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RESEARCH PAPER

A Study on the Preparation and Anti-Tumor Efficacy of Bovine Serum Albumin Nanospheres Containing 5-Fluorouracil

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ABSTRACT

The therapeutic profile of many anti-cancer drugs has been improved by their modified distribution through a colloidal carrier system. Hence, bovine serum albumin nanospheres containing 5-fluorouracil were prepared by pH-coacervation methods. To select the most suitable cryoprotector for the formulated nanosphere system, a study on the effect of cryoprotectors in the prevention of particle agglomeration was done. Using glucose and mannitol at various concentrations during freeze drying, glucose at a concentration of 5% was observed to be relatively more effective in the prevention of particle agglomeration than the other cryoprotectors. The carrier capacity was determined through the drug-to-albumin ratio. The particle size of all the drug-loaded batches was analyzed before and after freeze drying. The batch of nanospheres with uniform size distribution, and highest drug loading, was used for other subsequent studies. The effect of surfactant in drug loading was estimated through various concentrations of sodium lauryl sulfate, and it was observed that the surfactant has no influence on drug loading at the selected concentrations. The batch of nanospheres with highest drug loading was evaluated for its in-vitro release, and the drug release was found to be in a bi-phasic pattern. To evaluate the efficacy of 5-fluorouracil-loaded nanospheres against cancer cells, an in vitro cytotoxicity study was carried out using HEp-2 cell lines. The nanosphere-bound drug was observed to produce a

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better cytotoxic effect than the free drug. The anti-tumor efficacy of drug-loaded nanosphere was investigated in DLA tumor-induced mice models, and the percentage tumor inhibition was relatively higher in animals treated with nanosphere-bound drug than with free drug.

Key Words: *Anti-tumor study; Bovine serum albumin; Cytotoxicity; 5-Fluorouracil; In vitro release; Nanospheres*

INTRODUCTION

Several particulate drug carriers have been developed to improve the efficacy and reduce the toxicity of some anti-cancer agents.[1] Among the polymers proposed to produce nano- and microparticles, the natural polymers such as proteins have been largely employed because of their biocompatibility and biodegradability.[2] It has also been investigated that the gelatin nano- and microparticles, prepared by means of different processes and hardened by a suitable cross-linking agent such as glutaraldehyde, enhance tumoral cell phagocytosis.[3] Hence, these systems have been widely studied in parenteral formulations as carriers of cytostatic drugs such as doxorubicin and methotrexate.[4] However, one of the major problems with the current cancer therapy is that the drug is not retained in the tumor cells, because of the *p*-glycoprotein-dependent accelerated efflux mechanism.[5] Anti-cancer agent absorbed (or entrapped) inside the nanoparticle can bypass the efflux action of drug-resistant cancer cells, thus resulting in their enhanced cellular drug uptake, accumulations, and therapeutic effects.[6] Encouraging reports have been published about the anti-tumor efficacy of anti-cancer drugs loaded onto cyanoacrylic nanoparticles[7] and gelatin.[8] Hence to exploit the probable therapeutic role of bovine serum albumin as a carrier in the targeted delivery of cytostatic agents, the drug 5-fluorouracil, which has an inherent affinity for tumor, was selected and a preliminary study on the preparation and anti-tumor efficacy of drug-loaded nanosphere was investigated.

MATERIALS AND METHODS

5-Fluorouracil USP was donated by Cipla Pharmaceuticals, Bangalore, India. Bovine serum albumin powder was purchased from Sigma Chemical Co., St. Louis, MO. Millipore filter 1 μm (Bvsp 00010) and Millipore filter unit (cpsMM 75541) were obtained from Millipore Ltd., Bangalore, India. Other materials and equipment used include glutaraldehyde 25% (AR grade), acetone (AR grade), ethanol (AR grade), glucose, and mannitol (LR grade). Confluent monolayer culture of HEp-2 cells was kindly provided by the Pasteur Institute of India, Coonoor, India. Dalton's ascites lymphoma cells were obtained from the Amala Cancer Center, Thrissur, Kerala, India.

