLN dispersion contained uniformly distributed particles within the size range of nanometers.

2.4. Surface charge characterization

The zeta potential of the drug loaded SLNs was characterized in order to determine the surface charge property.

Commonly, zeta potential can be an index to the stability of the nanosphere. The zeta potential of the produced SLNs was studied and the findings are summarized in Table 2. The zeta potentials were around −35 mV. It has been suggested that full electrostatic stabilization requires a zeta potential of $> \pm 30$ mV, while potentials between 5mV and 15mV result in limited flocculation and potentials between −5 mV and +3 mV yield maximum flocculation (Schwarz et al. 1994). Therefore, the zeta potentials obtained in the present work were within the range of full electrostatic stabilization.

2.5. Thermal analysis of freeze dried SLNs

Drug loaded SLNs were analyzed by DSC to investigate the crystalline nature of incorporated paclitaxel. The DSC thermograms of paclitaxel, stearic acid, physical mixture of excipients, paclitaxel loaded SLNs (F3) are shown in Fig. 3. The thermogram of F3 did not show the melting peak of paclitaxel around 219.59 °C (m.p. of paclitaxel) suggesting that paclitaxel was present in the SLNs either in amorphous form or molecularly dispersed.

2.6. X-ray diffraction studies

X-ray diffraction pattern of paclitaxel and paclitaxel loaded SLNs (F3) were obtained and compared, which revealed marked differences in the molecular state of paclitaxel (Fig. 3). X-ray diffraction analysis was performed to determine whether the entrapped paclitaxel existed in the less water-soluble crystalline state or the more soluble amorphous state. In the case of paclitaxel, the diffractogram exhibited peaks at the following 2θ values: 5.7°, 9.0°, 10.2°, 11.3°, and 12.6° (Fig. 4a). Among these, the peak of highest intensity was located at 5.7° 2θ, and the peaks at 9.02° and 12.6° 2θ were broad. When the diffraction pattern of paclitaxel-loaded SLNs were compared with that of paclitaxel, the pattern differed to a large extent. Several high-angle diffraction peaks were observed in paclitaxel-loaded SLNs at the following 2θ values: 5.4°, 6.3°, 9.9°, 11.2°, 12.8°, 13.8°, 14.2°, 16.6°, 18.8°, 19.4°, 20.6°, 21.8° and 23.6° (Fig. 4b). Sharp peaks in X-ray diffraction spectra indicate a crystalline structure. Results show sharp peaks for free paclitaxel but not in paclitaxel-loaded SLNs, this indicates that the paclitaxel entrapped in the nanoparticles existed in an amorphous state.

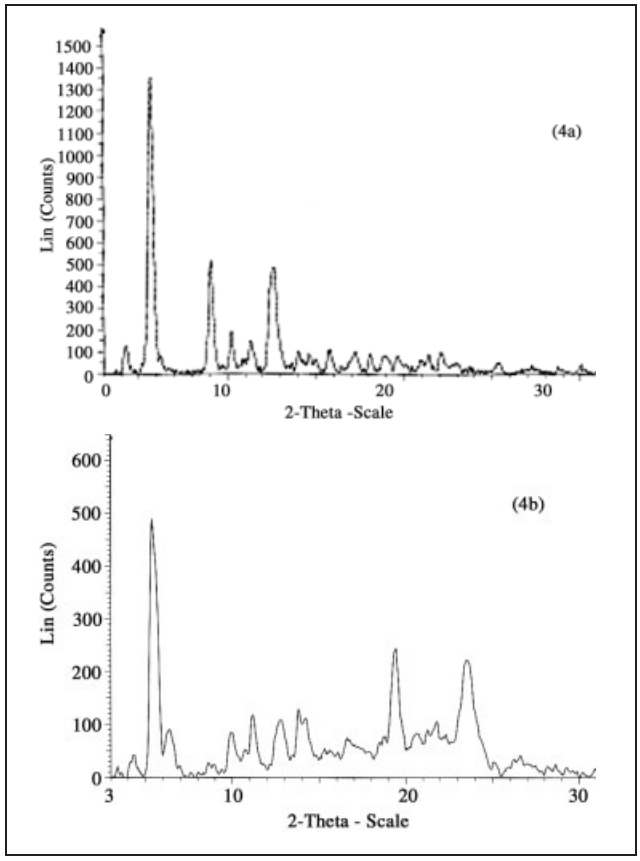

Fig. 4: (a) X-Ray diffraction pattern of paclitaxel. (b): X-Ray diffraction pattern of paclitaxel-loaded SLNs (F3)

2.7. Surface chemistry characterization

The paclitaxel-loaded SLNs were characterized by FTIR spectra for their surface chemistry. FTIR spectra of pacli-

