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Lipomer of doxorubicin hydrochloride for enhanced oral bioavailability

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ABSTRACT

The present study discusses design of doxorubicin hydrochloride (Dox) loaded lipid based nanocarrier (LIPOMER) for oral delivery. High entrapment (>90%) and high loading (38.11 ± 0.37% w/w) of hydrophilic Dox in lipid nanocarrier of polyglyceryl-6-distearate was achieved using poly(methyl vinyl ether-co-maleic anhydride) (Gantrez® AN 119) and a modified nanoprecipitation method. Dox-LIPOMER revealed nanosize (314 ± 16.80 nm) and negative zeta potential (−25.00 ± 2.41 mV). Dox-LIPOMER exhibits sustained release in vitro and was influenced by ionic strength of dissolution medium. DSC and XRD studies suggested amorphous nature of Dox in LIPOMER. TEM revealed spherical morphology of Dox-LIPOMER. Dox-LIPOMER was stable up to 12 months at 25 °C/60% RH. A 384% enhancement in oral bioavailability compared to Dox solution was observed following Dox-LIPOMER administration at 10 mg/kg body weight. Superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) assay data of heart and kidney tissues of rats treated with Dox-LIPOMER were comparable with untreated rats. Dox-LIPOMER represents a potential safe drug delivery system for oral administration.

1. Introduction

Anthracycline antibiotic doxorubicin hydrochloride (Dox) is one of the most important cytotoxic drugs used in the treatment of a variety of hematologic malignancies and a wide range of solid tumors (Chabner et al., 2006). Current chemotherapy using Dox is limited to intravenous administration of Dox as Dox solution or Dox-liposomes. Although liposomal Dox revealed significant decrease in cardio and renal toxicities (Patil et al., 2008a,b), administration of liposomal Dox necessitates hospitalization and is cost prohibitive.

Oral chemotherapy could maintain sustained therapeutic Dox concentration in the blood to improve efficacy with a possible decrease in side effects (Bromberg, 2005). Oral Dox nanocarriers could provide significant improvement in patient compliance with decreased cost of therapy. Dox, a BCS class III drug and P-glycoprotein (P-gp) substrate, exhibits high first pass metabolism in liver, hence successful oral chemotherapy continues to pose serious challenges. Bromberg and Alakhov (2003) report enhanced absorption of Dox in Caco-2 cell monolayers from microgels composed of cross-linked copolymers of poly(acrylic acid) and Pluronics. Concurrent administration of Dox with myricetin enabled a 1.51–2.17 fold enhancement in oral bioavailability. This was attributed to inhibition of P-gp and reduced first-pass metabolism by inhibition of CYP3A in the small intestine and/or in the liver (Choi et al., 2011). Increased oral bioavailability of Dox from PLGA nanoparticles (Kalaria et al., 2009) and Dox-PAMAM dendrimer (Ke et al., 2008) have been reported.

Various mechanisms have been proposed to explain the uptake of orally administered nanocarriers (O'Hagan, 1990; Florence, 2005). While nanoparticles <220 nm could be endocytosized by ordinary enterocytes (Sanders and Ashworth, 1961; Matsuno et al., 1983), paracellular transport of polyalkylcyanoacrylate nanocapsules (100–200 nm) loaded with an iodinized oil have also been reported (Aprahamian et al., 1987). Wells et al. (1988) demonstrated the intestinal translocation of fluorescent particles (1 μm) administered in drinking water to mice and suggested the role of intestinal macrophages in uptake. Volkheimer et al. (1968) described the passage of large particles (5–150 μm) across the intestine by a mechanism that was termed 'persorption'.

The preferred pathway for the uptake of orally administered nanocarriers in the size range 20 nm to 10 μm, through Peyer's patches (Joel et al., 1978) is reported to be significantly influenced by the hydrophobicity of nanocarriers (Eldridge et al., 1990). Lipid nanoparticles due to their highly hydrophobic nature could exhibit enhanced uptake through Peyer's patches (Bargoni et al., 2001). Li et al. (2009) reported enhanced lymphatic uptake of quercetin loaded solid lipid nanoparticles. Further, targeting orally administered nanocarriers to the lymphatics assumes great significance in cancer chemotherapy, as tumor cells frequently metastasize to secondary sites or organs through the lymphatic system (Maeda, 1991). Orally administered lipid based nanocarriers of Dox could therefore provide significant clinical advantage. Dox being a hydrophilic drug, overcoming the challenge of poor

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Dox loading in lipid nanocarriers is an important step to enable oral Dox therapy.

