Drug Development and Industrial Pharmacy, 26(12), 1293–1296 (2000)

COMMUNICATION

Study of Biodistribution of Methotrexate-Loaded Bovine Serum Albumin Nanospheres in Mice

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ABSTRACT

Nanospheres made from natural hydrophilic polymers have been proved efficient in terms of better drug-loading capacity, biocompatibility, and possibility less opsonization by reticuloendothelial system (RES) through an aqueous stearic barrier. Hence, nanospheres containing methotrexate were prepared from bovine serum albumin (BSA) by a novel pH coacervation method. A drug-to-polymer ratio study was carried out to determine the carrier capacity. The batch with the highest drug loading was subjected to in vitro analysis. It was found to provide a slow release after an initial burst release. Biodistribution of nanosphere-bound drug was compared with that of free drug in mice. It was observed that the percentage increase in drug distribution to the lungs, liver, and spleen was markedly high from the nanosphere when compared to free drug.

Key Words: *Biodistribution; Bovine serum albumin nanospheres; Methotrexate.*

INTRODUCTION

During the past few decades, much research has been carried out to investigate the potential applications of hydrophobic biodegradable polymers in colloidal drug delivery systems for controlled and targeted delivery of various therapeutic agents (1). However, most of these polymers present problems associated with the cost and use of toxic organic solvents in production; few of these polymers (like polylactic acid, polylactic glycolic acid, and polyalkyl cyanoacrylate) were observed to produce in vitro cytotoxic effects (2).

Many reports have been made about the phagocytic uptake of polymeric colloidal particles by the reticuloendothelial system (RES) through rapid opsonization (3). To overcome this major hurdle in drug targeting, a few investigators have proposed the concept of obtaining stealth colloidal particles directly prepared from natural hydrophilic carriers like sodium alginate (4). The use of natural carriers such as low-density lipoprotein (5) and

erythrocytes (6) has been investigated as a means to avoid RES uptake. The drug methotrexate, which is a versatile anticancer drug, has been largely studied and evaluated through liposomal carrier systems (7) and other hydrophobic polymeric colloidal carriers like polymethylmethacrylate copolymers (8).

The data pertaining to the evaluation of hydrophilic polymers as drug carriers for cytotoxic agents like methotrexate are minimal (9). Hence, in our present study, we investigated the significant role of bovine serum albumin (BSA) nanospheres containing methotrexate as a drug carrier in the reduction of dose.

EXPERIMENTAL

Materials

Bovine serum albumin (A 2153) was obtained from Sigma (St. Louis, MO); methotrexate USP was supplied by IDPL, Limited (Chennai, India). A membrane filter with a pore size of 1 μ (Bvsp 00010) was purchased from Millipore (Bangalore, India). Acetonitrile (high-performance liquid chromatography [HPLC] grade) and methanol (HPLC grade) were obtained from Supleco (Bangalore, India). Other chemicals, like acetone, glutaraldehyde, and ethanol, were analytical reagent grade.

Preparation of Bovine Serum Albumin Nanospheres

Bovine serum albumin nanospheres were prepared by a novel pH coacervation method (10) by which 2% aqueous solution of BSA was prepared in 100 ml of distilled water. The pH of the solution was adjusted to 9 using 0.5 M sodium hydroxide solution. The solution was stirred on a magnetic stirrer, and a suitable amount of acetone was added dropwise until the solution became just turbid. The BSA nanospheres so formed were cross-linked by adding 100 μl of a 4% glutaraldehyde-ethanol solution; stirring was continued at room temperature for 3 hr. After the cross-linking period, the nanospheres formed were filtered through a Millipore membrane filter with a cutoff range of 1 μm. The filtrate was centrifuged at 20,000 rpm at 25° for 30 min. After centrifugation, the supernatant was removed, and the suspension was washed three times with acetone. Finally, the nanospheres so obtained were suspended in an acetone-water mixture.

Determination of Particle Size

A concentrated aqueous suspension of nanospheres was spread over a slab and dried under vacuum. The sample was shadowed in a cathodic evaporator with a gold layer 20 nm thick. The diameters of all the spheres in each field were calculated using a JSM-6400 scanning electron microscope (Tokyo, Japan).