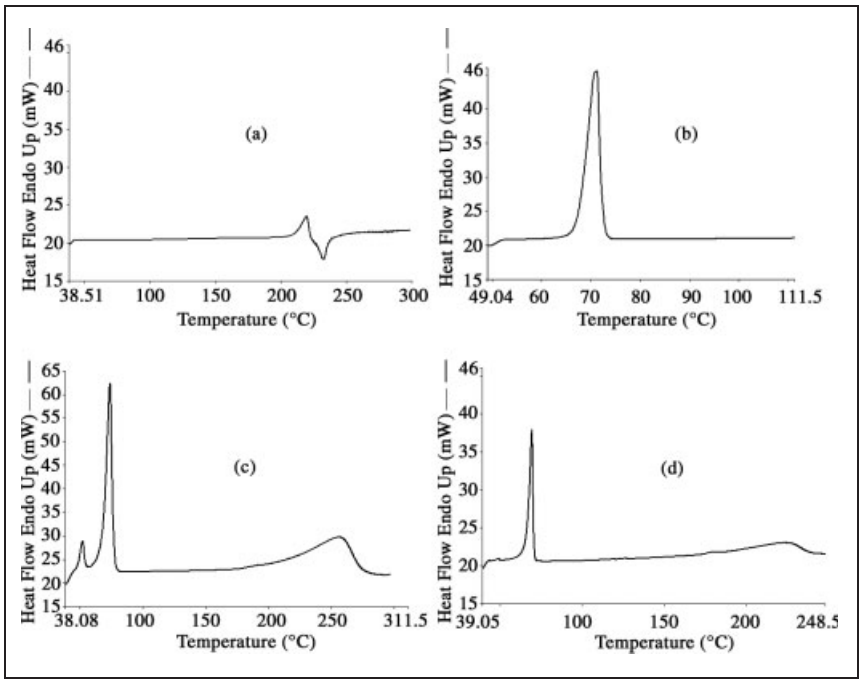

Fig. 3:
DSC thermogram of (a) paclitaxel, (b) stearic acid, (c) physical mixture, and (d) paclitaxel loaded stearic acid-SLNs

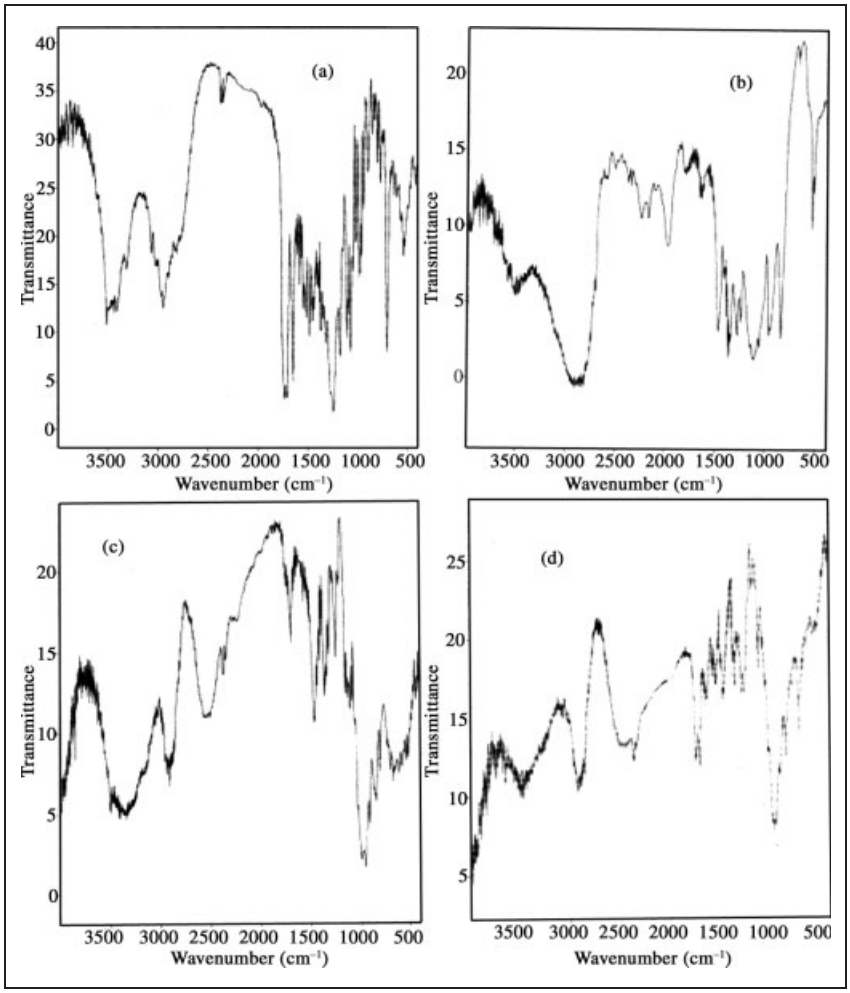

Fig. 5:
FTIR spectra of (a) paclitaxel, (b) poloxamer 188, (c) drug free SLNs, and (d) paclitaxel-loaded SLNs

taxel, poloxamer 188, drug free SLNs (F1) and paclitaxel-loaded SLNs (F3) were obtained and characterized to determine the chemical nature of the surface layer (Fig. 5). The samples used for the study were preserved in desiccator before use. The FTIR spectrums showed that the characteristic peaks of pure poloxamer were at 3500, 2884, and 1114 cm^{-1} due to the stretching of O−H, C−H, and C−O groups, and for paclitaxel at 1710, 1240 (>C=O), 850 (epoxy rings), 3020 (=CH− stretching), and 3500 (−OH). The spectra of F1 and F3 were compared and both exhibited a broad peak at 3500 cm^{-1} like the poloxamer spectrum. No significant difference was observed between the spectra of F1 and F3, further the FTIR spectra showed the characteristics peaks of poloxamer at 3500, 2884, and 1100 cm^{-1}. This made us draw the conclusion that poloxamer was present on the outer surface of the SLNs. Finally from the FTIR spectral interpretation this can be concluded that paclitaxel was absorbed to the inner lipid layer of nanoparticles and only a little amount of paclitaxel was present on the surface of SLNs. Since the solubility of paclitaxel in water is very low, this makes the drug tend to stay inside lipid particles rather than diffuse to the surface.

2.8. In vitro release kinetics of paclitaxel loaded SLNs

The ideal fate of SLNs *in vivo* is to eventually release their contents to the surrounding biological fluid or to the endosome/lysosome within the biological cell. Thus, the mechanism and kinetics of release of paclitaxel from SLNs was studied in detail.

