

Development of Novel lipid Nanoparticles for oral Bioavailability Enhancement of Irinotecan: *In-vitro* and *In-vivo* Investigations

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ABSTRACT

Background: Irinotecan is indicated as a first line treatment for metastatic colorectal cancer. However, chemotherapy with irinotecan is restricted to i.v. route owing to poor and erratic oral bioavailability due to its excessive intestinal efflux by P-glycoprotein. **Objective:** Aim of the present study is to improve the oral pharmacokinetic profile of irinotecan (IRT) by merging the attributes of nano-particulate system and P-glycoprotein (P-gp) modulation activity of excipients. **Methods:** Gelucire 44/14 solid lipid nanoparticles (SLN) were developed and optimized by Box-Behnken design. Optimized formulation was evaluated for various *in vitro* attributes and *in vivo* pharmacokinetic profile. **Results:** Size of optimized SLN was found to be 179.8 ± 15.3 nm with polydispersity index, 0.367 ± 0.029 and drug entrapment, $78.2 \pm 4.6\%$. SLN showed biphasic release profile i.e. initial burst release followed by sustained release. Differential scanning calorimetric (DSC) and X-ray diffractometric (XRD) analyses of SLN demonstrated the loss of drug's crystallinity in SLN. Further, confocal laser scanning microscopy showed higher permeation of Rhodamine 123 (P-gp substrate) across intestinal epithelium through SLN when compared with free rhodamine 123 solution. Furthermore, *in-vivo* studies exhibited superior pharmacokinetic profile; significantly high ($p < 0.001$) C_{max} (1471.6 ± 190.9 ng/mL) was attained through SLN when compared with C_{max} of oral suspension (1113.5 ± 125.5 ng/mL), similarly, high T_{max} ($p < 0.001$) was observed which revealed sustained effect of SLN. Conclusively, 2.79 folds improvement in oral bioavailability of IRT could be achieved through SLN when compared with oral suspension. **Conclusion:** Outcomes of studies suggested the potential of developed SLN for oral delivery of irinotecan and possibility to replace pre-existing intravenous therapy.

Key word: Gelucire 44/14, Lipid nanocarrier, Intestinal gut sac method, Pharmacokinetic study.

INTRODUCTION

Molecules with wide range of biological activities including antimicrobial, antiviral, and anticancer are available commercially however, oral administration of several of them is still a major challenge. In general, these molecules either exhibit a poor aqueous solubility or site specific permeability characteristics across gastro intestinal tract (GIT). In addition, most of these compounds are either substrate for the biological transporters (including P-glycoprotein) or a major metabolic enzyme, cytochrome P450 or both, resulting in a significant loss of drug due to expulsion or first pass metabolism. Furthermore, the unfavourable physicochemical properties as well as

hostile environment of the gastrointestinal tract possess major challenges for successful oral delivery.¹⁻⁵ A number of anticancer drugs suffer from these drawbacks and limit the possibilities of their oral administration. Despite these difficulties, the development of oral formulations for anticancer drugs is always viable due to a number of reasons including noninvasive nature, flexibility in designing of dosage form, dosing frequency, better patient compliance and being economic.⁶ Furthermore, oral delivery of cytotoxic drugs would eliminate or reduce the need of hospitalization, medical and nursing care as well as infusion equipments etc.⁷ It is generally assumed that long

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exposure of cancerous cell to a drug at modest concentrations is more effective than a pulsed supply at higher concentration.⁸ Unfortunately, most of the anticancer agents are administered via iv route which exhibit initial rapid increase followed by fast decay of plasma drug concentrations resulting into ineffective therapy and increased side effects.⁸⁻¹⁰ Orally administered drugs absorb gradually and can sustain an effective concentration of drug in plasma which may prolong the exposure to cancerous cells resulting into improved efficacy and lower adverse effects.

Irinotecan is indicated as a first line treatment for metastatic colorectal cancer. However, chemotherapy with irinotecan is restricted to i.v. route often causes patient incompletion. Till date no oral formulation of irinotecan is available commercially due to the poor and erratic oral bioavailability owing to its excessive intestinal efflux by P-glycoprotein. P-gp mediated efflux and cytochrome P450 mediated metabolism have remained a major reason of multi drug resistance consequently failure of chemotherapy. Nano-encapsulation of drugs allows them to evade reorganization by P-gp and extremely small size of systems facilitates its penetration across physiological barriers. Various studies have also revealed that nanoparticulate systems could be a good alternative for oral delivery of such type of drugs.^{8, 11-16} However, P-gp mediated efflux cannot be circumvented by use of nanoparticles alone because released drug from nanoparticles is effluxed out of the cell membrane by P-gp.¹⁷ To surmount these limitations, numbers of classical and natural P-gp and cytochrome P450 inhibitors have been investigated to improve the oral efficacy.¹⁸⁻²¹ However, these inhibitors have also been reported to have high toxicity profile, inhibitory effect on unintended efflux transporters and unpredicted pharmacokinetic interactions.²² Subsequently, 3rd generation P-gp inhibitors (Tariquidar, Zosuquidar) were specifically developed to overcome the P-gp mediated multi drug resistant (MDR). Third generation P-gp inhibitors were supposed to have lowest toxicity and high specificity. However, a number of investigations have demonstrated their cross-reactivity with mitoxantrone resistance protein (MXR) and/or drug-metabolizing enzymes.²³ Currently, number of excipients belonging to pharmaceutical grade have shown comparable P-gp inhibitory activity as well as lesser side-effects.²⁴ Hence, application of pharmaceutical excipients including polymer, lipid, oil, surfactant and co-surfactant as efflux pump inhibitors in the improvement of *peroral* drug delivery has drawn enormous attention.^{25,26} Therefore, development of nanosized delivery systems with excipients having P-gp modulation activity could be a more effective

approach for the cytotoxic drugs to bypass P-gp at tissue and cellular level.

