International Journal of Pharmaceutics 380 (2009) 96–104

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Proliposomes of exemestane for improved oral delivery: Formulation and *in vitro* evaluation using PAMPA, Caco-2 and rat intestine

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A R T I C L E   I N F O

Article history:
Received 10 April 2009
Received in revised form 27 June 2009
Accepted 2 July 2009
Available online 17 July 2009

Keywords:
Proliposomes
Calorimetry (DSC)
Dissolution
Permeability
Intestinal transport
Parallel artificial membrane permeability assay (PAMPA) and Caco-2 cells

A B S T R A C T

The aim of the present study was to develop proliposomal formulations to enhance the oral bioavailability of exemestane by improving solubility, dissolution and/or intestinal permeability. Proliposomal powder formulations were prepared using different ratios of drug (exemestane), distearoyl–phosphatidylcholine (DSPC), cholesterol and dimyristoyl–phosphatidylglycerol (DMPG) by solvent evaporation method. The effect of phospholipid composition and drug:lipid ratio on *in vitro* performance of proliposomes was studied. Proliposomes were characterized for their particle size distribution, thermal characteristics by differential scanning calorimetry (DSC) and dissolution behavior. Further, the formulated proliposomes were subjected to *in vitro* permeation or transport studies using different models such as rat intestine, parallel artificial membrane permeability assay (PAMPA) and Caco-2 cell line. Proliposomes provided enhanced exemestane dissolution due to incorporation into the phospholipid bilayers and change in the physical state from crystalline to amorphous. The *in vitro* transport studies in rat intestine, PAMPA and Caco-2 models revealed that the proliposomes were successful in enhancing the permeation of exemestane. These proliposomal formulations of exemestane could provide improved oral bioavailability due to enhanced solubility, permeability and hence absorption.

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1. Introduction

Liposomes are the most promising, broadly applicable, and highly researched of all the novel delivery systems. Liposomal encapsulation can be used to enhance lipophilicity; however the success rate of oral delivery of liposomes was limited due to erratic and unpredictable absorption profiles (Fukunaga et al., 1991; Arien et al., 1993; Chaudhari et al., 1994). Liposomes also suffer from several stability problems such as aggregation, susceptibility to hydrolysis and oxidation (Rajesh et al., 2001). However, these stability problems can be avoided by formulating liposomes as proliposomes (Payne et al., 1986; Betageri, 2005).

Proliposomes are dry, free-flowing granular products, which, upon the addition of water, disperse to form a multi-lamellar liposomal suspension (Payne et al., 1986). The oral delivery of liposomes could be improved by enhancing the ability of liposomes to retain their integrity at the site of absorption which could be achieved by formulating them into proliposomes (Deshmukh et al., 2008). Several studies have been reported which prove the utility of oral proliposomes in providing the enhanced solubility and bioavailability for insoluble/poorly soluble drugs (Brocks and Betageri, 2002). Proliposomal formulations of silymarin were prepared for oral administration. These silymarin proliposomes were reported to be stable and enhance the gastrointestinal absorption of silymarin (Yan-yu et al., 2006). Salmon calcitonin (sCT) proliposomes were prepared and evaluated for oral delivery. The apparent permeability of sCT across Caco-2 cell monolayers was increased as the result of incorporating sCT into the proliposomes, suggesting that the pharmacokinetics of sCT would be modified through the administration of proliposomes (Song et al., 2002). Further, the feasibility of using the sCT and bile salts containing proliposomes to improve the intestinal absorption of sCT was explored using rats and Caco-2 systems (Song et al., 2005).

Oral proliposomes have been extensively investigated in our laboratory to increase the permeation of highly hydrophilic compounds and to increase the solubility of poorly water-soluble drugs (Betageri, 2004, 2005). An insoluble drug glyburide was incorporated in a proliposomal formulation and a 3-fold increase in the dissolution of the drug was observed by our group previously (Rajesh et al., 2001). In another study a proliposomal formulation of halofantrine exhibited an increase of 41–47% in the area under curve, and 90–100% in $C_{\max}$ compared with the control groups (Brocks and Betageri, 2002). Phospholipid–surfactant proliposomal beads were formulated for improved oral delivery of cromolyn sodium (Deshmukh et al., 2008). Our previous studies have estab-

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doi:10.1016/j.ijpharm.2009.07.008

lished that proliposomes can serve as a better carrier for improving the dissolution and permeation of drugs.

Exemestane is a novel oral steroidal aromatase inactivator, which showed promising anti-tumor activity in postmenopausal women with hormonal sensitive (estrogen-dependent) breast cancer (Scott and Wiseman, 1999). Nevertheless, exemestane suffers from poor solubility, first pass metabolism and bioavailability problems (Valle et al., 2005). Oral bioavailability of exemestane is reported to be about 42% and plasma levels found to increase by approximately 40% after a high-fat breakfast. Also, the absorption of exemestane reported to be dependent upon formulation type and food where the suspension (compared to tablets) and fed state (compared to fasted state) provided increased absorption (Valle et al., 2005).

Thus, there is a scope of developing lipid-based formulations of exemestane for enhancing its solubility and bioavailability that result in the improved oral delivery. Recently, self-micro emulsifying drug delivery system (SMEDDS) of exemestane was reported (Singh et al., 2008). In this study, it has been reported that SMEDDS could be explored as potential carrier systems for dissolution enhancement of exemestane. Therefore, the purpose of our present research was to develop proliposomal formulations of exemestane. Another important objective of this study was to perform *in vitro* evaluation of proliposomal exemestane formulations using different models such as rat intestine, parallel artificial membrane permeability assay (PAMPA) and Caco-2 cell line. These *in vitro* studies could provide useful information about dissolution and permeation/absorption aspects of exemestane which further could be correlated to *in vivo* bioavailability studies. We hypothesize that these proliposomal formulations of exemestane might lead to improved oral bioavailability due to enhanced solubility, permeation and, thus absorption.

