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Research Article

DESIGN, OPTIMIZATION AND CHARACTERIZATION OF LAMIVUDINE LOADED SOLID LIPID NANOPARTICLES FOR TARGETED DELIVERY TO BRAIN

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ABSTRACT

The present study was started with aim to develop lamivudine mannose conjugated solid lipid nanoparticles for targeted drug delivery to brain. Mannosylated solid lipid nanoparticles enable improvement of brain bioavailability and reduction of lamivudine toxicity. The lamivudine loaded solid lipid nanoparticles were prepared by solvent injection method. The mannose conjugation on nanoparticles surface was done by reaction between aldehyde group of mannose and free amino function group on nanoparticles surface. The formulation variables were successfully optimized using Box Behnken design. The particle size, entrapment efficiency and zeta potential of optimized formulation were found to be 206.4 nm, 48.12% and -43.6 mV respectively. Nanoparticles showed sustained release profile up to 12 hrs. The mannosylated solid lipid nanoparticles showed low % hemolysis and better uptake inside the macrophages cell as compare to pure drug. Results of this study indicated that mannose conjugated solid lipid nanoparticles would be a promising therapeutic system for efficient delivery of the lamivudine into brain macrophages.

KEYWORDS: Human immunodeficiency virus, Brain targeting, Mannose conjugation, Cellular uptake study.

INTRODUCTION

AIDS is a virally mediated disorder represents a challenge for current antiviral therapies. Antiviral drugs must be gain access to central nervous system in sufficient concentration to eradicate the HIV. Such pharmacokinetic considerations are not met effectively because blood brain barrier prevent entry of antiretroviral hydrophilic drugs like lamivudine (LMV), thus viruses resides in the macrophages cannot be completely killed by conventional therapy. The brain bioavailability of LMV is 0.05%–1.14%, whereas the virologic IC50 of LMV in brain is 0.78%–4.90%[1] it is required to have sufficient LMV concentration in the brain macrophages for efficient therapy. Recently, increasing attentions has been focused on the solid lipid nanoparticles (SLNs); because it offers advantages like-possibility of controlled drug release, improve drug stability and low toxicity[2]. Incorporation of LMV in SLNs results in decrease bone marrow toxicity, increase bioavailability and improve antiviral activity. The active targeting of SLNs containing entrapped antiretroviral drug is possible approach to overcome the limitations of conventional therapy. Mannose receptors present on the surface of macrophages, and therefore mannose conjugated SLNs may target the macrophages, which may improve drug bioavailability in the brain, which improve the therapeutic outcomes[3, 4].

MATERIALS AND METHODS

Lamivudine was kindly gifted by Ranbaxy Laboratories Limited, Dewas, India. Stearic acid and was purchased from Loba chemical Ltd., India. Tween 20, Acetone AR and Ethanol AR were purchased from S.D. Fine chemical Ltd., India. Kolliphor RH 40, Kolliphor EL were purchased from BASF, India. All other reagents, solvents and chemicals were analytical grade and purchased locally.

Preparation of Lamivudine loaded solid lipid nanoparticles

LMV loaded SLNs were prepared by solvent evaporation technique[5]. In practice calculated quantity of LMV, stearylamine and stearic acid were dissolved in in 10 ml ethanol: acetone (1:1) and maintained at 60^{0}C. The ratio of stearylamine to stearic acid was maintained to 1:2. The aqueous surfactant solution containing 10 ml distilled water and Tween 20 was maintained at same temperature. The organic solution injected by using syringe (21 gauge) into aqueous phase and stirred at 4000 rpm for 10 minutes with heating using a hot plate at 60^{0}C. The resulting hot dispersion was subjected to probe sonication (VCX500, Sonics and materials, U.S.A.) at 20% amplitude for 10 minutes and cooled to room temperature.

Mannose conjugation on SLNs surface

Mannose conjugation of prepared SLNs was done by ring opening of D-Mannose followed by reaction of an aldehyde group of mannose with the free amino group of SLNs surface in sodium acetate buffer (pH 4.0)[6, 7]. Briefly Lamivudine SLNs were incubated with D-mannose in sodium acetate buffer (pH 4.0) for 12 hrs. After 12 hrs SLNs were subjected to extensive dialysis against distilled water for 30 minutes to remove unconjugated mannose. Furthermore mannosylated SLNs were dispersed in distilled water containing Tween 20.