Among the lipid-based nanocarriers including lipid core micelles, liposomes, microemulsions, nanoemulsions, solid lipid nanoparticles and self-nanoemulsifying drug delivery system, solid lipid nanoparticles and self nano-emulsifying drug delivery systems have emerged as preferred formulations for delivering poorly water-soluble and least bioavailable drugs. Solid lipid nanoparticle (SLN) is a nanoparticulate delivery system which has combined advantages of nanoparticles, liposomes, emulsion excluding the disadvantages of these systems.²⁷ SLN are up taken by Peyer's patches and follow the lymphatic route of absorption thus avoiding the hepatic first pass metabolism of drugs which may result into improved oral bioavailability.²⁸⁻³² The other advantages including controlled drug release, high drug stability, and high drug entrapment, negligible biotoxicity makes them a carrier of choice for drug delivery. Scaling up of solid lipid nanoparticles is possible and can be useful for industrial research in future.²⁷

The aim of the present study is to investigate the attributes of lipid nanocarriers as well as P-gp inhibitory effect of pharmaceutical excipients and explore their utilization for enhancement of oral bioavailability of irinotecan. In this perspective, solid lipid nanoparticles were developed using Gelucire 44/14 (lipid), a P-gp inhibitor and evaluated for various *in-vitro* attributes and *in-vivo* pharmacokinetic profile.

MATERIAL AND METHODS

Materials

Irinotecan hydrochloride was provided ex-gratia by Fresenius Kabi Pvt Ltd, India. Dichloromethane, (AR grade), Acetonitrile (HPLC grade), Orthophosphoric acid (HPLC grade), Tween 20, cetyl alcohol, stearic acid, glyceryl monostearate, sodium dihydrogen phosphate dihydrate, potassium dihydrogen phosphate and sodium hydroxide were purchased from SD fine chemical, Mumbai, India. Different grades of Gelucire, Precirol, and Compritol 888 ATO were obtained as a gift sample from Gattefosse, Gennevilliers, France. Water was obtained from Milli-Q water purification system (Millipore, MA, USA).

HPLC analysis

HPLC analysis was carried out by using in house developed and validated HPLC methods.³³ Separation module e2695 HPLC system equipped PDA detector 2998 (Waters Alliance, Milford, MA, USA) was utilized for the purpose. Chromatographic data was processed by LC solutions software Empower 2. Drug separation could

be done efficiently by C18 column (5 μm , 250 \times 4.6 mm) using mobile phase comprising of acetonitrile and sodium dihydrogen phosphate dihydrate buffer (0.045 μM) containing ion pair agent heptane sulphonic acid sodium salt (0.0054 μM), pH 3. Flow rate was maintained at 1 mL/min and analysis was carried out at 254.9 nm.

Screening of lipids

Lipids were screened on the basis of drug's solubility in it. Solubility of irinotecan in different lipids was determined by previous report with slight modification.³⁴ In brief, 200 mg each of different lipids was taken in small vials and excess amount of drug was added gradually in it until saturation observed. The vials were tightly closed and were kept for stirring at $75 \pm 0.5^\circ\text{C}$ and 400 rpm for 72 h in incubator shaker. Samples were analyzed for drug content by using high performance liquid chromatography.³⁵

Preparation and statistical optimization of drug loaded SLN

Solid lipid nanoparticles were prepared by single emulsion solvent evaporation method.³⁶ In this method, required quantities of lipid (Gelucire 44/14) and drug were dissolved in 2 ml dichloromethane and emulsified in 20 ml of aqueous solution of Tween 80 of different strength (Table 1) followed by sonication using Probe sonicator (Vibra cell™ VC 505, 500 watts, 30% amplitude). The resultant emulsion was subjected to solvent evaporation under mild stirring (400 rpm). The obtained formulation was subjected to analysis for various *in-vitro* attributes.

Box-Behnken design was applied for statistical optimization of the formulation parameters and to study the main effects, interaction effects and quadratic effect of the formulation ingredients on the SLNs formulation. For the optimization of SLN, Design was applied at 3 independent variable (factors) each variable with 3 levels (-1, 0, +1, low, medium and high respectively) and design matrix was constructed which comprising of 17 experimental runs. Selected independent variables were sonication time (X_1), surfactant concentration (X_2) and Lipid: drug ratio (X_3) and dependent variables (responses) were size (Y_1) and entrapment efficiency (Y_2) of SLN. Generation and evaluation of the statistical experimental design were performed with the Design Expert 8.1 software.

Particle size and polydispersity index

The particle size and size distribution (PDI) of the SLN was determined by using dynamic light scattering

(DLS) method with a computerized inspection system (Malvern Zetasizer, Nano-ZS, Malvern, UK) and analysed by 'DTS nano' software.

Particle shape and morphology

Surface morphology of solid lipid nanoparticles was studied with the help of transmission electron microscope (TEM, TOPCON 002B) operating at 200 KV, capable of point to point resolution. A drop of suspended solid lipid nanoparticles was allowed to deposit directly on the circular copper film grid of 300 meshes and sample was negatively stained with a 1% aqueous solution of phosphorous tungstic acid. After drying, the specimen was viewed under a microscope.³⁷

Entrapment efficiency

Entrapment efficiency of SLN was determined by using Amicon R ultracentrifuge filtration tubes with slight modifications.³⁴ Filtration tube consisting of an upper detachable donor compartment mounted with semi-permeable membrane (molecular weight cut-off of 12 KD) which can be fixed over the lower receiver centrifuge tube. 1 mL of SLN suspension was diluted 10 times with water to dissolve any un-entrapped and un-dissolved drug. 4 mL of this diluted formulation was then placed in the upper compartment of the centrifuge filtration tube and centrifuged for 15 min at 3000 rpm. All the colloidal species (SLN) were retained over the membrane of upper compartment. Retained SLN were digested with 5 mL dichloromethane, filtered through 0.22 μm membrane filter and analysed for drug content (entrapped). Similarly, filtrate retained in the receiver compartment was also analysed by the HPLC.³⁵ The entrapment efficiency was expressed as the percentage of the total drug entrapped

$$\text{Percentage entrapment} = \frac{T - C}{T} \cdot 100$$

where T is the total amount of drug that is detected in both the filtrate and the sediment and C is the amount of drug detected only in the filtrate (un-entrapped drug) and T-C represents the encapsulated drug.

