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Research Article

FORMULATION AND EVALUATION OF SUBMICRONIC EMULSIONS OF AMPHOTERICIN B

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ABSTRACT

Amphotericin B is a polyene antifungal agent, which despite its nephrotoxicity remains drug of choice for most severe systemic fungal infections. The present study was hypothesized to test whether submicronic emulsions of Amphotericin B would be suitable for decreasing the nephrotoxicity of the drug and improve the uptake by reticulo endothelial system. Submicronic lipid emulsions of Amphotericin B have been prepared using spontaneous emulsification technique. The physical parameters and stability of emulsions were evaluated. The *In-vitro* release was investigated in pH 7.4 phosphate buffer at 37˚C. The concentrations of Amphotericin B in various organs were determined by third order derivative spectroscopy after IV administration of Amphotericin lipid emulsion and marketed Amphotericin B solution in tested organs. The AUC and MRT of Amphotericin B lipid emulsion were significantly higher than those of Amphotericin B solution especially in liver, spleen, heart and lung tissues. A much lower drug levels were obtained in kidney after administration of Amphotericin B lipid emulsion as compared to Amphotericin B solution. Biodistribution studies revealed that increasing concentration of Amphotericin B in liver with peak levels at 10h after administration of Amphotericin B lipid emulsion followed by decline in tissue levels after administration of marketed intravenous injection of Amphotericin B solution peak concentrations in liver were immediately obtained at 1h followed by decline in the drug tissues. The AUC of Amphotericin B lipid emulsions in liver was increased 4.85 times as compared to Amphotericin B solution. The higher concentration of Amphotericin B in the tissues (1.13 and 1.55 in spleen and lung respectively) indicated that Amphotericin B lipid emulsion accumulated in RES organ. In conclusion, our results suggest that an emulsion obtained by spontaneous emulsification process would be suitable for use as a drug carrier and which ultimately decreases the nephrotoxicity of Amphotericin B.

Keywords: Amphotericin B, Submicronic emulsions, Spontaneous emulsification

INTRODUCTION

Intravenous emulsions containing emulsified vegetable oils have been in clinical use for over 30 years. They were originally used for parenteral nutrition [1]. They were only resorted to when a patient was unable to obtain sufficient nourishment orally. The use of injectable emulsions in parenteral nutrition is justified by the fact that their caloric content per unit volume is greater than that of conventional carbohydrate or protein solution [2]. Emulsions must be sterile, isotonic, non pyrogenic, nontoxic and stable physically and chemically [3, 4, 5]. The particle size of the droplets must be less than 1µm [4, 6]. Recently submicronic lipid emulsions have been employed as colloidal carriers for the intravenous administration of lipophilic drugs [7, 8, 9,10].

The objective of this study was to prepare an emulsion whose particles mimic both physically and chemically the properties of natural lipoproteins. Lipoproteins are endogenous particulate carriers responsible for transport of cholesterol and other lipids in blood circulation. LDL which is major carrier of cholesterol to extra hepatic tissue and liver is particularly active in the uptake of LDL, accounting for the clearance of at least 70% circulating LDL[6].

Amphotericin B is a strong antifungal polyene antibiotic but its low solubility leads to poor bioavailability through oral route [5, 9]. The commercially available injectable forms contain deoxycholate as a solubilizer but drugs poor haematological tolerance and nephrotoxicity limits their usefulness. Lipid formulations are captured by reticuloendothelial system and are delivered to site of infection thus reducing their side effects [9, 10]. Amphotericin B an ampiphilic drug, insoluble in water and oil and therefore presents formulation problems. The present work reports, studies to prepare submicronic emulsions of Amphotericin B for intravenous use.

An ideal method for the preparation of injectable emulsion should be simple, reproducible and easy to scale up[6]. There exists a wide variety or techniques for the formulation of emulsions[11]. The techniques used to prepare such injectable emulsions involve ultrasound [12] and/or two stage homogenizations and more recently micro fluidizer [13, 14].

In this work, we present efficient method for preparing injectable emulsions of Amphotericin B based on spontaneous emulsification. Submicronic emulsions are prepared by adding an organic phase, containing oil and lipophilic surfactant to an aqueous phase containing the surfactant.