Lipid-polymer hybrid nanoparticles (LIPOMER) of a highly water soluble drug doxycycline hydrochloride with high drug loading (≈20%) designed using different lipids and Gantrez® AN 119 (Gantrez) as entrapment enhancer revealed enhanced reticulo-endothelial system (RES) uptake following intravenous administration (Patil et al., 2008a,b). Further, with glyceryl monostearate as the lipid, asymmetric nanostructures were formed, which revealed high splenic uptake (Devarajan et al., 2010). We have recently reported high Dox loading in nanoparticles of a hydrophobic polymer polyethylene sebacate (PES) using Gantrez (Guhagarkar et al., 2010). In the present study we explore design of Dox-LIPOMER using the same approach. Dox-LIPOMER was designed with the dual objective of enhancing Dox loading in a lipid based nanocarrier and the possibility of facilitating oral absorption of Dox.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (RPG Life Sciences Pvt. Ltd., Mumbai, India), poly(methyl vinyl ether-co-maleic anhydride) (Gantrez® AN 119 – Anshul Agencies, Mumbai, India/International Specialty Products Inc., Spain), polyglyceryl-6-distearate (Plurol® Stearique WL 1009 – Gattefosse, France), acetonitrile HPLC grade Azeocryst Organics Pvt. Ltd., Mumbai, India and trehalose 100 (Gangwal Chemicals Pvt. Ltd., Mumbai, India/Hayashibara Co. Ltd., Japan) were obtained as gift samples. Sodium chloride AR, di-sodium hydrogen orthophosphate anhydrous AR, calcium chloride LR, potassium dihydrogen orthophosphate AR, tetrahydrofuran AR, methanol for HPLC, orthophosphoric acid AR (specific gravity 1.75), glacial acetic acid AR, thiobarbituric acid AR grade, n-butanol, sodium lauryl sulphate extrapure and pyridine were purchased from S. D. Fine-Chem Limited (Mumbai, India). Magnesium acetate tetrahydrate pure and sodium acetate trihydrate crystal pure was purchased from MERCK (Mumbai, India). Superoxide dismutase (SOD) and catalase (CAT) assay kits were purchased from BioVision, CA, USA. 1,1,3,3-Tetramethoxypropane was purchased from Sigma–Aldrich, Mumbai, India. All other chemicals and solvents were either spectroscopic or analytical grade.

2.2. Preparation of Dox-LIPOMER

Dox-LIPOMER was prepared by a modified nanoprecipitation method (Guhagarkar et al., 2010). Briefly, polyglyceryl-6-distearate (PGDS) as lipid and Gantrez (Table 1) were dissolved in 7 ml tetrahydrofuran (THF) (organic phase) and added dropwise to an aqueous phase (30 ml) containing Dox (10 mg) with continuous stirring at room temperature (28 °C) using a magnetic stirrer. This was followed by addition of 2 ml of 0.25% w/v of an aqueous solution of magnesium acetate tetrahydrate to cross link Gantrez® AN-119 (Guhagarkar et al., 2010). The resulting dispersion was stirred for 8 h to allow evaporation of THF. All the experiments were performed in triplicate.

2.3. Entrapment efficiency (EE) and drug loading (DL)

EE was determined by centrifuging the LIPOMER dispersion at 15,000 rpm for 45 min at 25 °C. The supernatant containing free Dox was withdrawn and the pellet was washed twice with distilled deionized water. The washout liquids were pooled with the supernatant and Dox concentration measured by UV–vis spectrophotometry at 478 nm. EE was calculated using Eq. (1):

$$\mathrm{EE}\,(\%) = \frac{\mathrm{Dox_{total}} - \mathrm{Dox_{supernatant}}}{\mathrm{Dox_{total}}} \times 100 \quad (1)$$

Dox loading in LIPOMER was determined by a reported method (Wong et al., 2006). The amount of Dox entrapped in the LIPOMER was determined as mentioned above in the determination of EE. Percent Dox loading (DL% w/w) was then calculated using Eq. (2):

$$\mathrm{DL}\,(\%\mathrm{w/w}) = \frac{\mathrm{W_{DL}} \times 100}{\mathrm{W_{LIPOMER}}} \quad (2)$$

where W_{DL} = weight of Dox in LIPOMER and $W_{LIPOMER}$ = weight of LIPOMER.

2.4. Particle size

Particle size was determined by Photon Correlation Spectroscopy (PCS) using the N4 plus submicron particle size analyzer (Beckman Coulter, USA). The analysis was performed at a scattering angle of 90° and a temperature of 25 °C. LIPOMER dispersions were centrifuged at 15,000 rpm for 30 min at 25 °C. The resultant pellet was redispersed in distilled deionized water using ultrasonic probe system for 5 min with 15 s pulse at 200 V over an ice bath. Dispersions were then appropriately diluted with filtered water (0.2 μm filter, Millipore India Pvt. Ltd.) to obtain 5×10^4–1×10^6 counts per second. Each sample was analyzed in triplicate and average particle size and polydispersity index (PI) measured.

2.5. Zeta potential

The zeta potential of the LIPOMER dispersion was measured by determining the electrophoretic mobility using the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). LIPOMER dispersions were centrifuged at 15,000 rpm for 30 min at 25 °C. The resultant pellet was redispersed in distilled deionized water (50 μg/ml of Dox-LIPOMER) using ultrasonic probe system for 5 min with 15 s pulse at 200 V over an ice bath. Samples were filled into the folded capillary cell and zeta potential was measured. Each sample was analyzed in triplicate.

2.6. In vitro drug release studies

Drug release studies were performed by a dialysis method (Guhagarkar et al., 2010). Briefly, 1 ml of the aqueous LIPOMER dispersion corresponding to 5 mg Dox was sealed in a dialysis tubing (MWCO: 12,000–14,000 Da, Himedia Laboratories Pvt. Ltd., Mumbai, India). Drug release was initiated by immersing the dialysis tube in 150 ml of release media preheated to 37 ± 0.5 °C in a USP dissolution apparatus I (Electrolab, Mumbai, India) at 50 rpm. The various release media evaluated include acetate buffer pH 4.5, 0.15 M NaCl solution and 0.05 M $CaCl_2$ solution. Aliquots (5 ml) were withdrawn at specified time points and Dox concentration was determined by UV–vis spectrophotometry at 478 nm. Percent cumulative drug release versus time profiles were plotted.