DSC analysis

It was performed with Perkin-Elmer Pyris 1 DSC, equipped with Intracooler 2P cooling accessory. Accurately weighed (5 mg), placebo SLN, pure drug, physical mixture and drug loaded solid lipid nanoparticles were separately placed in standard aluminum pans and sealed with a lid. Scanning from 10 to 300 $^\circ\text{C}$ was performed keeping the heating rates of 10 $^\circ\text{C}/\text{min}$ with a nitrogen purge of 20 mL/min.

XRD analysis

X-ray diffractometry of drug, placebo SLN and drug loaded solid lipid nanoparticles was carried out with a Philips Analytical X-Ray B. V. diffractometer (PW 3710) using Nifiltered, CuK α radiation (1.54056 Å), at 35 kV voltage and 30 mA current. The XRD patterns were recorded using diffraction angles (2θ) from 5 to 40° with step size 2° per minute.

In-vitro release study

Release study of irinotecan was performed using dialysis bag method. 2 mg of the drug suspension and SLN formulation (equivalent to 2 mg of irinotecan) were taken into a dialysis bag (5 mL) and immersed in 250 mL of release media under stirring at 37°C. The dissolution media consisted of simulated gastric fluid (pH 1.2) for first 2 h and simulated intestinal fluid (pH 6.5) thereafter for further 48 h. A sample of 2.5 mL was removed at each time interval (0.083, 0.167, 0.5, 1, 2, 3, 6, 12, 24 and 48 h) and analyzed by HPLC.³⁵

Intestinal permeation study by confocal laser scanning microscopy

Permeation potential of SLN loaded with Rhodamine B, a P-gp substrate as well as fluorescent probe was evaluated by using intestinal gut sac method. 5-7 cm long rat ileum was taken and tied from one side while another side was kept open to introduce the formulation. From the open side of the intestine, 1 mL formulation (SLN suspended into Tyrode's solution) was filled into intestinal sac followed by hanging it into beaker containing Tyrode's solution with proper aeration for 60 min. After 60 min the intestine was removed and made a cut to open the sac. Sectioned intestinal pieces were mounted on the slide facing apical side up followed by washing with running water to remove the un-penetrated surface particles. Treated sections were analyzed under confocal scanning laser microscope (CLSM) to measure the depth of penetration. Similar procedure was adopted for free Rhodamine B solution.

Pharmacokinetic (PK) studies

Irinotecan loaded solid lipid nanoparticles of Gelucire 44/14 and irinotecan suspension were taken for *in-vivo* pharmacokinetic studies. The animal protocol to carry out pharmacokinetic study was reviewed and approved by the Institutional Animal Ethics Committee of Jamia Hamdard (436, 2012). Animals used for the pharmacokinetic study were male Albino wistar Rats, weight 200-220 gm. The animals were kept under standard laboratory conditions (temperature 25 ± 2°C

and relative humidity 55 ± 5%) and were housed in polypropylene cages with free access to standard laboratory diet (Lipton feed, Mumbai, India) and water *ad libitum*. An oral dose of 10 mg/kg of Irinotecan was taken for the study.³⁸

The rats were divided into 2 groups (n=6). Group I received Irinotecan loaded Gelucire 44/14 solid lipid nanoparticles and Group II received oral drug suspension. The rats were anesthetized using diethyl ether and blood samples (0.2 mL) were withdrawn from the retro orbital plexus at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, and 48 h and collected in EDTA coated microcentrifuge tubes. The collected blood samples were mixed gently and centrifuged at 5000 rpm for 10 min to separate plasma. Separated plasma was stored at -20°C until analysis. Drug analysis was carried out using HPLC.³⁹

Statistical analysis

The PK data of free drug suspension and drug loaded SLN was compared for statistical significance by one way ANOVA followed by two tail paired test using GraphPad Instat software (GraphPad Software Inc., CA, USA).

RESULTS AND DISCUSSION

Screening of lipids

A wide variety of lipids including natural, semi-synthetic and synthetic like waxes triglycerides, higher fatty acids and steroids are available which can be exploited for production of lipid based nano-carrier systems. Selection of lipid is a very important criteria as it affects the entrapment, leaching of entrapped drug during storage, release profile as well as performance of the lipid systems. In the present study, lipids were screened on the basis of drug solubility in it as it may affect the above said parameters. Solubility was determined by adding excess amount of drug in different lipids. Irinotecan exhibited maximum solubility in Gelucire 44/14 (93.5 ± 3.41 mg/gm) followed by Compritol 888, Gelucire 50/13, precirrol ATO, glyceryl monostearate, cetyl alcohol, stearic acid and gelucire 39/01 (Figure 1). Gelucire 44/14 was chosen for the SLN development as it possesses various advantages over other lipids. It showed the highest solubilizing capacity of drug in it which may result into highest drug entrapment. Additional benefits of having P-gp modulation activity^{40,41} as well as relatively lower crystalline in nature hence the lower probability of expulsion of drug during storage.^{25,34} Therefore, better *in-vitro* and *in-vivo* parameters could be anticipated with the use of Gelucire 44/14.

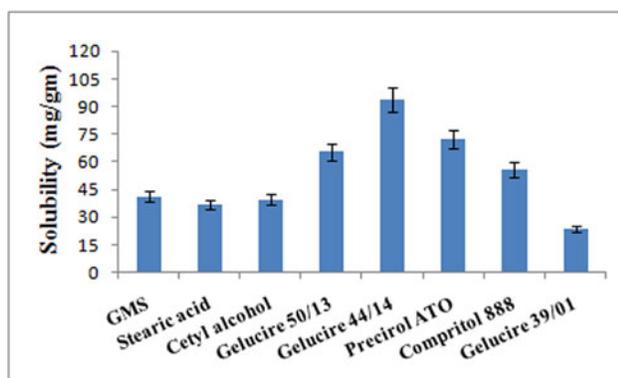


Figure 1: Solubility profile of irinotecan in different lipids

Preparation and statistical optimization of drug loaded SLN

Particle size and drug entrapment are the two most important aspects of nanoparticulate systems for the efficient delivery of drug. Size is a crucial factor for the cellular uptake of particles across the intestine via enterocytes as well as through payer's patches while drug entrapment affects the availability of drug within the

cells, tissues, or organs. Therefore, different independent variables were studied by using response surface methodologies to achieve particles size minimum and drug entrapment maximum. Among all the response surface methodology, Box Behnken design requires lesser number of runs and reduces the number of experiments. Therefore, it was chosen for optimization of formulation. 3-factor, 3-level Box-Behnken design was employed to construct polynomial models for the optimization process. Experiments were designed to study the effect of three independent variables i.e. sonication time (X_1), surfactant concentration (X_2), and lipid drug ratio (X_3) at three levels (-1, 0 +1, low, middle and high values, respectively) on their dependent variables/responses i.e. Y_1 (particle size) and Y_2 (entrapment efficiency). Total 17 experiments including 5 repetitions at center point were suggested. Responses obtained from the experiments have been shown in the Table 1.

