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Research Article

Development, Optimization and Characterization of Sterically Stabilized Liposomes of Irinotecan

Krupali A. Thacker, Bhupendra G. Prajapati*

Department of Pharmaceutics, Shree S. K. Patel College of Pharmaceutical Education and Research, Ganpat University, Ganpat Vidyanagar, Mehsana, Gujarat, India

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ABSTRACT

The present investigation work summarizes the development of irinotecan liposome injection for intravenous infusion, 4.3 mg/mL (10 mL fill), a generic equivalent of reference product onivyde irinotecan liposome injection for intravenous infusion, 4.3 mg/mL (10 mL fill), which is indicated for colorectal cancer dosing for camptosar. Quality by design (QbD) approach has been used to develop generic irinotecan liposome injection and manufacturing process that ensures the quality, safety and efficacy of irinotecan liposome injection. Preliminary screening trials for drug (API) loading suggest that active loading process with extrusion process using ammonium dihydrogen phosphate shows better drug entrapment with less free drug. A 2(5-1) fractional factorial design of experiments (DoE) adopted to evaluate the effect of lipids concentration in ethanol, hydration temperature, hydration time, extruder temperature and extruder pressure on particle size. Based on design space, optimized process parameters were defined for maintaining the required particle size. A 2(4-1) fractional factorial DoE adopted to evaluate the drug loading heating temperature, drug loading heating time, drug loading cooling temperature and drug loading cooling time on particle size and free drug. Based on design space, optimized process parameters were defined for maintaining the required particle size and % free drug. Stability study of the final formulation suggest no any degradation of impurity and no any % free drug is increasing with respect to stability time. Also all characterization was performed on final formulation and all data is comparable with the reference formulation. The developed formulation of irinotecan liposome injection for intravenous infusion, 4.3 mg/mL (10 mL fill) shows less free and high entrapped drug compared to reference product with new remote loading agent except patent of onivyde.

Introduction

Liposomes are small vesicles of spherical shape which are created from natural phospholipids and cholesterol. Due to their size, hydrophobic and hydrophilic character (besides biocompatibility), liposomes are favorable for drug delivery. Liposome formulation of anticancer drug is less toxic than the available free drug of the anticancer molecule.[1] Anthracycline drugs working with the principle of stopping the growing of separating cells by interposing into the DNA and, because of that kill mainly rapidly separating cells. Hair, gastrointestinal mucosa, and blood cells are also having these cells. So, class of drug/molecule is highly toxic.[2-3]

Liposomal encapsulation technology is the latest technique adopted by medical investigators to transmit drugs that act as healing promoters to the assured body organs. This type of delivery system proposes the site-specific delivery of vital amalgamations to the body. Microscopic foams known as liposomes are generated by method called encapsulation, this method encapsulate numerous materials and drugs inside the liposome.[4-6]

Liposomes may form a layer around their molecules, protect it from the enzymes in mouth and stomach,

***Corresponding Author:** Dr. Bhupendra G. Prajapati

Address: Department of Pharmaceutics, Shree S. K. Patel College of Pharmaceutical Education and Research, Ganpat University, Ganpat Vidyanagar, Mehsana, Gujarat, India

Email ✉**:** bhupendra.prajapati@ganpatuniversity.ac.in

Tel.: +919429225025

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Table 1: Preliminary screening for active/remote drug loading agents.