Experimental design

For the optimization of the formulation, concept of design expert was used. There were three major factors affecting the formulation, total lipid (%w/v), Lipid: drug ratio, surfactant concentration (% w/v) as well as two responses to be optimized viz., particle size and % entrapment efficiency. A three-level three- factor Box –Behnken design (Design Expert, version 10, Stat-Ease) was used. The design consists of center points in replicate and the set of points lying at the midpoint of each edge

of the multidimensional cube that defines the region of interest. The independent variables selected along with their levels are shown in table 1. The seventeen batches of Lamivudine SLNs were prepared as suggested by software and responses were measured.

Particle size and zeta potential measurement

Particle size, polydispersity index and zeta potential of the SLNs dispersions were determined using Zetasizer Nano ZS (Malvern Instruments Ltd., UK) equipped with 5-mV He-Ne laser. Lamivudine loaded SLN dispersions were diluted ten times with distilled water and placed in polycarbonate cuvette. The analysis was carried out at an angle of 90° at a temperature of 25°C.

Percent entrapment efficiency

The percentage entrapment efficiency of Lamivudine in the lipid matrix was measured using the indirect method. The Lamivudine loaded SLN dispersions were diluted ten times and subjected to ultra-centrifugation at 80,000rpm for 1 hour at 4°C using Optima Max XP ultracentrifuge (Beckman Coulter, U.S.A.) to separate the unentrapped drug. The pellet of lipid was formed at the bottom. The aqueous phase above the pellet (i.e., the supernatant) was carefully separated and analyzed by UV spectrophotometry at λmax of 270 nm after suitable dilution with water. Percentage entrapment efficiency was calculated by following equation.

$$Percent\ entrapment\ efficiency = (\mathrm{WL} - \mathrm{WF}) \times 100 \div \mathrm{WL}$$

Where, WL = Theoretical content of LMV in SLNs dispersion
WF = Free LMV in supernatant as quantified by UV spectrophotometry.

In-vitro release study

The *in-vitro* release of LMV from LMV solution and LMV M-SLNs were performed by a dialysis diffusion technique in phosphate buffer pH 7.4 at 37±0.5^{0}C[8]. Briefly, 3mg of LMV and equivalent of LMV SLNs were separately dispersed in 4 ml of phosphate buffer pH 7.4. The resulting dispersion was put in the dialysis bag (MWCO 13000-14000 Da, HiMedia, India) and was dialyzed separately against 150 ml of phosphate buffer (pH 7.4). At predetermined intervals 5ml of aliquots were withdrawn, filtered through 0.45 µm membrane filter and Lamivudine content was determined spectrometrically. The release medium was replenished with an equal volume of fresh phosphate buffer maintained at same temperature. Each experiment was performed in triplicate and the mean value of percent cumulative release and standard deviation at each time point were calculated.

Hemolytic toxicity study

Hemolytic study was performed as per earlier reported procedure with slight modification[9, 10]. Briefly whole human blood was collected using EDTA in blood collecting vials (HiMedia, India) and centrifuged at 7000 rpm for 10 minutes. The RBCs were collected and resuspended in normal saline. In 2 ml RBCs suspension, 4ml distilled water was added, which was considered as 100 % toxic. Similarly 4 ml of normal saline was added in 2 ml RBCs suspension assumed to produce no hemolysis. 2 ml of LMV solution, SLNs and LMV M-SLNs were added separately in In 2 ml RBCs suspension and incubated for 2 hrs. After 2 hrs. the formulations were centrifuged at 7000 rpm for 10 minutes and supernatants were collected. The absorbance of supernatant measured spectrometrically and % hemolysis was calculated, using following equation. All experiment were performed in triplicates and values expressed graphically as mean± SD.

$$\%\ Hemolysis = \frac{Abs - Ab50}{Ab100 - Ab50} \times 100$$

Where *Abs* is the absorbance for the sample. *Ab50* is the absorbance for 0% hemolysis. *Ab100* is the absorbance for control.