MATERIALS AND METHODS

Amphotericin B was procured from Synbiotic Ltd. (Gujarat). The oily phase, a mixture of saturatedchain fatty acids (Miglyol 812) a gift sample from Sasol (Germany gmbH). The lipophilic surfactants Epikuron 100 and Ovothin were gift samples from Degussa Bioactive (Deutschland gmbH). Ethyl oleate and glycerol were purchased from S.D. fine chemicals (Mumbai). Soyabean oil and Corn oil were purchased from Rajesh chemicals (Mumbai). The hydrophilic surfactant Pluronic F68, copolymer of polyethylene oxide and polypropylene oxide was a gift sample from BASF (Mumbai). All other chemicals were of analytical grade. The *in-vivo* studies were conducted according to the protocols approved by the Institutional Animal Ethical Committee (IAEC) Regn. No. 1358/ac/10/CPCSEA, Vishnu Institute of Pharmaceutical Education & Research, Narsapur, Medak District, Andhra Pradesh, India.

Preparation of Injectable Emulsion

The percentage of Miglyol 812 was varied (5, 7.5, 10%) and different combination of surfactants were tested. An organic phase containing the Amphotericin B, Ovothin, Epikuron 100 and the oil was prepared, as was an aqueous phase containing Pluronic F 68, glycerol and distilled water. The oily alcohol solution was then slowly added into the aqueous phase under moderate magnetic stirring. The light yellow milky emulsion formed spontaneously. The emulsion forms because the two solvents are completely miscible while lipophilic component became progressively less soluble. The organic solvent was removed by using rotary evaporator under reduced pressure.

Characterization of Emulsions[15, 16]

1. Macroscopic and Microscopic appearance

The color and homogeneity of emulsion and presence of creaming or phase separation were observed visually. The homogeneity of emulsion was also examined by light microscopy.

2. Particle Size distribution analysis[17, 18]

The mean diameter of oil droplets was estimated by photon correlation spectroscopy using a Mastersizer (Malvern Masterisizer) S ver. 2.19. Malvern Mastersizer with analysis model set at poly-disperse sample with Range lens: 300 RF mm, Beam length: 2.40mm and Obscuration in between 16-20%.

3. Percent Drug loading

Accurately measured quantity of emulsion was taken and to it 1ml concentrated Hydrochloric acid, Dimethyl sulfoxide and trichloroacetic acid was added and centrifuged. To the clear supernatant solution methanol and trichloroacetic acid were added and absorption was read at 332 nm.

4. In-Vitro Drug Release

In-vitro drug release from emulsion was determined by using specially assembled apparatus. A tube of 3.5cm length and 2.5 cm diameter was fixed with parchment paper. Accurately measured quantity of emulsion corresponding to 1mg of drug was diluted to 3mlwith saline phosphate buffer pH 7.4 and filled into the tubes. The tubes containing the emulsion was then tied to the shaft of the basket type USP XII dissolution apparatus which was then lowered into 100ml beaker containing 50 ml saline phosphate buffer pH 7.4. The contents of the beaker were stirred at 100 rpm at a temperature 37˚± 2˚C throughout the experiment. 5ml of sample were withdrawn periodically after every 1hr. After every withdrawal the samples were replaced by fresh medium. The withdrawn samples were analyzed on Jasco UV Spectrophotometer at 332nm.

5. Accelerated Stability Studies

A. Centrifugation

The emulsions were centrifuged in a 10cm radius centrifuged tube for 1h at 3000 rpm. The treated emulsions were then evaluated for creaming, cracking and phase separation.

B. Freeze thaw cycling

The emulsions were subjected to freeze thaw cycling, by cycling them between 4˚C and 45˚C each for 24h alternatively and observed for creaming, cracking, and sedimentation and phase separation after 10 such cycles.

C. Medium term stability

The particle size, pH, drug content and *In-vitro* release of emulsions were measured after 15, 30 and 60 days of storage in the dark at 4˚C.

Bio-Distribution studies in rats[19, 20]

Bio-distribution studies were carried out in rats weighing 150±20g. The animals were fasted overnight and allowed to free access to water. Amphotericin B lipid emulsion (A.B.L.E) and marketed preparation (Amphotericin B solution A. B. Sol.) were injected intravenously into rats through tail vein (Table-2). For each preparation and each sampling time point, six mice were treated with a single dose of 250µg/ml of A.B.L.E and A. B. Sol. respectively. The rats were sacrificed at the end of the 1h, 2h, 4h, 8h, 12h, 16h, and 24h. Liver, kidney, spleen, lung and heart were isolated and washed with normal saline. All organs were homogenized, extracted with dimethylsulfoxide and sonicated and centrifuged at 3000 rpm. The extracts obtained were further diluted and subjected to third order derivative spectroscopy[21, 22] at 399 nm. Tissue concentration data of Amphotericin B obtained from rats were pooled to provide mean concentration data. The area under tissue concentration – time curve (AUC) was calculated by using linear trapezoidal rule[23, 24]. The area under the first moment curve (AUMC) was calculated similarly using the trapezoidal rule. The value of F was calculated as follows