2.7. Differential Scanning Calorimetry (DSC) study

Thermal analysis was conducted using the Pyris 6 DSC differential scanning calorimeter (PerkinElmer, Netherlands). Powder samples were weighed in standard aluminum pans and heated from 35 to 300 °C at a heating rate of 10 °C/min using an empty pan as reference under a purge of nitrogen (20 ml/min).

Table 1
EE, particle size, PI, drug loading and zeta potential of Dox-LIPOMER (mean ± S.D.; $n = 3$).

Dox (mg)	Lipid (mg)	Gantrez (mg)	EE (%)	DL (% w/w)	Particle size (nm)	PI	Zeta potential (mV)
	10	–	4.88 ± 1.00	4.65 ± 0.91	>1000	–	−2.51 ± 1.10
	25	–	6.28 ± 2.26	2.44 ± 0.86	>1000	–	−2.62 ± 1.31
	50	–	6.45 ± 2.74	1.27 ± 0.54	>1000	–	−2.33 ± 0.93
10	10	1.25	58.66 ± 1.93	34.27 ± 0.74	791 ± 38.97	0.341 ± 0.019	−6.50 ± 1.93
	10	2.5	83.15 ± 1.77	39.94 ± 0.51	650 ± 29.87	0.236 ± 0.015	−14.07 ± 2.37
	10	5	92.37 ± 1.43	38.11 ± 0.37	314 ± 16.80	0.230 ± 0.016	−25.00 ± 2.41
	10	10	79.55 ± 1.49	28.46 ± 0.38	281 ± 13.32	0.215 ± 0.020	−28.43 ± 2.10

2.8. Powder X-ray diffraction (PXRD) study

Powder X-ray diffraction pattern of various samples was recorded at room temperature with a PANalytical X'Pert PRO MPD $\theta/2\theta$ diffractometer (Almelo, Netherlands). The common Cu-Kα radiation source was operated at a voltage of 40 kV and a current of 30 mA. Secondary side of the optical path consists of X'Celerator RTMS detector equipped with diffracted beam monochromator. Samples were scanned from 7° to 60° (2θ).

2.9. Transmission electron microscopy (TEM)

TEM of LIPOMER was performed following negative staining with uranyl acetate. A drop of LIPOMER dispersion (1 mg/ml) was placed on Formvar®-coated copper grids (Ted Pella, Inc., Redding, CA) followed by the addition of a drop of 2% w/v uranyl acetate. At the end of 3 min, excess liquid was removed, the grid air-dried and imaging conducted, using a transmission electron microscope (CM200 TEM; Philips/FEI, Inc., Briarcliff Manor, NY, USA).

2.10. Stability study

Dox-LIPOMER freeze dried using trehalose 100 as cryoprotectant was stored at 25 °C/60% RH and 40 °C/75% RH in stoppered glass vials. Dox-LIPOMER was evaluated for particle size, PI, zeta potential, drug content and *in vitro* drug release at the end of 3, 6 and 12 months. To quantify Dox in the LIPOMER, Dox-LIPOMER (equivalent to 1 mg of Dox) was dispersed in THF (5 ml) by sonication. An aqueous solution of sodium chloride (5 ml, 4% w/v) was added to facilitate ion exchange mediated release of Dox from the Dox-Gantrez ionic complex, wherein sodium chloride served as counter ion. The resulting dispersion was further sonicated for 2 min and centrifuged at 15,000 rpm for 45 min at 20 °C and the supernatant collected. The pellet was resuspended in THF and the procedure repeated till a colorless pellet was obtained indicating complete extraction of Dox from the LIPOMER. The supernatants were pooled together, filtered through 0.22 micron filter (Millipore, Mumbai, India) and the concentration of Dox was measured by HPLC. Analysis was performed at room temperature (25 °C) using a Jasco Instrument (PU-2080, Japan) equipped with a Agilent Zorbax SB-C18 column and a UV-Visible detector (UV-2075, Japan) at 254 nm. The mobile phase comprised acetonitrile:water (30:70), pH adjusted to 3 with orthophosphoric acid at a flow rate of 1 ml/min. Sample (20 μl) was injected into the system and the drug content was extrapolated from a standard plot.

2.11. Bioavailability study

Dox-LIPOMER (equivalent to 10 mg/kg of Dox) dispersed in 1 ml of potable water and Dox aqueous solution (10 mg/kg) were administered by oral gavage needle to female Sprague-Dawley rats weighing between 200 and 220 g ($n = 4$). The animal protocol was duly approved by the Institutional Animal Ethics Committee of Institute of Chemical Technology (ICT), Mumbai, India. Blood samples (around 0.5 ml) were collected from the retro-orbital plexus under mild anesthesia using combination of halothane and oxygen at 0.5, 1, 2, 6, 12 and 24 h in micro-centrifuge tubes containing 20 μL of EDTA solution. Plasma was collected by centrifuging the blood samples at 5000 rpm for 10 min at 25 °C. To plasma (0.2 ml), acetonitrile (0.2 ml) was added and the resulting mixture vortexed vigorously for 2 min and centrifuged at 15,000 rpm for 15 min at 25 °C and 0.1 ml of the supernatant was injected into the HPLC system. The detector was operated at a wavelength of 254 nm. Agilent Zorbax SB-C18 column (5 μm, 4.6 mm × 250 mm) was used. Mobile phase consisted of 50% acetate buffer (pH 5), 30% methanol and 20% acetonitrile. The flow rate was maintained at 1 ml/min.