Cytotoxicity study

Macrophages cells were seeded in 96 well plate at density 1*10^4 cells in 100 µl of Dulbecco's modified Eagle's Medium (DMEM) high glucose with 10 % Fasting blood sugar (FBS) medium. The plated were incubated in anerobic condition with 5 % CO2 at 37 ^{0}C for 48 hrs to obtained complete monolayer. Cells were then incubated with serial dilutions (1, 50, 100 µM) of samples (LMV, LMV SLNs, LMV M-SLNs), medium as negative control and dimethyl suphoxide as positive control for 37 ^{0}C, 5% CO2 for 48 hrs. After incubation times, the methyl thiazole tetrazolium test (MTT) was performed as per procedure described in[11]. The results were expressed as percentage of cell viability.

Cellular uptake study

The cells were seeded in 12 well plates, so that each well contained 5 x 10^5 cells in 2 ml DMEM H/G medium containing 10% FBS. Culture plate containing cells, was incubated under anaerobic conditions, bubbled with 5% CO_2 at 37°C for 48h to obtain complete monolayer of cells in well. Medium containing serial dilutions (50 & 100ppm) of LMV SLNs and LMV M-SLNs was added to wells in triplicate and plates were incubated at 37°C and 5% CO_2 for 12 hrs. After incubation supernatant was carefully collected and cells were given washing to remove any adherent formulation. 1 ml of 1% w/v SDS was added to each well to break the cells. The plate was shaken for 5 mins on plate shaker and cell lysate was collected. The lysate samples were processed and LMV content was analyzed using HPLC. The % uptake of formulation by Macrophages cells was calculated.

Stability studies

Stability studies of SLNs dispersion were conducted according to International Conference on Harmonization guideline (Q1AR2). To conduct stability study, 60 ml batch was prepared and it was divided into six different portions each of 10 ml and filled into glass vials, sealed with rubber stopper and metal clips. Of these, three portions were stored at 25^{0}C/60% RH ± 5% RH and remaining portions stored at 40^{0}C/75% RH ± 5% RH in stability chamber (Thermolab, India) for a period of three months. After three months the samples were analyzed for drug content and *In-vitro* release profile.

RESULT AND DISCUSSION

Optimization of formulation variables

Seventeen batches of NLCs were prepared as suggested by software DESIGN EXPERT 11 (Statease) and analyzed for particle size and entrapment efficiency. Results are shown in following table 2. The selected independent variables were found to influence the two dependent variables. All batches showed particle size in the range between 184-250 nm, entrapment efficiency 75-40 %. The models fitted for each response were linear, cubic, quadratic and two factor interaction. The results found are shown in table 3. The linear model was found to fit best for all two responses. Using the ANOVA, the equation involving main factors were determined based on the estimation of various statistical parameters.

Table 1: Variables in Box Behnken design

S.No.	Independent variables	Levels		
		-1	0	+1
1	X_1 = total lipid (% w/v)	1	2	3
2	X_2 = lipid : drug ratio	20	50	80
3	X_3 = surfactant concentration (%w/v)	1	1.5	2

Table 2: Observed responses in design

Run	A = total lipid (% w/v)	B = lipid : drug ratio	C = surfactant concentration (%w/v)	Response 1 Particle size (nm)	Response 2 Entrapment efficiency (%)
1	1	20	1.5	192	43.25
2	2	50	1.5	207	53.4
3	1	80	1.5	189	49.96
4	2	50	1.5	208	57.21
5	2	20	1	215	47.41
6	3	80	1.5	220	75.20
7	2	20	2	200	44.38
8	1	50	1	200	47.10
9	2	50	1.5	211	51.34
10	3	50	2	225	52
11	3	20	1.5	236	47.87
12	2	50	1.5	208	58.351
13	3	50	1	250	54.208
14	1	50	2	184	40.045
15	2	50	1.5	210	60
16	2	80	2	198	72
17	2	80	1	218	75