2. Materials

Distearoyl–phosphatidylcholine (DSPC) and dimyristoyl–phosphatidylglycerol (DMPG) were obtained from Genzyme Pharmaceuticals (Cambridge, MA, USA). Cholesterol was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Caco-2 cells were obtained from the ATCC (American type culture collection) cell repository (Manassas, VA, USA) and transwell 6-well cell culture plates were obtained from Corning Corp. (Corning, NY, USA). Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA, USA). L-*R*-phosphatidylcholine (for PAMPA) and *n*-dodecane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MultiScreen-IP PAMPA assay plates and PTFE Receptor plates were purchased from Millipore Corp. (Billerica, MA, USA). Dulbecco's MEM, nonessential amino acids, penicillin, and L-glutamine, EDTA, and trypsin/EDTA were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sprague–Dawley rats were obtained from Harlan Inc. (Indiana polis, IN, USA). All the organic solvents were of HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA).

3. Methods

3.1. Preparation of proliposomal powder

Proliposomal powder formulations were prepared with different ratios of drug (exemestane), distearoyl–phosphatidylcholine (DSPC), cholesterol and dimyristoyl–phosphatidylglycerol (DMPG). The lipid composition of the formulations with their corresponding formulation code is depicted in Table 1. The drug, lipid(s) and cholesterol were dissolved in ethanol and ethanol was evaporated to get a dry powder. The dried material was passed through the #60 mesh sieve to obtain a free-flowing proliposomal powder. The exemestane content of the proliposomes is determined using the analytical method described in the following section.

Table 1
Formulation composition of proliposomes.

Formulation code	Lipid composition[a]			Formulation composition[a]
	DSPC	Cholesterol	DMPG	
I	1	0	0	1:1, drug:lipid
II	1	1	0	1:1, drug:lipid
III	7	2	1	1:1, drug:lipid
IV	1	0	0	3:1, drug:lipid
V	1	1	0	3:1, drug:lipid
VI	1	1	0	1:3, drug:lipid

[a] Molar ratio.

3.2. Analysis of exemestane

Drug analysis was carried out by reversed phase HPLC method using the method reported by Breda et al. (1993) with minor modifications with respect to mobile phase. Water's HPLC system with the UV–visible detector was used for the analysis. The mobile phase consisted of 70:30 acetonitrile:0.02 M phosphate buffer (pH 4.0) set at a flow rate of 1.0 ml/min (isocratic method). The elution was done on a Prodigy C-18 column (5 μm, 250 × 4.66 mm) and the chromatographic analysis was carried out at 247 nm.

3.3. Particle size analysis of proliposomes after hydration

Proliposomes (powder) were hydrated in pH 7.4 phosphate buffered saline (PBS) by sonication and vortex to obtain liposomes, these liposomes were then analyzed for the size distribution. Particle size analysis was performed by dynamic light scattering (DLS) using a NICOMP 370 particle sizer.

3.4. Differential scanning calorimetric (DSC) analysis

DSC studies were performed to comprehend the physical state of the drug in the proliposomal formulations. The analysis was carried out using PerkinElmer Pyris 1 differential scanning calorimeter (with Pyris Manager software) (PerkinElmer Corporation, CT, USA). The peak transition temperature (T_m) and heat of fusion (enthalpy) (ΔHf) were determined and used in the analysis. Indium (T_m = 159.2 °C; ΔHf = 28.8 J/g) was used as a standard for routine calibration. An empty aluminum pan was used as reference, and nitrogen (purity > 99.99%) was used as the purge gas. The samples were scanned at 10 °C/min from 20 °C to 400 °C and DSC chromatograms were recorded for comparison.

3.5. Dissolution studies

Dissolution studies were conducted using USP type-II apparatus. The dissolution medium used was 900 ml of pH 7.4 PBS. The studies were carried out at 100 rpm and at 37 ± 0.5 °C. Each experiment was performed in triplicate and the samples were withdrawn at fixed time intervals up to 3 h. After appropriate dilutions, the samples were analyzed. Cumulative percent of drug released was calculated, and mean of three samples was used in the data analysis.

3.6. Rat intestinal permeation studies

Intestinal permeability of the formulations was assessed by using mucosal sheets from the rat intestine at 37 °C in pH 7.4 PBS. On the day of the experiment, Sprague–Dawley rat was anesthetized and a midline abdominal incision was made and the entire length

of the intestine was removed; the first 15-cm segment distal to the pylorus was discarded. The excised intestine was flushed with ice-cold saline to remove any intestinal contents. The intestine was cut open and cut into strips (about 0.5 inch size) large enough to fit between the two half cells of a modified diffusion chamber. The tissue was then clamped between the two half cells of a diffusion chamber. The diffusion apparatus then placed in well-oxygenated buffer. On the mucosal side was added about 400 μl of hydrated proliposomal (liposome) solution, in pH 7.4 PBS, containing 1000 μg/ml of exemestane. To the serosal side was added about 5 ml of pH 7.4 PBS. The diffusion apparatus was then placed in an incubator with a continuous supply of 5% carbon dioxide (CO_2) and 95% oxygen (O_2) during the entire length of the experiment. At predetermined time intervals, 1 ml samples of serosal fluid were withdrawn for up to 2 h. The samples were then analyzed by HPLC. Experiment was performed in triplicate and mean of three samples was used in the data analysis.

The apparent permeability coefficients (P_{app}) are calculated from the following equation:

$$\text{Apparent permeability}(P_{app}) = \frac{dQ/dt}{C_0/A}$$

where dQ/dt is the flux across the monolayer or intestinal sac (μg/s), C_0 the initial concentration in the mucosal side of the intestinal sheet (μg/ml), and A the surface area of the sheet available for diffusion/transport.

3.7. Parallel artificial membrane permeability assay (PAMPA) studies

PAMPA studies were carried out using the following protocol. The 96-well filter plate was used as the permeation receptor and the 96-well receiver plate was used as the permeation donor. PBS, pH 7.4 has been used both as donor and receptor buffer throughout the study. A 5% solution (w/v) of lecithin in dodecane was prepared and the mixture sonicated to ensure complete dissolution. The lecithin/dodecane solution, 10 μl, was then carefully added into each donor plate well. Immediately after the application of the artificial membrane, 150 μl of proliposomal solution (equivalent to 100 μg/ml of exemestane) was added to each well of the donor plate. Buffer (PBS, pH 7.4), 250 μl, was added to each well of the receptor plate. Then the drug-filled donor plate was placed into the receptor plate, making sure the underside of the membrane was in contact with the buffer. The filter (donor) plate was then coupled with the receiver plate and the plate assembly was incubated at room temperature without agitation for 4 h. The assembled plate was placed into a sealed container with wet paper towels to avoid evaporation. After incubation, samples from the donor and receptor plate were analyzed for exemestane concentration by HPLC method. Experiment was performed in triplicate and mean of three samples was used in the data analysis.