$$F = \frac{AUC_{A.B.L.E.}X\ dose_{A.B.Sol.}}{AUC_{A.B.Sol.}X\ dose_{A.B.L.E}}$$

REUSLTS AND DISCUSSION

The formulations prepared with Soyabean oil were more viscous and difficult to disperse during the emulsification process, even at the lower concentrations (2.5, 3.75%) of the oil. The stable emulsions could not be obtained with Soyabean oil and resulted in creaming after centrifugation and total phase separation after freeze thaw cycling. The formulations prepared with Miglyol were yellow and fluid in nature. A 5% oil concentration resulted in stable emulsion. Increasing the oil concentration from 5% to 7.5% resulted in creaming of emulsion and phase separation after freeze thaw cycling.

Use of single surfactant did not result in stable emulsion and therefore combination of surfactants was used. A combination of two surfactants Pluronic F68 and Epikuron 100 were found to be less effective as compared to combination of Pluronic F68 and Ovothin. Addition of 0.5%-0.6% of Epikuron 100 improved the stability of emulsion showed no phase separation even after 10 freeze thaw cycles and was stable up to two months at 2˚C as indicated by medium term stability studies.

pH of all formulations was in range of 4-6 and was not affected by change in oil or surfactant concentration.

The emulsion prepared with Soyabean oil had much lower drug loading as compared to those prepared with Ethyl oleate and Miglyol, which had good drug loading in the range of 90-95%.

BatchE2 showed 90% of particles were less than 0.91µm, however 10% of particles were above submicronic range. This formulation would not be suitable for parenteral administration. Batches E3 and E4 had wide particle size distribution with 25% of particles above submicronic range, similar results were obtained with batch E1 also. Batch E11 showed all the particles were in submicronic range, 10% of particles were less than 1.17µm, 50% of particles were less than 0.29µm, 75% of particles were less than 0.36 µm and 90% of particles were less than 0.46 µm, thus making suitable for intravenous administration figure 1.

M1 and M3 batches prepared with Miglyol had 50% of particles above submicronic range, however batch M2 had all particles in submicronic range 10% of particles were less than 0.54 µm, 50% of particles were less than 0.74 µm, 75% of particles were less than 0.78 µm, 90% of particles were less than 0.94 µm. figure.2

In-vitro release profiles of batches E11figure.3 and M2 figure4, showed burst release with about 58% of drug releasing at the end of 1h and followed by constant release up to 5h Table I

Third order derivative spectroscopy was used to determine the drug concentration in the tissues as it eliminates spectral interference arising from other endogenous compounds and permits direct quantification of Amphotericin B on the basis of intensity of peak appearing at 399nm.

Derivative processing enhances sharp features transforming the normal spectra of Amphotericin B into series of peaks and troughs that differ in position and intensity with the order of derivatization.

Biodistribution studies indicated increasing concentration of Amphotericin B in liver with peak levels at 10h after administration of Amphotericin B lipid emulsion followed by decline in tissue levels after administration of marketed intravenous injection of Amphotericin B solution peak concentrations in liver were immediately obtained at 1h followed by decline in the drug tissues figure. 5 Table 2