S. no.	*Ingredients*	*Quantity used (mg/mL)*								
		009	*010*	*011*	*012*	*013*	*014*	*015*	*016*	*017*
1	Irinotecan free base	4.3	2.0	4.3	4.3	2.0	4.3	2.0	4.3	2.0
2	Sucrose octasulfate salt	250 mM	250 mM	-	-	-	-	-	-	-
3	Sucrose	-	-	250 mM	-	-	-	-	-	-
4	Ammonium sulfate	-	-	-	250 mM	250 mM	-	-	-	-
5	Copper sulfate	-	-	-	-	-	250 mM	250 mM	-	-
6	Ammonium dihydrogen phosphate	-	-	-	-	-	-	-	250 mM	250 mM
7	1,2-Disteraroyl-sn-glycero-3-phophocholine (DSPC)	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81	6.81
8	Cholesterol	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22	2.22
10	Methoxy-terminated polyethylene glycol (MW 2000)-distearoylphosphatidyl ethanolamine (MPEG-2000-DSPE)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
11	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid (HEPES)	4.05	4.05	4.05	4.05	4.05	4.05	4.05	4.05	4.05
12	Sodium chloride	8.42	8.42	8.42	8.42	8.42	8.42	8.42	8.42	8.42
13	Ethanol	79.80	79.80	79.80	79.80	79.80	79.80	79.80	79.80	79.80
14	Water for injection	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL	q.s. to 1 mL

Table 2: Formulation and process variables of irinotecan liposome injection.

Formulation/Process variables		*Levels*	
		-1	*+1*
A	Lipids Concentration in ethanol (mg/g)	100.00	300.00
B	Hydration temperature (°C)	60.00	70.00
C	Hydration time (min)	10.00	50.00
D	Extruder temperature (°C)	60.00	70.00
E	Extruder pressure (psi)	300.00	800.00
Responses		*Acceptance criteria*	
R1	PSD D90 after hydration	500–4000 nm	
R2	PSD D90 after extrusion	100–175 nm	

digestive juices basic solutions, intestinal flora, bile and other components in the human body, and free activists and protect them from all such body fluids. The composition of the liposomes like drugs are shielded from the some of the degradation like oxidation and degradation in normal tissues. This protective phospholipid layer remains intact until the composition of the liposome are transported to the appropriate target tissue, organ, or system where the contents need to be consumed.[7]

All the anticancer molecule used in therapy are very toxic to cancerous and the normal cells. So, site-targeting to the tumor is important for tumor reduction. Encapsulation of anticancer molecules into liposome formula offers targeted delivery of anticancer drugs for the treatment of cancer.

Irinotecan is an anti-neoplastic drug/enzyme inhibitor mainly used to treat colorectal cancer. Irinotecan was approved for the treatment of advanced pancreatic cancer. Irinotecan is rapidly converted into its active metabolite SN-38 in body which is degraded in its inactive form which results in toxicity and discontinuation of the drug, but the development of more potent and less toxic liposomal formulation of such anticancer drugs can help improve the cancer treatment.[8-11]

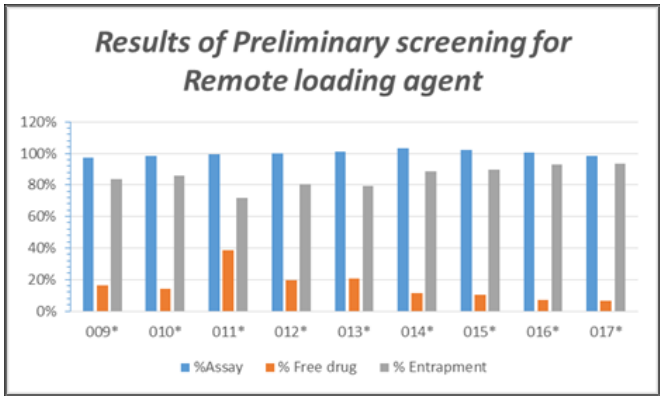

Figure 1: Results of preliminary screening for remote loading agents.

Materials And Methods

Materials

Irinotecan, lipids like 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), MPEG-2000-DSPE and cholesterol, 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES), sodium chloride, sucrose octasulfate salt, sucrose, ammonium sulfate, copper sulfate, ammonium dihydrogen phosphate and other materials were obtained as free of charge from Emcure Pharmaceuticals ltd., Ahmedabad, India. Other analytical grade chemicals, solvents and reagents were used.