The quadratic equations were generated for obtained responses from the experiments by applying software, Design Expert version 8.1. The generated equations are

Table 1: Level of independent variables and observed responses obtained after running experiment as per the Box-Behnken Design for irinotecan loaded SLN

Factors (Independent variables)	Levels				
	-1	0	+1		
X_1 : Time of sonication (min)	1	1.5	2		
X_2 : Surfactant concentration (%w/v)	0.0375	0.0750	0.1125		
X_3 : Lipid: Drug ratio	10	15	20		
Observed responses					
Formulation code	X_1	X_2	X_3	Y_1 Size (nm)	Y_2 % EE
F ₁	-1	-1	0	223.1 ± 14.56	80.23 ± 4.23
F ₂	+1	-1	0	183.9 ± 9.98	68.2 ± 2.67
F ₃	-1	+1	0	203.2 ± 13.68	76.6 ± 3.24
F ₄	0	0	0	175.2 ± 12.89	75.00 ± 3.54
F ₅	+1	+1	0	172.1 ± 10.36	67.40 ± 2.87
F ₆	-1	0	-1	206.4 ± 11.76	73.53 ± 3.42
F ₇	+1	0	-1	175.9 ± 9.56	62.23 ± 2.84
F ₈	0	0	0	174.1 ± 13.56	75.9 ± 3.69
F ₉	-1	0	+1	205.8 ± 10.48	80.04 ± 4.11
F ₁₀	0	0	0	176.8 ± 13.58	76.65 ± 4.08
F ₁₁	+1	0	+1	176.1 ± 11.36	72.88 ± 3.09
F ₁₂	0	-1	-1	185.2 ± 10.53	68.90 ± 2.73
F ₁₃	0	0	0	172.2 ± 12.59	76.30 ± 2.99
F ₁₄	0	+1	-1	173.9 ± 10.23	66.40 ± 2.56
F ₁₅	0	-1	+1	192.2 ± 7.96	78.93 ± 4.25
F ₁₆	0	+1	+1	186.1 ± 9.86	76.78 ± 2.76
F ₁₇	0	0	0	170.8 ± 11.23	77.20 ± 4.1

given below and in these equations only significant term has been included.

$$\text{Particle Size}(Y_1) = +173.80 - 16.25X_1 - 6.12X_2 + 14.22X_3 + 7.47X_2^2 \quad \text{Eq. 1}$$

$$\%EE (Y_2) = 76.21 - 4.96X_1 - 1.13X_2 + 4.70X_3 + 1.03X_1X_3 - 1.84X_2^2 - 2.2X_2^3 \quad \text{Eq. 2}$$

For the response Y_1 , the Model F-value 26.45 implies the model is significant ($P < 0.0001$). In this case X_1 , X_2 , X_1^2 , X_2^2 are significant model terms. The Lack of Fit F-value of 5.01 is not significant ($P < 0.077$). Non-significant lack of fit is good for the model to fit. Similarly, for response Y_2 the model F value 67.47 implies that the model is significant ($P < 0.0001$) and the lack of Fit F value 1.11 ($P < 0.44$) is not significant. Adequate precision measures the precision signal to noise ratio. This model can be used to navigate the design space if ratio is greater than 4. In our case its values were found 15.27 and 29.71 for Y_1 and Y_2 respectively.

Positive values of factors reflect the synergistic relationship i.e. an effect that favours a higher value, while a negative value represent an antagonistic effect between the independent variable (factor) and dependent variable.⁴² It can be understood clearly from the equation, sonication time and (X_1) and surfactant concentration (X_2) favours a smaller size particle. However, no significant effect of lipid drug ratio was observed on particle size. The entrapment efficiency was found increase with lipid drug ratio (X_3) and decrease by remaining two factors (sonication time and surfactant concentration). The relationship between dependent and independent variables is presented through contour plots and response surface analysis generated by the Design Expert version 8.1 software (Figure 2 and 3).

With respect to particles size (Y_1), factors X_1 , X_2 and X_3 exhibited more or less curvilinear relationship from lower to middle level then nonlinear (Figure 2a-c). A more clear-cut effect of various factors on size of SLN can be understood in three-dimensional response surface plots (Figure 2e-f). The size of the SLNs was found decrease with increase in the sonication time (Figure 2d), surfactant concentration (Figure 2e) and lipid drug ratio (Figure 2f). Size of the solid lipid nanoparticles directly depends upon the size of the globules formed during emulsification process thus smaller size of SLN at higher sonication time can be featured to production of smaller oil globules during the emulsification process at high sonication time owing to high energy input.⁴³ Similar to sonication time, reduction in particles size was observed at higher surfactant concentration and vice versa. Plausible reason for production

of larger size particles at lower surfactant concentration can be attribute to insufficient surfactant molecules to stabilize the globules formed during emulsification process while smaller size SLN at higher surfactant concentration can be attributed to availability of required surfactants molecules to stabilize the droplets. Observations are in agreement with previously reports.^{44,45} Furthermore, mild change in particles size was observed at different lipid-drug ratio. To study the effect of this variable, the amount of lipid was kept constant while the amount of drug was changed. As lipid was kept constant therefore only entrapped drug molecules would affected the particle size. Thus, smaller change in particle size at different lipid drug ratio can be attributed to distribution of drug into large number of particles. The results are agreement with previous report.⁴⁶

