Methods for particle size reduction of liposomal amphotericin B

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Abstract:

Liposomal amphotericin B were prepared by thin film hydration technique. Particle size was reduced by ultrasonic device and high pressure homogeneous device. The size distribution was determined by dynamic light scattering by the Zetasizer ZS90 equipment. The morphology of amphotericin B liposomes was observed by Transmission electronic microscopy (TEM) with negative staining technique. The result shows that combining high-pressure homogenization and membrane extrusion provided monolayer liposomal amphotericin B with particle size less than 200 nm and homogenously (PDI < 0.3).

Keywords: ***amphotericin B, liposomes, particle size.***

Classification number: ***3.3***

Introduction

Liposome is a drug carrier with outstanding advantages of controlling the dissolution and of carrying drug to targeted organs [1]. There are many methods for the preparation of liposomes in which thin film hydration method is popularly used because of its advantages, such as: relatively simple pharmaceutical technique, uncomplicated implementation, applicable to all phospholipid, high efficiency in liposomes performance with other lipid-soluble drug substances, etc. However, one of the disadvantages of this method is that the received liposomes usually has big dimension, many layers and is heterogeneous (from about 50-1,000 nm) [1, 2]. As a result, in order to use liposome in the injection dosage form, it is necessary to make it smaller and more homogeneous in order to facilitate the sterile filter procedure and enhance the carrying of drugs to its target.

Liposomal amphotericin B were prepared using the thin film hydration method [3]. This study presents the results of the investigation of some methods' particle size reduction and homogenization of liposomal amphotericin B. This is an important and decisive step for the next study for the preparation of injection dosage form of liposomal amphotericin B.

Ingredients and methodology

Ingredients and equipment

- *Ingredients:* amphotericin B was purchased from Dr. Ehrenstorfer GmbH (Germany), phosphatidylcholin soybean hydrogenation (HSPC) and distearoyl phosphatidylglycerol (DSPG) were purchased from Lipoid (USA), cholesterol (Chol) was purchased from Sigma Chemical Co. (St. Louis, Mo.), Polycarbonate membrane was purchased from Whatman (USA) and other chemicals which met the standards of USP, producers or pure chemistry.

- *Equipment:* Rovapor R-210 Rotary distillation system (Buchi, Germany), NS 29/32 2,000 ml globular jar, high-pressure membrane extruter EmulsiFlex-C5 (Avestin, Canada), Wiseclean 40 kHz Ultrasonic bath (Korea), Ultrasonic probe UP200Ht (Hielscher, Germany), Zetasizer nano ZS90 analyzer size system (England), Transmission electronic microscopy (TEM) JEOL 1010 (Japan) and other standardised equipment, devices were used.

Methodologies

Liposome preparation: the liposomal amphotericin B were prepared by the thin film hydration method as previously described [3] with HSPC/DSPG/Chol at the molar ratio of 2.0/0.8/1.9. The amphotericin B/total phospholipid ratio was 9/100 (mol/mol), citrate-buffer [pH 5.0] was used as the hydration solution. The evaporation conditions were as follows: the solvent mixture was removed from liquid phase by rotated evaporator at 40°C and 150 rpm in the first 30 minutes, then continued at 50 rpm in the remaining time. The hydration conditions were as follows: the temperature was 50°C, the speed of rotation was 200 rpm.

Size reduced method:

+ Polycarbonate membrane extrusion method with mini-extruder device [1, 4].

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+ Ultrasonic methods with ultrasonic bath and ultrasonic probe [1].

+ High-pressure homogenization method in combination with membrane extrusion method [1].

Particle size distribution were determined by dynamic light scattering method with Zetasizer ZS90, morphology of particles by transition electronic microscope with negative staining technique [5-7].

Results and discussion

After being prepared by the thin film hydration method, liposomal amphotericin B's (orignal L-AmB) size ranged from 738 to 1,026 nm, the size distribution was 0.451-0.868. Those liposomal amphotericin B were used to investigate the effects of equipment and parameters in particle size reduced process in liposomal amphotericin B formulation.

Results from membrane extrusion method with mini-extruder device

Liposomal amphotericin B prepared in the previous step was extruded sequentially through 1,000-400-200 nm polycarbonate membrane with a manual mini-extruder device. Each of the samples were extruded 29 times at 60^0C. Extruded liposome had low PDI (from 0.111-0.227), indicating homogeneous particle distribution. However, the particle size was still large (> 200 nm), the volume after each extrusion was only 1-10 ml, so it was not applicable to the mass scale.

Results from ultrasonic methods

Using ultrasonic probe: 200 ml original L-AmB was homogenized by UP200Ht ultrasonic probe (200 w, 26 kHz). After 1, 2, 3,... and 10 minutes, 1 ml of the sample was withdrawn and particle size distribution properties were characterised. The results were presented in Table 1.