Preparation of Irinotecan Liposomes

The present investigation work summarizes the development of irinotecan liposome injection for intravenous infusion, 4.3 mg/mL (10 mL fill), a generic equivalent of reference product onivyde irinotecan liposome injection for intravenous infusion, 4.3 mg/mL (10 mL fill), which is indicated for colorectal cancer dosing for camptosar. Quality by design (QbD) approach has been used to develop a generic irinotecan liposome injection and manufacturing process that ensures the quality, safety and efficacy of irinotecan liposome injection. In present investigation, irinotecan liposome was prepared using ethanol injection for vesicle preparation, extruder as a size reduction, tangential flow filtration for exchanging the outer surface of liposome and active drug loading process for drug encapsulation.

Evaluation of Irinotecan Liposomes

Preformulation Studies

Preliminary screening for active/remote drug loading agents was performed with different salt (like sucrose octasulfate, sucrose, ammonium sulfate, copper sulfate, ammonium dihydrogen phosphate, magnesium sulfate, iron sulfate and sodium meta bisulfate) at different concentration (4.3 mg/mL and 2.0 mg/mL) of saturated drug solution to check the precipitation of drug with salt. Precipitation of API with salt suggests that ammonium dihydrogen phosphate and copper sulfate show sufficient precipitation to entrap the drug in liposome.[12-14]

Preliminary screening for manufacturing process was carried out with ammonium dihydrogen phosphate and copper sulfate as an active loading agent. Formulation was prepared using different manufacturing technique (like active loading, passive loading with spray dryer and passive loading with rotary evaporator) to check %drug

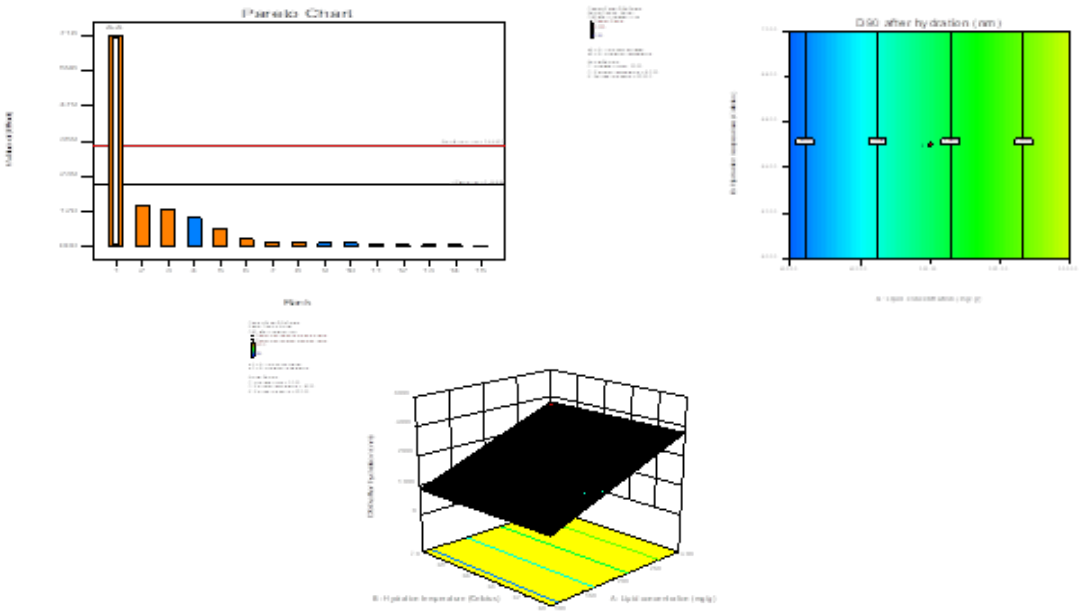

Figure 2: a) Pareto chart, b) Contour plot c) 3D surface plot for effect of lipid concentration and hydration temperature on D90 after hydration.

Table 3: Process optimization study for active loading.

