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Improved Biological Activity and Stability of enzyme L-Asparaginase in Solid Lipid Nanoparticles Formulation

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ABSTRACT

To protect the biological activity of an enzyme during the development of formulations is one of the biggest challenges. The tetrameric form of L-Asparaginase is used to treat Acute Lymphocytic Leukaemia. It possesses shorter in vivo half–life. Using a modified (water/oil)/water-emulsion method followed by solvent evaporation L-Asn was successfully encapsulated at the core of Solid Lipid Nanoparticles made of lipid glyceryl monostearate. This study elucidated that the preparation of L-Asn loaded SLN develop a colloidal formulation with enhanced activity. The *in-vitro* release profile of the enzyme revealed first bursts has been increased. The study of the lyophilised formulation also shows that the enzyme holds its biological activity and retains its particle size distribution. Consequently, by using an apt combination of homogenisation speed, temperature and additives the storage and biological activity of L-Asn in SLN formulation can be improved.

Keywords: L-Asparaginase, Solid Lipid Nanoparticles, Controlled release, Lymphocytic Leukaemia

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1. INTRODUCTION

Proteins are known for their delicate molecular configuration and its relation to their biological activity hence development of a drug delivery system for proteins is an all-time big challenge to the pharmaceutical Industry. Enzyme L-Asparaginase is used specifically in the treatment acute lymphoblastic leukaemia. L-Asparaginase is well known for its ability to catalyse the biochemical reaction that converts proteinogenic amino acid L-Asparagine and produces L-Aspartic Acid and ammonia. This amino acid L-Asparagine is required in the biosynthesis of glycoproteins and other proteins. The ultimate mechanism of action of the L-ASN is that when L-Asparagine that cannot be auto synthesized by cancer cells unlike normal cells and upon degradation in the blood circulation turns to a state of malnourishment and ultimately famishing malignant cells. Short in vivo circulation is one of the prominent problems of L-asparaginase just like other proteins so to meet the desired therapeutic levels it turns inevitable to have multiple intravenous injections [1]. Oscillating L-Asparaginase concentration in the blood accompanying variety of side effects extending from minor allergic reactions to fatal anaphylaxis [2,3]. Anaphylactic authors have also proposed the administration of the L- asparaginase incorporated in liposomes [4–6]. Encapsulation protects the protein from its proteolysis and can extend the drug release. This encapsulation as liposomes of as SLN always comes with a biggest challenge of retaining the stability of the protein entrapped and it may be due unfavourable micro environment which interferes the integrity of the protein and affect its biological activity. Encapsulation still at its best loses the biological activity but at the worst can produce immunogenic or toxic products. As an alternative carrier for the delivery of therapeutic proteins, Solid lipid particulate systems have a lot of potential to explore. In this research work an optimum particulate carrier system of Solid Lipid Nanoparticles with L-ASN was developed through optimisation of various parameters. The aim of this research was to incorporate hydrophilic proteins using optimum particulate system formulation and nanospheres (Gaspar et al., 1998). The formulation development process was examined to characterise the influence of optimisation parameters in context to the type and concentration their excipients used, (lipids and surfactants), mechanical parameters like the type of stirrer and speed used and the impact of temperature on the size and biological activity of the enzyme. SLN formulation of L-Asparaginase protects the

enzyme from antigen determinants and increased the circulating time in blood confers its sustained release with intact biological activity. The biological activity of L-asparaginase was studied during its formulation development, storage, and release. L-Asn loaded SLN was prepared using melted lipid glyceryl monostearate, surfactants Span 80 and Tween 20 and 10000 rpm stirring speed at a constant temperature of 50℃ throughout produces nanospheres, with 86 % (w/w) recovery and a mean diameter of 164 mm and a narrow size distribution.

2. MATERIALS AND METHODS

2.1 Materials

L-Asparaginase was purchased from United Biotech (P) Ltd. as a lyophilised powder. Apart from enzyme other chemicals procured from HIMEDIA Laboratories (Mumbai, India) were of analytical grade.

