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Study on Formulation and Targeting Efficiency of Amphotericin-B Nanospheres

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Colloidal drug carriers such as nanospheres are gaining importance by achieving reduced toxicity, enhanced efficacy and site-directed action. Hence, bovine serum albumin nanospheres were prepared by pH coacervation method. The potential antifungal agent amphotericin-B was incorporated into nanospheres at various concentrations. The drug-loaded batches were subjected to *in vitro* analysis and found to exhibit a bi-phasic release pattern. The batch with optimum drug-loading and satisfactory release profile was selected as ideal batch and it was used for *in vivo* targeting study in mice using HPLC analysis. The targeting efficiency of drug loaded nanospheres was compared with that of free drug in terms of percentage, increase in targeting to various organs like liver, lungs and spleen. The increase in targeting percentage was found to be 56.5%, 40.4% and 31.8% in lungs, liver and spleen respectively.

Splendid achievements have been made in the management of diseases through the invention of drugs over the past decade. Drug and drug deliveries have got equal importance in the pharmaceutical field. Recently much interest has been generated by various drug delivery systems like microspheres, liposomes, neosomes, polymeric beads, nanocapsules and nanoparticles from biodegradable polymers because of the possibilities for controlled release, increased drug efficacy and reduced toxicity after parenteral administration[1,2]. Among various other colloidal carriers, the nanoparticles are gaining more popularity because of their stability and ease of preparation[3]. But most of the nanoparticles used as drug carriers are rapidly taken up from the blood by the targeting macrophages. This phenomenon is of great value in macrophages of reticulo-endothelial system (RES) mainly in the liver and spleen a drug to organs and to treat diseases of reticulo-endothelial system[4]. This prompted us to incorporate amphotericin-B that is used in manifestations of RES like candidiasis[5] into bovine serum albumin nanospheres. Thus in our present study we compared the targeting efficiency of drug loaded nanospheres with that of free drug in terms of percentage increase in targeting to various organs of reticulo endothelial system.

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EXPERIMENTAL

Bovine serum albumin (BSA) powder was purchased from Sigma chemicals Co., USA. Amphotricin-B has been generously supplied by Synbiotics Ltd., Baroda, Millipore filter unit (CPSMM-7554) was obtained from Millipore, Bangalore HPLC cartridges were supplied by Supleco-Ltd., Bangalore. Acetone and glutaraldehyde 25% were of AR grade. Phosphate buffer (pH 7.4), acetonitrile, methanol and ethanol were of HPLC grade and supplied by Supleco-Ltd., Bangalore.

Preparation of bovine serum albumin nanospheres containing amphotericin B:

It was accomplished by pH coacervation method, where 30 ml of 2% solution of BSA was first filtered through a millipore membrane filter with a size of 1 μm, the filtrate was collected and its pH was adjusted to 9 using 0.5 M sodium hydroxide solution. Five milligram of drug amphotericin-B was mixed with albumin solution using a magnetic stirrer. The batch loaded with 5 mg of drug was named as BSA-1. Then a suitable amount of acetone was added drop wise from a syringe until the

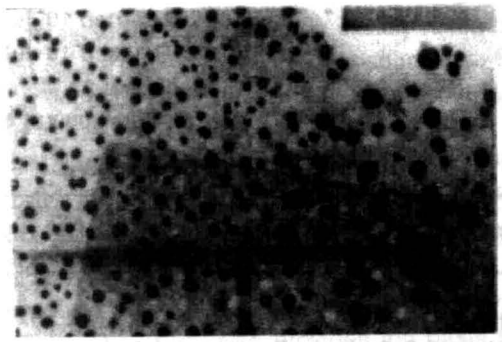

Fig. 1: Shows the transmission electron micrograph of bovine serum albumin Nanospheres containing Amphotericin B

solution became just turbid. The nanospheres so formed were cross-linked by adding 100 µl of 4% glutaraldehy de-ethanol and the solution was mixed continuously at room temperature for 3 h. After the cross linking stage, the drug-loaded nanospheres were filtered through a Millipore filter. The filtrate was centrifuged for 30 min at a speed of 20,000 rpm. The supernatant was decanted and the suspension was washed three times with acetone until a clear sediment of nanospheres was obtained, which was suspended in acetone-water mixture to prevent particle clumping on storage. Transmission electron micrograph of drug loaded nanospheres is depicted in Fig. 1.

By using the aforementioned procedure four other batches of nanospheres viz., BSA-2, BSA-3, BSA-4 and BSA-5 were prepared each containing 10, 15, 20 and 30 mg of amphotericin-B respectively. The percentage yield of nanospheres in each batch was determined to be in the range of 20-23%.

Estimation of amount of drug incorporated into albumin nanospheres[7]:

Five milligrams of drug loaded nanosphere from each batch was incubated with 5 ml of 5% hydrochloric acid in absolute ethanol at 4° for 24 h. After 24 h of incubation, the nanospheres were separated by high-speed centrifugation and the drug content was analysed in the supernatant by UV spectrophotometer.