Permeability of the compounds was calculated using the following formula (Chen et al., 2008):

$$P_e = -\ln[1 - C_A(t)/C_{equilibrium}]/A \times (1/V_D + 1/V_A) \times t$$

where P_e is permeability in the unit of cm/s. A = effective filter area = $f \times 0.3\,cm^2$, where f = apparent porosity of the filter (discussed below), V_D = donor well volume = 0.3 ml, V_A = receptor well volume = 0.2 ml, t = incubation time (s), $C_A(t)$ = compound concentration in receptor well at time t, $C_D(t)$ = compound concentration in donor well at time t, and

$$C_{equilibrium} = \frac{[C_D(t) \times V_D + C_A(t) \times V_A]}{(V_D + V_A)}$$

For the apparent porosity of the PVDF filter, the assumption of f = 1 for PVDF filter has been used. However, f = 0.76 has been used by some researchers so that the results obtained from PAMPA based on PVDF filter can be directly compared to the results obtained from PAMPA based on polycarbonate filter (Wohnsland and Faller, 2001). As polycarbonate filter was used in the present study, f = 0.76 was used for the permeability calculations.

3.8. Caco-2 permeability studies

Caco-2 cells were grown in polystyrene cell culture flasks until confluent. Seeding was done in the 6-well polystyrene plates with polyester inserts having an insert membrane growth area of 4.7 cm^2. Transepithelial electrical resistance (TEER) was measured using an EVOM voltmeter with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA). The cell monolayers with average TEER values of about 150 Ω were used in the study.

The proliposomal formulations were suitably reconstituted and equilibrated using PBS (pH 7.4) prior to yield exemestane concentration of 500 μg/ml. Transport studies were performed at 37 °C on mature Caco-2 cell monolayers from apical to basolateral side with 1.5 ml of the test suspension/solution added to the donor compartment and 2.5 ml of PBS (pH 7.4) in the receiver compartment. At predetermined time points, 250 μl samples were withdrawn from the receiver compartment and replenished with an equal amount of PBS (pH 7.4) for up to 4 h. These samples were analyzed for exemestane content by HPLC method. Experiment was performed in triplicate and mean of three samples was used in the data analysis.

The apical to basal apparent permeability coefficient was calculated according to the equation (Bermejo et al., 2004):

$$\text{Apparent permeability}(P_{app}) = \frac{V/(\text{area} \times \text{time}) \times [\text{drug}]\text{receiver}}{[\text{drug}]\text{initial, donor}}$$

where, V is the volume of the receptor well in ml (2.5 ml), A is the area of the membrane insert (4.7 cm^2), [drug]receiver is the concentration of drug in the receiver compartment, [drug]initial, donor is the initial drug concentration added to the donor compartment, and time is the total transport time in seconds.

3.9. Statistical analysis

Statistical analysis of the dissolution profiles was carried out by one-way analysis of variance (ANOVA), and by comparing the dissolution profiles using a model-independent method (Costa and Lobo, 2001). The mean dissolution time (MDT) of the formulations were determined and compared subjecting the MDT values to one-way analysis of variance (ANOVA) to examine the statistical difference. The MDT values were calculated using the following formula:

$$\text{MDT} = \frac{\sum_{j=1}^{n} \hat{t}_j \Delta M_j}{\sum_{j=1}^{n} \Delta M_j}$$

where, j is the sample number, n is the number of dissolution sample times, $\hat{t}_j$ is the time at midpoint between t_j and t_{j-1} (easily calculated with the expression $(t_j + t_{j-1})/2$), and ΔM_j is the additional amount of drug dissolved between t_j and t_{j-1}. Similarly, the apparent permeability values (P_{app}) of rat intestinal and Caco-2 studies and effective permeability values (P_e) of PAMPA study were subjected to ANOVA analysis for statistical validation of the results. A confidence limit of $P < 0.05$ was fixed and the theoretical and calculated values of F (F_{crit} and F_{cal}) were compared for the interpretation of results and to examine the statistical difference.

Dissolution results were further evaluated for f_2 factor (similarity factor values) to assess the similarity or difference in the dissolution profiles (Shah et al., 1998).

4. Results and discussions

4.1. Formulation of proliposomes

Dimyristoyl phosphatidylcholine (DMPC) and dipalmitoyl phosphatidylcholine DPPC possess lower phase transition temperatures, 23 °C and 41 °C respectively, compared to DSPC (56 °C). Also, it has been reported that the dissolution enhancement of water-insoluble drug was higher in case of DMPC formulations compared to DPPC and DSPC (Betageri, 2002). DSPC was preferred over other phospholipids in the present investigation, as DSPC formulations would be expected to perform in a superior manner *in vivo* over other phospholipids. The *in vivo* preference originates from the fact that the DSPC offers improved rigidity and increased stability to the liposomal membrane against attack by the bile salts and enzymes of the intestine. This factor is of paramount importance especially at the *in vivo* conditions as liposomes are known to be unstable orally towards acidic (gastric) pH, bile salts and pancreatic enzymes.

4.2. Particle size analysis

Particle size is reported as a Gaussian mean diameter (intensity weighting) with standard deviation. Particle size of formulation II, III, IV, V and VI were found to be 1219.0 ± 159.8 nm, 963.1 ± 130.2 nm, 1161.7 ± 112.5 nm, 1003.6 ± 99.8 nm and 992 ± 145.3 nm respectively. The particle size in the range of about 1000 nm and above indicated that the multi-lamellar vesicles might have formed. Formation of multi-lamellar vesicles would be advantageous for the incorporation of insoluble drug like exemestane as there would be increased hydrophobic volume within the liposomal lamellae.