With respect to entrapment efficiency, two dimensional contour plots demonstrated a more or less curvilinear relationship between response (% EE) and factors X_1 , X_2 and X_3 (Figure 3a-c) while a more clear-cut relationship has been demonstrated through three-dimensional response surface plots (Figure 3e-f). The response surface plots demonstrated a reduction in entrapment efficiency with increase in sonication time, and surfactant concentration (Figure 3d and 3e). Reduction in entrapment efficiency of SLN at higher sonication time can be featured to more expulsion of drug owing to sonication induced acoustic cavitation while poor entrapment efficiency at higher surfactant concentration can be explained by increased leaching of drug from SLN owing to enhanced solubility of drug into external aqueous media.⁴⁷⁻⁴⁹ An inverse relationship was observed upon increasing lipid drug ratio. To study the effect, here lipid was kept constant while the amount of drug was varied hence the observation are explained on the basis of relative amount of lipid (higher or lower) with respect to amount of drug. Higher entrapment of drug at high lipid drug ratio (i.e. relatively higher lipid and lower amount of drug) can be justified by the availability of relatively more number of lipid molecules to interact with drug molecules (lower amount of drug) and solubilise them within it which resulting into more efficient entrapment while low entrapment at lower lipid drug ratio (higher amount of drug) can be attributed to insufficient lipid molecules to interact with drug molecules which resulting into crystallization and leaching of drugs thus lower entrapment.⁵⁰

Optimization and validation

As the performance of nanoparticulate system directly depends on particles size and drug entrapment there-

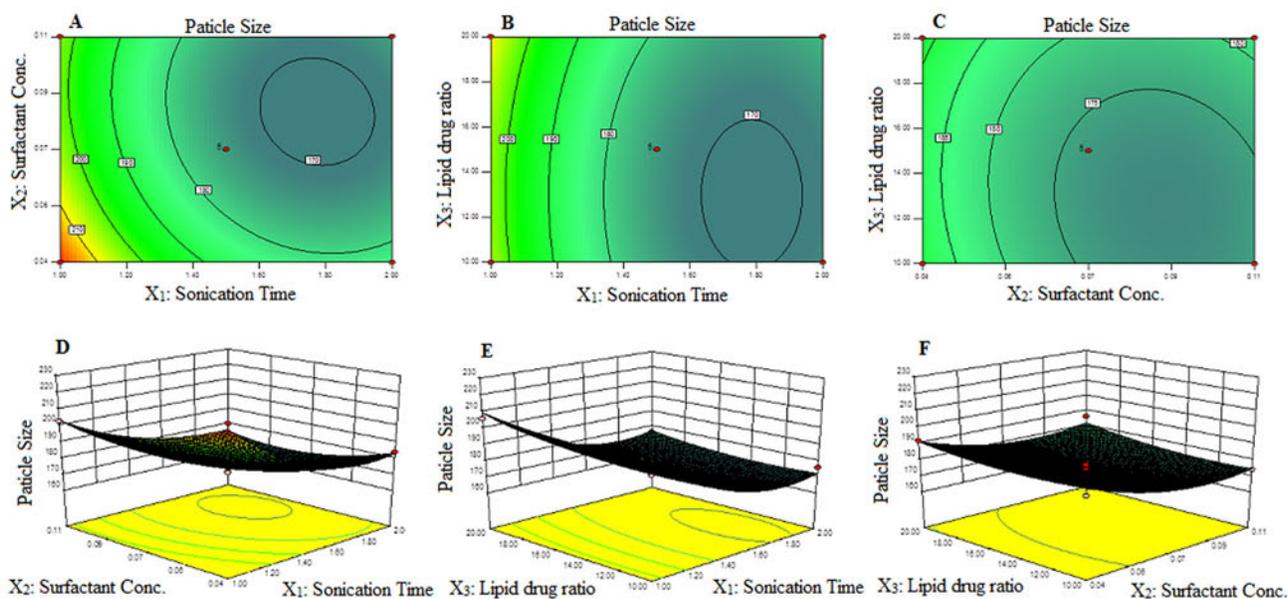


Figure 2: Contour plots and Response surface diagrams showing the effects of different variables on the size of the SLN

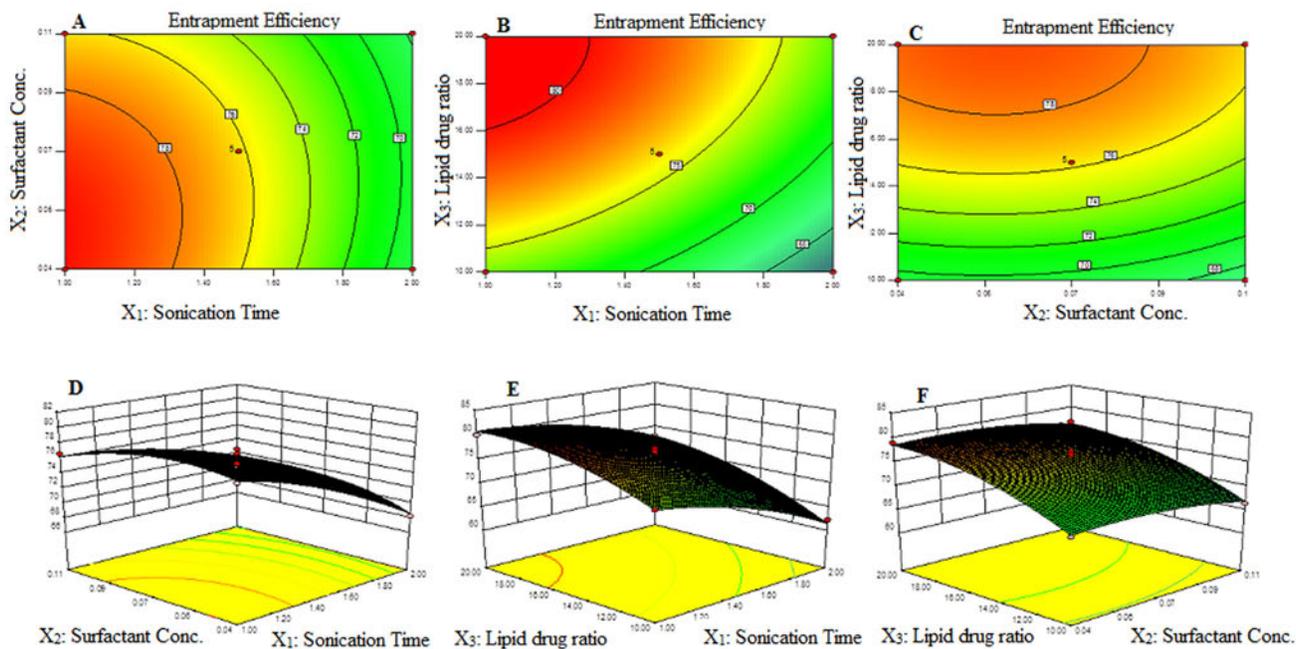


Figure 3: Contour plots and Response surface diagrams showing the effects of different variables on the entrapment efficiency of SLN

