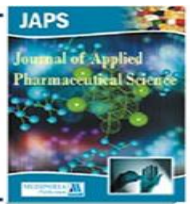
Journal of Applied Pharmaceutical Science
Available online at www.japsonline.com



ISSN: 2231-3354
Received on: 24-07-2012
Revised on: 09-08-2012
Accepted on: 17-08-2012
DOI: 10.7324/JAPS.2012.2818

Design and evaluation of liposomal delivery system for L-Asparaginese

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ABSTRACT

Despite of its effective anti-tumour activity,L-Asparaginase has limited clinical application due to the high rate of clinical hypersensitivity. In an attempt to develop a liposomal drug delivery for L-Asparaginase, enzyme loaded liposomes were formulated using soy lecithin, cholesterol and charge inducers by thin film hydration method. The effect of various components of the liposomes including the concentration of lecithin and cholesterol with or without the charge inducers on the entrapment efficiency and short term invitro cytotoxicity study was systematically investigated. The average particle sizes of the vesicles were found to be 43.2, 35.6 and 65.8 µm respectively for neutral, positive and negative liposomes. The percentage of drug loading was found to be 1.95, 2.39 and 2.35 % respectively for neutral, positive and negative liposomes.The *invitro* release study of L-Asparaginase was carried out using normal saline as dissolution medium and the release was found to be 86.88, 78.29 and 82.04 % respectively for neutral, positive and negative liposomes. The release of L-Asparaginase from liposomes was followed first order kinetics obeying non-Fickian diffusion. A short term cytotoxicity study was carried out using Ehrlich Ascites Carcinoma cells (EAC cells) which revealed that the cytotoxicity concentration CTC_{50} for pure drug was found to be 64 mcg as compared to liposomal formulation of 50 mcg.

Keywords: L-Asparaginase, liposomes, charge inducers, release kinetics, *in vitro* cytotoxicity studies.

INTRODUCTION

Liposomes are the phospholipids microscopic vesicles usually composed of one or more concentric lipid bilayer, separated by water or aqueous buffer normally with diameter ranging from 80nm to 10µm(Allen et al, 1995)Liposomes are gaining popular areas of research interest owing to the irinherit nature which includes high degree of biocompatibility, flexibility in their fabrication techniques and encapsulation of both the hydrophilic and hydrophobic drugs at large amount inside the vesicles (Babuet al, 1995 , Balasubshiniet al, 2006 and Banghamet al, 1995) . Because of their structural versatility in terms of size, composition, surface charge, bilayer fluidity and the ability to deliver at the surface cell specific ligands. It also includes a controlled retention and entrapped drugs in the presence of biological fluids, prolong vesicles residence in circulation and enhance the vesicles uptake by target cells. Various *in vivo* studies revealed but, particularly in areas such as cancer chemotherapy, anti microbial therapy, vaccine and certain ophthalmic disorder has clearly recorded that the liposome entrapped drug and vaccines exhibits superior pharmacological response as compared to conventional formulation (Bangham, 1974).

Liposomes can serve as adeport form which the entrapped molecules are released once a period of times. This release behavior shall be exploited to maintain therapeutic drug level in the blood for the prolong period of time leading to an increased duration of action and decreased the frequency of administration (Bi et al, 1999). Conversely liposomes can protect the patients against the incidence of side effect of anti-neoplastic agents (Chang et al.,2000)L-Asparaginase is an effective neoplastic agent for the treatment acute lymphoblastic leukemia chemotherapy, has limited clinical application due to its high rate of clinical hypersensitivity with an approximate of 3-78% of patient treated with unmodified forms of enzyme (Cleiner et al, 1989 ,Dante 2006 and Ertel et al, 1979) upon administration in a pure form it eliminated very rapidly (T_{50} – 2.88 hr), which reflects in the increased dosing level and dosing interval. A most promising development in the field of enzyme replacement therapy involves the usage of artificial lipid vesicles for the encapsulation of enzymes and the delivery to cells, both *invitro* and *in vivo(*Gabizon et al, 1989 and Gasper et al, 1996. However to protect the enzyme against the biodegradation upon infusion and to minimized the incidence of a systematic immunological reaction, many researchers have adopted the entrapment of enzymes by different techniques prior to infusion (Gregoriadis et al, 1993 and Higuchi 1963). However few interaction are available on liposomal formulation of L-Asparaginase with respect to its prolongation of circulating time without inhibition its enzymatic activity and prevention of inductionof anti L-Asparaginase antibodies and to mitigate anaphylactic reaction as compare to the free enzymes and prolongation of enzyme in general circulation without effecting its enzymatic activities (Ihler et al,.1973 and Ishii 2001) When enzymes are incorporated in the liposomes, they can be submissively targeted for the organs like liver, spleen and the bone marrow. (Jorge *et al.,* 1994and Kirby *et al.,* 1980more over the usage of natural phospholipids such as soy lecithin in liposomal formulation can significantly reduce the formulation cost of liposomes. (Koresmeyer *et al.,* 1983) Hence, the objective of the present study was to develop charged and neutral liposomal containing L-Asparaginase and to evaluate its efficiency with short term *invitro* cytotoxicity studies.