2.2 L-asparaginase-loaded Solid Lipid Nanoparticles formulation

L-Asparaginase encapsulated in Solid Lipid Nanoparticles were prepared by adjusting and optimising a double emulsion method (w/o)/w followed by solvent evaporation [7]. In this process 1000 ul of l-asparaginase was added in 275 mg of melted lipid glyceryl monostearate (GMS) solubilised in acetone and emulsified by using high speed homogeniser (homogeniser: Heidolph Instruments GmbH & CO. KG Walpersdorfer, Silent Crusher M, Z664952, Schwabach, Germany) at a speed of 10000 rpm at 40°C for 5mins on a thermostat hot plate (magnetic stirrer cum hot plate, Heidolph Instruments GmbH & CO. KG Walpersdorfer, Hei-Tec P/N: 505-30000-00, Schwabach, Germany). This primary emulsion was converted to secondary emulsion (w/o)/w by adding 20 ml of an aqueous solution containing Tween 20 (2%) under continuous stirring on the magnetic stirrer for one hour at 1000 rpm followed by evaporation of acetone. The nanospheres were centrifuged at 4°C, 12000 rpm for 20 min. It was resuspended in distilled water. The centrifuged nanoparticles were filtered through1 µm pore size microfilter and stored at -20°C. The particle size and zeta potential were studied by laser diffraction particle analyser (Delsa™Nano Submicron Particle Size and Zeta Potential, Beckman Coulter INC. Brea, CA).

2.3 Quantification of biologically active enzyme in SLN

L-Asparaginase encapsulated in Solid Lipid Nanoparticles was estimated for its biological activity using different methods of extraction. Nanospheres were added into PEG 200, PEG 400 to dissolve lipids and to make the enzyme free. In addition to it mechanical methods, organic solvents and alkaline solutions were also used to disintegrate and further release the biologically active form of the enzyme. The enzyme was finally extracted from the suspension at a pH 7.4 in the phosphate buffer. The disintegrated nanoparticles were centrifuged to collect enzyme and multiple extractions were done. Total protein content in the suspension was determined. Most importantly, the biological activity of enzyme in the supernatant was also determined. 45 mg of SLN nanoparticles were suspended in 5 ml distilled water and sonicated with ultasonicator for 30 min.

2.4 Determination of the total protein content

The disintegrated sample was further characterised using two different method to estimate the total protein content and the biological activity of the enzyme released from SLN micro BCA protein-assay was used to estimate the total protein content. In this method protein was kept in alkaline solution and reduces Cu^{2+} to produce a complex bicinchonic acid–Cu with a characteristic violet colour which was later quantified using spectrophotometer at wavelength of 562 nm. In the protocol, protein sample and reagent in a proportion of 1:1 was incubated for 60 min at 60°C in the darkness followed by cooling of the reaction mixture to normal room temperature for next 30 min and the absorption was observed at 562 nm. It was done in triplicate and repeated as and when required.

2.5 Determination of biologically active protein of SLN

The biological activity of the enzyme released after disintegration was measured using an established method of Wriston with little modifications [8]. L-Asparagine converted to L-aspartic acid and ammonia through a biochemical reaction catalysed by L-asparaginase. Ammonia thus released reacts with Nessler's reagent produces a light orangish-yellow coloured complex and its reading was estimated using UV spectrophotometer at 425 nm wavelength. The reaction mixture assay consisted of 1.7ml of 0.01 M L-asparagine which was prepared in 0.05 M Tris-HCl buffer (pH 7.4), 500 ul of enzyme containing sample was incubated at 37°C for 60 min and the reaction was stopped by adding 100 µL of 1.5 M Trichloroacetic acid was used to abort the reaction. It was followed by centrifugation at 14000 rpm for 15 min. 0.5 ml of clear supernatant collected was added to 7.0 ml reagent grade water. 1ml of Nessler's reagent was added and vortexed. It was further kept for 10 minutes. OD was measured at 425 nm using UV visible spectrophotometer. It was also done in triplicate and repeated as and when required.

2.6 In vitro release studies

Nanospheres were resuspended in 0.05 M Tris-buffer pH 7.4. PEG 400 (1%) was added followed by Sodium Lauryl Sulphate (0.01%). 1.25 ml aliquots of suspension containing 1.5–3 mg SLN were transferred to microtubes and sealed tightly. The samples were placed in shaker incubator at 37°C at 70 rpm. From 72 hrs, for every 24hrs the pH of the release medium was adjusted by 5N NaOH to 7.0–7.5. 03 samples were collected at different time intervals. The sample immediately after collection at a specific time interval was centrifuged at 8000 rpm for 15 min using refrigerated centrifuge machine at temperature of 4°C. The clear supernatant was then assayed for the biological activity of the enzyme and for the determination of total protein content as mentioned earlier.