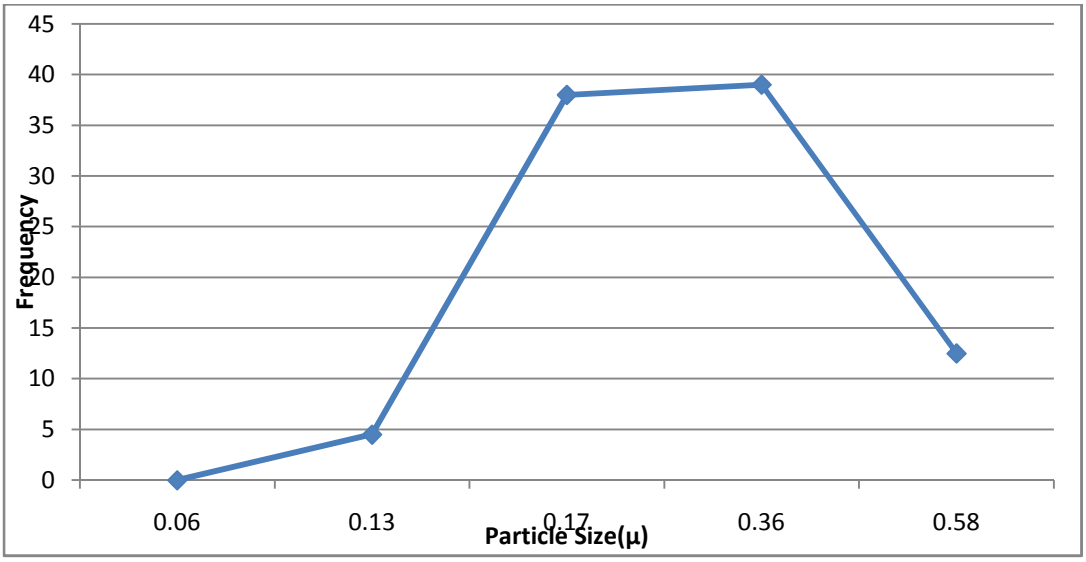
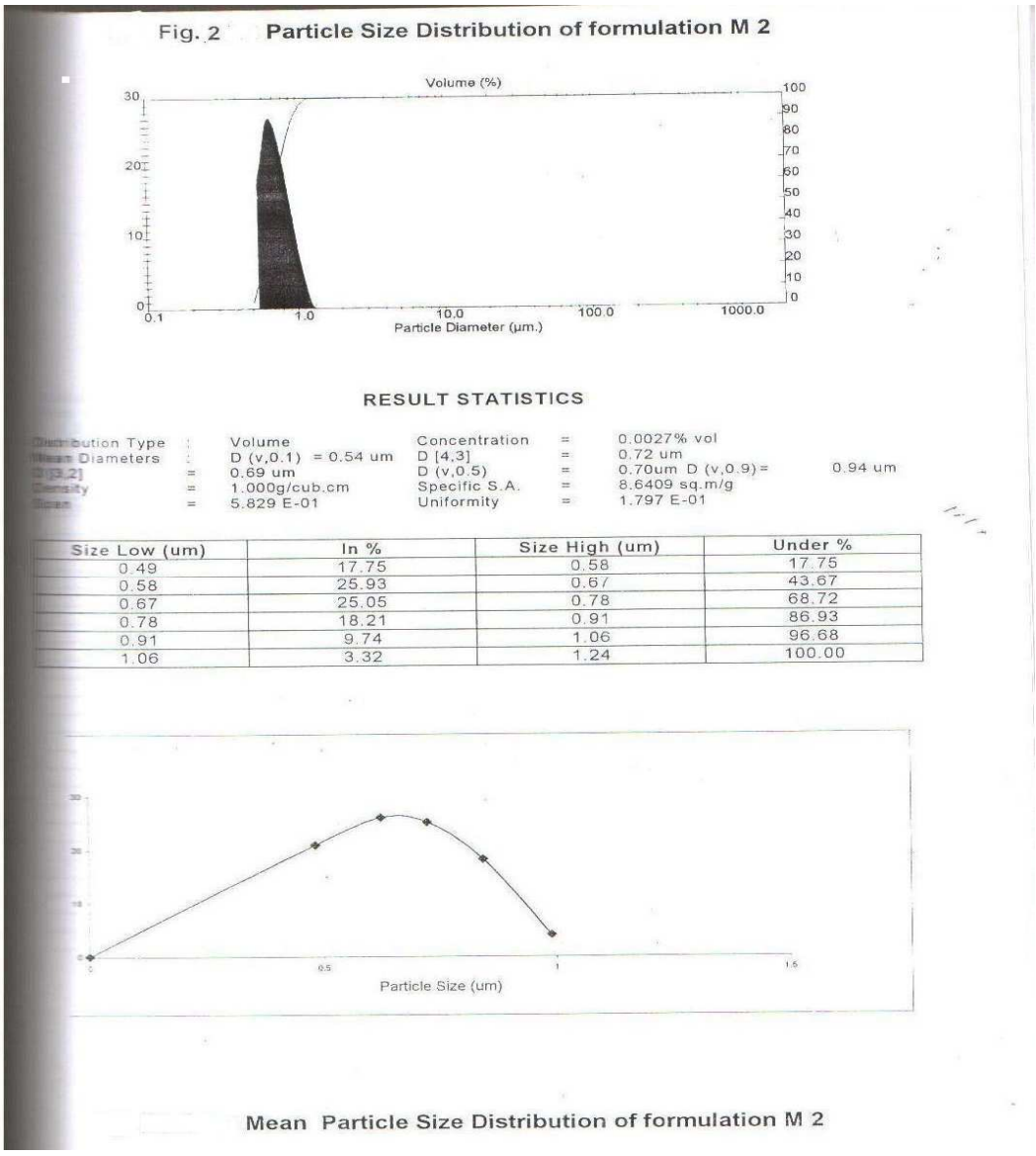


Fig. 1: Particle size distribution of E11



Size Low (um)	In %	Size High (um)	Under %
0.49	17.75	0.58	17.75
0.58	25.93	0.67	43.67
0.67	25.05	0.78	68.72
0.78	18.21	0.91	86.93
0.91	9.74	1.06	96.68
1.06	3.32	1.24	100.00