MATERIALS AND METHODS

L-Asparaginasewas obtained as a gift sample from Biochem Laboratories Mumbai, India. Soy lecithin and cholesterol were obtained as a gift sample from Lipoid, Germany. Stearylamine and Dicetyl phosphate were procured from Sigma chemicals, USA. All other chemicals and solvents were analytical grade and procured fromS.D.Fine chemicals, Mumbai, India.

Procedure for the preparation of l-asparagineseliposomes

L-aparaginasewas incorporated in the liposomes by thin film hydration method (Morris *et al.,* 1978).Briefly,the hydrophobic excipients such as soya bean lecithin, cholesterol and stabilizers (dicetyl phosphate and sterylamine) were dissolved in chloroform and stirred mechanically to form homogeneous mixture and the mixture is dried in rotary flask evaporator (Superfit, India)under an aspirate vacuum (25 mm Hg) and a water bath with the temperature maintained at 25^0 C. The thin film layer formed was flushed with nitrogen gas for 5 min and maintained overnight under vacuum to remove traces of chloroform.The thin film was resuspended in 50ml of normal saline containing L-Asparaginase and rotated without vacuum at 100 rpm, to get the homogeneous liposomal suspension. Then the liposomal suspension was passed through 0.45 µm pore size (Millipore) filter under nitrogen gas for 5 min. Untrapped L-aparaginase was removed from the liposomal dispersion by centrifugation at 10000 rpm for 30 min and supernatant is discarded and the liposomal pellets were washed two times with normal saline. The concentrated liposomes were freezing dried (Christ, Germany). The final liposome powder was stored in a tight container at 4^0C for further studies. The composition of the different types of liposomes is shown in table no: 1

Physic chemical evaluation of liposomes

Particlessize, Surface morphology and Zeta potential ofliposomes

The mean particle size and the particle size distribution of various liposomal formulations were determined using zeta-sizer after re-suspending and dilution of freeze dried liposomes in distilled water.The particle size distribution was analyzed or processed with computerized inspection system. Data were automatically analyzed by Light scattering system spectroscopy using a Beckman Coulter DELSA Nano C – Nano Particle Size Analyzer with Zeta potential measurement facility which works on the FST Method- Forward Scattering through Transparent electrode.

The surface morphologyof L-aparaginase liposomes were carried out by scanning electron microscopy (SEM) (Geol, Japan). The liposome Samples were mounted on metal stands and coated with gold to thickness of 200—500 A. then the plates were magnified to X 200 to take the morphological photograph of the prepared liposome.

Table. 1: Composition and different type of liposome.

Sl no.	Type of liposomes	Lecithin (%)	Cholesterol (%)	Sterylamine (%)	Dicetyl phosphate (%)
1	Neutral	5	5	-	-
		6	4	-	-
		7	3	-	-
		8	2	-	-
2	Positive	5	4	1	-
		6	3	1	-
		7	2	1	-
		8	1	1	-
3	Negative	5	4	-	1
		6	3	-	1
		7	2	-	1
		8	1	-	1

Encapsulation efficiency and content of L-asparaginase in liposomes

The encapsulation efficiency (EE) is determined as the ratio of the amount of the L-asparaginase encapsulated in the liposomes to that of the total L-asparaginase in the liposomes suspension. The amount of the L-asparaginase encapsulated in liposomes was measured fallowing the ultra-centrifuge method with slight modification (Nesbit *et al.,* 1979) briefly, liposomes containing 50 mg of the L-asparaginase was divided in to two parts. The first part was diluted to the 1ml with normal saline immediately after preparation were centrifuged at 1000 rpm for 10 min to remove the L-asperaginase already released from the liposomes. The 1ml of liposomes supernatant was determined by uv – visible spectrophotometer at 595 nm.