Formulation process variables (independent variables)		*Levels*	
		-1	*+1*
A	Drug loading heating temperature	60.00	70.00
B	Drug loading heating time	30.00	90.00
C	Drug loading cooling temperature	2.00	15.00
D	Drug loading cooling time	10.00	50.00
Responses		*Acceptance criteria*	
R1	Particle size (Z- Avg)	50-150 nm	
R2	% Free drug (Irinotecan)	0-5 %	

Table 4: Analytical result of DoE batches of irinotecan liposome injection

Batch no.	*Formulation process variables (independent variables)*					*Responses (dependent variables)*	
	A: Lipid concentration (mg/g)	*B: Hydration temperature (°C)*	*C: Hydration time (min)*	*D: Extrusion temperature (°C)*	*E: Extrusion Pressure (PSI)*	*Particle size -D90 After hydration (nm)*	*Particle size -D90 After extrusion (nm)*
018	100.0	70.0	10.0	60.0	300.0	1050	160
019	100.0	70.0	10.0	70.0	800.0	591	156
020	100.0	70.0	50.0	70.0	300.0	746	154
021	100.0	60.0	10.0	60.0	800.0	839	161
022	300.0	60.0	50.0	70.0	300.0	2240	164
023	300.0	60.0	10.0	60.0	300.0	2420	160
024	300.0	70.0	50.0	70.0	800.0	3090	148
025	100.0	60.0	50.0	70.0	800.0	727	147
026	200.0	65.0	30.0	65.0	550.0	3750	150
027	300.0	60.0	50.0	60.0	800.0	2550	148
028	100.0	60.0	10.0	70.0	300.0	708	146
029	300.0	70.0	10.0	70.0	300.0	2750	157
030	200.0	65.0	30.0	65.0	550.0	1380	157
031	300.0	60.0	10.0	70.0	800.0	2270	152
032	100.0	70.0	50.0	60.0	800.0	793	151
033	300.0	70.0	10.0	60.0	800.0	3360	150
034	100.0	60.0	50.0	60.0	300.0	1040	179
035	300.0	70.0	50.0	60.0	300.0	3170	164

entrapment, %free drug and particle size (z-avg). Active loading process was selected based on highest entrapment of irinotecan, which is also supported by literature (Patent) for irinotecan liposome.

Preliminary screening for size reduction process for dummy liposomes was carried out with extruder at different pressure (250–350 psi and 750–850 psi) and high-pressure homogenizer at different pressure (15000 psi and 25000 psi) to check %drug entrapment, %free drug and particle size (z-avg). Size reduction by extruder shows better control of %free drug as soft dummy liposomes of lipids which breaks during high shear process. Also active loading process confirms high amount of drug entrapment with less free drug.

Finalization of active/remote drug loading agents were done using trials shown in Table 1 with different remote loading agent with different concentration of API with active loading method with extruder for size reduction method was carried out and evaluated for %drug entrapment and %free drug.

As shown in Fig. 1 ammonium dihydrogen phosphate shows the highest entrapment of irinotecan inside liposome compared to other remote-loading agents. So, ammonium dihydrogen phosphate as a remote loading agent was evaluated for further process optimization study.

Formula and Process Optimization Study for Dummy Liposomes

A formulation optimization study was conducted to evaluate the effect of lipids concentration in ethanol, hydration temperature, hydration time, and extruder temperature