The *in vitro* release profiles of paclitaxel were obtained by representing the cumulative percentage of paclitaxel release with respect to the amount of paclitaxel loaded in SLNs. The *in vitro* release behavior of paclitaxel from SLNs in PBS (pH 7.4 at 37 °C) was studied using a hydrophilic dialysis membrane as the donor compartment which retained the nanoparticles and allowed the transfer of drug into the receptor compartment. The release behavior of paclitaxel from all the developed SLNs exhibited a biphasic pattern characterized by initial burst during the first 24 h, followed by a slower and sustained release. The most important effect of emulsifiers on the properties of the produced SLNs was found to be that on the release behavior. We can explain this phenomenon by the preparation procedure, after the organic solvent was evaporated, the paclitaxel was dissolved in the lipid, the rapid quenching of the nanoemulsion might not have allowed the drug to crystallize, and the paclitaxel was trapped in the solid lipid. This prolonged release behavior was desirable that makes possible to bypass gastric and intestinal degradation of the encapsulated drug. The higher load of paclitaxel resulted in its improper encapsulation, thus more initial burst effect followed by slow release.

The release rate of drug can be altered by changing the drug concentration in the lipid matrix. It has been reported that the increase of drug concentration up to a certain level may enhance the release of drugs due to an increase in the drug thermodynamic activity (Rao et al. 1998). Three different paclitaxel-loaded SLNs (0.05, 0.25, 0.5 mmol) were used to study the effect of drug load on the release of paclitaxel from the nanoparticles. Figure 6a shows the

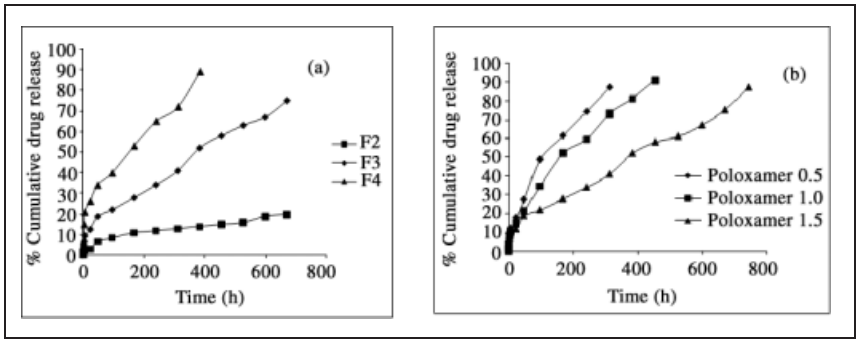

Fig. 6:
(a) *In vitro* drug release from paclitaxel loaded SLNs with 0.05, 0.25, 0.5 mmol of paclitaxel loading. (b) *In vitro* drug release from paclitaxel loaded SLNs (F3) with 0.5, 1.0, 1.5%w/v poloxamer

percentage release of paclitaxel from F2-, F3- and F4-SLN formulations. Within 24 h, 2.8%, 12.4% and 26.2% of paclitaxel was released from 0.05, 0.25, 0.5 mmol drug-loaded stearic acid SLNs, respectively. The lower burst release with poloxamer 188 might be related to the surfactant's hydrophilicity, which reduces a possible localization of paclitaxel on the SLNs surface, consequently decreasing its availability for immediate dissolution, this indicated that paclitaxel incorporated in the delivery system is associated with the nanoparticles. This lower initial burst was probably caused by the drug adsorbed on the nanoparticle surface or precipitated from the superficial lipid matrix. The slow release was probably due to diffusion of drug from the lipid matrix in which it was molecularly dispersed. The release by diffusion is greatly dependent on the surface morphology of the product (Feng et al. 2001). The release rate of high drug-loading sample seemed to be slightly higher than that of low drug-loading sample. The higher load of paclitaxel resulted in its improper encapsulation, thus more initial burst effect followed by slow release was seen.

As the poloxamer concentration in the SLN preparation was increased from 0.5 to 1.5%, the thickness of the poloxamer coating increased thereby increasing the length of diffusion resulting in decrease in the drug release from 17.63 to 12.0% for stearic acid SLNs, as shown in Fig. 6b.

The *in vitro* release mechanism of paclitaxel from these lipid systems was further evaluated by using zero order, first order and Higuchi release kinetic models (Table 3). The release profiles of all the SLNs best fit into the Higuchi equation that describes the diffusion of drug from homogenous and granular matrix systems. The drug release from a matrix system is said to follow Higuchi's release kinetics if the amount of drug released is directly proportional to the square root of time. The slopes obtained from the plots were proportional to an apparent diffusion coefficient. It was found that the *in vitro* drug release of paclitaxel was best explained by Higuchi's equation, as the plots showed the highest linearity (r^2), followed by zero order and first order. This explains why the drug diffuses at a comparatively slower rate as the distance for diffusion increases, which is referred to as square root kinetics (or Higuchi's kinetics) i.e. matrix diffusion controlled mechanism. Considering these observations it was concluded that all the batches of SLNs had the potential for sustained drug delivery. Formulation F3 ($r^2 = 0.9813$) was found to have the highest entrapment efficiency 89.0 ± 1.23% and the lowest particle size 113 ± 6.6 nm, therefore, F3 was chosen as the optimized paclitaxel loaded SLNs of stearic acid lipid matrix and was taken for stability studies.