To the second part, Triton X-100 (0.001%) solution was added to disrupt the liposomal bilayer. The sample was analysed after adding 1ml Bradford's reagent. The bound drug gets liberated in to the solution and the amount of the drug present in the whole solution was determined by UV—Visible spectrophotometer. (Shimadzu, Japan).

The encapsulation efficiency was calculated according to the reported method:

$$\textbf{Encapsulation efficiency} = \frac{C_{total} - C_{out}}{C_{total}} \times 100$$

Where $\mathbf{C_{out}}$ is the liposomal suspension diluted with the normal saline and ultra-centrifuged to remove the liposome and the $\mathbf{C_{total}}$ is the liposome suspension added with Triton X-100 in order to disrupt the liposome completely to release the encapsulated drug to the solvent.

The normal saline solution was filtered through a Millipore filter. Concentration of the drug in the filtrate C_{out} and C_{total} were quantitatively analyzed after adding 1ml Bradford's reagent by UV –Visible spectrophotometer at 595nm (Shimadzu ,Japan)

In vitro dissolution studies of L-asparaginase liposomes

A quantity of liposomal formulation equivalent to 5 mg of L-asparaginase was taken in 50 ml of dissolution media containing normal saline maintaining at 37^0 C. Periodically 5ml aliquots were taken and diluted in to two parts. The first part treated with Triton X-100 (0.001%) solution to liberate the bound drug. The second part was filtered through Millipore centrifugal filters**.** Concentration of the drug in the filtrates were determined after adding 1ml Bradford's reagent and the absorbance of the filtrates was determined at wavelength of 595 nm.

Short term stability study of L-asparaginase liposomes

The stability study was carried out using lyophilized positive liposomes. The stability of L-asparaginase loaded of enzyme liposomes was evaluated in term of its leakage in to the storage medium (0.9% normal saline) and by the change in the *in vitro* characteristics at two different time intervals. To determine the stability of the liposomes, the percentage of drug loading and *in-vitro* release characteristics have been checked in six sample batch, stored at 4^0C and at the room temperature (25^0C) for 3 month. During the stability study, each sample was withdrawn at the end of the 3 month and the difference in drug content and changes in the entreat of the *in vitro* release profiles before stability study and after the stability study were considered to be proportional to the enzyme leakage into the storage medium and also the extent of change in the stability of *(in vitro)*of liposomes.

***Short term in vitro cytotoxicity study* (**Sessa*et al.,* 1968) and Shieh *et al.,* 1997)

The Ehrlich Ascites Carcinoma (EAC) cells were obtained from the peritoneal fluid of infected mice. Aliquots (1ml) and EAC (1× 105/ml) were washed twice with Hank's buffer salt solution (HBSS), centrifuging at 1000 rpm and cell count was adjusted to 20 lakhs/ml seeded into 24 cell micro liter plates subsequently varying amount of L-asparaginase (7.8, 15.6, 31.25, 62.5 and 125 µg/ml) L-asparaginase liposomal formulation (equivalent to 7.8, 15.6, 31.25, 62.5 and 125 µg/ml of L-asparaginase) were added along with control and incubated at 37^0C under 5% atmospheric CO_2 for 3 h. The numbers of viable cells remaining in the each well were determined by trypan blue (0.4%) dye exclusion technique. Any compound which is cytotoxic to cells inhibits the cell proliferation and kills the cells. The trypan blue dye capable of penetreating in dead cells, therefore, the dead cells tube up the blue strain while the viable cell don't. This method gives an exact number of dead and viable cells