Drug-to-Polymer Ratio

To carry out the study on the drug-to-polymer ratio, five different batches of nanospheres containing various concentrations of drug (10 mg, 20 mg, 30 mg, 40 mg, and 50 mg) were prepared by following the general procedure. The drug methotrexate was incorporated into the aqueous BSA solution before adjusting the pH of the medium to 9. Each drug-loaded batch had a constant pH and concentration of BSA with variation only in the drug concentration.

Estimation of Amount of Drug Incorporated into Bovine Serum Albumin Nanospheres

Drug-loaded nanospheres (10 mg) were incubated with 10 ml of hydrochloric acid in ethanol at 4° for 24 hr. The nanospheres were separated by centrifugation at 3000 rpm, and the drug content in the supernatant was analyzed by HPLC.

Estimation of Drug by High-Performance Liquid Chromatography

A Waters HPLC system was used for the HPLC analysis. A Kromasil C_8 column (5 μ, 25 cm × 4.6 mm i.d.) was used. A mixture of acetonitrile (50 mm) and phosphate buffer (3.0) was used as the mobile phase at a flow rate of 1.5 ml/min with an operating pressure of 3000 psi. A rheodyne 7125 injector with a 20-μl loop was used for the injection of samples. Detection was done at 302 nm with a sensitivity of 0.001 AuFs. The mobile phase was filtered through a 0.45-μ membrane filter and degassed.

In Vitro Analysis

In vitro release of methotrexate from nanospheres was performed by the centrifugal ultrafiltration technique (11). The quantity of methotrexate-loaded nanosphere

suspension equivalent to 5 mg of drug from the batch loaded with 40 mg of drug was put into a conical flask containing 100 ml of phosphate buffer with a pH of 7.4. The flask was kept in a shaker/incubator at 37°C. Drug-releasing media (2 ml) was withdrawn at various time intervals (15 min, 30 min, 45 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, and 24 hr). The samples were passed through a membrane filter (Millipore with a pore size of 1 μ Bvsp 00010) and then subjected to centrifugation at 6000 rpm for 20 min. After centrifugation, 100 μl of the ultrafiltrate were taken, and the methotrexate content was determined by HPLC. The same study was conducted in triplicate.

In Vivo Biodistribution Studies in Mice Models

Fifty healthy adult mice weighing 25 to 30 g were selected and fasted for 12 hr and then divided into three groups. Group 1 consisted of 20 mice; they have received free drug equivalent to 97.5 μg/30 g in 0.3 ml of sterile normal saline. Group 2 consisted of another 20 mice; they have received methotrexate-loaded naonspheres equivalent to 97.5 μg/30 g of drug in 0.3 ml of sterile normal saline. The remaining 10 mice were group 3; they served as the solvent control. The free drug and the nanosphere-bound drug were injected through the tail vein (12). Mice were sacrificed 24 hr after the administration of samples, and various organs (lungs, liver, spleen, and kidney) were isolated and homogenized with 30 ml of 0.2 M sodium hydroxide. The homogenized organs were centrifuged at 6000 rpm for 15 min, and the supernatant was collected and analyzed for drug content by HPLC.

Extraction and Isolation of Drug from the Isolated Organs

Extraction and isolation of methotrexate from the various organs was accomplished using sep-pak Vcc 3 cc (C_{18}) cartridges connected to a vacuum extraction manifold. The packing material in the cartridges was conditioned with acetonitrile (3 mm), 3 ml of water, followed by phosphate buffer (pH 3) (3 ml × 2) before loading the samples. The homogenized sample (1 ml) was loaded onto the cartridges for isolation of methotrexate. The drug was eluted using a mixture of acetonitrile and phosphate buffer.