3. RESULTS

3.1 Stability studies of L-Asparaginase in the preparation of SLN formulation

Preparation of Solid Lipid Nanoparticles by modified double emulsion method followed by solvent evaporation is a multi-step process critical for biological activity of enzymes. First, during formation of the primary elusion (w/o), the hydrophilic enzyme can get denatured at the interface of aqueous/organic solvent. It may get adsorbed at this interface leads to the unfolding of the protein results in partial or total loss of catalytic activity. As mentioned in (Table 1).

Table 1: Influence of acetone on the biological activity of L-Asparaginase in aqueous solution

State of Study	Enzyme L-Asparaginase (µg/ml)	Biological Activity (%)	Protein Content (%)
Without Acetone	118.00	100± 6.1	100.0 ± 0.1
With Acetone	226.00	91.1 ± 0.2	94.2 ± 1.7
	189.78	80.9 ± 2.6	102.0 ± 5.3
	118.00	72.8 ± 1.6	98.0 ± 2.1
	59.00	65.1 ± 5.1	97.2 ± 4.1
	29.50	56.3 ± 2.1	99.5 ± 2.4
	14.75	44.9 ± 0.7	98.3 ± 2.3
	7.38	18.8 ± 0.8	99.0 ± 4.7
	3.69	9.2 ± 1.7	97.9 ± 3.4

When the aqueous solution of L-Asparaginase emulsified with melted glyceryl monostearate lipid dissolved in acetone at a volume ratio of 1:2 leads to a considerable decline in enzyme activity with dilution of the protein solution. This loss of biological activity was strongly dependent on the area of interface and the concentration of the enzyme used and was found highly comparable, the total quantity of biological activity lost found to be constant in all enzyme dilutions. So, it was found that the higher enzyme concentrated retains more biological activity that the diluted one. The enzyme's biological activity also got affected due to processing conditions for getting the desired particulate size like temperature and stress generated due to shear forces. Unlike the biological activity of the enzyme the protein content remains unaffected even at the interface. Although loss of biological activity was observed but emulsion was found stable as no insoluble aggregated were seen. Although reduction of exposure from 30 to 15 s increased the L-asparaginase activity by 25.7 ± 1.3%, insufficient emulsification resulted in immediate coalescence of the droplets. Thus, ultrasonication for 30 s at the minimum was required. In the process of emulsification, a decrease in the loss of biological activity of the protein with the increasing concentrations of the enzyme in aqueous solution were found concurrent (Table 2).

Table 2: Influence of emulsification with acetone by high speed homogenisation on the activity of L-Asparaginase

Enzyme L-Asparaginase in Primary phase of emulsion (mg/0.5ml)	Enzyme activity prior emulsification (U)	Enzyme activity after emulsification (U)	Enzyme activity after emulsification (%)
2.126	232.13±0.8	198.34±0.2	85.4
1.063	149.78±0.4	105.21±1.2	70.2
5.31.5	76.1±1.6	48.05±2.2	63.1
0.266	33.50±4.2	15.05±1.2	44.9

3.2 Stability studies of L-Asparaginase in the aqueous solution

The preparation of enzyme loaded SLN formulation L-Asparaginase as double emulsion requires high speed homogenisation, temperature and acetone as organic solvent. The stabilising effect of processing parameters (homogenisation speed, homogenisation time and temperature); additives like acetone and pH on the activity of l-asparaginase in aqueous solution was investigated (Table 3). The protein possesses maximum biological activity at pH 8.6 although biological activity of L-Asparaginase ranging between pH 7.0 to 9.25 [9]. The enzyme observed to show maximum biological activity in 0.05 M Tris Buffer at 8.6. The activity got decreased to almost 30 % in tris buffer at pH 8.0 and further decreased to approx. 70% in phosphate buffer at pH 7.4 [10]. Homogenisation speed was used to make a stable emulsion and shear stress to reduce the particle size, but it had a great impact on the stability of the activity of the enzyme. To attain the desired particle size of nanospheres (< 200nm), 10000 rpm homogenisation speed was used for making w/o primary emulsion. It was observed that the decrease in homogenisation speed increase the biological activity as less speed produces comparatively lesser shear stress on the tetrameric structure of the enzyme. The process of producing w/o primary emulsion due to higher melting point the lipid used glyceryl monostearate was carried out at a constant temperature of 30°C. The biological activity of the enzyme was completed retained at 30°C but started decreasing as the temperature increased and completely lost at 47°C.