RESULT AND DISCUSSION

The main difficulty in the application of liposomes has been the stability of the latter that results in the aggregation and fusion on storage and instability in the plasma. However many techniques were developed to overcome this problems. In the present work L-asparaginase liposomes using soy lecithin, cholesterol with different charge includes were prepared by the thin film hydration method, yielded particles with discrete nature. The average particle size of neutral , positive and negative liposomes were 43.2µm± 2.3 , 35.2 µm± 4.5 and 65.8 µm ±5.2 the mean zeta potential of neutral , positive and negative liposomes were -1.87, 26.4 , -17.89 mV. Although there was no significant difference in the encapsulation efficiency tends to increase as the degree of saturation of the lecithin used for the liposomal membrane increased. It is well known that regardless of the liposomes surface charge, cholesterol rich liposomes are more stable than cholesterol free liposomes [33] but, cholesterol can increased the ordered arrangements of lipid membrane. Thus, high content of cholesterol in the liposomes can decreased the flexibility of the membrane in the preparation process and thereby decreased the permeation of L-asparaginase in to liquid bilayer.(Zhang et al.,2005) The entrapment efficiency of L-asparaginase also decreased with the increased in the concentration of the cholesterol. Different ratios of soylecithin and cholesterol (1:1, 1.5:1, 2.3:1 and 4:1) were employed in the study. Since the increase of cholesterol in the liposomal preparation did not

significantly increase the entrapment efficiency of L-asparaginase, therefore the ratio of lecithin and cholesterol was fixed to be 2.3:1 in the preparation of charged and neutral liposomes. Conversely when the lipid content was increase than 12%, the hydration of lipid film become incomplete and difficult for the liposomal suspension to pass through the extrusion membrane due to its high viscosity. This is also another reason for which the lipid content was fixedto 2.3:1 with or without charge inducer. There was no significant increase in the entrapment efficiency of the L-asparaginase in to the neutral and the charged liposomes. The percentage drug loading or the entrapment efficiency was found to be 1.95, 2.39 and 2.35 respectively for the neutral, positive and negative liposomes. The zeta potential indicates the degree of repulsion between, similarly charged particles in dispersion. For the particles that are small, a high zeta potential will offer stability and resist aggregation. When the potential is low leads to attrition of particles and the dispersion will break and flocculate. The mean zeta potential of neutral, positive and negative liposomes was -1.87, 26.4 and -17.89 mV.

The polydispersity index (PI) is the measure of size distribution of the liposomes using Malvern zetasizer. Polydispersity values range from 0.000 to 1.000 was accepted and regarded as the particle size distribution was unimodal. The polydispersity values of neutral, positive and negative liposomes were within the prescribed limits revealing that the particle size distribution was unimodel. Fig 1 Showsthe in vitro release profile of L-asparaginase from liposomes. It was observed that irrespective of the charge on the liposomes, the drug release was found to be 86.68%, 78.29% and 82.04% respectively for neutral , positive and negatively charged liposomes at the end of 8 h. There was no much difference in the release of the enzyme for the neutral and the negative liposomes. There was a significant decrease in the release of the L-asparaginase from positive liposomes containing sterylamine. The presence of the sterylamine in the positive liposomes offers positive charge which leads to liposome-liposome interaction, there by destabilizing the membrane. This may be due to the cross linking of stearylamine with L-asparaginase retarded the release significantly.

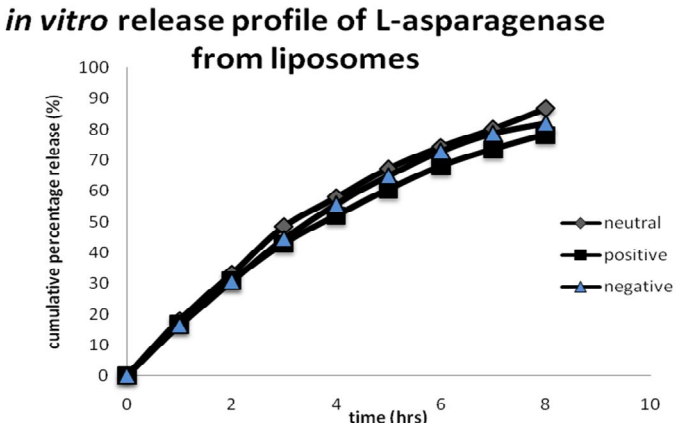

Fig. 1: in vitro release profile of L-asparagenase from liposomes.