2.12. Toxicity study

Female Sprague-Dawley rats of uniform body weight (180–220 g) with no prior drug treatment were used for the toxicity study. The animal protocol was duly approved by the Institutional Animal Ethics Committee of Institute of Chemical Technology (ICT), Mumbai, India. The animals were randomly divided into three groups of 5 rats in each group. Group-I was the control group without any treatment; Group-II was administered a solution of Dox in 5% w/v dextrose (0.4 ml) by the intravenous (i.v.) route, while Group-III received Dox-LIPOMER in 1 ml potable water by oral gavage. Two doses of Dox (4 mg/kg) were administered to the groups on day 1 and on day 15. The weight of all the animals was recorded prior to treatment. At the end of day 28, all the animals were weighed, euthanized and the heart and kidneys isolated and weighed. The tissues were preserved at −20 °C till the complete analysis was performed. Dox-induced oxidative stress was monitored by measuring the malondialdehyde (MDA), a marker of lipid peroxidation and superoxide dismutase (SOD) and catalase (CAT) level in these organs. To determine SOD and CAT activities, tissues (0.5 g) were homogenized in 5 ml PBS (pH 7.4) whereas, to determine MDA level, tissues (0.5 g) were homogenized in 2.5 ml PBS (pH 7.4) using tissue homogenizer at 20,000 rpm for 5 min. SOD and CAT activity in homogenized tissues were determined using assay kits while MDA was assayed in the form of thiobarbituric acid reacting substances assay (TBARS) (Ohkawa et al., 1979).

2.13. Statistical analysis

All values were expressed as mean value ± standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using the one-way ANOVA with Dunnet's test and Student's *t*-tests. $p < 0.05$ was the criterion for statistical significance.

3. Results and discussion

3.1. Preparation of Dox-LIPOMER by modified nanoprecipitation method

The modified nanoprecipitation method resulted in Dox loaded polymeric nanoparticles with high entrapment efficiency and drug

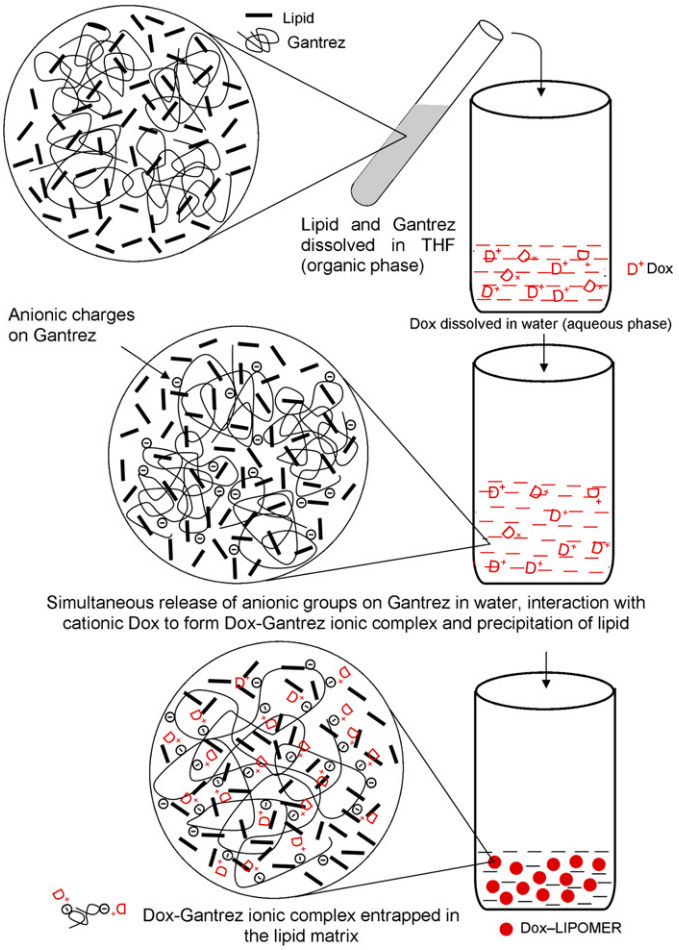

Fig. 1. Schematic representation of formation of Dox-LIPOMER by modified nanoprecipitation method.