Table 3: Kinetics of optimized formulation of paclitaxel loaded SLNs

Formulations	Zero order	Higuchi		First order
	r^2	r^2	slope	r^2
PTX-SASLN$^{0.05}$	0.9213	0.9866	0.72	0.4950
PTX-SASLN$^{0.25}$	0.9744	0.9813	2.70	0.5361
PTX-SASLN$^{0.5}$	0.9306	0.9816	4.08	0.5995
PTX-SASLN$^{P\text{-}0.5}$	0.9560	0.9931	4.88	0.7533
PTX-SASLN$^{P\text{-}1.0}$	0.9681	0.9915	4.09	0.7365
PTX-SASLN$^{P\text{-}1.5}$	0.9739	0.9815	2.69	0.5362

2.9. Stability in simulated gastric medium

Taking into account that the primary goal of our work was to design a nanoparticulate drug delivery system intended for oral administration of paclitaxel, prior to the physicochemical stability evaluation on storage, surface modified nanoparticles i.e. poloxamer coated paclitaxel loaded SLNs were evaluated for their stability in simulated gastric fluid.

This protective coating was found to prevent the molecular degradation of the lipid since negligible degradation was observed in gastric medium, as the samples showed negligible amount of drug when subjected to HPLC analysis after extraction with DCM. For comparison, non-coated paclitaxel loaded SLNs were also prepared and subjected to a similar procedure, which displayed an instantaneous and massive aggregation following their incubation in gastric medium, whereas protective coating of poloxamer 188 reduced this process. More specifically, for poloxamer-coated SLNs size remained unchanged.

Consequently, the absence of degradation observed in all the poloxamer-coated nanoparticles suggested that neither acidity nor gastric protease was able to cause lipid degradation, indicative of the protective poloxamer coating around the nanoparticles. The acid pH of the gastric medium is a key factor in the aggregation process of SLNs and the different behavior of the SLNs tested could be explained by the different stabilization mechanism of the surfactant employed. At low pH, lecithin is not ionized, and consequently under these conditions, ionic stabilization is not strong enough to prevent aggregation, thus for SLNs without poloxamer coating aggregation was observed. In contrast, the sterically stabilizing properties of poloxamer 188 were not affected by the pH. Thus the poloxamer prevented SLN lipids from getting oxidized by providing a layer of protection, preventing their aggregation and preserved the structural integrity of the SLNs.

2.10. Physicochemical stability studies

In the field of drug targeting, delivery systems are often poorly defined in physicochemical terms and little atten-

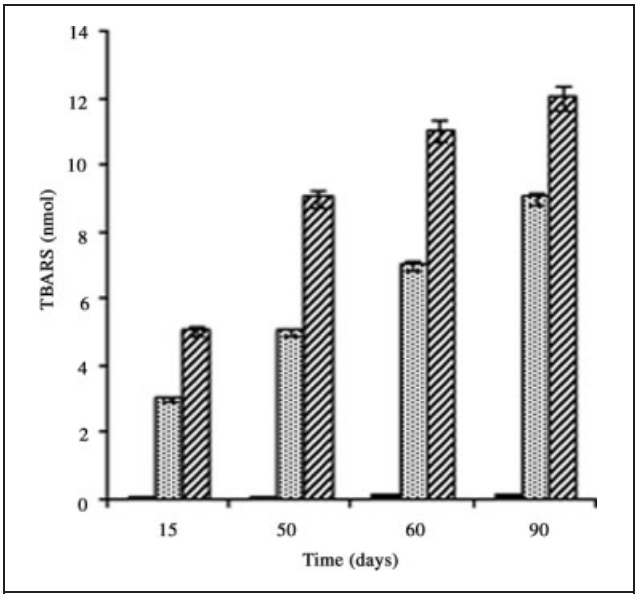

Fig. 7: Effect of storage conditions on particle size of paclitaxel loaded SLNs (mean ± SD; n = 3)

tion is paid to issues related to long-term stability and reproducibility in preparation and performance. This often makes results difficult to reproduce. These issues are critical to the success of these new delivery systems. The stability of SLNs on storage is of great concern, as it is the major hindrance to the development of marketed preparations. Aggregation and fusion, which lead to changes in particle size and particle size distribution, are the main results of the physical instability of lipid systems. Such processes occur to a significant extent over long periods of storage. Chemically, phospholipids used in the study were susceptible to oxidation and hydrolysis, the unsaturated acyl chains of phospholipids may be oxidized in the absence of specific oxidants by a free radical chain mechanism. Oxidation and oxidative effects can be minimized by storing the lipids at a low temperature and in an inert atmosphere and through careful handling. The effects produced by both these instabilities could influence the *in vivo* behavior of SLNs (targeting, cell uptake, and clearance), therefore extensive studies were required before a lipid-based formulation was used for pharmacological therapy. As discussed earlier, paclitaxel loaded SLNs (F3) were prepared at room temperature, which contained α-tocopherol as the anti-oxidant in the lipid phase. In this study, the physical stability of developed paclitaxel loaded SLNs were evaluated following storage at 4 ± 2 °C, 25 ± 2 °C/60 ± 5% RH and 40 ± 2 °C/75 ± 5% RH for various lengths of time, using particle size, shape, zeta potential, drug content, and TBARS assay as the determining criteria.

No significant change in the particle size of all the SLNs was observed when they were stored at 4 ± 2 °C and 25 ± 2 °C/60 ± 5% RH up to 90 and 60 days, respectively. A significant increase in the particle size of SLNs was observed at 40 ± 2 °C/75 ± 5% RH after 15 days (Fig. 7). The increase in size could be due to the aggregation of particles. These results indicate that aggregation is temperature dependent. This phenomenon of particle aggregation is shown in the TEM photomicrographs of the SLN formulation (Fig. 8).