Among the different liposomal preparation positive liposomes shows the better entrapment efficiency than that other batches, because this batch was subjected to *in-vitro* cytotoxicity study. Before lyophilisation, the liposomes were stored in 0.9% normal saline.

To evaluate the stability of L-asparaginase liposomes in the storage media at the 4^0 C and the room temperature 25^0C. The stability study was carried out interms of percentage drug leakage into the media for 3 month. The results of the stability studies on L-asparaginase loaded liposomes clearly indicated that the percentage of leakage of enzyme in the media was comparatively high when the when the liposomes were stored at room temperature. The mean percentage decrease in the drug loading at the 4^0 C was found to be 3.4± 0.15, which was considered to be very minimum and negligible. Similarly,the mean percentage of leakage of enzyme into the storage media was 14.01±2.83 at the room temperature. The loss of the drug to the storage media from liposome at the room temperature was found to be significant ($p>0.05$) when the data was statistically analyzed. Hence, the formulated L-asparaginase liposomes were found to be stable at 4° C before lyophillisation.

The short term *in-vitro* cytotoxicity study was done using EAC cell line showed a comparatively better efficiency through the enzyme loaded liposomes, than the free drug (Table 3 and Fig: 5 and 6). The percentage viability of the enzyme loaded liposomes was determined to be 15, 43, 58, 62 and 74 % at concentrations of 7.8, 15.6, 31.25, 62.5 and 125µg/ml respectively. Whereas the percentage viability through the free drug observed to be 19, 52, 62, 76 and 84 % at the concentrations of 7.8, 15.6, 31.25, 62.5 and 125 µg /ml respectively. It is also interesting to note that the addition of the positive charge inducer significantly increase the cytotoxicity of the liposomes against cell lines when compared with the free drug. It is well known that the positive charge liposomes have higher affinity against the tumor cell as compared to the neutral and negatively charged liposomes. Our result complies with the result reported by the previous method [35-36]. Therefore, it may be possible to value L-Asparaginase easily uptaken by the tumor cell resulting in increased cytotoxicity.

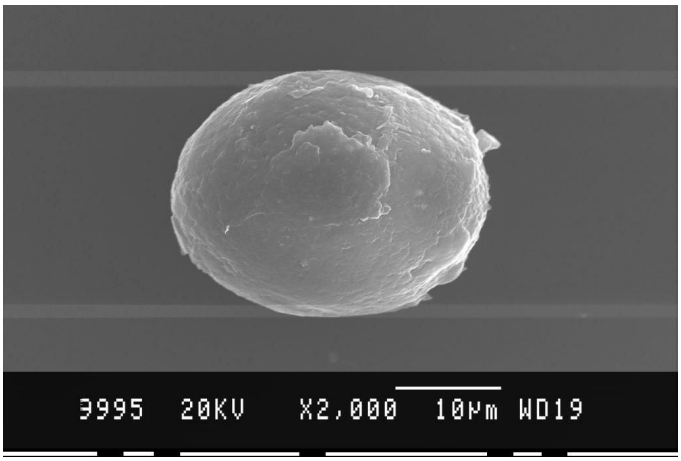

Fig. 2: scanning electron micrograph of positive liposome.

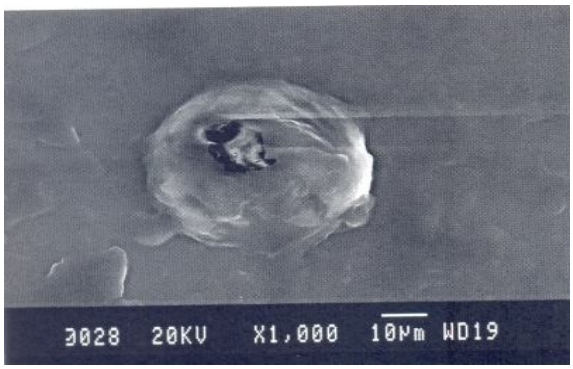

Fig. 3: scanning electron micrograph of negative liposome.