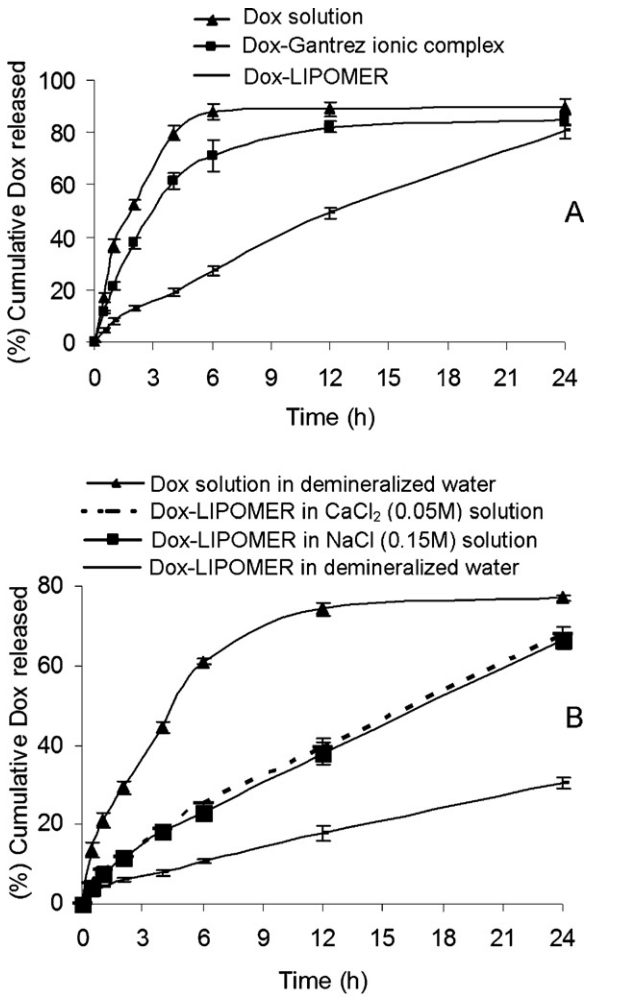

Fig. 2. *In vitro* drug release in (A) acetate buffer pH 4.5 and (B) demineralized water, NaCl (0.15 M) solution and $CaCl_2$ (0.05 M) solution (mean ± S.D.; $n = 3$).

loading (Guhagarkar et al., 2010). Cavalli et al. (1993) prepared microemulsion based solid lipid nanoparticles (SLN) of Dox using ion pairing agents to improve Dox loading. Subedi et al. (2009) converted doxorubicin hydrochloride into doxorubicin base and incorporated into SLN, while Wong et al. (2004, 2006) report polymer–lipid hybrid nanoparticles (PLN) based on microemulsion method using anionic polymers in combination with lipids. Nevertheless, Dox loading in all the above studies was low.

In our study the lipid PGDS and Gantrez were dissolved in the organic phase (THF), the high molecular weight Gantrez was in a state of chain extension and in intimate contact with the dissolved lipid. Hydrophilic Dox dissolved in the aqueous phase was in the ionized state. Since THF has low surface tension (26.40 J/m^2) in comparison to water (71.98 J/m^2), addition of the organic phase to the aqueous phase, causes high interfacial turbulence at the interface, resulting in rapid inter diffusion of organic and aqueous phase. These results in rapid nanoprecipitation of the lipid to entrap Gantrez coupled with simultaneous and instant hydrolysis of the anhydride groups on Gantrez, to expose free $-COO^-$ groups. Instantaneous interaction of cationic Dox with the $-COO^-$ groups of Gantrez results in the formation of the hydrophobic ionic complex which rapidly partitions into the lipid matrix, resulting in high EE of Dox in LIPOMER. This process is schematically depicted in Fig. 1.

Dox being highly water soluble, high concentration of Dox could be dissolved in the aqueous phase to facilitate high drug loading. High drug loading is also facilitated by the high molecular weight (200 kDa) of Gantrez which offers large free spaces due to a disordered state (Layre et al., 2006; Guhagarkar et al., 2010). High Dox loading up to 38.11 ± 0.37% is observed in our study.

In the absence of Gantrez, EE was very poor and large lipid aggregates formed (Table 1). Inclusion of Gantrez not only enhanced the EE of Dox in LIPOMER but also enabled nanosize particles due to its amphiphilic nature. Gantrez due to large number of $-COO^-$ groups imparted a negative surface charge as reflected by the negative zeta potential (Table 1).

3.2. *In vitro* drug release studies

Typical drug release profiles of Dox solution, Dox-Gantrez ionic complex and Dox-LIPOMER in acetate buffer pH 4.5 are shown in Fig. 2A. While Dox solution revealed rapid release, Dox-LIPOMER revealed slow and sustained release. Sustained Dox release from the LIPOMER is attributed to the lipid matrix imparting a barrier to drug release. To confirm the mechanism of drug release from Dox-LIPOMER, release was also carried out in demineralized water and media containing NaCl (0.15 M) and $CaCl_2$ (0.05 M). Higher release of Dox in ionic media (>70%) compared to demineralized water (<35%) suggested the predominant mechanism of drug release from the LIPOMER as ion exchange followed by diffusion (Fig. 2B).

The kinetics of Dox release from LIPOMER was fitted to various kinetic models such as zero-order, first-order, Higuchi equation, and Korsmeyer–Peppas equation. The data in Table 2 indicated that

Table 2
In vitro Dox release data – Model Fitting (mean ± S.D.; $n = 3$).

Dox formulation	Model →	Zero order			First order			Higuchi			Korsmeyer–Peppas		
	Release media	r^2	Slope	Intercept	r^2	Slope	Intercept	r^2	Slope	Intercept	r^2	Slope	Intercept
Dox solution	Water	0.721	2.574	27.654	0.588	0.026	1.402	0.887	16.221	8.933	0.956	0.476	1.327
	Acetate buffer (pH 4.5)	0.471	2.398	47.665	0.373	0.019	1.610	0.676	16.370	27.364	0.816	0.417	1.521
Dox-Gantrez ionic complex	Acetate buffer (pH 4.5)	0.621	2.764	33.325	0.469	0.027	1.441	0.815	17.995	11.909	0.895	0.535	1.340
Dox-LIPOMER	Water	0.994	1.146	3.249	0.841	0.039	0.648	0.974	6.448	−3.319	0.987	0.606	0.593
	Acetate buffer (pH 4.5)	0.990	3.226	5.853	0.793	0.046	0.949	0.980	18.24	−12.84	0.997	0.741	0.872
	NaCl (0.15 M) solution	0.992	2.565	5.970	0.790	0.043	0.921	0.979	14.479	−8.845	0.997	0.689	0.848
	$CaCl_2$ (0.05 M) solution	0.991	2.615	6.653	0.808	0.041	0.958	0.981	14.786	−8.508	0.997	0.661	0.892