fore responses obtained after running experiments were fitted into software and finally particles size and drug entrapment were optimized by point prediction method (in built solution of software) keeping following criteria; minimum particles size and maximum drug entrapment. Eventually, Design Expert® software suggested a following levels of factors to achieve the goal; sonication time (1.35 min), stabilizer concentration (0.077 % w/v),

and lipid drug ratio (16.77), and anticipated particle size 180.8 nm and drug entrapment 78.74%. The anticipated response (size and EE) were further validated by performing experiments. Experimental values for particle size and entrapment efficiency were found 179.8 ± 15.3 nm and $78.2 \pm 4.6\%$, respectively. The low standard deviations between anticipated and experimental values confirmed the reproducibility of the model.

In-vitro characterization of optimized formulation

Particles size, size distribution, shape and surface characteristic play a pivotal importance on *in-vivo* behavior of systems. Therefore, developed system was characterized for various *in-vitro* attributes. Particle size and polydispersity index were confirmed by Malvern zeta sizer. The average particle size of optimized solid lipid nanoparticles was found 179.8 ± 15.3 nm and their distribution is depicted in Figure 4a. Shape of particles was determined by TEM which revealed almost spherical, spindle in shape, discrete and appearing dark.³⁴ Size of some particles was measured by TEM which were found in nanometer range varying from 120 nm to 200 nm, showing an agreement with the particle size measured by dynamic light scattering (Figure 4b). The developed system is within the nanometer range (<200 nm) having low PDI (0.37), high entrapment and almost spherical in shape. The size of nanoparticles is within the range (50-500 nm) which is considered most appropriate for the endocytic uptake.^{51,52} Thus, better performance of the developed system can be anticipated. % Entrapment efficiency of the optimized 44/14 SLN was found $78.2\% \pm 4.6$ is considered reasonable and high drug content can be anticipated at the absorption site.

Entrapped drug may exist either in amorphous or crystalline form or molecularly dispersed. Physical state of the drug in the final formulation was confirmed by DSC and XRD. DSC thermogram of placebo Gelucire SLN exhibited a small peak around 45°C which corresponds to melting endotherm of Gelucire (Figure 5a). Pure drug showed an intense endothermic peak at 234.7°C (Figure 5b) which can be attributed to melting point of drug. When drug was physically mixed lipid, DSC thermogram of physical mixture demonstrated two peaks at 45°C and 234.7°C corresponding to melting endotherm of lipid and drug (Figure 5c) while DSC thermogram of irinotecan loaded SLN showed only one peak around 45°C and peak corresponding to melting endotherm of drug was disappeared (Figure 5d). For further confirmation of physical state of drug in the SLN, XRD analysis was also performed. The diffraction pattern of drug exhibited sharp and intense peaks at 2θ scattered angles indicated crystalline nature of irinotecan (Figure 6a). The characteristic peaks for irinotecan were absent in diffractograms of placebo (Figure 6b) as well as drug loaded SLN (Figure 6c). Disappearance of characteristic intense peaks of drug in DSC thermogram and XRD diffractogram of drug loaded SLN indicates that the drug existed either in amorphous or solid solution or solid dispersion form in the final nanoparticulate formulation. Physical form of the drug within the formulation influences the *in-vitro* and *in-vivo* release of the

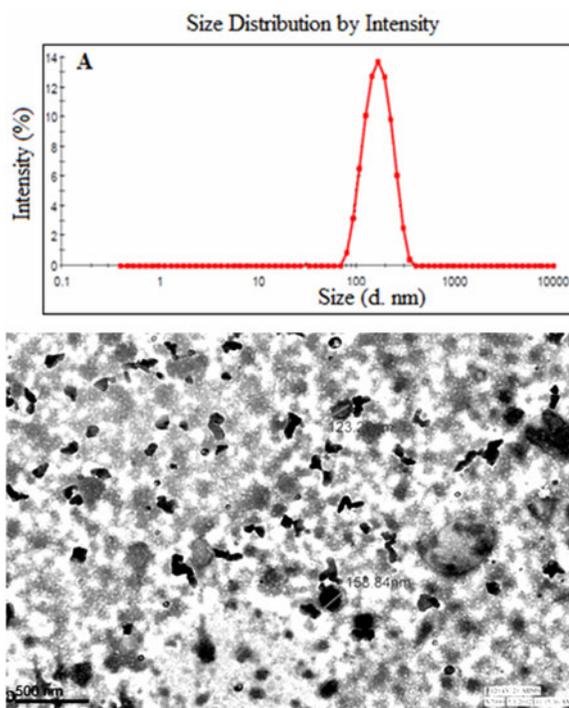


Figure 4: (A) Particle size and size distribution by DLS; (B) shape and surface morphological analysis by TEM.

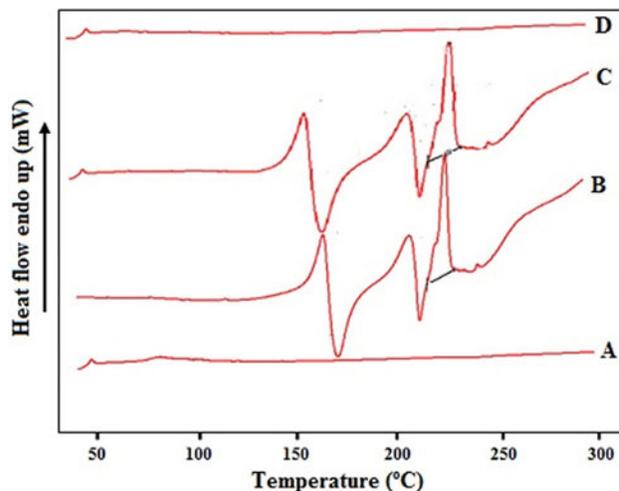


Figure 5: DSC thermogram of; (A) placebo SLN; (B) pure drug; (C) physical mixture; (D) drug loaded SLN

drug from the prepared systems. The less-ordered crystals and the amorphous state also contribute to high drug loading.⁵³