Table 3: Influence of pH and additives on the biological activity of the enzyme L-Asparaginase

Study Parameter	Enzyme Activity (%)
Water pH 8.6	95.6 ±1.4
Water pH 7.4	91.9±8.9
Water pH 6.6	79.1±0.3
0.5 M Tris Buffer pH 8.6	74.3±0.2
0.05 M Tris Buffer pH 8.6	68.1±1.4
0.05 M Tris Buffer pH 7.4	60.5±5.1
0.5 % aqueous glycerol	65.9±1.7
1% aqueous glycerol	46.2±2.8
10% aqueous glycerol	21.6±1.6
1% aqueous Pluronic F127	98.3±0.2
1% aqueous PEG 400	68.0±9.8
10% aqueous PEG 400	41.8±2.6

3.3 Stability studies of L-Asparaginase in nanoparticles

L-Asparaginase loaded SLN formulations were prepared and studies in the presence of well-established stabilising agents PEG 400, Pluronic 127, Glycerol and Tris In practice, the average particle size of all the formulations prepared using different stabilisers were found to be 318± 8 nm (Table 4). All formulations were monodispersed, and huge particles lost upon its filtration through microfilter and affect the yield in the presence and absence of stabilisers. The encapsulation of enzyme was low with PEG 400 based formulation in comparison to that of glycerol, Pluronic 127 and Tris buffer. The activity of the enzyme incorporated in nanospheres was also studied when stabilisers were a component of the inner

and outer aqueous phase (Table 5). Tris-buffer denaturated the entrapped enzyme give less biological activity followed by Pluronic 127. In contrast, formulation prepared with stabiliser glycerol and PEG 400, more enzyme activity was reported in comparison to the additive-free nanoparticles.

Table 4: Characterisation of enzyme L-Asparaginase loaded SLN in the presence of stabilisers

Inner aqueous phase	Median Particle Size	Yield Recovered (%)	Final Loading (%)	Entrapment Efficiency (%)
L-ASN in water	310	51.5±0.3	0.36	76.1
L-ASN in 0.5 M Tris Buffer pH 8.6	234	36.9±0.6	0.51	73.9
L-ASN in Glycerol	326	56.34±0.1	0.38	80.6
L-ASN in 10% PEG 400	360	57.46±0.1	0.16	24.7
L-ASN in 1 % Pluronic F127	198	39.2±1.2	0.32	62.5

Table 5: Biological activity of L-Asparaginase in the presence and absence of different stabilisers

Inner core aqueous phase (L-Asparaginase in)	Biological activity of L-Asparaginase encapsulated in SLN (%)
Water	16.2±1.4
1% aqueous Pluronic F127	9.2±1.8
10% PEG 400	43.5±0.4
0.5% Glycerol	53.2±7.9
0.5 M Tris Buffer pH 8.6	Not detected

3.4 In vitro release studies of biologically active L-Asparaginase protein from SLN formulation prepared with stabiliser

Without any stabiliser and preservative in 60 mM Phosphate buffer at pH 7.4, a huge loss of the biological activity in the first 24 hrs of in vitro release studies has been already reported. PEG 400 (1%) as stabiliser and Sodium Lauryl Sulphate (0.01%) as preservative were added in 50 mM Tris-buffer at pH 7.4 to study the in vitro enzyme release studies. The in vitro release studies of the biologically active enzyme from Solid Lipid Nanoparticles with comparable size in the presence of different stabilisers was also studies and it showed that different stabilisers produce their different effects on the initial burst (Fig. 1). As the surface characteristics of the nanoparticles change with the stabilisers that in turn hampers or increase initial first burst. In this initial burst study, least loss of SLN was witnessed with glycerol followed by PEG 400, Pluronic 127 and Tris buffer respectively. Hence the biological activity was also highest in nanoparticles with glycerol and lowest in Tris buffer.