Fig. 2: Particle Size distribuion of formulation M2

Table 1: characterization of amphotericin b emulsions obtained after spontaneous emulsification

Batch No:	Oil	Visual appearance	Appearance under light microscope	Particle size range(µm)	Percentage drug loading (%)	pH	Percentage drug release up to 5h (%)	Stability	
								After centrifugation	Freeze thaw cycling
E1	EO	Fluid	Homogenous	0.23-9.24	90.12	4.20	88.97	Creaming	Phase separation
E2	EO	Fluid	Homogenous	0.14-0.91	92.5	5.24	94.17	Creaming	Phase separation
E3	EO	Fluid	Homogenous	0.18-6.74	93.0	5.49	90.79	Creaming	Phase separation
E4	EO	Fluid	Homogenous	0.24-6.86	89.14	5.63	88.2	Creaming	Phase separation
E5	EO	Fluid	Heterogenous	0.17-0.44	95.0	5.43	893.3	Stable	Stable
S1	SO	Viscous	Heterogenous	0.22-4.24	63.6	5.17	89.0	Creaming	Phase separation
S2	SO	Viscous	Heterogenous	0.24-6.86	72.48	5.32	87.0	Creaming	Phase separation
M1	M	Fluid	Homogenous	0.62-2.0	94.0	5.40	91.2	Creaming	Phase separation
M2	M	Fluid	Homogenous	0.54-0.94	95.5	5.45	90.02	Stable	Stable
M3	M	Fluid	Homogenous	0.61-7.84	92.0	4.84	90.69	Creaming	Phase separation