4.3. Dissolution studies

Encapsulation of the water-insoluble drug exemestane into proliposomes resulted in the enhanced dissolution (Figs. 1 and 2). Proliposomes enhanced the dissolution of exemestane 4–5 times compared to pure drug. At the end of 60 min, exemestane dissolution was about 40% from proliposomes compared to about only 9% for pure drug. This might be due to the enhanced solubility of the drug by the phospholipid molecules or possible change in the physical state of the drug (crystalline to amorphous). However,

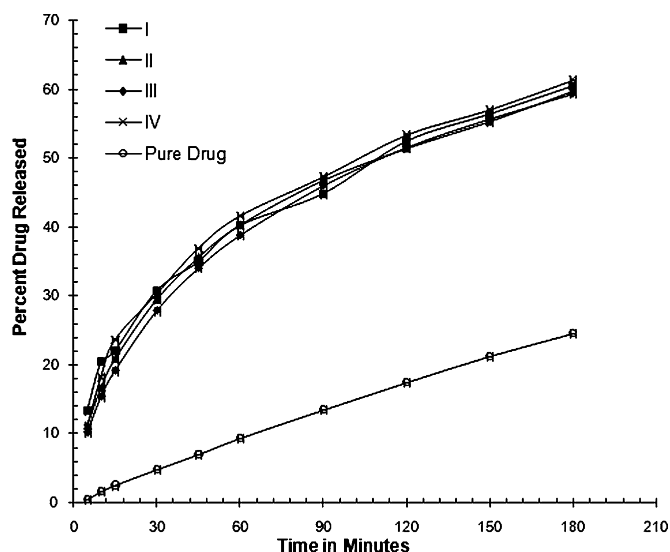

Fig. 1. Comparative exemestane dissolution from proliposomal formulations (each data point represents the average of six samples).

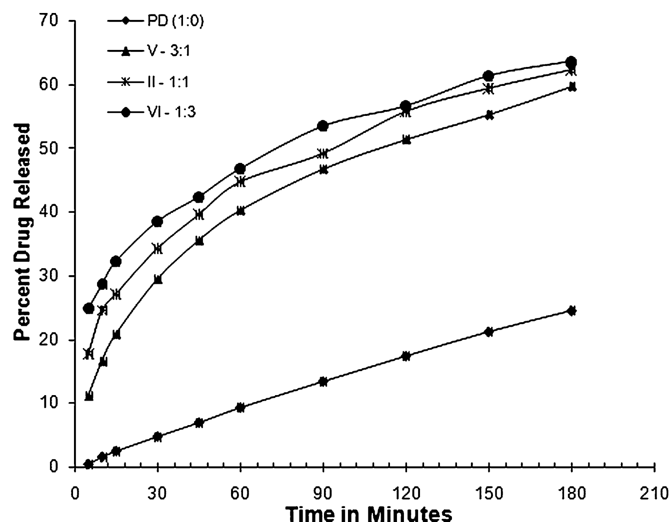

Fig. 2. Effect of drug:lipid ratio on exemestane dissolution from proliposomes (each data point represents the average of six samples).

there was no significant difference between the dissolution profiles of formulations I, II, III and IV. The non-significant difference between dissolution profiles of formulations I, II, III and IV were further confirmed statistically ($P < 0.05$) (data not shown).

From the dissolution studies it was observed that formulations I–VI were indistinguishable in terms of dissolution enhancement of exemestane. However, the formulation II with 1:1, DSPC:cholesterol is preferred over other formulations for oral delivery for the following reasons. It has been reported that the DSPC liposomes containing higher molar ratios of cholesterol exhibited enhanced stability at pH 2.0 and in bile solution (Yukihiko et al., 1993). Also the increased solidification provided by the cholesterol is expected to provide improved resistance towards the pancreatic lipase (Yukihiko et al., 1993). Thus, formulation II can be speculated to provide improved oral delivery of exemestane compared to I, III and IV. Hence, formulation II was considered for further study to evaluate the effect of lipid ratio on exemestane dissolution from proliposomes.

The effect of drug to lipid ratio is depicted in Fig. 2. It can be observed from Fig. 2 that increase in the drug to lipid ratio resulted in the increased exemestane dissolution. This might be due to increased amount of lipid (with respect to drug) available for entrapment of the water-insoluble exemestane within its phospholipid bilayers to enhance the dissolution. The dissolution profiles of formulation II, V and VI were further analyzed by subjecting the mean dissolution time (MDT) values to one-way analysis of variance (ANOVA) to examine the statistical difference (Costa and Lobo, 2001). The ANOVA analysis of MDT values for formulations V and II ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 5.3$), formulations II and VI ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 4.5$) and, formulations V and VI ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 17.6$) proved that the drug:lipid ratio has a significant influence on the exemestane dissolution from the proliposomal formulations. Although the exemestane dissolution from V and II and, II and VI found to be statistically insignificant ($P < .05$); there was a significant difference in the exemestane dissolution among formulations V and VI. Similar trend was observed with f_2 factor analysis. The f_2 values were found to be 25, 65 and 53 for pure drug and formulation V; formulation V and formulation II and; formulation II and formulation VI; respectively. These f_2 values indicated that there was a significant difference in the dissolution profiles of pure drug and formulation V; and no significant difference between V and II and, II and VI. Nevertheless, there was a significant difference in the dissolution profiles of formulation V and formulation VI