Table 3: Model summery for two responses

Models	R^2	Adjusted R^2	Predicted R^2	Standard deviation	Remark
Response 1					
Linear	**0.9364**	**0.9217**	**0.8708**	**4.69**	Suggested
2FI	0.9517	0.9227	0.7683	4.66	
Quadratic	0.9783	0.9505	0.6882	3.73	
Cubic	0.9976	0.9904		1.64	Aliased
Response 2					
Linear	**0.7178**	**0.6527**	**0.4430**	**6.37**	Suggested
2FI	0.7859	0.6575	0.0402	6.32	
Quadratic	**0.9296**	**0.8390**	**0.2967**	**4.34**	Suggested
Cubic	0.9707	0.8826		3.70	Aliased

Table 4: ANOVA for responses

Source	Response 1		Response 2	
	F	P>F	F	P>F
Model	35.14	< 0.0001	10.27	0.0028
A	247.81	< 0.0001	15.97	0.0052
B	2.91	0.1316	53.88	0.0002
C	51.94	0.0002	1.50	0.2608
AB	3.04	0.1248	6.44	0.0388
AC	1.46	0.2666	0.3326	0.5822
BC	0.4496	0.5240	0.0000	1.0000
A^2	4.20	0.0795	10.28	0.0149
B^2	3.25	0.1145	4.49	0.0719
C^2	1.50	0.2603	0.1346	0.7246

Table 5: Predicted and observed responses for the optimized formulation

Response	Predicted value	Observed value	% error
Particle size	208	206.4	0.769231
% EE	49.164	48.12	2.123505

Table 6: Uptake of LMV SLNs and LMV M-SLNs in macrophages cell-line

LMV Concentration (ppm)	% Uptake	
	LMV SLNs	LMV M-SLNs
50	11.5055 ±1.961	27.271 ±2.7503
100	12.1536 ±1.463	30.487 ±1.369

Table 7: One way ANOVA table for responses (Drug content in SLNs before stability study and after exposure to R.T.)

Source	SS	df	MS	F=
Between treatments	37.3649	1	37.3649	100.8322
Within treatments	2.9645	8	0.3706	
Total	40.3294	9		

Table: 8 One way ANOVA table for responses (Drug content in SLNs before stability study and after exposure to Accelerated storage condition)

Source	SS	df	MS	F=
Between treatments	65.5462	1	65.5462	222.3715
Within treatments	2.3581	8	0.2948	
Total	67.9043	9		

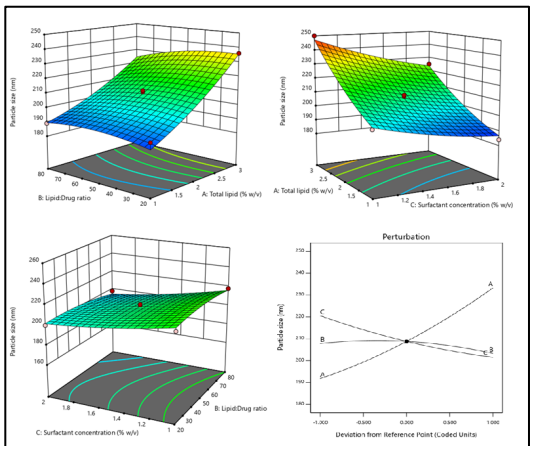

Figure 1: Response surface plot three-dimension showing effect of independent variables on particle size

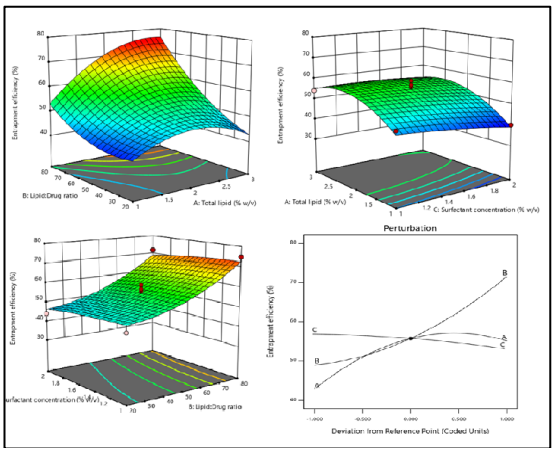

Figure 2: Response surface plot three-dimension showing effect of independent variables on % entrapment efficiency