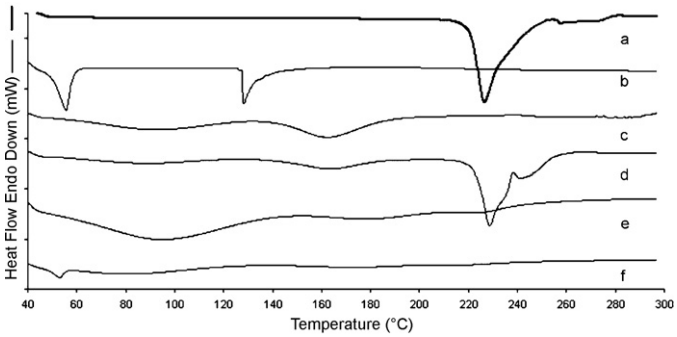

Fig. 3. Differential scaning calorimetry thermograms of (a) Dox, (b) PGDS, (c) Gantrez, (d) Dox-Gantrez physical mixture, (e) Dox-Gantrez ionic complex and (f) Dox-LIPOMER.

Dox release followed zero order kinetics ($r^2 = 0.990$) (Thakkar et al., 2009).

3.3. DSC

DSC thermograms (Fig. 3) revealed sharp melting endotherms of Dox and lipid indicating their crystalline nature. Dox-LIPOMER however showed no sharp endotherm suggesting decrease in crystallinity of Dox and PGDS, the lipid.

3.4. PXRD

The diffractograms shown in Fig. 4 further confirmed the results of DSC thermal analysis. Sharp diffractions of Dox in the diffractograms of Dox (curve 1) and Dox-Gantrez physical mixture (curve 4) revealed a crystalline nature. Absence of all the diffractions of Dox in the diffractograms of ionic complex (curve 4) and Dox-LIPOMER suggested amorphization of Dox. Further, the diffraction peak intensity of lipid was reduced in the diffractogram of Dox-LIPOMER compared to bulk lipid.

The partial amorphization of lipid in LIPOMER as seen from DSC and PXRD could have additionally contributed to the high Dox loading. The above results are in agreement with a previous study (Hou et al., 2003).

3.5. Morphological characterization by TEM

The transmission electron micrograph (TEM) of Dox-LIPOMER is shown in Fig. 5. The particles were spherical in shape with smooth surface. The particle size was observed to be 250–350 nm and comparable to the results of particle size by PCS method.

3.6. Stability study

Lyophilization of Dox-LIPOMER showed marginal but acceptable increase in particle size (S) ($S_{final}/S_{initial}$: 1.13). Values of $S_{final}/S_{initial}$ 1.3, are considered as not being significant (Saez et al., 2000). LIPOMER stored at 40 °C/75% RH showed a significant increase in particle size (687 ± 27 nm) and PI (0.787 ± 0.04) at the end of one month. However, LIPOMER stored at 25 °C/60% RH showed good physical and chemical stability up to 12 months (Table 3). Further f2 value >50 for *in vitro* drug release confirms no significant change in *in vitro* release of Dox-LIPOMER over 12 months (Table 3).

3.7. Bioavailability study

The plasma concentration versus time curves of Dox and Dox-LIPOMER after oral administration are reported in Fig. 6. The various

Table 3
Stability data of Dox-LIPOMER (mean ± S.D.; $n = 3$).

	Particle size (nm)	PI	Zeta potential (mV)	Drug content (%)	*In vitro* release (f2 value)
Initial	357 ± 15.63	0.275 ± 0.011	−23.52 ± 1.56	96.76 ± 1.40	–
1 Month	368 ± 20.50	0.270 ± 0.018	−24.65 ± 2.80	96.71 ± 1.26	89
3 Months	344 ± 21.59	0.263 ± 0.014	−22.95 ± 1.44	96.94 ± 1.61	81
6 Months	381 ± 17.01	0.271 ± 0.011	−23.35 ± 3.17	97.14 ± 1.65	87
12 Months	377 ± 16.92	0.282 ± 0.016	−23.64 ± 2.32	95.90 ± 1.02	85

Table 4
Pharmacokinetic parameters of aqueous Dox solution and Dox-LIPOMER following oral administration (mean ± S.D.; $n = 4$).