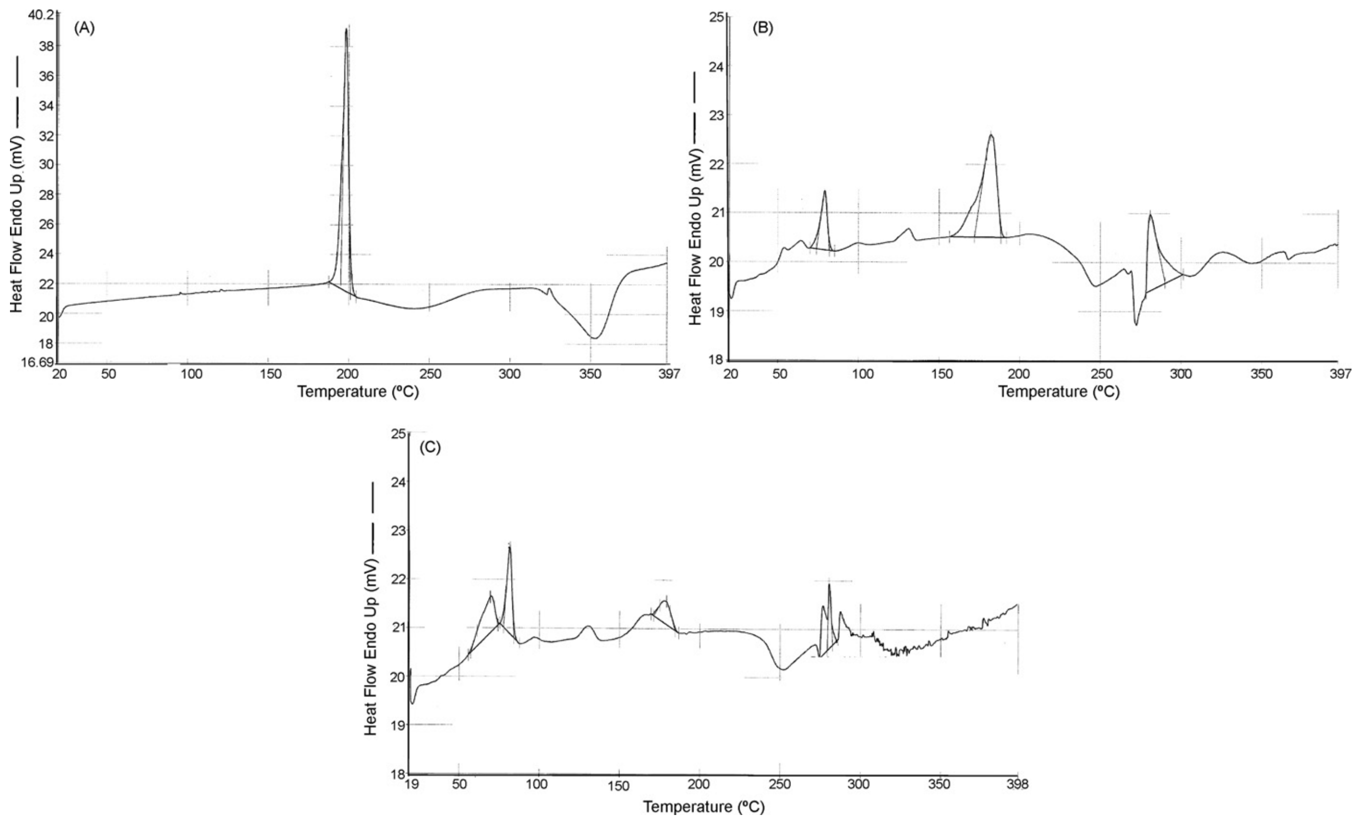

Fig. 3. A. DSC profile of exemestane (pure drug). B. DSC profile of formulation II. C. DSC profile of formulation VI.

($f_2 = 20$), further confirming the importance of effect of drug:lipid ratio on exemestane dissolution from proliposomes.

4.4. DSC studies

DSC studies were performed to evaluate the physical state of the drug in the formulation and to analyze the thermal properties of the drug. DSC profiles of pure drug (exemestane), formulation II and formulation VI are shown in Fig. 3A, B and C respectively.

The phase transition peak for pure exemestane appeared at 198 °C and the enthalpy (ΔH) for phase transition temperature of pure exemestane was found to be 86.8 J/g. However, the phase transition peak for exemestane shifted to 182 °C and 179 °C in case of proliposomal formulations II and VI respectively. Also, as the lipid ratio in the proliposomes increased the phase transition peak of the exemestane broadened and eventually disappeared (formulation VI). There was a significant influence of proliposomal formulation of exemestane on the ΔH of exemestane. It can be observed from Fig. 3A that as the exemestane was present in the proliposomes, there was a decrease in the ΔH. Also, as the drug:lipid ratio increased from 1:1 (formulation II) to 1:3 (formulation VI) the ΔH decreased from 43.6 J/g to 5.9 J/g respectively.

These results indicated that incorporation of insoluble drug exemestane within proliposomes and an increase in the lipid ratio in proliposomes might have resulted in the conversion of the drug from crystalline to amorphous form in the proliposomes. The DSC results compliment the dissolution of exemestane from proliposomal formulations (Figs. 1 and 2), which exhibited an increase in the dissolution of exemestane with an increase in the lipid ratio of the formulation. Also, the extent of dissolution enhancement increased as the drug:lipid ratio increased due to enhanced conversion of exemestane from crystalline to amorphous form.

4.5. Rat intestinal permeation studies

The liposomal exemestane (Figs. 4 and 5) exhibited an increased transport across the rat intestinal membrane compared to pure drug. There was about 3–4 times increase in the intestinal transport of exemestane from proliposomes compared to the control (pure drug). It can be observed from Fig. 4 that the order of transport enhancement was II > III > I > pure drug. Formulation IV exhibited very poor permeability, which could not be detected/analyzed. Cholesterol was added to the lipid phase to improve stabil-

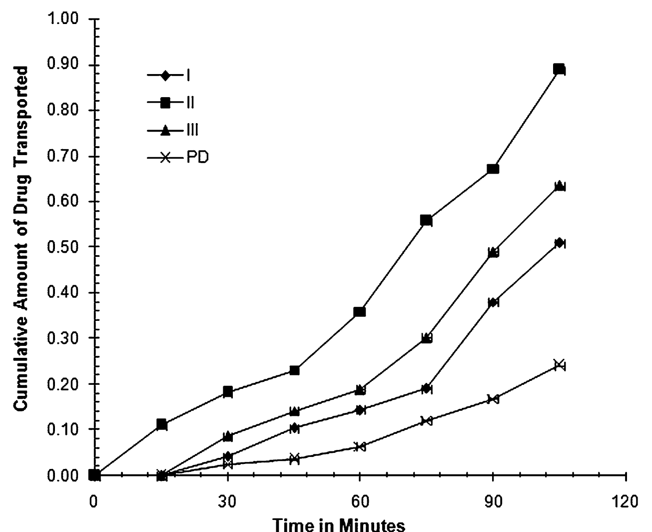

Fig. 4. Comparative rat intestinal transport data of exemestane from proliposomes (each data point represents the average of three samples).