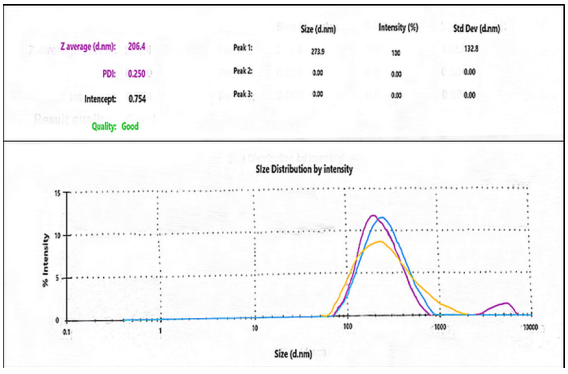

Figure 3: Particle size of optimized SLNs formulation

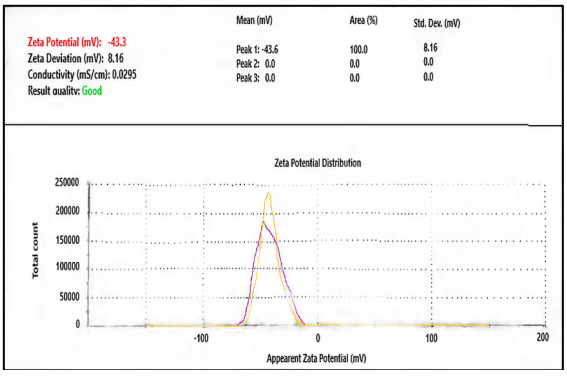

Figure 4: Zeta potential of optimized SLNs formulation

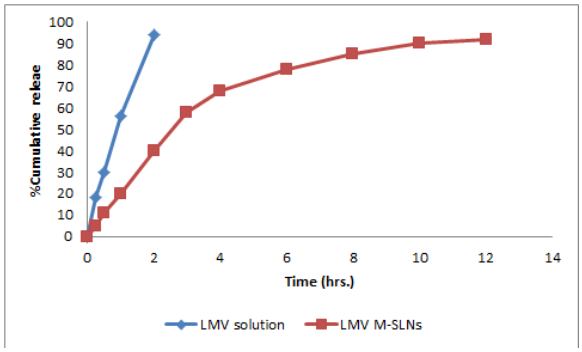

Figure 5: *In-vitro* release profile of LMV solution and LMV M-SLNs in phosphate buffer (pH 7.4)