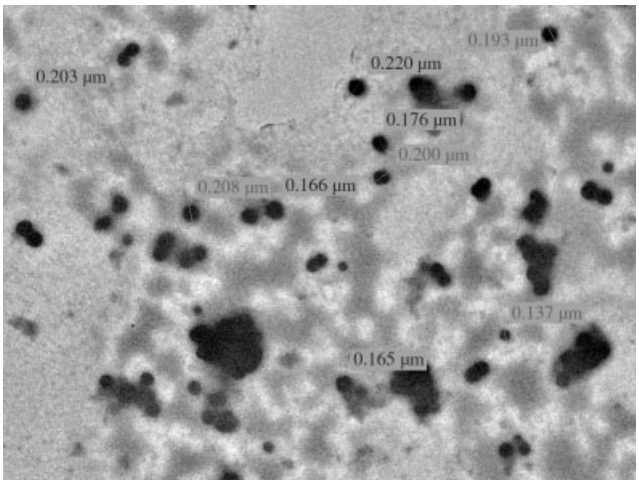

Fig. 8: TEM photominograph of the paclitaxel loaded SLNs at 40 °C ± 2 °C/75% ± 5% RH after 15 days of sterage

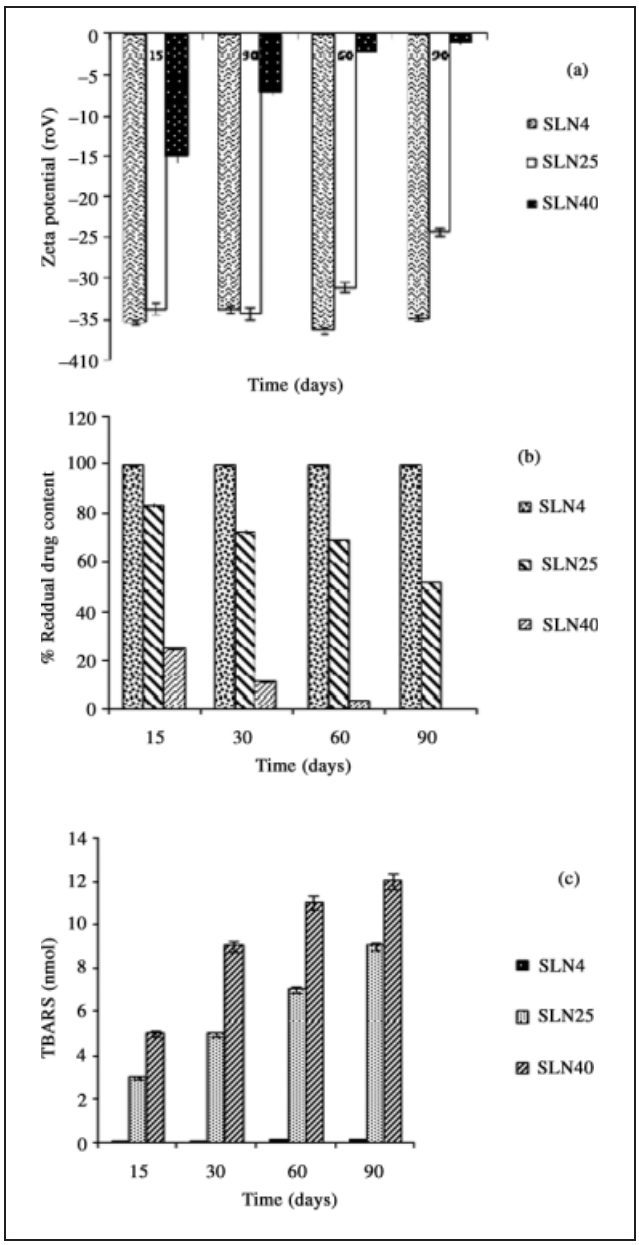

Fig. 9: (a) Effect of storage conditions on zeta potential of paclitaxel loaded SLNs (mean ± SD; n = 3). (b) Effect of storage conditions on the percentage of residual drug content of paclitaxel loaded SLNs (mean ± SD; n = 3). (c) Effect of storage conditions on TBARS formed in various SLN formulations (mean ± SD; n = 3)

The surface charge of particles played an important role in the rate of aggregation and fusion of particles and thus in the physical stability of SLNs. No significant change in the zeta potential of SLNs was observed when they were stored at 4 ± 2 °C and 25 ± 2 °C/60 ± 5% RH up to 90 and 60 days, respectively. The decrease in zeta potential of SLNs at 40 ± 2 °C/75 ± 5% RH was found to be a function of time up to 90 days (Fig. 9a), i.e. with increasing days of storage the zeta potential decreased, as at high temperature and relative humidity, the outer hydrophilic poloxamer coating dissolved out with time leading to system's reduced steric stability.

More than 99.99% (<100%) of the drug was retained for 90 days in all the SLN formulations during storage at 4 ± 2 °C. While 50% to 60% of the initial drug was present after 90 days of storage at 25 ± 2 °C/60 ± 5% RH, at 40 ± 2 °C/75 ± 5% RH, SLN could retain ~8 to 10% of their initial drug, after 30 days (Fig. 9b).

The lipid peroxidation was measured as MDA which is the end product of lipid peroxidation and reacts with TBA as a thiobarbituric acid reactive substance (TBARS) to produce a red colored complex which has peak absorbance at 532 nm. No red colored complex formation was observed for the formulations stored at 4 ± 2 °C. TBARS production i.e. formation of a red colored complex was significantly higher for SLNs stored at 4 ± 2 °C/75 ± 5% RH (Fig. 9c).