Evaluation of *in vitro* drug release :

Ten milligrams of drug-loaded nanosphere from each batch was taken into a 250 ml conical flask and 50 ml of 7.4 pH phosphate buffer saline was added to it, then the flask was kept in a shaker cum incubator and the shaker was adjusted to 80 horizontal strokes per minute at 37°. Five milliliters of drug releasing media was withdrawn at

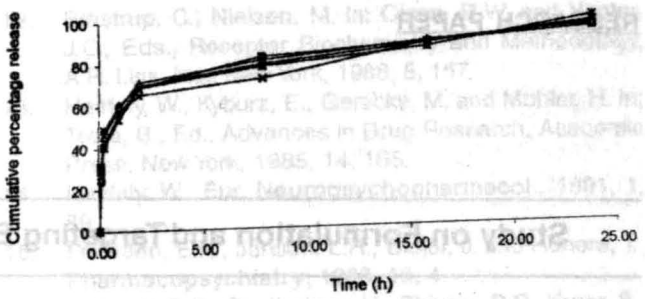

Fig. 2: Cumulative percent release of Amphotericin B from various drug loaded batches of Nanospheres

Nanospheres containing amphotericin B —◆— 5 mg —■— 10 mg —△— 15 mg —✳— 20 mg —✳— 30mg were incubated in PBS at 37°. Samples withdrawn at different time intervals and amphotericin B content was determined spectrophotometrically

various time interval of 0 and 30 min, 1, 2, 8, 16 and 24 h. These samples were vacuum filtered through the membrane filter of 1 µm pore size. The drug was estimated in each batch by UV spectrophotometer. Figure 2 shows the *in vitro* release profile of all the drug-loaded batches.

***In vivo* Studies** :

Thirty six healthy mice with an average weight of 25-30 g were selected, a constant day and night cycle was maintained and the temperature of the animal room was kept at 25° throughout the study. Then the animals were divided into 3 groups, each containing 12 mice. Group I received nanospheres containing drug equivalent to 218.4 µg/20 g of animal. Group II received free drug equivalent to 218.4 µg/20 g of animal. Group III served as solvent control with 0.2 ml of phosphate buffer solution. After 3 h the mice were sacrificed and the liver, the lungs, the spleen and the kidney were isolated and homogenised by using a tissue homogeniser with 5 ml of dimethyl sulphoxide.

Estimation of drug from isolated organs[9]:

Extraction and isolated of amphotericin-B from various organs each accomplished using sep. pak, vac 3 cc (C18) cartridges connected to a vacuum extraction manifold. The packaging material in the cartridges was conditioned with acetonitrile (3 ml), 3 ml of water followed by phosphate buffer (pH 3.0) before loading the samples. One milliliter of homogenised samples was loaded into the cartridges for isolation of amphotericin-B, after the samples had completely passed through the packing materials in the cartridge. The cartidge was then washes with phosphate bufffers (3 ml x 3 times). Amphotericin-B

TABLE 1 : TISSUE DISTRIBUTION OF AMPHOTERICIN B FROM NANOSOHERES AND FREE DRUG

S. No.	Organ	Amphotericin B Nanospheres Amount detected in targeted organ ng/organ	Free Drug Amount detected in targeted organ ng/organ
1.	Liver	83.41 ± 0.91 (38.19%)	36.31 ± 0.63 (16.62%)
2.	Lungs	9.75 ± 0.65 (4.46%)	5.81 ± 0.94 (2.66%)
3.	Spleen	38.6 ± 0.56 (17.69%)	26.37 ± 0.45 (12.07%)

retained on the cartridge was then eluted using 1.5 ml of acetonitrile-50 mm, phosphate buffer (pH 3.0) 2:1 ratio. The eluted samples were analysed. The retention time of amphotericin-B was found to be 7.15 min. The calibration curve was plotted. using the areas of the standard solution versus concentration in ng/ml. The peak area of the sample chromatogram was calculated for various organs. The targeting efficiency of drug loaded nanosphere was compared with that of free drug in terms of percentage increase in targeting to various organs.

RESULTS AND DISCUSSION

The drug to polymer ratio was studied in 5 batches of BSA with varying drug concentration that included 5, 10, 15, 20 and 30 mg. The amount of drug bound per mg of nanosphere was determined in each batch. It was found to be 4.64 µg/mg in BSA-1 batch and 11.5, 14.5, 26.8 and 28.8 µg/mg respectively in BSA-2, BSA-3, BSA-4 and BSA-5. It was observed that the drug to polymer ratio was found to be increasing proportionally with increase in concentration up to a concentration of 20 mg. It complies with the results of earlier investigators[10], but after a concentration of 20 mg there is no significant increase in the loading capacity.

The size of the drug-loaded nanospheres was found to be increased than the plain nanospheres, and it was lying in the size range between 120.7 nm to 446.4 nm with an average of 278.5 nm. The *in vitro* release of all the five batches of BSA showed an interesting bi-phasic release with an initial burst effect after 1h. The initial burst is due to the release of the drug loaded on the nanosphere surface, and the second part of the release may be due to the slow diffusion of the drug as the matrix erodes slowly. The cumulative percentage drug release was found to be 90% and above for all the batches. The comparison between the amount of drug targeted from nanospheres and free drug in various organs is presented in Table 1. When compared with that of free drug, the targeting efficiency of drug loaded nanosphere was 56.5% higher in lungs, 40.4% higher in liver and 31.8% more in spleen. The drug-loaded nanospheres showed preferential drug targeting, to lungs, which is essentially required in the treatment of candidiasis.

Thus, our present study shows the targeting efficiency of drug loaded nanospheres over the free drug interms of more drug distribution to certain tissues, this may provide an increased therapeutic efficacy. Consequently, more amount of drug targeted to various organs may help in the reduction of dose required for the therapy, and thereby the dose related systemic side effects like neuro and renal toxicity can also be minimised.

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