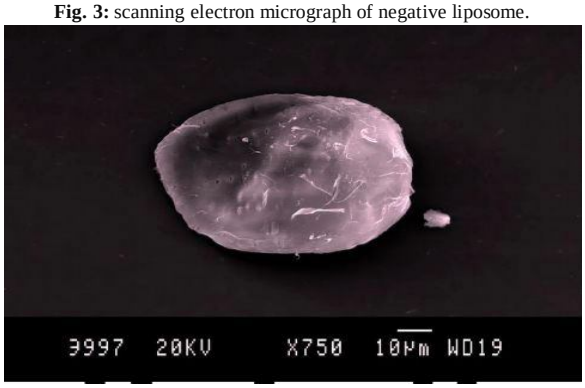

Fig. 4: scanning electron micrpgraph of neutral liposomes.

Table. 2: physico chemical and kinetic model of different type of liposomes.

Type ofliposome	Average particle size (µm)	Zeta Potential (mV)	Polydispersity	% drug loading
Neutral	43.2±2.3	-1.87	0.292± 0.03	1.95
Positive	35.2±4.5	26.4	0.266 ±0.01	2.39
Negative	65.8 ±5.2	-17.89	0.256± 0.01	2.35

Table. 3: comparative in vitro cytotoxicity study of L-asparaginase loaded liposomes using EAC cells.

Group		Concentration of drug (mcg/ml)	Percentage viability of ECA Cells (%)
Control asparaginase	L-	125	19
		62.5	52
		31.25	62
		15.62	76
		7.81	84
Liposomal asparaginase	L-	125	15
		62.5	43
		31.25	58
		15.62	62
		7.81	74

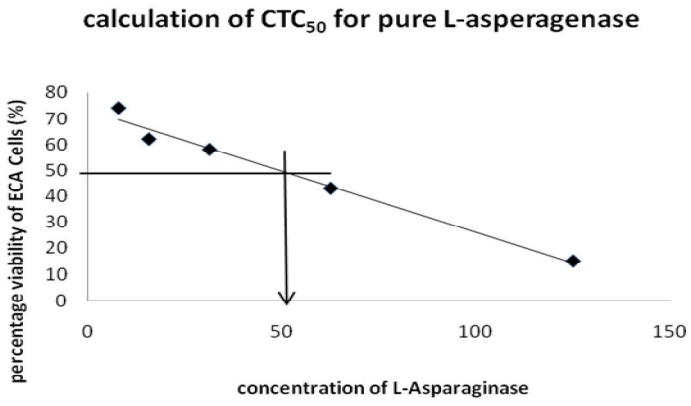

Fig. 5: calculation of CTC_{50} for pure L-Aspergenase.

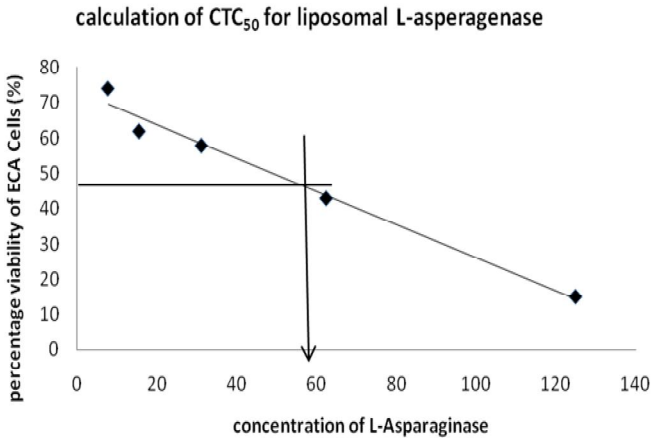

Fig. 6: calculation of CTC_{50} for liposomal L-Aspergenase.

CONCLUSION

The positive liposomes containing L-asparaginase is found to be a suitable and potential carrier interms of their particle size, drug loading capacity *invitro* release characteristics, physical stability and *in vitro* cytotoxicity. Hence, it may be used as an alternative in site specific delivery of anticancer enzyme. In turn it may be useful in reduction of toxicity and better patent compliance. However, suitable *invivo* study needed to be carried out to establish its potential effect.

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