RESULTS AND DISCUSSION

Preparation of BSA nanospheres by the pH coacervation method enabled obtaining spherical and discrete

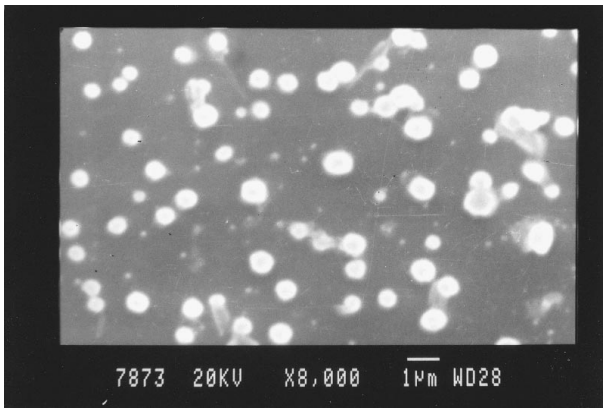

Figure 1. Scanning electron micrograph of bovine serum albumin nanospheres containing methotrexate.

nanospheres with a size ranging from 445.2 nm to 835 nm. The average size was 712.5 nm. The average size of the drug-loaded nanospheres increased compared to the plain nanospheres; it increased from 712.5 nm to 826 nm (Fig. 1). The drug carrier capacity of BSA with respect to methotrexate was determined through the study of the drug-to-polymer ratio. The drug-loading capacity was 11.2%, 16.2%, 22.4%, 33.1%, and 34.6%, respectively, for batches with 10 mg, 20 mg, 30 mg, 40 mg, and 50 mg of methotrexate. It is evident that there was a linear increase in drug-loading capacity with the increase in concentration of drug. There was no significant increase in drug loading after a drug concentration of

Table 1

In Vitro Release Study of Cumulative Percentage of Drug Release

Time Interval (hr)	Cumulative Percentage of Drug Release in Three Sample Batches			Average
	I	II	III	
0.15	14.80	11.03	15.94	13.92
0.30	17.22	17.19	20.13	18.18
0.45	22.17	22.53	22.94	22.54
1	29.40	26.00	27.50	27.63
2	30.97	28.67	30.59	30.07
4	32.50	30.96	41.20	34.88
8	40.84	36.28	43.94	40.35
12	43.94	41.25	47.39	44.1
24	90.51	89.72	93.18	91.13

Table 2

Amount and Percentage of Drug Distribution to Various Organs Through Bound Drug and Free Drug

Organ	From Nanosphere (Bound Drug)		Free Drug		Increase in Percentage Distribution of Drug from Nanospheres in Comparison with Free Drug
	Quantity of Drug Distributed	Percentage of Drug Distributed	Quantity of Drug Distributed	Percentage of Drug Distributed	
Liver	682.84	70.03	459.44	47.12	32.71
Lungs	120.34	12.34	85.34	8.25	33.14
Spleen	147.64	15.14	107.40	11.01	27.27
Kidney	24.48	2.51	24.18	2.48	1.19

Amount of drug distribution is expressed in terms of micrograms per milliliter of organ suspension.

40 mg. Hence, a concentration of 40 mg of drug was considered ideal to achieve the highest loading among the various chosen concentrations of drug. The same batch was selected for the subsequent in vitro and in vivo studies.

The in vitro study of the sample batches showed an initial burst release within 15 min, and 50% of the drug was released within the first 12 hr; afterward, the drug was released in a slow manner over 24 hr. The cumulative percentage release was 90% for all the sample batches (Table 1).

Investigations through the in vivo biodistribution studies showed that there is a marked difference in the percentage drug distribution from the bound drug through the nanosphere when compared to the free drug (Table 2). The percentage increase in drug distribution to the lungs, liver, and spleen from nanospheres was 33.4%, 32.71%, and 27.25%, respectively. This could be due to the phagocytic uptake of nanospheres by the macrophages through the passive targeting. The percentage increase in drug distribution in these organs, which contain abundant macrophages, may allow a reduction in dose to reduce the systemic toxicity.

CONCLUSION

Nanospheres containing methotrexate developed by the pH coacervation method were found to be effective in terms of better drug-loading capacity, satisfactory release characteristics, and reduction of dose. However, the corresponding stability of this system and its significant role in targeted delivery through stearic hindrance are yet to be investigated.

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