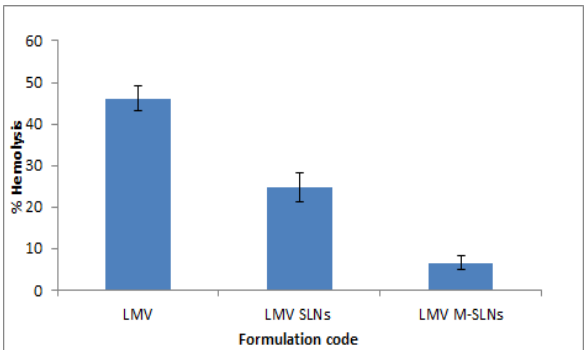

Figure 6: Data of hemolysis study of different SLNs formulation

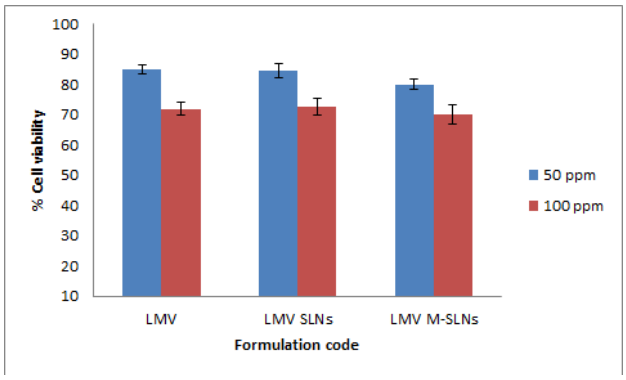

Figure 7: MTT test on cell line incubated with various SLNs formulations

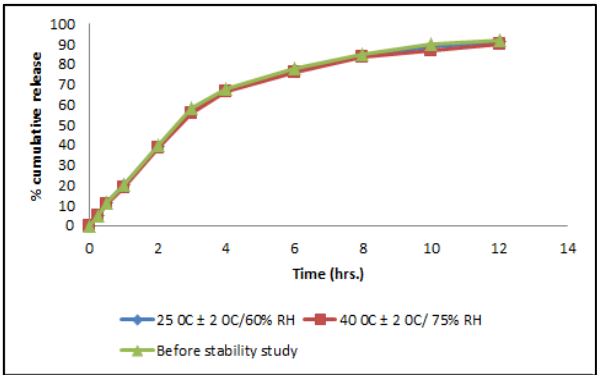

Figure 8: Comparative plot of *in vitro* drug release profile of LMV SLN stored at room temperature, accelerated storage condition and before stability study.

Influence of independent variables on particle size

Obtained particle size of all the batches, is shown in table 2. The most significant factor contributing to the variation in particle size was A as shown by value of the coefficient. The factor A showed positive effect on the particle size. The increase in particle size is a logical consequence of the increase in amount of lipid (factor A) since the particles are composed of this lipid[12]. The factor C showed negative effect on the particle size which means an increase in the value of C will show decrease in the value of particle size[13]. This observed decrease in the particle size with increase in surfactant concentration can be explained by greater number of surfactant molecules available to emulsify the lipid particles, leading to more efficient emulsification which results in smaller particle size. The quadratic model explaining the effect of various factors on particle size was;

$$\text{Particle size} = 208.80 + 20.75 * A - 2.25 * B - 9.50 * C - 3.25AB - 2.25AC - 1.25BC + 3.72A^2 - 3.28\,B^2 + 2.23\,C^2$$

Factor B not showed significant effect on the particle size. Further analysis using ANOVA (table 4) showed significant effects of the independent variable (P>F, 0.0001) on response 1. The model F-value 35.14 implies that the model is significant. A good correlation between predicted and observed value as indicated by R^2 value of 0.9783. The surface response plot and perturbation plot generated by software are shown in figure 1.

Influence of independent variables on percent entrapment efficiency

The obtained % EE of all the batches is shown in table 2. The most significant factor contributing in variation of % EE was B. Increase in the level of B (lipid: drug ratio) result in increase in EE. This is because of increase in amount of drug in the formulation with respect to lipid.. The second factor after B contributing to the variation in % EE was A as shown by value of the coefficient. Increase in the level of factor A (total lipid % w/v) from -1 to +1 result in increase in the EE. This increase in EE due to increase in factor A is observed because there is a greater amount of lipid available to accommodate the added drug. Factor C showed non-significant negative effect on % EE. The negative effect on % EE was due to extraction of LMV out of the lipid matrix with increase in concentration of surfactant. The quadratic model explaining the effect of various factors on % EE was;

$$\%\,EE = 55.80 + 6.13 * A + 11.25 * B - 1.87 * C - 5.50AB + 1.25AC + 0.00BC - 6.87A^2 + 4.47B^2 - 0.7750\,C^2$$

Further analysis using ANOVA (table 4) showed significant effects of the independent variable (P>F, 0.0001) on response 2. The model F-value 10.27 implies that the model is significant. A good correlation between predicted and observed value as indicated by R2 value of 0.9296. The surface response plot and perturbation plot generated by software are shown in figure 2.

To get optimized formulation, numerical optimization was performed using Design expert software. The optimization of formulation was based on criteria of minimum particle size, and maximum drug entrapment. The predicted levels of formulation factor obtained by the software were 2% w/v/ of total lipid, lipid: drug ratio of 21.64, 1.5% w/v of surfactant concentration. The optimized batch of NLCs was prepared and predicted values of responses were compared with observed values and % error was calculated (table 5). The observed values of responses were found to be close to the predicted value evident from less value of % error. By this the validity of the design was proven[14].

The particle size and zeta potential of optimized SLNs formulation were found to be 206.4 nm and -43.6 mV respectively (Figure 3 and 4). The high value of zeta potential indicates the stability of nanoformulation.