Formulation	C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (ng/ml)*h	Relative bioavailability
Dox solution	44.77 ± 4.85	2	3.49 ± 1.02	270 ± 41.71	–
Dox-LIPOMER	81.14 ± 16.32	6	6.86 ± 0.85	1037 ±186	384%

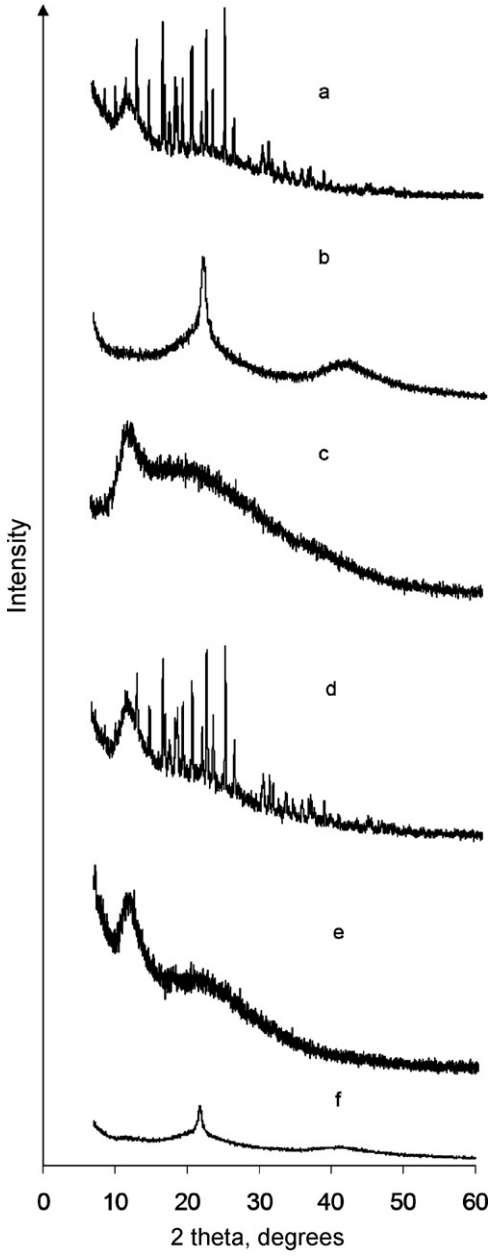

Fig. 4. Powder X-ray diffraction crystallographs of (a) Dox, (b) PGDS, (c) Gantrez, (d) Dox-Gantrez physical mixture, (e) Dox-Gantrez ionic complex and (f) Dox-LIPOMER.

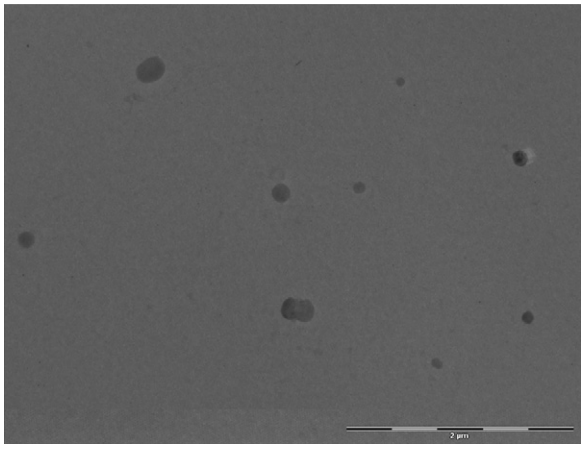

Fig. 5. Transmission electron micrograph of Dox-LIPOMER.

pharmacokinetic parameters are reported in Table 4. At all time points, plasma Dox concentrations were consistently higher for rats treated with Dox-LIPOMER. Moreover, sustained Dox plasma concentration was observed from LIPOMER as revealed by high $t_{1/2}$ (6.86 h) in comparison to Dox solution ($t_{1/2}$ – 3.49 h). Further, even at the end of twelve h, the plasma Dox concentration with the LIPOMER was comparable ($p > 0.05$) to the C_{max} of Dox solution (33 ng/ml). Dox-LIPOMER revealed a 384% enhancement in bioavailability with the added advantage of sustained release.

In the past three decades, nanocarriers have been extensively studied to enhance the bioavailability of poorly absorbable drugs. Drugs, whose oral bioavailability has been improved by means of

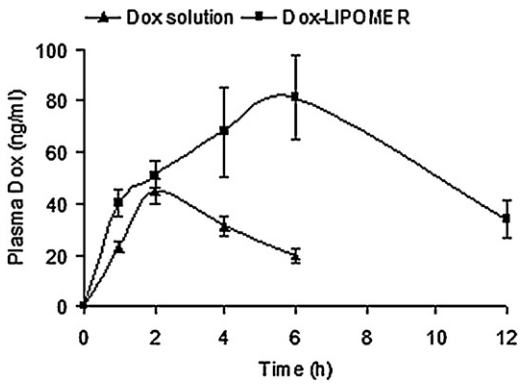

Fig. 6. Dox plasma concentration versus time profiles of Dox solution and Dox-LIPOMER after oral administration (mean ± S.D.; $n = 4$).

Table 5
Percent body weight gain and weight of heart and kidneys of rats (mean ± S.D.; $n = 5$).

Treatment group	Weight (g) prior to treatment	Weight (g) on 28th day post treatment	% Weight gain	Heart weight (g)	Kidneys weight (g)
Control	202 ± 10.73	277 ± 17.24	37.12 ± 4.879	0.897 ± 0.044	2.062 ± 0.099
Dox solution i.v.	197 ± 7.64	215 ± 4.03	9.13 ± 2.821	0.828 ± 0.024	2.040 ± 0.135
Dox-LIPOMER oral	197 ± 10.31	261 ± 6.65	32.48 ± 7.294	0.886 ± 0.046	2.036 ± 0.197

their loading in nanocarriers include plasmid DNA (Mathiowitz et al., 1997), proteins like calcitonin (Garcia-Fuentes et al., 2005) and insulin (Damagè et al., 1988; Ma et al., 2005), heparin (Hoffart et al., 2006) and idarubicin (Zara et al., 2002). Multiple mechanisms have been proposed by which nanocarriers could enhance the absorption of drugs including the protection of the loaded drug against degradation, establishment of a drug concentration gradient from the drug nanocarrier towards the absorptive membranes (Salman et al., 2006) and absorption of intact nanocarriers (Mathiowitz et al., 1997).