Table 1. The particle size and particle distribution of liposomal amphotericin B samples using ultrasonic probe (n = 3).

Time (minute)	Particle size (d.nm)	PDI
Origin	566.0 ± 11.5	0.607 ± 0.019
1	256.7 ± 5.3	0.453 ± 0.031
2	202.9 ± 4.3	0.411 ± 0.031
3	175.4 ± 4.5	0.303 ± 0.008
4	148.1 ± 0.1	0.255 ± 0.008
5	146.6 ± 1.3	0.212 ± 0.025
6	139.1 ± 0.1	0.220 ± 0.028
7	139.4 ± 0.1	0.227 ± 0.001
8	140.6 ± 0.4	0.248 ± 0.004
9	141.3 ± 4.7	0.253 ± 0.001
10	145.9 ± 1.0	0.272 ± 0.007

As illustrated in Table 1, with ultrasonic probe method, after 4 minutes the samples with a particle size of under 200 nm were distributed relatively homogeneously (PDI < 0.3). The limitation of this method is that the samples usually have impurities (caused by releasing titan metal from probe) and are not appropriate for scale up.

Use ultrasonic bath: 200 ml of liposomal amphotericin B was put into a glass beaker and scanned in Wiseclean (40 kHz, 22 litres in capacity, containing 6 litres of water) ultrasonic bath for 20 minutes with an interrupting procedure: scanned for 30 seconds and stopped for 30 seconds (sample A1), scanned for 1 minute and stopped for 1 minute (sample A2), scanned for 2 minute and stopped for 2 minute (sample A3) (using iced water during the procedure to avoid heating of liposome suspension). After the homogenization process, the sample was taken to measure the particle size and particle distribution. The results are presented in Table 2.

Table 2. The particle size and particle distribution of liposomal amphotericin B samples using ultrasonic bath (n = 3).

Sample	Particle size (d.nm)	PDI
A1	522.13 ± 26.05	0.727 ± 0.075
A2	507.97 ± 33.62	0.713 ± 0.104
A3	662.30 ± 59.01	0.542 ± 0.051

As shown in Table 2, after 20 minutes, the samples had a particle size of above 500 nm, heterogeneous distribution (PDI > 0.5). The results indicated that ultrasonic bath was not an effective method to reduce particle size. These results were consistent with other studies [8-10] showing that reducing process was more effective when liposome was poured direct into the bath, with higher ultrasonic frequency (at least 80 kHz) and it should be adjusted during the operation.

Results from high-pressure homogenization method in combination with membrane extrusion method

Prepared liposomal amphotericin B was passed through Emulsiflex-C5 equipment alone or conjoined with extruder holder (placed 400 nm polycarbonate membrane) under a pressure at 5,000 psi (350 bar). The number of compressed cycles was investigated to determine suitable parameters.

The effect of the homogenization cycle: the results are presented in Table 3.

Table 3. The particle size and particle distribution of liposomal amphotericin B samples according to homogenization cycle.

Homogenization cycle	Particle size (d.nm)	PDI
0	882.6	0.508
1	174.0	0.408
2	149.2	0.364
3	141.9	0.376
4	135.1	0.382
5	130.5	0.371
6	121.1	0.376
7	112.5	0.306
8	110.0	0.294

Table 4. The particle size and particle distribution of liposomal amphotericin B samples according to the membrane extrusion times.

Times of extrusion	Particle size (d.nm)	PDI
0	149.2	0.364
1	134.3	0.337
2	134.9	0.281
3	131.8	0.274
4	131.6	0.275

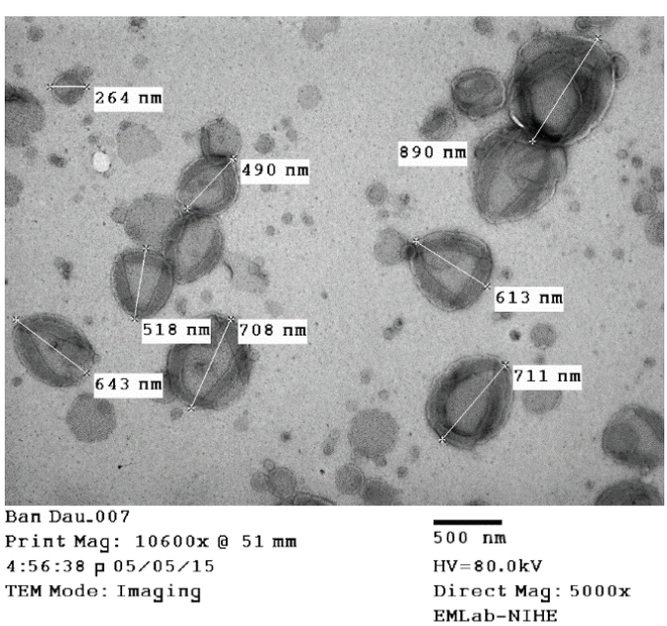
The results in Table 3 show that after the first cycle, the particle size was reduced to less than 200 nm. However, the particle distribution was still heterogeneous (PDI > 0.4). From the second cycle onwards, particle size gradually decreased and the PDI remained at about 0.3. Therefore, there was a requirement to combine with membrane extrusion method for getting more homogeneous system.