ity of the vesicles (Betageri et al., 1993). Biophysical studies of phospholipid–cholesterol bilayers have also shown that the addition of 30–50 mol% cholesterol to phosphotidylcholine liposomes can increase the hydrophobicity in the interfacial region of the liposome bilayer (Bernsdorff et al., 1997). In the present investigation the incorporation of cholesterol with DSPC in the proliposome found to provide enhanced transport of exemestane. As the cholesterol concentration was increased in the proliposomes from formulation I (no cholesterol), formulation III (8:2, phospholipid:cholesterol) to formulation II (1:1, phospholipid:cholesterol) the intestinal transport of exemestane enhanced. Addition of negatively charged phospholipid DMPG found to be unsuitable for increasing the dissolution and intestinal transport of exemestane from proliposomes. Although the dissolution data showed no significant difference between the formulations I, II and III; the transport study indicated the remarkable difference in the rat intestinal transport of exemestane among I, II and III ($P < 0.05$) (data not shown). Formulation II with 1:1, DSPC:cholesterol showed highest intestinal transport for exemestane. Hence, this lipid composition was selected for further studies.

Effect of drug:lipid ratio of proliposomes on exemestane transport is depicted in Fig. 5. As the drug:lipid ratio in proliposomes increased from 3:1 to 1:1 exemestane transport increased. But, further increase to 1:3 did not significantly affect the transport. Although, it appears that there was a slight decrease in the exemestane transport when the drug:lipid ratio was increased from 1:1 to 1:3 (Fig. 5), statistically no significant difference was observed when the apparent permeability (P_{app}) values were analyzed by ANOVA. The P_{app} values are shown in Table 2. The ANOVA analysis of P_{app} values indicated that Formulation V did not show significant improvement in the exemestane permeability compared to pure drug ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 1.6$). However, formulation II (1:1, drug:lipid) exhibited significant increase in exemestane permeability compared to V ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 36.2$). Also, there was no significant change of further increase in drug:lipid ratio to 1:3 as indicated by ANOVA ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 4.1$ among formulation II to VI). Thus the statistical analysis of permeability results indicated 1:1 as the optimum drug:lipid ratio for enhanced intestinal transport of exemestane from proliposomes.

4.6. PAMPA permeability studies

PAMPA, a non-cell-based permeability model provides an estimate of the passive transcellular permeability because it lacks transporter- and pore-mediated permeability. An adequate lipophilicity (log P) is required for a permeate to travel across the phospholipids membrane by passive diffusion (Balimane et al., 2006). PAMPA assay is a robust and reproducible. It is relatively fast (4–16 h), inexpensive, and straightforward. The majority of drugs (>80%) enter the blood stream by passive diffusion, and the permeation values obtained correlate with human drug absorption values from published methods (Sandhya et al., 2008).

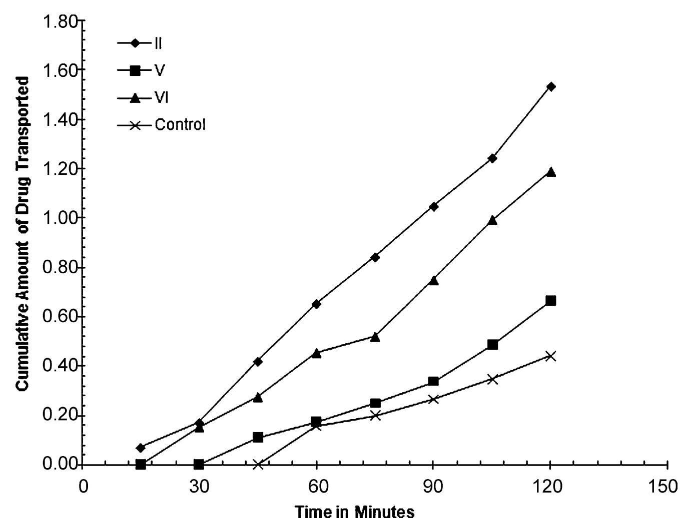

Fig. 5. Effect of drug:lipid ratio on rat intestinal transport of proliposomal exemestane (each data point represents the average of three samples).

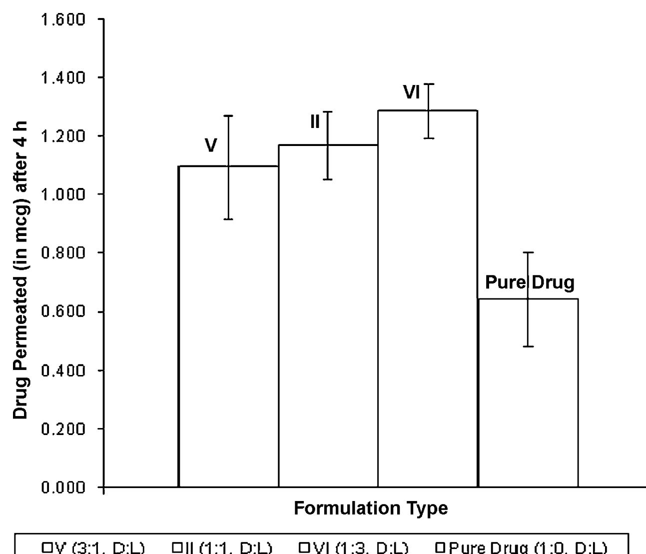

Fig. 6. Comparative PAMPA permeability: effect of drug:lipid ratio of proliposomes (each data point represents the average of six samples).