***In-vitro* release study**

In-vitro drug release study is a measurement of release of active pharmaceutical ingredient (API) from the formulation matrix, is important evaluation parameter for product development and quality control. In present study in-vitro release study of LMV from LMV suspension and LMV SLNs were performed using dialysis tube diffusion technique by using dissolution apparatus. Percent cumulative release of Percent cumulative release of LMV from LMV solution and LMV SLNs is graphically represented in figure 5. SLNs showed sustained release profile up to 12 hrs.

Hemolytic toxicity study

Free drug i.e. LMV showed hemolytic toxicity up to 46.06±0.29%. The LMV SLNs exhibited hemolytic toxicity up to 24.71±3.37% and LMV M-SLNs showed toxicity up to 6.55±1.64% (Figure 6). The reduction in hemolytic toxicity of LMV SLNs and LMV M-SLNs was in comparison to free LMV was possibly due to the entrapment of LMV in a biocompatible lipid matrix. The hemolytic toxicity of LMV SLNs is may be due to surface amino group induced by stearylamine and this is the major limitation of such polycationic systems[6]. Mannose conjugation to the SLNs surface significantly lowered hemolytic toxicity due to inhibition of interaction of the charge amine group and RBCs related with nonconjugated SLNs.

Cytotoxicity study

In-vitro cytotoxicity of different SLNs formulations was evaluated by using MTT assay. The % cell viability of cells after exposure to various concentrations of SLNs are shown in figure 7. Since cell viability was found to always above 70% even at the higher concentration (100 ppm). There is no significant difference of cell viability between LMV, LMV SLNs and LMV M- SLNs group. These results proved the *in-vitro* safety of the SLNs. However long term in-vivo toxicity should be evaluated.

Cellular uptake study

Results of *in-vitro* cellular uptake study of different SLNs formulations are shown in table 6. Mannosylated LMV SLNs showed significantly higher uptake inside the cells as compare to unconjugated SLNs. This showed Mannose conjugation did increase the accumulation of LMV entrapped in SLNs in brain.

Stability study

Stability studies were carried out for the developed LMV M-SLNs in two storage conditions, i.e. 25 ± 2°C, 65 ± 5% RH and 40 ± 2°C, 75 ± 5% RH, for 3 months according to ICH guidelines. The results of *in-vitro* studies are shown in figure 8. It was observed that at the end of 12 hours almost equal i.e. 91.02% and 90.12% of LMV was released from LMV M-SLNs stored at R.T. and accelerated storage conditions respectively, which was also close to drug release from SLNs before stability study i.e. 92.12%. Similarity factors were calculated and found to be 81.4438 and 62.606. The values of similarity factors above 50 indicate no significant difference in release behavior of formulation after storage.

Percent LMV content in M-SLNs stored at R.T. and accelerated storage conditions was determined by UV spectroscopy. The drug content before stability study was considered as 100 %. The % LMV content in M-SLNs stored at R.T. and accelerated storage conditions was found to be 96.134 ± 0.785% and 94.880 ± 0.6815% respectively. The data treated with one way ANOVA to find out significant difference between the %drug content of formulation stored at different storage conditions. The results of ANOVA showed in table 7 and 8. In table 7 the f value was 100.8322. The p value was less than 0.00001, indicated significant difference between drug content in M-SLNs before stability and stored at R.T. In table 8 the f value was 222.3715. The p value was less than 0.0001, indicated significant difference between drug content in M-SLNs before stability and stored at accelerated storage conditions. The both results showed significant difference in drug content indicated instability of LMV.

CONCLUSION

In this study, LMV loaded mannosylated SLNs as a brain targeted drug delivery system was prepared and characterized. The formulation variables were successfully optimized by Box Behnken design. The SLNS showed particle size around 206.4 nm and zeta potential -43.6 mV. LMV Man SLNs showed low % hemolysis and better uptake inside the macrophages cells as compared to unconjugated LMV SLNs. Accelerated stability study conformed the stability of SLNs formulation. Thus study concluded that mannose conjugated LMV SLNs are a potential drug delivery system for brain targeted delivery of anti-retroviral drug.

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