Lipid peroxidation processes start with the hemolytic fission of the phospholipid acyl chains or as a consequence of the attack of reactive oxygen species, such as superoxide anion, hydrogen hydroperoxide, and hydroxyl radical, which are able to abstract hydrogen from an acyl chain, leading to acyl radical formation. All these chemical processes require energy, which can be supplied by electromagnetic radiation, heat, redox reactions, and so on. The first acyl radicals formed react with oxygen, producing acyl peroxyl radicals, which react with other acyl chains. These mechanisms are involved in the propagation phase of lipid peroxidation, leading to the formation of hydro and cyclic peroxides and later MDA, short-chain aldehydes, esters, ketoacids, and so on. TBA reacts with carbonyl substances (aldehydes, ketones). The obtained results indicated that stearic acid SLNs remained stable at 4 ± 2 °C.

2.11. In vitro cytotoxicity

The cytotoxicity of the free paclitaxel and paclitaxel-loaded SLNs (F3) were evaluated *in vitro* on HepG2 cell lines by the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (Gagandeep et al. 1999). The assay was based on mitochondrial dehydrogenase cell activity as an indicator of cell viability. The assay determined cell viability based on the mitochondrial conversion of a water-soluble tetrazolium salt, MTT to the water-insoluble blue formazan product. The growth medium was replaced with a fresh one containing varying concentrations of distinct paclitaxel formulations investigated in the study.

SLNs had a negative surface charge, which could affect the cellular uptake of the prepared nanoparticles due to electrostatic repulsion forces between the nanoparticles and the rather negatively charged surface of cells. The interaction of SLNs with cells appears to depend significantly on the stabilizer used. In the present study, the purpose of using poloxamer to coat SLNs was not only to improve stability but also to target it to the intestinal mucosa for uptake by Peyers' patches followed by cellular absorption. As a result of its amphiphilic nature, poloxamer 188 had the capability to interact with the cell membrane (Wu et al. 2005).

The drug loading of the paclitaxel-loaded SLNs was found to be 25%. The findings indicate that paclitaxel is cytotoxic to cultured hepatocellular carcinoma cells. The drug formulated in the SLNs showed advantages in achieving lower cell viability or equivalently, higher cytotoxicity in comparison to the drug alone as shown in Table 4. The cell viability was measured at different drug concentrations (0.02–100 μg/ml) and decreased significantly ($P < 0.001$, ANOVA) when the drug was in the SLNs compared to free paclitaxel after 24 h incubation within the cells. Table 4 shows that the CC_{50} value for HepG2 cells decreased from 0.58 μg/ml for paclitaxel to 0.085 μg/ml for the F3 SLN formulation after 48h of incubation. A significant difference in the CC_{50} value of paclitaxel and F3 SLN formulation was also observed even after 72 h incubation with HepG2 cells. It can be seen from Figure 6 that the drug release from F3 SLN formulation was only 25% of the total amount encapsulated in SLNs, which shows that 25% of the drug encapsulated in the SLNs can have comparative effects on CC_{50} to that of the full amount of paclitaxel.

With SLNs, the uptake in HepG2 cells increased which indicates that the SLNs had a specific interaction with HepG2 cells due to the outer most poloxamer coating, poloxamer 188 was preferentially adsorbed on the particle surface and was desorbed, leaving the surface "unprotected". This could explain the more extensive uptake of poloxamer stabilized paclitaxel loaded SLNs than of paclitaxel alone.

It was speculated that such enhanced interaction would result in enhanced absorption of the encapsulated lipophilic drug. The SLNs prepared in the study were first internalized into HepG2 cells followed by the release of encapsulated paclitaxel inside the cytoplasm to inhibit the growth of the cells. Thus, the active targeting nature of these SLNs may enhance their cellular uptake, with a consequent decrease in systemic toxicity. The effect of adsorbed poloxamer 188 and 407 surfactants on the intestinal uptake of 60-nm polystyrene particles after oral administration in the rat was studied by Hillery et al. They observed that it was possible to manipulate the uptake profile of the polystyrene particles by modifying their surface properties with adsorbed poloxamer 188 and 407 surfactants (Hillery et al. 1996).

The present study for designing the paclitaxel-loaded lipid nanoparticles differs from the earlier reported methods in various ways, like this modified solvent injection approach proved to be a faster method for producing the SLNs, where a low boiling solvent, diethyl ether had been used for dissolving the lipid materials, which evaporated very fast resulting in SLNs. A mixture of lecithin and polox-

Table 4: CC_{50} of paclitaxel and its formulation in HepG2 cell lines after 48 h and 72 h by MTT assay ($n = 3$, $p > 0.05$)

Formulation	CC_{50} (μg/ml)	
	Incubation time (h)	
	48	72
Paclitaxel	0.58 ± 0.02	0.29 ± 0.05
F3	0.085 ± 0.005	0.061 ± 0.003

amer188 was used as surfactant which resulted in smaller sized paclitaxel-loaded nanoparticles, when compared to the study by Chen et al. (2001) where paclitaxel-loaded SLNs had been developed using F68 and stearic acid as the lipid material and also other studies, where different lipid materials have been used for the production of paclitaxel-loaded SLNs (Cavalli et al. 2000; Betul et al. 2006). The SLNs prepared in the present study were concluded to be a good system to be tested in future for oral absorption, *in vivo*.

Because *in vitro* and *in vivo* release might be very much different, further research is needed to confirm such preliminary results.