Effect of drug:lipid ratio of proliposomes on exemestane permeability in PAMPA model is shown in Fig. 6. It can be observed from Fig. 6 that proliposomes provided improved permeability of exemestane in PAMPA model. This may be due to the increased log P values of liposomal exemestane compared to the control (pure drug) which ultimately resulted in the enhanced passive permeation across PAMPA membrane. The effective permeability (P_e) values for the proliposomes and pure drug are given in Table 2. Pure exemestane demonstrated the lowest permeability (P_e value of 0.49×10^{-6} cm/s) whereas the formulation VI with higher lipid ratio showed highest permeability (P_e value of 1.00×10^{-6} cm/s). The ANOVA results further demonstrated that formulation V showed significantly increased exemestane permeability compared to pure drug ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 8.0$). Further increase in the drug:lipid ratio did not significantly impact the exemestane permeability [($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 0.3$ for formulations V and II) and ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 3.4$ among formulations II to VI). However, as could be seen from Fig. 6, there was a slight increase (though statistically insignificant) in the exemestane permeability as the drug:lipid ratio increased from 3:1 (V) to 1:1 (II) to 1:3 (VI). Hence, the statistical analysis of the PAMPA permeability results (P_e values) indicated that formulation V (with 3:1, drug:lipid ratio) was adequate to provide enhanced permeability of exemestane, beyond (beyond 1:3, drug:lipid ratio) which there was no significant effect.

4.7. Caco-2 permeability studies

Caco-2 cells are most widely and successfully used permeation models which are used to study passive diffusion, active transport, paracelllular permeability and active efflux (Balimane et al., 2006). It can be observed from Fig. 7 that the proliposomal formulations provided enhanced exemestane permeability in Caco-2 model compared to the pure drug (exemestane). The exemestane

Table 2
Permeability measurement of proliposomes.

Permeability values for different models	Formulation type			
	PD (control)	V	II	VI
P_{app}[a] for rat int. permeability (10^{-6}) (cm/s)	0.48 ± 0.12	0.60 ± 0.11	1.56 ± 0.25	1.20 ± 0.18
P_e[b] for PAMPA permeability (10^{-6}) (cm/s)	0.49 ± 0.10	0.84 ± 0.19	0.91 ± 0.17	1.00 ± 0.22
P_{app}[a] for Caco-2 permeability (10^{-6}) (cm/s)	0.67 ± 0.09	0.99 ± 0.10	1.09 ± 0.17	1.01 ± 0.19

[a] Apparent permeability value.
[b] Effective permeability value.

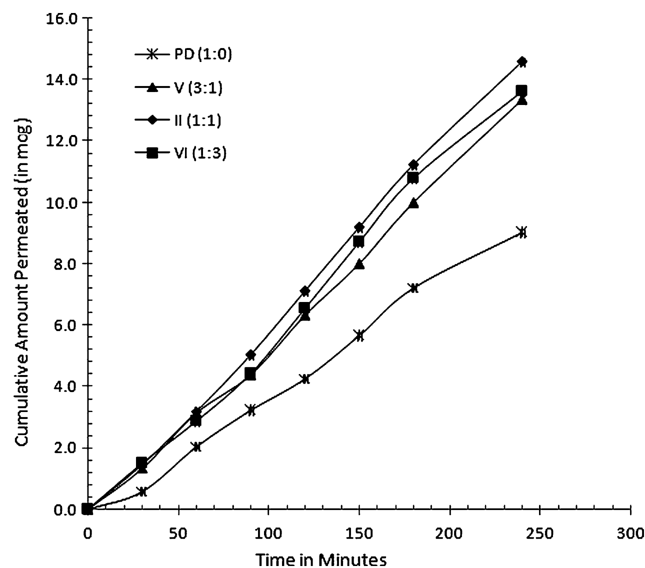

Fig. 7. Effect of drug:lipid ratio of proliposomes on Caco-2 permeability of exemestane (each data point represents the average of three samples).

permeability slightly increased as drug:lipid ratio increased from 3:1 to 1:1 and further increase to 1:3 resulted in the slight decrease in the permeability. The apparent Caco-2 permeability values for exemestane from proliposomes are given in Table 2. The apparent permeability values of the formulations were significantly greater for proliposomal formulations when compared to the pure drug and the permeability was found to be highest in case of formulation II. Thus the proliposomes exhibited the P_{app} values that are reported to be necessary for favorable (and complete) intestinal absorption in humans (i.e., $\geq 1 \times 10^{-6}$ cm/s) (Artursson and Karlsson, 1991). Whereas, the pure drug by itself was not able to exhibit permeability characteristic (P_{app} value of 0.67×10^{-6} cm/s) that favors complete intestinal absorption in humans. These results are in good agreement with the previous report that the oral bioavailability (absorption) of exemestane is reported to be about 42% (pure exemestane, P_{app} value of $>0.1 < 1 \times 10^{-6}$ cm/s) and, the plasma levels found to increase after a high-fat breakfast (proliposomes, P_{app} value of $\geq 1 \times 10^{-6}$ cm/s) (Valle et al., 2005). ANOVA analysis indicated that formulation V demonstrated significantly increased exemestane permeability compared to pure drug ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 16.4$). Similar to PAMPA results, further increase in the drug:lipid ratio did not significantly influence the exemestane permeability [($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 0.8$ for formulations V and II) and ($P < .05$, $F_{crit} = 7.7$ and $F_{cal} = 0.4$ among formulations II to VI). In this case also (similar to PAMPA), the statistical analysis of the permeability results (P_{app} values) indicated that formulation V with 3:1, drug:lipid ratio was sufficient to provide enhanced permeability of exemestane, beyond (beyond 1:3, drug:lipid ratio) which the effect was non-significant.

It was observed that the permeability values (P_{app} and P_e), in case of all three models studied (rat intestine, PAMPA and Caco-2), found to initially increase with increase in the drug:lipid ratio to some point and then decrease. It is difficult to point out the exact reason for such observation; however, authors could provide the following explanation. It is known that the permeability increases with increase in the lipophilicity. Thus, as the drug:lipid ratio was increased initially there was a significant increase in the exemestane permeability. However, beyond certain point (drug:lipid ratio) there was no significant effect of drug:lipid ratio on exemestane permeability. This may be due to the hindered diffusion through stagnant aqueous diffusion layers (unstirred water layer) (Camenisch et al., 1997). The unstirred water layer (UWL) adjacent to the surface of the intestinal/cell membrane presents a significant barrier to the permeation/absorption of lipophilic solutes (Lennernas, 1998). The unstirred water layer is approximately 30–100 μm thick in man (human intestinal membrane) (Lennernas, 1998). However, in the *in vitro* studies, it has been reported that the UWL thickness varies from 128 to 1544 μm depending upon the stirring rates employed during the study (Karlsson and Artursson, 1991). An increased stirring rate reduces the influence (thickness) of the unstirred water layer at the epithelial interface and enhances mixing in the donor and receiver chambers (Lakeram et al., 2008). But, authors performed the permeability measurements with no stirring of the donor and/or receptor chamber. Therefore, the UWL might have influenced the permeability of exemestane from proliposomes with relatively higher drug:lipid ratio due to the absence of stirring effect. Thus, the observed no significant effect after certain drug:lipid ratio may be due to hindered diffusion through UWL with increase in the lipophilicity of formulation.