In-vitro release study

Since solid lipid nanoparticles were developed for oral delivery therefore release studies were performed both in simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.5). Free drug suspension and SLN exhibited pH dependent release profile. Free drug suspension showed faster release at lower pH 1.2 (in SGF) and

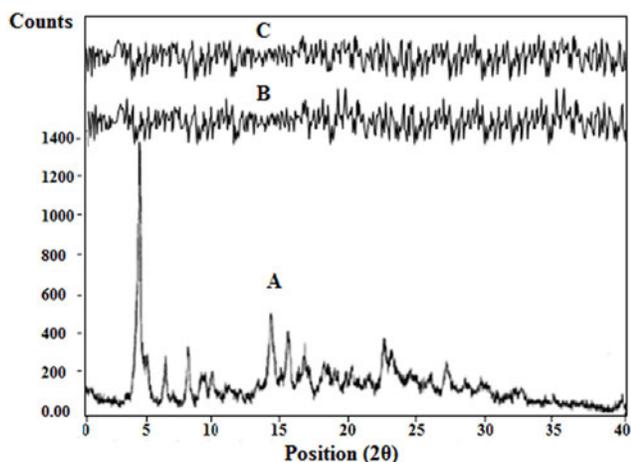


Figure 6: X-ray diffractogram; (A) pure drug; (B) drug loaded SLN; (C) placebo SLN

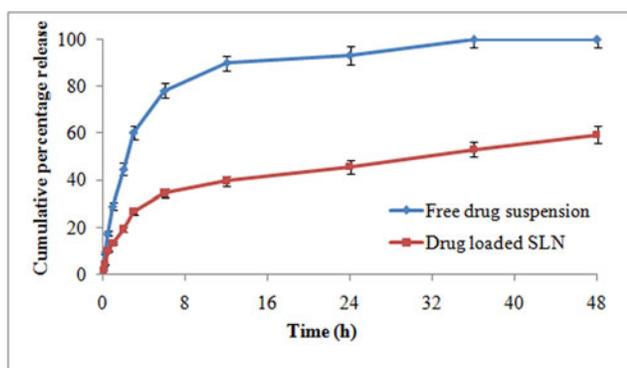


Figure 7: *In-vitro* release profile of pure drug suspension and drug loaded SLN

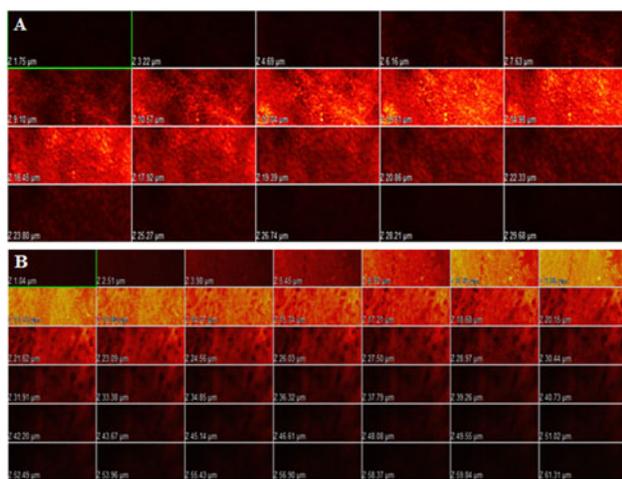


Figure 8: Confocal laser scanning microscopic images of intestine revealing depth of penetration of Rhodamine 123 after treatment with (A) Rhodamine-123 solution; (B) Rhodamine-123 loaded Solid Lipid Nanoparticles

slower release at higher pH 6.5 (in SIF). Approximately $42.93 \pm 2.69\%$ of the drug was released in 2 h at acidic condition and subsequent release of the drug in SIF pH 6.5 was relatively slower. $94.2 \pm 4.06\%$ of total drug

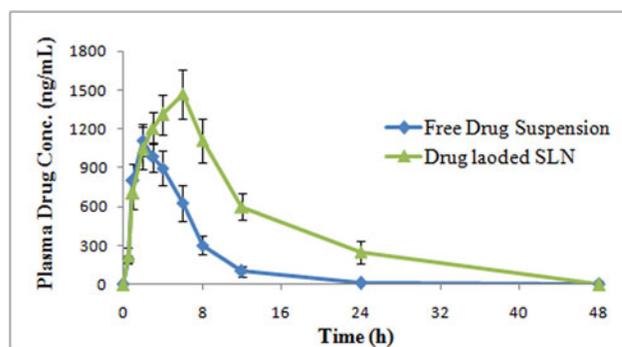


Figure 9: Plasma drug concentration time profile of free drug suspension and drug loaded Gelucire 44/14 SLN (Mean \pm SD; n = 6)

was released in 24 h. Similar to free drug suspension, SLN also showed a biphasic release pattern, initial burst release followed by sustained drug release in SGF and SIF. The initial fast release ($19.07 \pm 2.62\%$ in first 2 h) followed by slow release, $59.32 \pm 3.3\%$ in 48 h (Figure 7). pH dependent drug release from free drug suspension as well as from the formulation can be attributed to the higher solubility of drug at low pH as compared to higher pH due to the basic nature of irinotecan. Free drug suspension showed initial burst release followed by slower release. Similar pattern was also observed with SLN but more sustained in comparison to free drug suspension, it is due to encapsulation of drugs within the lipid matrix. The initial fast release can be attributed to release of the drug present at the surface of SLN and the subsequent slow release due to entrapped drug within the core of SLN as well as its higher affinity to the lipid. Sustained release profile is desirable to provide protection to drug molecules from P-gp efflux pumps. Furthermore, absorption could be improved via uptake of SLN in intact form through payer patches and enterocytes following endocytosis.