Many investigators have reported that hydrophobic particles are better absorbed from Peyer's patches. Eldridge et al. (1990) reported high uptake of microspheres made up of hydrophobic polymers into the Peyer's patches of the small intestine of mice, whereas no or very little uptake was observed with microspheres made up of hydrophilic polymers. Gantrez although a hydrophilic polymer, absorption of Gantrez nanoparticles through Peyer's patches has been reported (Salman et al., 2005, 2006, 2008). LIPOMER being a combination of lipid and Gantrez could therefore have enabled high bioavailability by facilitating uptake through Peyer's patches and thereby, bypass of first pass metabolism of Dox in the liver.

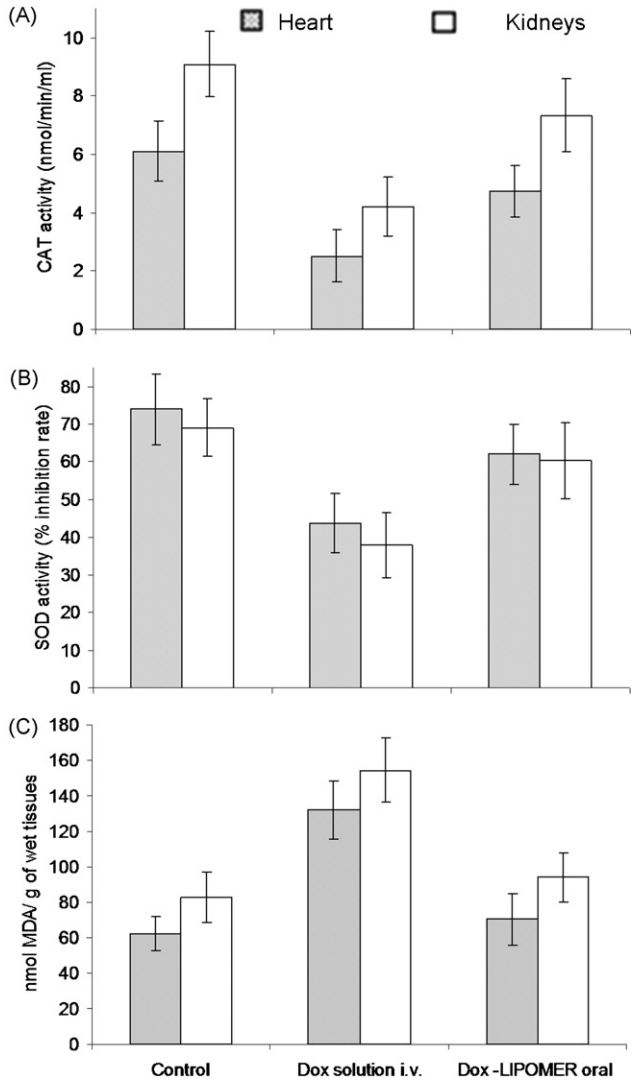

Fig. 7. Levels of (A) CAT, (B) SOD and (C) MDA in heart and kidney tissues of rats (mean ± S.D.; $n = 5$).

3.8. Toxicity study

3.8.1. Percent weight gain

Literature reports loss in body weight following exposure to Dox (Kalaria et al., 2009), however no weight loss, nor significant difference ($p > 0.05$) in weight of heart and kidneys of rats of various treatment groups was observed in our study. On the other hand, weight gain comparable ($p > 0.05$) with untreated rats was observed in rats treated with oral Dox-LIPOMER. Rats treated with Dox solution i.v. appeared weak, with hair erection, a hunched posture and significantly ($p < 0.05$) low percent weight gain compared to untreated and oral Dox-LIPOMER treated rats (Table 5). This suggests that oral Dox-LIPOMER was well tolerated by rats.

3.8.2. Oxidative stress markers

Cardiac and renal toxicities are the major complications of Dox therapy (Patil et al., 2008a,b). These toxicities are mediated by oxidative stress and the formation of an iron anthracyclin free radical which causes severe damage to the plasma membrane of these organs (Xu et al., 2001). Moreover high affinity of Dox for cardiolipin phospholipid species which are mainly present in the mitochondrial membranes of heart, leads to high accumulation of Dox in the heart tissues (Kalaria et al., 2009). Levels of these biochemical oxidative stress markers MDA, CAT and SOD in heart and kidney tissues were carried out to investigate the potential of Dox-LIPOMER in reducing these toxicities in comparison to intravenous Dox solution.

A marked decrease in CAT and SOD activity and increased MDA level (Fig. 7) in cardiac and renal tissues of rats treated with i.v. Dox solution compared to control group ($p < 0.001$) reflect toxicities in these tissues. In contrast, the CAT, SOD and MDA levels of cardiac and renal tissues of rats treated with oral Dox-LIPOMER were comparable with the control group ($p > 0.05$). The results of the toxicity studies therefore suggest the potential safety of oral Dox-LIPOMER.

4. Conclusion

Dox-LIPOMER provides the advantages of high EE and loading of Dox, high oral bioavailability and reduced toxicity, presenting itself as a safe delivery system with potential for oral administration of Dox. Efficacy studies however, are needed to confirm the same.

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