3. Experimental

3.1. Chemicals

Paclitaxel (MW 853.9) (99.87% w/w) was obtained as a gift sample from Dabur Pharma Ltd., India. Stearic acid (MW 284.48 g/mol) was purchased from Merck Schuchardt, Germany and soya lecithin 95% was obtained from BDH Laboratory, England. Poloxamer 188 (Pluronic® F-68) was obtained from Pluronic® BASF Corp., Sigma (USA). D-Trehalose dihydrate extrapure was resourced from Sisco Research Laboratories Pvt. Ltd., India. Dialysis tubing cellulose membrane, D9277 (avg. flat width 10 mm (0.4 in.), which retains most proteins of molecular weight 12,000 or greater) was purchased from Sigma, USA. Other chemicals were of analytical reagent grade and used without further purification.

3.2. Preparation of solid lipid nanoparticles

3.2.1. Formulation-1 (F1)

The method used for preparation of SLNs was modified from the solvent injection technique reported earlier by Schubert et al. (Schubert and Müller-Goymann 2003; Betul et al. 2006). Briefly, 5 ml of ether solution containing stearic acid (0.23 mmol), soya lecithin (0.175 mmol) and α-tocopherol (0.025 mmol) was injected through an injection needle (single use $30G^1/_2$ PrecisionGlide needle) into poloxamer solution (1.5% w/v) at 40 ± 2 °C under continual stirring in order to form SLNs. The mixed system was evaporated in a 40 ± 2 °C bath for 30 min and homogenized at 20000 rpm for 1 h using SilentCrusher M with dispersion tool 8F (Heidolph instruments GmbH and Co. KG, Germany). The resulting nanosuspension was ultracentrifuged twice at $60000 \times g$ for 1 h at 4 °C (Beckman L-80 ultracentrifuge equipped with a Ti-70 rotor, Beckman Instruments Inc., USA). The supernatant was discarded and the SLNs were redispersed in a 25 ml trehalose solution (15% w/v), used as a cryoprotectant and freeze-dried for 24 h (CHRIST ALPHA 1–2, Vaccubrand Type RZ2, Germany). The lyophilized SLNs were resuspended in distilled water prior to evaluation. This formulation was designated as F1 i.e. blank SLN formulation.

3.2.2. Formulation-2, 3 and 4 (F2, F3 and F4)

Paclitaxel-loaded SLNs designated as F2, F3 and F4 were fabricated by the addition of 0.05, 0.25 and 0.5 mmol of paclitaxel, respectively, dissolved in dichloromethane (DCM), to 5 ml of ether solution containing stearic acid (0.23 mmol), soya lecithin (0.175 mmol) and α-tocopherol (0.025 mmol). The remaining procedure was followed as described for the fabrication of F1.

3.3. Physicochemical characterization of SLNs

3.3.1. Transmission electron microscopy

The morphology of SLNs was examined by transmission electron microscopy (TEM) (FEI Philips, Morgagni 268D, USA). Before measurement, SLN dispersions were diluted with distilled water, stained with a 2% solution of sodium phosphotungstic acid for contrast enhancement and sprayed on copper grids. The air-dried samples were then directly examined under TEM and magnification upto 22000x, was used.

3.3.2. Photon correlation spectroscopy

The average diameter and polydispersity index of drug free and drug loaded SLNs were determined by photon correlation spectroscopy (PCS) using Zetasizer Nano ZS90 (Malvern Instruments, UK). SLN dispersions were diluted 1 : 20 with filtered water prior to analysis. The values were measured as an average of triplicate experiments. The polydispersity index measures the size distribution of the nanoparticle population.

3.3.3. Determination of Zeta potential

The surface charge studies of blank (F1) and drug loaded (F2, F3 & F4) formulations were carried out by Zetasizer Nano ZS90 (Malvern Instruments, UK). The nanosuspensions were dispersed in distilled water (1 : 100) prior to all analyses. Each sample was analyzed in triplicates.

3.3.4. Thermal analysis of freeze dried SLNs

Differential scanning calorimetry (DSC) analysis was performed using Perkin Elmer Pyris 6 DSC, USA. The instrument was calibrated with indium (calibration standard, purity > 99.999%) for melting point and heat of fusion. A heating rate of 10 °C/min was employed in the temperature range of 35–300 °C. Analysis was performed under a nitrogen purge (50 ml/min). Standard aluminium sample pans were used. About 10 mg sample was taken for analysis. An empty pan was used as reference.

3.3.5. X-ray diffraction

Samples were investigated by placing the dried lyophilized SLNs on a zero-background plate and by measuring the XRD pattern (Bruker D8 ADVANCE). Copper was the source of radiation obtained at 40 mA and 30 kV, passed through nickel filter. The diffractometer was equipped with a 2θ-compensating slit. The scans were run from 3° to 40° 2θ, increasing at a step size of 0.050° with a counting time of 2 s for each step. Al_2O_3 corrandum was the standard used for calibration and poly-methyl-methyl acrylate was used as the holder.

3.3.6. Surface chemistry characterization using Fourier transform infrared (FTIR) spectroscopy

Surface chemistry was examined by FTIR spectroscopy, the FTIR spectra were obtained using the potassium bromide disc method for, paclitaxel, poloxamer 188, drug-free SLNs (F1), and paclitaxel loaded SLNs (F3) on BIO-RAD, FTIR spectrometer (Win-IR software). The infrared spectra were acquired to draw information on the molecular state of lipids and paclitaxel.