Intestinal permeation study by confocal laser scanning microscopy

The CLSM studies for assessment of intestinal permeability were carried out using Rhodamine-123 as fluorescent probe which is itself a P-gp substrate hence chosen for permeation study. The study was carried out for 60 min and the tissue slides were observed by scanning along the 'Z' axis at various depths. The depth of fluorescence with free Rhodamine-123 solution was found $22.33 \mu\text{m}$ (Figure 8a) while SLN showed significantly higher value of penetration, $61.31 \mu\text{m}$ (Figure 8b). The permeation of free dye solution was hampered by P-gp receptors present at the surface of intestinal epithelium. Better penetration of dye approximately 3 fold by using SLN can be attributed to encapsulation of

Table 2: Pharmacokinetic parameters of Irinotecan obtained after single dose (10 mg/kg) oral administration of free drug suspension and drug loaded SLN (Mean \pm S.D; n=6)

Formulation	T _{max} (h)	C _{max} (ng/mL)	AUC _{0-t} (ng.h/mL)
Free drug suspension	2.33 \pm 0.52	1113.5 \pm 125.5	7311.16 \pm 388.7
Gelucire 44/14 SLN	5.67 \pm 0.52	1471.6 \pm 190.9	20412.83 \pm 1343.4

dye which evade reorganization by P-gp at the plasma membrane as well as endocytic uptake of SLN facilitated improved delivery across the deeper intestinal layers.

Pharmacokinetics and statistical analysis

Encouraging observations of intestinal transport study were further strengthened by *in-vivo* studies. Hence, Pharmacokinetic parameters after single dose administration of irinotecan (10 mg/kg) were determined by applying non-compartmental model. The plasma drug concentration versus time profile was illustrated in Figure 9. The values of C_{max} and T_{max} were read directly from the arithmetic plot of time versus plasma concentration of Irinotecan. The AUC was calculated by using the trapezoidal method. The calculated PK parameters were shown in Table 2.

C_{max} and T_{max} of irinotecan oral suspension were found 1113.5 \pm 125.5 ng/mL and 2.33 \pm 0.52 h while C_{max} and T_{max} for irinotecan loaded SLN were found 1471.66 \pm 190.92 ng/mL and 5.67 \pm 0.52 h, respectively. C_{max} and T_{max} were found significantly high when compared with free drug (p<0.001). Similarly, irinotecan loaded SLN showed significantly high AUC_{0-t} (20413.1 \pm 1633.04 ng.h/mL) when compared with free drug, 7313.07 \pm 523.8 ng.h/mL (p<0.0001).

Significantly high T_{max} for irinotecan loaded SLN can be attributed to sustained release profile. In addition, it be explained by endocytic uptake of lipid systems by enterocytes and M cells of Payer's patches thus they releases drug at lymphatic system which subsequently reaches into blood circulation after a certain time lag.^{54,55} Significantly higher C_{max} and AUC_{0-t} of irinotecan through SLN can be justified by improved permeation across intestinal epithelia consequently enhanced absorption owing to encapsulation of irinotecan into SLN resulting into improved stability in hostile GI milieu as well as avoiding the recognition by cellular P-gp transporter and CYP-450. Furthermore, altered absorption i.e. direct uptake of nanoparticles via endocytosis through enterocytes and M cells of Payer's patches resulting into reduced first pass metabolism and P-gp mediated efflux.^{15,28,54-57} Moreover, P-gp modulation property of Gelucire 44/14 also would have imparted a significant

role in improved bioavailability. Thus cumulative effect resulting into 2.79 folds higher oral bioavailability.

CONCLUSION

The spirit of the present study revealed that lipidic nano-carrier was capable of enhancing the oral bioavailability of Irinotecan. A careful screening of lipids, statistical optimization and development of lipidic nano-carrier was successfully accomplished. Developed SLN was found effective as suggested by *in-vitro* and *in-vivo* assessments. Hence, it can be inferred from outcomes of the studies that a suitably designed nano formulation with excipient having P-gp modulation activity can be beneficial in improving the oral bioavailability of P-gp substrate molecules without using additional modulating agent and would be cost effective and showing better patient compliance.

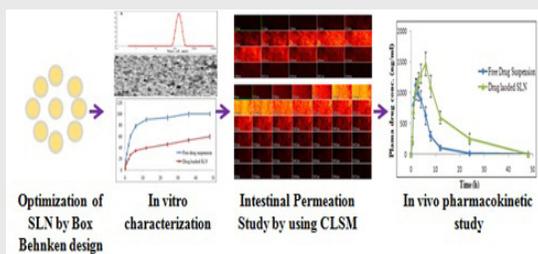
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PICTORIAL ABSTRACT



SUMMARY

- Irinotecan loaded solid lipid nanoparticles were statistically optimized by Box Behnken Design.
- Developed formulation demonstrated better penetration across rat ileum demonstrated by confocal microscopy.
- Developed formulation showed significantly high C_{max} , T_{max} and AUC_{0-t} when compared with free drug.
- Developed formulation demonstrated 2.79 folds improvement in oral bioavailability of irinotecan when compared with free drug high.

ABBREVIATIONS USED

IRT: Irinotecan; **P-gp:** P-glycoprotein; **SLN:** Solid lipid nanoparticles; **DSC:** Differential scanning calorimetric; **XRD:** X-ray diffractometric; **CLSM:** confocal laser scanning microscopy; **GIT:** Gastro intestinal tract; **CYP 450:** Cytochrome P450; **MDR:** multi drug resistant; **MXR:** Mitoxantrone resistance protein; **PDI:** Polydispersity index; **TEM:** Transmittance electron microscopy; **EDTA:** Ethylenediamine tetra acetate; **PK:** Pharmacokinetic; **EE:** Entrapment efficiency; **SGF:** Simulated gastric fluid; **SIF:** Simulated intestinal fluid.

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Dr Sushama Talegaonkar: Is working as Assistant Professor in Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard since September 2000. She has about 15 years experience in teaching and research. She obtained her Ph. D. Degree in Pharmaceutics from Dr. Hari Singh Gour Vishwavidyalaya, SAGAR (MP). Her research interests include development of various nano drug delivery systems for various biomedical applications, Bioavailability enhancement of various anticancer drugs by efflux transporter modulation, and enhanced topical and transdermal drug delivery. She is actively involved in developing wide variety of smart and functionalized nano drug delivery systems for targeted anticancer drug delivery.