EO-Ethyl oleate SO-Soyabean oil M- Miglyol 812

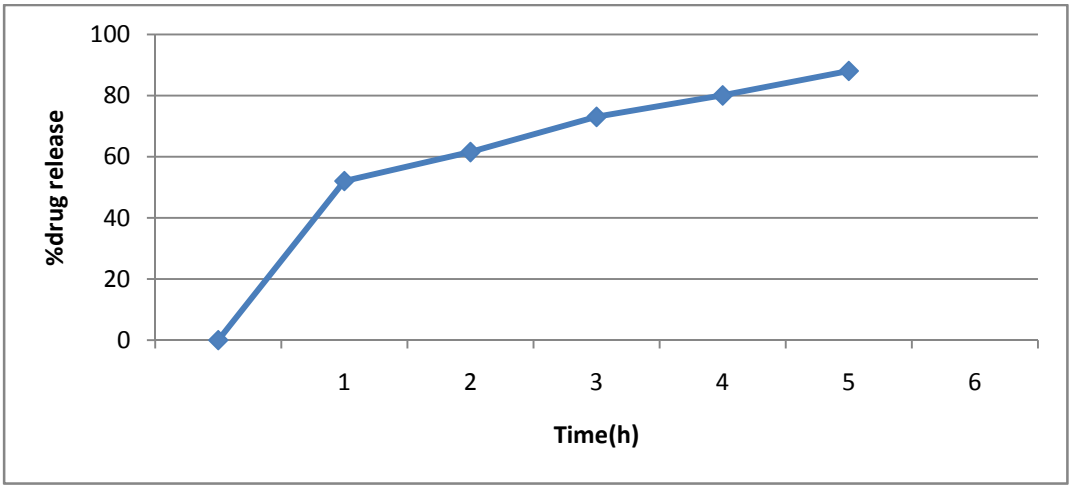


Fig. 3: *In Vitro* drug release profile of e11

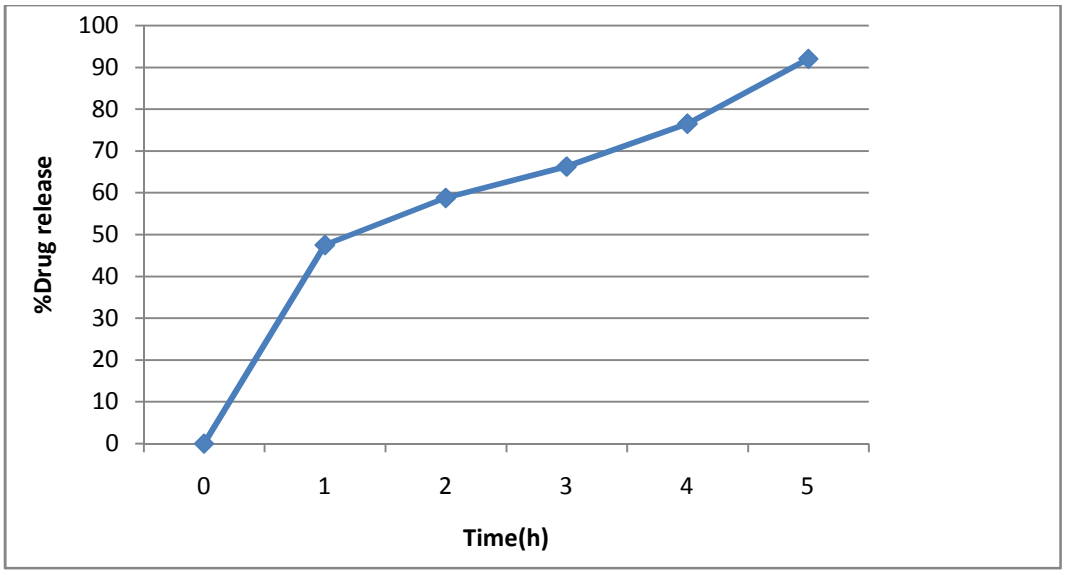


Fig. 4: *In Vitro* drug release profile of m2

Table 2: Concentration of Amphotericin B (µG/ML) In rats After I.V. adminstration of A.B.L.E and marketed A.B.SOL

Time(H)	Organ									
	Liver Mean	S.D.	Kidney Mean	S.D.	Spleen Mean	S.D.	Lungs Mean	S.D.	Heart Mean	S.D.
A.B.L.E.										
1.	387.5	12.5	91.66	31.54	320.8	75.34	816.66	166.45	-	-
2.	408.3	7.21	179.16	26.02	450.0	12.50	800.0	173.20	-	-
4.	400.0	97.62	266.66	19.09	495.8	59.07	816.5	155.13	62.66	1.84
8.	566.6	26.02	197.5	58.25	579.2	28.83	566.6	14.43	170.8	32.80
10.	666.6	26.02	195.83	7.21	600.0	43.30	504.16	81.33	179	5.89
16.	529.1	31.45	24.66	170.63	616.6	97.09	529.16	38.18	162.5	17.67
24.	341.6	68.84	175.0	43.30	625.0	137.5	866.0	114.99	112.5	12.5
A.B.SOL										
1.	275.0	33.07	131.83	38.60	216.6	137.6	558.3	62.91		
2.	250.0	50.0	154.16	31.45	189.1	69.02	395.8	121.4		
4.	212.5	97.62	191.66	38.188	445.1	101.80	354.16	34.08	112.5	36.79
8.	183.3	68.84	283.33	26.02	687.5	159.0	425.0	45.06	133.3	11.78
10.	1625.5	21.65	458.33	38.18	585.0	110.7	462.5	21.65		
16.	-	-	629.16	17.67	733.0	43.8	508.33	26.51		
24.	-	-	675	152.06	158.0	38.18	529.16	50.51		

A.B.L.E- Amphotericin B Lipid Emulsions A.B.Sol- Amphotericin B Solution (Marketed) S.D.-Standard Deviation

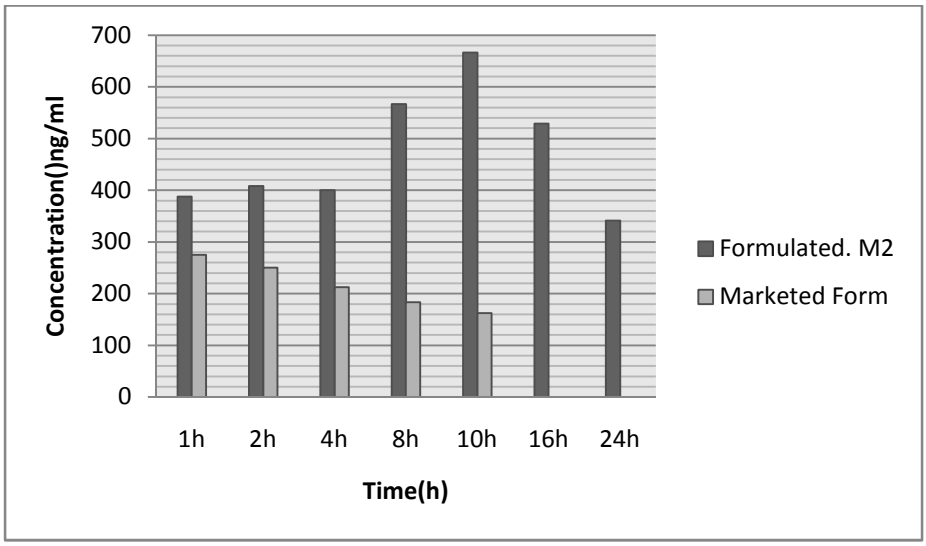


Fig. 5: Histogram showing tissue concentration of Amphotericin B lipid emulsion and Amphotericin B solution at different time intervals in liver

In kidney the drug levels were maintained at much lower concentration after administration of lipid emulsion, where as there was a constant increase in drug levels after administration of Amphotericin B solutions. Amphotericin B solution showed 4 times higher drug levels in kidney as compared to Amphotericin B lipid emulsion figure6.