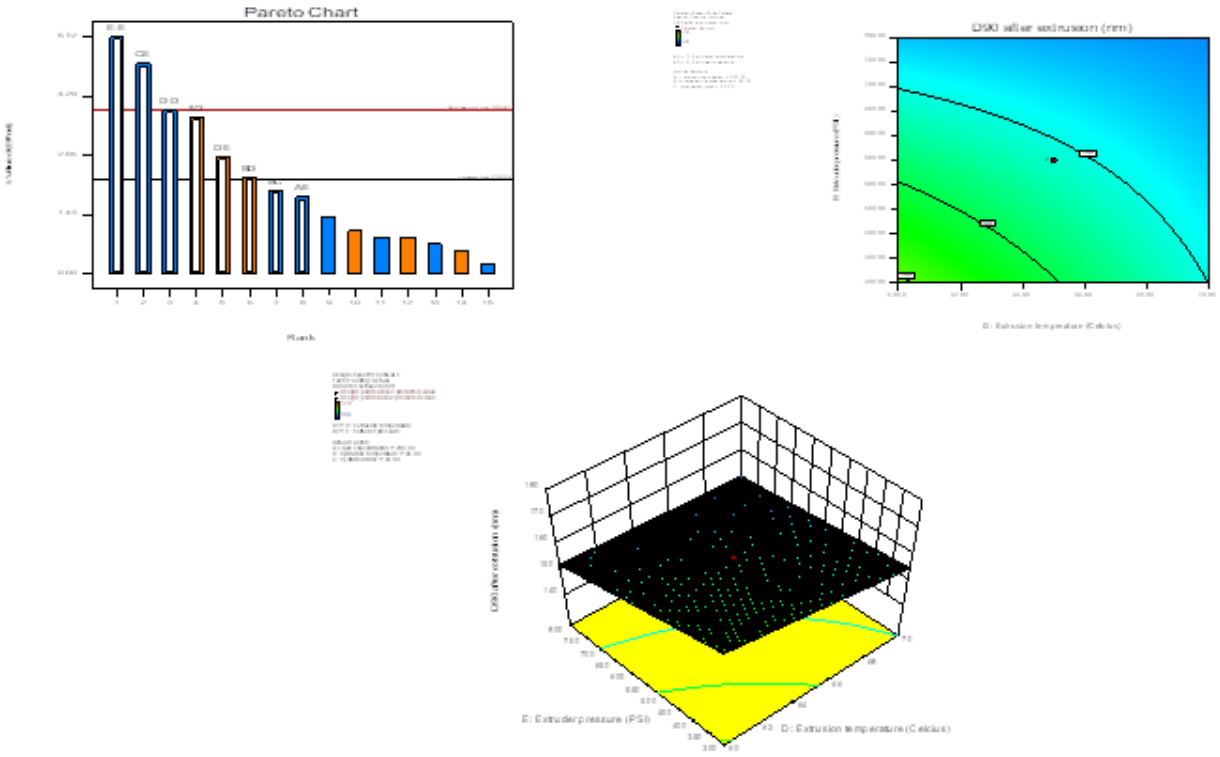

Fig. 3: a) Pareto chart, b) Contour plot c) 3D surface plot for effect of extruder pressure and extrusion temperature on D90 after extrusion.

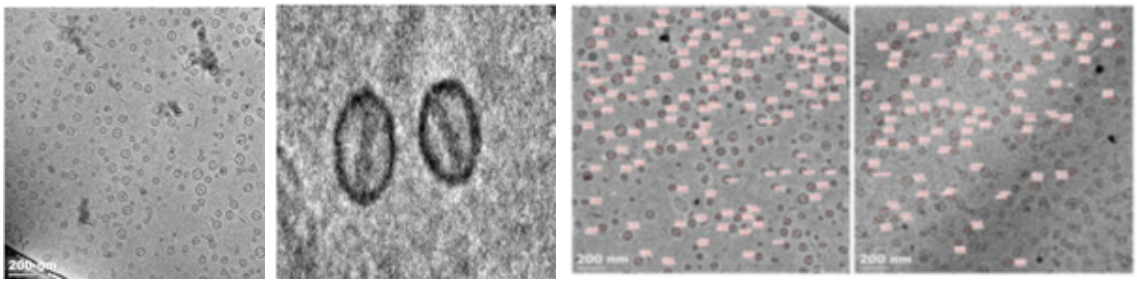

Fig. 4: Cryo TEM images of final formulation of irinotecan liposome injection.

Table 5: Results of DoE trials for optimizing process of active drug loading.