3.4. Assay and entrapment efficiency (EE) of SLNs

The EE% of paclitaxel in SLNs was analyzed by HPLC (Thermo Finnigan, USA), a reverse-phase LiChroCART® RP 18 column (250 × 4 mm i.d., pore size 5 µm, Merck, LiChrospher®100) was used. The column was protected with a LiChroCART® RP 18 pre-column (4 × 4 mm i.d., pore size 5 µm Merck, LiChrospher® 100). Chromatographic analysis was done on a LC surveyor system (Thermo Finnigan, USA) consisting of a quartenary LC pump with auto sampler and surveyor photo diode array detector. Mobile phase consisted of acetonitrile (ACN) and purified water (70 : 30), and inbuilt degasser present in the system degassed it (Crosasso et al. 2000; Chen et al. 2001.). The flow rate was kept at 1 ml/min and system was maintained at an ambient temperature of 25 ± 1 °C and the detection was carried out at a $\lambda_{max} = 227$ nm.

A modification of the procedure used by Dong et al. (2004) and Feng et al. (2004) was used to determine the content of paclitaxel in SLNs. Briefly, 3 mg of lyophilized SLNs were dissolved in 1 ml of DCM. The mixture was then vortexed vigorously for 5 min followed by centrifugation (Remi Equipments, India) at 10000 rpm for 10 min. The DCM layer was evaporated under vaccum using Centrifugal Vaccum Concentrator (Christ, Germany). The residue was then reconstituted with 50% ACN and was mixed on a vortex mixer for 90 s. A portion (20 µl) of the reconstituted sample was injected into the chromatograph. Data was acquired and processed by Chromquest software (Thermo Finnigan, USA). EE of the drug was expressed as the percentage of the paclitaxel in the produced nanoparticles with respect to the initial amount (mg) used for the preparation of SLNs (Eq. (1)). All the measurements were performed in triplicate.

$$EE\% = (\text{amount of drug in SLNs}_{(mg)}/\text{initial amount of drug}_{(mg)}) \text{ X } 100 \quad (1)$$

3.5. Stability studies

The lyophilized SLNs were divided into 3 sample sets and subjected to stability studies, in triplicate, as per ICH guidelines. We observed the effects of storage conditions at 4 ± 2 °C (in a refrigerator), 25 ± 2 °C/60 ± 5% relative humidity [RH], 40 ± 2 °C/75 ± 5% RH designated as SLN4, SLN25 and SLN40 respectively (in a stability chamber maintained at respective temperature and RH) for a period of 15, 30, 60 and 90 days, on the particle size, shape, zeta potential, drug content, and TBARS assay by keeping the SLNs in sealed amber-colored vials after flushing with nitrogen.

3.6. In vitro release kinetics of SLNs

The in vitro release profile of paclitaxel in SLNs was determined using a multicompartmental rotating cell system with donor and receptor compartments, a hydrophilic dialysis membrane with a molecular weight cut-off of

12000 Da, was used as the donor compartment. The freeze-dried paclitaxel-loaded SLNs (containing 1 mg paclitaxel) were added in 1ml of phosphate buffered saline (PBS, pH 7.4) and placed in the donor compartment, and the receptor compartment was filled with 15 ml PBS (pH 7.4) and placed on a magnetic stirrer maintained at 37 °C and stirred at 120 rpm. At fixed time intervals, the receptor solution was withdrawn and the dialysis tubing was resuspended in 15 ml of fresh buffer. To determine the drug concentration, the HPLC method was applied to analyze the receptor solution, after extraction with DCM.

3.7. Stability study in simulated gastric fluid

The specific aim was to investigate the stability of sterically stabilized paclitaxel loaded SLNs in GI fluids. The *in vitro* optimized paclitaxel loaded SLNs were incubated at 37 °C in a simulated gastric medium (USP XXIV, pH 1.2, pepsin 0.32% w/v). Whenever needed, the pH was readjusted during the experiments using NaOH or HCl. Samples were collected at times 0, 1 and 3 h and centrifuged for 5 min at 20000 rpm to precipitate particle aggregates and enzymes. The stability of poloxamer-coated SLNs in simulated gastric medium was evaluated in terms of particle aggregation, and drug content (i.e. lipid degradation). Size distribution of the particles in dispersion was measured by PCS. The samples were then subjected to extraction procedure of paclitaxel and analyzed by HPLC to test for the leak/presence of paclitaxel and thus lipid degradation.

3.8. In vitro cytotoxicity

The cell line studies of paclitaxel and paclitaxel loaded SLNs for the evaluation of cytotoxicity were done using human hepatoma cell line, HepG2 (ATCC No.HB-8065) by MTT assay. HepG2 (ATCC No.HB- 8065) was cultured in minimum essential medium (MEM) supplemented with Earl's salt (1.5 gL^{-1}), fetal calf serum (10%), L-glutamine (2 mM), sodium pyruvate (1%), streptomycin (100 $mgmL^{-1}$), penicillin (100 $IUmL^{-1}$) and amphotericin B (0.25 $mgmL^{-1}$). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were seeded in 96-well microtitre plates at a density of 1×10^5 cells per millilitre in serum-containing media and left for 24 h for recovery. Test lipid systems were added (0.001–1 mg/ml) in fresh complete media to microtitre plates and incubated for 72 h. Five hours before completion of the incubation period, 20 μL MTT (5 mg/mL) was added and the incubation was continued. The medium was removed and 100 μL DMSO was added to dissolve the formazan crystals. The optical density was measured at 550 nm using a plate reader (PowerWave X; BIO-TEK Instruments, Inc). The experiment was carried out in triplicate. The 50% cell cytotoxic concentration (CC_{50}) of paclitaxel and its F3 SLN formulation against HepG2 cell line at 48 h and 72 h was determined.

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