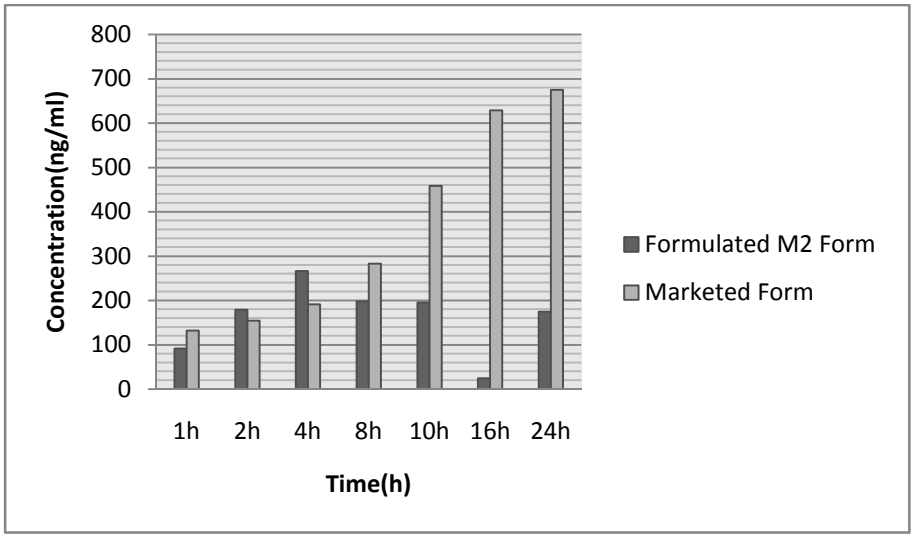


Fig. 6: Histogram showing tissue concentration of Amphotericin B lipid emulsion and Amphotericin B solution at different time intervals in kidney

As the drug enters the reticuloendothelial system, much higher concentrations of drug was present in lungs, spleen and liver figure7& figure8. In heart much lower concentration of drug was observed as compared to other tissues figure 9. Drug levels were observed at the end of 4h and were maintained up to 24h after administration of lipid emulsion but in marketed preparation lower concentration of drug levels were observed at 4h and 8h only.

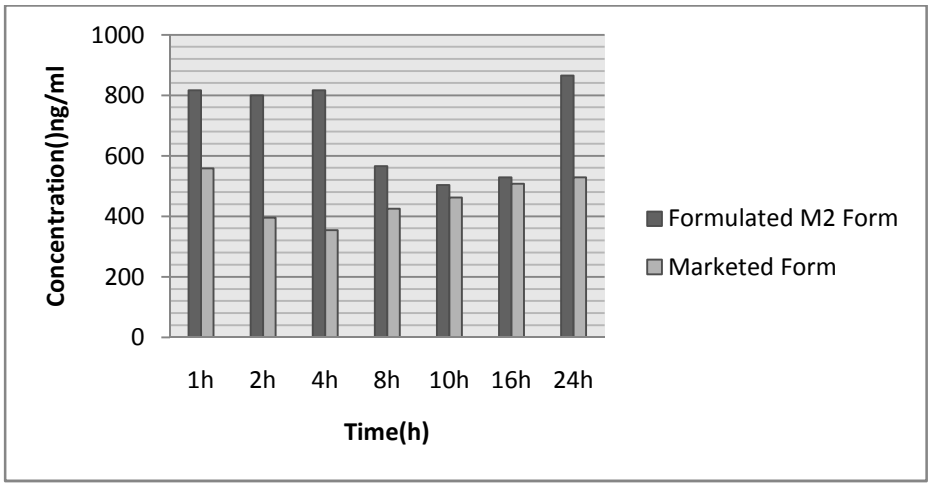


Fig.7: Histogram showing tissue concentration of Amphotericin B lipid emulsion and Amphotericin B solution at different time intervals in lungs