The effect of the membrane extrusion times: after passing through Emulsiflex-c5 equipment for 2 cycles as conducted above, samples were extruded through 400 nm polycarbonat membrane under a pressure of 500 psi. The results are presented in Table 4.

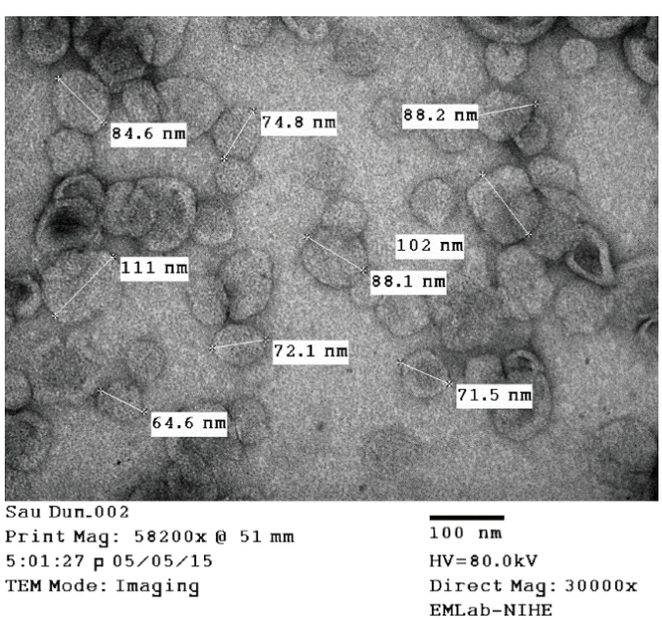
The TEM figures of samples taken after high-pressure homogenization method in combination with membrane extrusion are presented in Fig. 1.

It is illustrated in Table 4 and Fig. 1 that in the second cycle, after extruded through 400 nm membranes, the samples have a more homogeneous distribution with PDI < 0.3. There was not a significant difference in particle size and particle-size distribution between the second and the next cycles. Liposomes still had spherical shape and almost all of them had one layer, small particle size and relatively homogenous distribution.

The operation principle of the high-pressure homogenization method is the same as that of the polycarbonate membrane extrusion method with a gradually reduced pore size. Normally, high-pressure homogenization usually requires a number of cycles in order to obtain small and homogeneous liposome. However, the process of peeling the layers of liposome took



(A) Origin.



(B) After particle reduction.

Fig. 1. Original sample (A) and after particle reduction using high-pressure homogenization method in combination with membrane extrusion (B).

place under high-pressure and multi-cycle homogenization can break liposome resulting in loss of product. In addition, the temperature of the device may increase the effect on the stability of the liposome. In order to reduce the number of homogenization cycle (2 times), the combination of high-pressure homogenization and membrane extrusion method (using high-pressure membrane extruter EmulsiFlex-C5) selected in this study showed relatively optimal results and could be applied in practice.

Conclusions

The investigation into some methods used to reduce liposomal amphotericin B particle size was done; specifically, Membrane extrusion method using mini-extruder device resulted in highly homogeneous distribution (PDI < 0.2) but the particle size of liposomal amphotericin B was still larger than 200 nm. Ultrasonic method using ultrasonic probe after 4 minutes, resulted in small liposomal amphotericin B particle size (< 200 nm) and homogenous distribution (PDI < 0.2). Ultrasonic method using ultrasonic bath was used and all the investigated conditions resulted in large liposomal amphotericin B particle size (> 500 nm) and heterogeneous distribution (PDI > 0.5). The combined method of high-pressure homogenization method and membrane extrusion method under pressure of 5,000 psi and with 2 homogenization cycles, 2 times of extrusion through 400 nm polycarbonate membrane resulted in spherical liposomal amphotericin B, mostly with 1 layer, small particle size (< 200 nm) and relatively homogeneous distribution (PDI < 0.3).

These results will be the fundament for further studies on preparation of injection dosage form of liposomal amphotericin B.

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