Batch no.	*Formulation process variables (independent variables)*				*Responses (dependent variables)*	
	Drug loading heating temperature (°C)	*Drug loading heating time (°C)*	*Drug loading cooling temperature (°C)*	*Drug loading cooling time (°C)*	*Particle size (nm)*	*%Free drug*
036	70.00	30.00	2.00	50.00	89.9	0.6
037	70.00	90.00	2.00	10.00	91.3	0.7
038	70.00	30.00	15.00	10.00	89.7	20.8
039	60.00	30.00	2.00	10.00	90.5	3.3
040	65.00	60.00	8.50	30.00	89.2	0.64
041	60.00	30.00	15.00	50.00	89	1.52
042	70.00	90.00	15.00	50.00	90.8	0.65
043	60.00	90.00	15.00	10.00	90.1	0.4
044	65.00	60.00	8.50	30.00	90.8	0.7
045	60.00	90.00	2.00	50.00	90.5	0.5

Table 6: Initial and stability results final finished product.

S. no.	*Test*	*Specification*	*Initial*	*1M 2–8°C*	*3M 2–8°C*	*6M 2–8°C*
1.	Description	White to slightly yellow opaque isotonic liposomal dispersion				
2.	Assay of irinotecan	Between 90.0 to 110.0%	99.6%	99.8%	98.9%	98.5%
3.	Assay of DSPC	Between 85.0 to 115.0%	99.2%	98.4%	98.2%	98.7%
4.	Assay of MPEG DSPE 2000	Between 85.0 to 115.0%	97.1%	96.2%	97.1%	97.2%
5.	Assay of cholesterol	Between 85.0 to 115.0%	95.6%	95.2%	95.0%	94.8%
6.	pH of liposome	Between 5.0 to 7.0	5.64	5.60	5.63	5.68
7.	%drug entrapment	NLT 90%	93.8%	92.8%	93.0%	92.7%
8.	% Free irinotecan	NMT 10.%	6.2%	7.2%	7.0%	7.3%
9.	Particle size (z-avg)	NMT 150 nm	110 nm	106 nm	109 nm	112 nm
10	Zeta potential	Between -10 to -40 mV	- 26 mV	- 24 mV	-26 mV	-28mV

and pressure on particle size. The manufacturing process optimization study aimed to understand if there were any interactions of these variables on studied responses. This study also sought to establish the robustness of the proposed formulation. A $2^{(5-1)}$ fractional factorial design of experiments (DoE) with two centre points was used to study the impact of these process factors on the response variables listed in Table 2. Particle size after hydration and extrusion were the parameters for evaluation.

Process Optimization Study for Active Loading

As studied in extrusion process, the temperature of liposomal suspension plays a major role in the drug loading into liposomes. The drug enters into the liposome and forms a crystalline gel structure. The loading of the drug into the liposome should be done at a temperature above the glass transition temperature of the lipids used. In order to check the effect of loading temperature on the CQAs like particle size, free drug at different temperatures were evaluated. A $2^{(4-1)}$ fractional factorial DoE with one centre point was used to study the impact of these formulation factors on the response variables listed in the Table 3. Particle size after (z-avg) and %free drug (irinotecan) were the parameters for evaluation.

The final irinotecan liposomal formulation were evaluated for description, %assay of lipid, particle size, %assay of drug, zeta potential, pH of liposome, %drug entrapment, %free irinotecan and cryo-TEM and charged for the real-time stability study.

Physicochemical Characterization

Description

Prepared liposomal formulation were inspected for description visually.

pH of Liposome

Transfer about 10 mL of liposomal formulation in a clean, dry suitable glassware. Check the pH of the suspension by using a suitable calibrated pH meter at 25 ± 2°C.[15]

Particle Size (Z-avg) and Zeta Potential

The particle size (Z-avg) and zeta potential of irinotecan-loaded liposomes were determined using particle size analyser (Nano ZS 90), based on photon correlation spectroscopy at room temperature angle of detection at 90° angle.[16-18]

%Assay of Irinotecan

The high-performance liquid chromatographic (HPLC) method was developed of the determination of irinotecan ine of its degradation products. The samples were analyzed using YMC, PACK-PRO C18, 150 x 4.6 mm, 3 µm column. The mobile phase comprised of mixture of buffer, acetonitrile and methanol in the ratio of 300:250:500% v/v/v. The flow rate was maintained at 1.2 mL/min and detection wavelength was 383 nm using UV detector. [20-25]