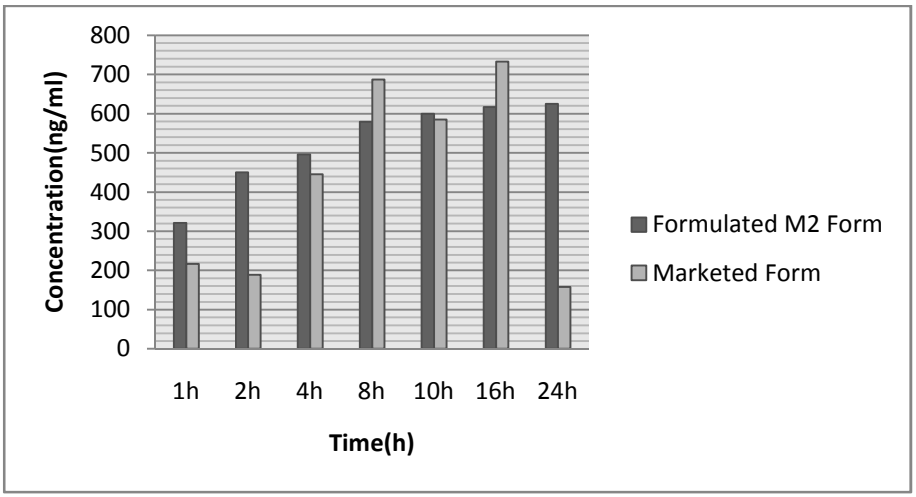


Fig. 8: Histogram showing tissue concentration of Amphotericin B lipid emulsion and Amphotericin B solution at different time intervals in spleen

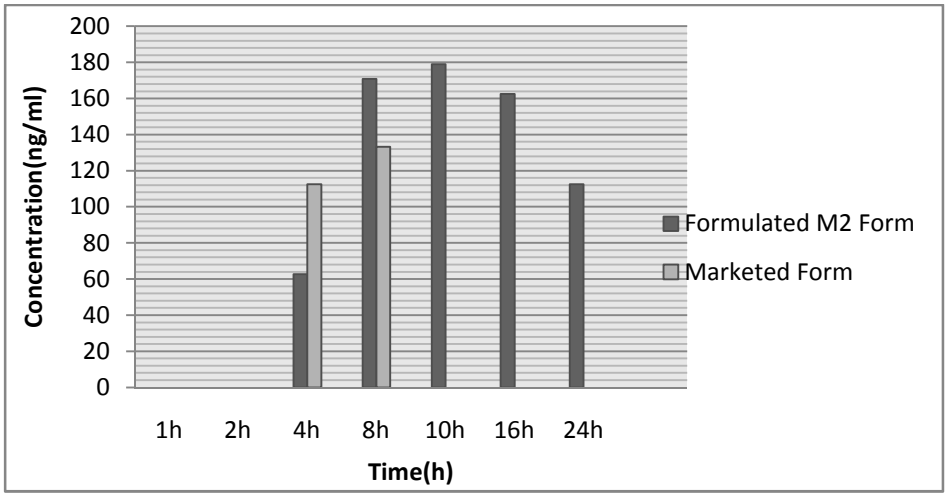


Fig. 9: Histogram showing tissue concentration of Amphotericin B lipid emulsion and Amphotericin B solution at different time intervals in heart

The AUC of Amphotericin B lipid emulsions in liver was increased 4.85 times as compared to Amphotericin B solution. The higher concentration of Amphotericin B in the tissues (1.13 and 1.55 in spleen and lung respectively) and F indicated that Amphotericin B lipid emulsion accumulated in RES organ. Table III.

Lipid emulsions are rapidly taken by RES and thereafter showed prolonged residence in these organs. The Amphotericin B lipid emulsion internalized inside these tissues can release their content at a slower rate. Lower concentrations of Amphotericin B in kidney and also a low F value with Amphotericin B lipid emulsion as compared to Amphotericin B solution showed decreased renal accumulation of the drug Table III. Thus lipid emulsions could prove useful in reducing the nephrotoxicity of the drug.

Table 3: Pharmacokinetic parameters of amphotericin B Lipid emulsions and marketed Amphotericin B solution after I.V. administration

Organ	AUC(mg/ml)ng.hr/ml	MRT(h)	F
A.B.L.E.			
Liver	12075.0	2.68	4.85
Kidney	4812.5	2.67	0.43
Spleen	13418.75	2.90	1.13
Lung	17150.0	2.92	1.55
Heart	2650.0	2.91	5.72
A.B.Sol			
Liver	2487.5	2.02	
Kidney	10962.5	3.23	
Spleen	11868.75	2.43	
Lung	11006.25	2.88	
Heart	462.5	3.13	

MRT - Mean Resident Time, F- Factor.

CONCLUSION

The present study suggests the preparation of submicronic emulsions of Amphotericin B suitable for intravenous application, the nature of surfactants, their relative concentration as well as that of oil were critical factors for the stability of emulsion. Submicronic emulsions of Amphotericin B would be suitable for decreasing the nephrotoxicity of the drug and improved uptake by RES and infected macrophages.

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