3.5 Stability studies of lyophilisation on the particle size and biological activity of L-Asparaginase nanoparticles

Lyophilisation of enzyme loaded nanoparticles resulted in formation of aggregates of size ranging in the micrometres that was also difficult to dissociate by ultrasonication. The effect of three lyoprotectans viz Tris buffer, glycerol and Pluronic F127 were studied on the enzyme loaded nanoparticles. Glycerol increases the particle size to 3 times with a considerable loss of biological activity. Tris buffer also increases the particle size during lyophilisation, but very little enzyme activity got lost. Pluronic F127 witnessed to be the best among the three in retaining the size and biological activity of L-Asparaginase.

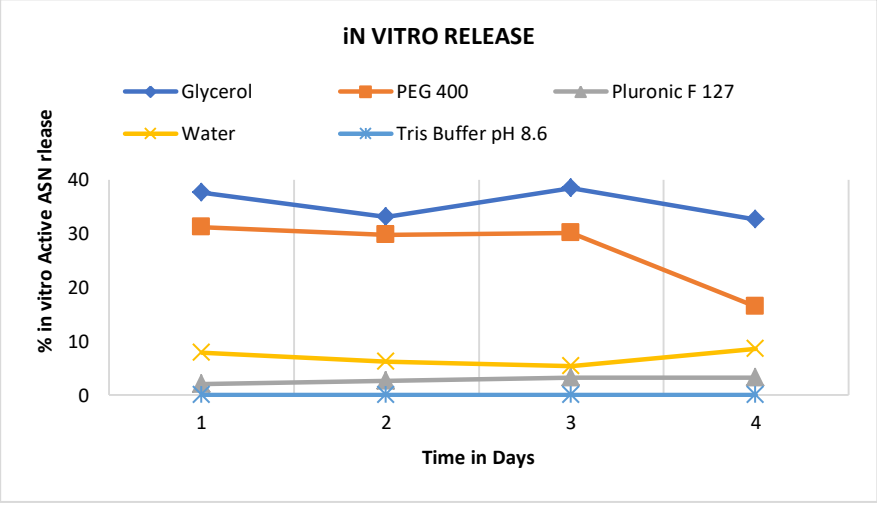

Figure 1: In vitro release study of L-ASN loaded SLN with different stabilisers

Table 6: Influence of stabilisers on particle size and enzyme activity of L-ASN loaded SLN during lyophilisation

L-ASN loaded SLN	Stabiliser Used	Particle Size (nm)	Biological activity (%)
After Preparation	-	164.3	
Before Lyophilisation	-	198.1	34.6
After Lyophilisation	No stabiliser	15479	11.2
	0.5 % Glycerol	6378	1.5
	0.05 M Tris Buffer	8392	9.7
	1% Pluronic F 127	307	24.3

4 DISCUSSIONS

The biological activity and structural stability of an enzyme is of utmost importance for any pharmaceutical formulation. To overcome all the possible sources of enzyme denaturation were identified in the study. A loss of biological activity of L-asparaginase was observed high at low protein concentrations at aqueous organic solvent interface. The method of double emulsification requires high speed which also affects the biological activity of the protein by generating shear stress on the enzyme in aqueous phase. At high protein concentrations these effects got comparatively reduced [11]. The formulation was further characterised after the involvement of stabilisers. Pluronic F127 was found to be the most successful stabilising agent in the stability studies performed in the aqueous solution of the enzyme [12]. The maximum biological activity of the enzyme was at pH 8.6 although it ranges fairly between pH 7.0 - 9.0. PEG 400 was used as in the release medium and gives good first burst but the enzyme lost its activity in the first 72 hrs and the enzyme found instable in PEG 400. Initially glycerol observed to be supportive in enzyme's stability, but it is not found suitable in maintaining particle size distribution and enzyme activity during lyophilisation.

5 CONCLUSIONS

A combination of stabilisers may improve stability of enzyme l-asparaginase in SLN e.g. the use of PEG 400 or glycerol to maintain enzyme activity during SLN preparation Pluronic F127 as a potent lyoprotectant. To retain the complete biological activity with the use of stabilisers is highly desirable.

Declaration

All authors declare no conflict of interest

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