%Drug Entrapment and %Free Drug

The %drug entrapment and %free drug analysis was carried out with manifold extraction assembly (positive pressure) using Oasis® HLB 1cc (30 mg) extraction cartridge to differentiate free drug and encapsulated drug and then differentiated solution was analyzed for assay of irinotecan with above method.[19,25]

%Assay of Lipids

The HPLC method was developed to determine lipid component (DSPC, MPEG 2000 DSPE, cholesterol) in its degradation products. The analysis of samples was carried out using zorbax eclipse XDB C8,150 x 4.6 mm, 5 µ column. The mobile phase comprised water as a mobile phase A and 3.0 g of ammonium acetate in 1000 mL of methanol as mobile phase B. The flow rate was maintained at 1-mL/min and detection wavelength was 215 nm using UV/PDA detector.[21-26]

Cryo-TEM

The cryo-TEM study was conducted at National Forensic Sciences University, India; liposomal irinotecan sample was weighed 8 mg and diluted in 1-mL HPLC grade water.

Cryo-TEM analysis was done using Final formulation samples.

Stability Study

Final irinotecan liposomal formulation were filled and sealed USP type I glass vial with teflon-coated rubber stopper and kept at 2 to 8°C for 6 months. Various stability tests like description, %assay of lipid, particle size, %assay of drug, zeta potential, pH of liposome, %drug entrapment, %free irinotecan were analysed at different time interval.

Results

Irinotecan liposome was prepared with ethanol injection of lipid solution into the buffer and allowed to hydrate in the buffer at higher temperature for certain period of time and the effect of hydration time at higher temperature on the CQAs like particle size etc. were studied. The temperature of the liposomal suspension plays a major role in the ease of extrusion. The temperature to be used will depend on the lipid composition of the liposomes. The operational temperature for extrusion should be above the glass transition temperature of the liposomal suspension for easy size reduction. Different temperatures were used to check the effect on CQAs like particle size during the extrusion process.

The size reduction of the liposomes is based on the pressure at which it is pushed into the extruder containing nucleopore membranes. In general, more the pressure applied, faster the extrusion process. Experiments were carried out by performing extrusion at various pressures to check the effect of pressure on CQAs. As shown in Fig. 2, pareto chart, contour plot and 3D surface plot suggest lipid concentration in ethanol have major impact on the particle size after hydration

Results of formula and process optimization study for dummy liposomes for D90, after hydration (nm) and D90 after extrusion (nm) are listed in below Table 4.

Effect of Factors on D90 After Extrusion

The overlay contour plots of selected independent variable upon the response under study are shown in preceding section. The yellow zone indicates the design space, where all selected response was estimated to be within desired acceptance criteria. The overlay contour plots demonstrated that all the selected design's center points were within the design space. As shown in Fig. 3, pareto chart, contour plot and 3D surface plot suggest extrusion pressure and temperature have major impact on the particle size after extrusion

Results of Process Optimization Study for Active Loading

Results of process optimization study for active loading for particle size (nm) and %free drug are listed in below Table 5.

Effect of Independent Variables on Particle Size (Z-average)

The particle size of final formulation was controlled in desired range using extrusion process, thus particle size of the final formulation was found to be independent of all the processing parameters during active drug loading of irinotecan liposome injection, concluding that the selected factors have insignificant effect on response.

Effect of Independent Variables on Free Drug Content

Irinotecan is highly prone to entrapped inside the liposome because of pH gradient, so within a specified process variable, it is able load inside the liposome. Due to loading mechanism of irinotecan, free drug content of final formulation was found insignificant in studied range of all the processing parameters (independent variables).

Stability Study

Stability study of final formulation of irinotecan liposome, injection was performed for 6 months at 2 to 8°C condition. Stability study final formulation was performed for mainly reason of its degradation and content of %free drug, %assay, %lipid content and particle size.[27-29] Stability study of optimized irinotecan liposome injection was carried out as per the ICH guideline. As this is a liposomal formulation containing lipids with lower TG (less than 50°C), a stability study at higher temperature is impossible. So batch was charged for stability study at refrigerated condition. The results stability study after 6 months with initial were shown in Table 6.