Another possible explanation for the observed effect of drug:lipid ratio on exemestane permeability could be provided as follows. Lipophilic/hydrophobic permeates are known to get adsorbed to the membrane during permeability studies (Camenisch et al., 1997). Therefore, it may be possible that the formulations with relatively higher drug:lipid ratio might have adsorbed (to some degree) to the membrane leading to the decreased concentration of permeated drug at receptor side/chamber. Nevertheless, further studies are required to validate the effects of UWL and membrane adsorption before arriving at the definitive conclusions.

Further, it was observed that the exemestane permeability results did not follow an identical trend in PAMPA, rat intestine and Caco-2 models. The observed dissimilarities may be due to the differences in the permeation mechanisms operating in these *in vitro* models. PAMPA is a passive absorption model that captures uncontaminated transcellular diffusion across the lipid bilayers (Kansy et al., 1998). It is completely devoid of any influx/efflux transporters or paracellular pores. On the other hand, cell tight junctions and various drug transporters (influx and efflux) are expressed in the rat intestine and Caco-2 cell models along with the paracellular transport. However, there was a good correlation observed between the PAMPA and Caco-2 permeability results. In both of these models, formulation V (with 3:1 drug:lipid ratio) found to be adequate to provide enhanced exemestane permeability. Good correlation between PAMPA and Caco-2 data indicated that the passive diffusion may be a predominant mechanism for exemestane permeation.

On the contrary, formulation V was not sufficient to provide significantly increased exemestane permeation in rat intestine study. Formulation II (with 1:1, drug:lipid ratio) found to be optimum for improved exemestane permeability in rat intestinal transport study. The exact reasons for this variation, compared to PAMPA and Caco-2, are not known. This variation again may be due to the differences in the permeation mechanisms operating in these models and due to the fact that both PAMPA and Caco-2 are artificial/cultured membranes where as rat intestine membrane is actually a part of animal model. Hence, the thickness of UWL, extent of membrane adsorption during permeation and membrane structural differences among PAMPA, Caco-2 and rat intestine might have manifested in the observed variation in the exemestane permeability. It has been reported that the PAMPA and Caco-2 permeation results have a good correlation to human oral absorption (Kerns et al., 2004). On the other hand, for drugs with low solubility (like exemestane), it has also been shown that the rat intestinal epithelium is more effective in predicting human fraction absorbed than using Caco-2 cells (Watanabe et al., 2004). Thus, the permeability measurements using multiple *in vitro* models such as rat intestine, PAMPA and Caco-2 can synergistically provide invaluable information about the permeability/absorption assessment of the formulations. In the present study, the proliposomal formulations found to provide enhanced exemestane permeation in all the *in vitro* models studied. Overall, formulation II could be preferred over other formulations which provided the enhanced exemestane permeability irrespective of the model studied. Thus, from the *in vitro* models studied, the proliposomes proved to be the promising oral delivery systems for enhancing exemestane absorption and bioavailability.

5. Conclusions

Proliposomes provided enhanced exemestane dissolution due to incorporation into the phospholipid bilayers and change in the physical state from crystalline to amorphous which was confirmed from DSC studies. The *in vitro* transport studies in rat intestine, PAMPA and Caco-2 models revealed that that the proliposomes were successful in enhancing the permeation of exemestane. The enhancement of *in vitro* permeation appears to be due to the combined effect of improved solubility/dissolution (especially true in case of PAMPA studies) and increased uptake of liposomes through intact cell membranes (particularly in case of rat intestinal and Caco-2 transport studies). The dissolution and *in vitro* transport of exemestane from proliposomes was dependent upon the formulation composition and drug:lipid ratio. The effect of drug:lipid ratio on permeability of exemestane was not same in the rat intestinal transport, PAMPA and Caco-2 models. The observed variations in permeability in different *in vitro* models studied were attributed to the differences in the permeation mechanisms operating in these models. However, the good correlation between the PAMPA and Caco-2 permeability results indicated that the permeability/absorption of exemestane was primarily by passive diffusion mechanism. Most importantly, irrespective of the models used for permeation studies, the proliposomes were successful in enhancing the *in vitro* permeation of exemestane.

The *in vitro* results obtained are promising and convincing in demonstrating the utility of these proliposomal formulations for improved oral delivery of exemestane. The free-flowing proliposomal powder formulation of the present investigation could be incorporated into the capsules or compressed into the tablets after processing with other conventional excipients commonly used in the manufacture of capsules and tablets. Also, these formulations could further be considered for enteric coating or gastric protective coating to further enhance the oral bioavailability of liposomal exemestane. An understanding of the clinical relevance of the *in vitro* data will be very valuable in understanding the commercial utility of the proliposomal formulations. Further, the *in vivo* bioavailability studies in human volunteers are necessary to demonstrate the clinical relevance of the *in vitro* data and to assess the commercial utility of the proliposomal formulations. In this regard, the enteric coating of these proliposomal formulations, formulation into tablet/capsule dosage forms and *in vivo* bioavailability studies are in progress in author's laboratory. The findings of these studies would be reported in the forthcoming section of this article.

Acknowledgements

The authors are thankful to Dr. Santos B. Murty from Murty Pharmaceuticals, Inc., Lexington, Kentucky for his help with DSC studies. The authors express their sincere gratitude to Western University of Health Sciences, Pomona, California for awarding post-doctoral fellowship to carry out this project.

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