Stability study of final formulation suggest no any degradation of impurity and no any %free drug increase with respect to stability time. Data suggest, there is no any %free drug increment in final formulation. So, based on above data, it can be concluded that final formulation is more stable in liposomal formulation.

Cryo TEM

The Cryo-TEM study was conducted at National Forensic Sciences University, India; liposomal irinotecan sample was weighed 8 mg and diluted in 1-mL HPLC grade water. Following Fig. 4 is the different scale images of Cryo-TEM for final formulation of irinotecan liposome injection.[30-32]

A Cryo TEM image also confirms that %free drug is much less in test product compared to reference product. Also Cryo TEM images confirms the sphericity (more than 90%), particle size (around 90 nm), pegylation (PEG layer on liposome surface) and state of an encapsulated drug (crystalline) inside the liposome.

Discussion

A systematic development was carried out to develop irinotecan liposome injection by assessing various parameters to control liposome's free drug and particle size. Preliminary screening trials for drug (API) loading

suggest that active loading process is more relevant and useful compare to passive loading for producing liposome with higher entrapment and less free drug. Preliminary screening trials for particle size reduction suggest that extruder is more relevant and useful compare to high-pressure homogenization for producing soft liposome with higher entrapment and less free drug. Preliminary screening trials for active/remote drug loading agents suggest that ammonium dihydrogen phosphate is best remote loading agent compare to sucrose octasulfate, sucrose, copper sulfate and ammonium sulfate. Data suggest that batches with an ammonium dihydrogen phosphate show better drug entrapment than all other remote loading agents. Even ammonium dihydrogen phosphate shows better drug entrapment with less free drug compare to sucrose octa sulfate, which innovator uses. So, formula was finalized with ammonium dihydrogen phosphate and other ingredients are same as innovator.

Formula and process optimization study for dummy liposome was conducted to evaluate the effect of lipids concentration in ethanol, hydration temperature, hydration time, extruder temperature and extruder pressure on particle size. A $2^{(5-1)}$ fractional factorial DoE with two centre points was used to study the impact of these process factors on the response variables. Data suggest that all the parameters have impact on particle size of liposome. So, based on design space, optimized process parameters were defined for maintaining the required particle size. Process optimization study for active loading was conducted to evaluate the effect of drug loading heating temperature, drug loading heating time, drug loading cooling temperature and drug loading cooling time on particle size and free drug. A $2^{(4-1)}$ fractional factorial DoE with one center points was used to study the impact of these formulation factors on the response variables. Data suggest that all the parameters have impact on particle size of liposome and %free drug. So, based on design space, optimized process parameters were defined for maintaining the required particle size and %free drug. Stability study of final formulation suggest no any degradation of impurity and no any %free drug is increasing with respect to stability time. Data suggest, there is no any %free drug increment in final formulation, whereas in reference formulation, free drug is increasing with respect to time. So, it can be concluded that final formulation is comparable and more stable than the reference formulation. All characterization was performed on final formulation and all data is comparable with reference formulation. Cryo TEM images also confirm that %free drug is much less in test product compared to a reference product. Also, Cryo TEM images confirm the sphericity, particle size, pegylation and state of encapsulated drug inside the liposome, which shows encapsulation of irinotecan into the liposome.

Conclusion

A $2^{(5-1)}$ fractional factorial DoE with two centre points confirms the impact of particle size of liposome. A $2^{(4-1)}$ fractional factorial DoE with one center points confirms the impact particle size of liposome and %free drug for encapsulation of drug in to the liposome. Stability study, chemical analysis and Cryo TEM study confirm the stability of final formulation and encapsulation of drug in to the liposome and comparable to available reference product. So, based on above all results, it can be concluded that test product is more stable than reference product with very less %free drug in irinotecan liposome injection.

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