Preparation of Bovine Serum Albumin Nanospheres Containing 5-Fluorouracil by pH-Coacervation Method and Study on the Effect of Cryoprotector in Freeze Drying

The preparation of nanospheres by pH-coacervation method,[9] was done completely under aseptic conditions, where 2% sterile aqueous solution of bovine serum albumin in 100 mL of sterile water was prepared, and then the pH of the solution was adjusted to 9 by using 0.5 M sodium hydroxide solution. The solution was then stirred on a magnetic stirrer, and a suitable amount of acetone was added dropwise until the solution became just turbid. The bovine serum albumin nanospheres so formed were cross-linked by adding 100 μL of 4% glutaraldehyde–ethanol solution, and stirring was continued at room temperature for 3 hr. After the cross-linking period, the formed nanospheres were filtered through a Millipore membrane filter having a size range of 1 μm. The filtrate was centrifuged at 20,000 rpm at 25°C 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 sterile acetone–water mixture and then subjected to the test for sterility by USP method.[10] After assuring the sterility of the formulated nanospheres, they were evaluated for the effect of cryoprotectors.

Nanospheres are normally isolated and stabilized by freeze drying, hence to assure the redispersibility of this colloidal system, various sugars have been used in preventing the aggregation of nanoparticles during the freeze-drying process. To select the suitable cryoprotectors for the formulated nanosphere system, and to identify the effective concentration of that cryoprotector, a preliminary study was carried out using two different cryoprotectors, such as glucose and mannitol at a concentration of 1%, 3%, and 5%, and they were added to the nanosphere suspension before freeze drying. All six batches of freeze-dried nanospheres with six different concentrations of cryoprotectors were redispersed in normal saline solution by hand shaking, and then evaluated for their particle aggregation through scanning electron microscopy (SEM).

Preparation of Bovine Serum Albumin Nanospheres Containing 5-Fluorouracil by pH-Coacervation Method

Through the preliminary study on cryoprotectors, glucose at 5% concentration was selected as an effective cryoprotector. Hence all the drug-loaded batches were subjected to freeze drying only after the addition of 5% glucose as a cryoprotector. To determine the carrier capacity of bovine serum albumin, with respect to 5-fluorouracil (5-FU), a study on drug-to-polymer ratio was carried out, by adding various concentrations of drug to a constant amount of polymer. Here 2% aqueous solution of bovine serum albumin in 100 mL of sterile water was prepared, to which 10 mg of 5-FU was added, resulting in a concentration of 100 μg/mL of solution. Then the pH of the solution was adjusted to 9, by using 0.5 M sodium hydroxide solution, and the resulting solution was stirred on a magnetic stirrer to enhance the complete dissolution of drug in the carrier. A suitable amount of acetone was added dropwise, until the solution became just turbid. The bovine serum albumin nanospheres so formed were cross-linked by adding 500 μL of 4% glutaraldehyde–ethanol solution and stirring was continued at room temperature for 3 hr. After the cross-linking period, the formed nanospheres were filtered through a sterile Millipore membrane filter, having a size range of 1 μm, in aseptic conditions. The filtrate was centrifuged at 20,000 rpm at 25°C for 30 min. After centrifugation, the supernatant was removed, and the suspension was washed three times with acetone. After the sterile filtration, subsequent steps were also carried out in aseptic conditions. Finally, the nanospheres so obtained were suspended in sterile acetone–water mixture and then transferred to sterile vials. They were subjected to freeze drying, then named batch B.

Five other batches of nanospheres containing various concentrations of 5-FU, such as 50 μg/mL, 150 μg/mL, 250 μg/mL, 300 μg/mL, and 500 μg/mL, were also prepared following the aforementioned procedure, and named batches A, C, D, E, and F, respectively. All the drug-loaded nanospheres were checked for their sterility and evaluated for their size, before and after freeze drying.

Determination of Particle Size

A concentrated aqueous suspension of nanosphere was prepared from each batch before and after freeze drying, and then the samples were spread over a slab and dried under vacuum. The samples were shadowed in a cathodic evaporator with a gold layer of thickness 20 nm. The diameter of a minimum of 50 spheres in each batch was calculated using a JSM-6400 scanning electron microscope. The particle size determinations in each batch were done in triplicate in three different fields, and the mean was calculated.

Estimation of Amount of Drug Incorporated into Bovine Serum Albumin Nanospheres

Twenty milligrams of freeze-dried 5-FU-loaded nanospheres from each batch were taken and incubated with 20 mL of hydrochloric acid in ethanol at 4°C for 24 hr. The disrupted and broken nanospheres were separated by centrifugation at 3000 rpm and the drug content in the supernatant was analyzed by high-performance liquid chromatography (HPLC).

Estimation of Drug by HPLC Method

To extract the drug from the nanospheres, they were treated with hydrochloric acid in ethanol, as mentioned in the above procedure. After the separation of broken nanospheres by centrifugation, 50 μL of supernatant was injected (U6K injector) into a high-performance liquid chromatograph (Waters M 5000A HPLC system) composed of a pump

(LC-5A, Shimadzu Co., Japan), and an ultraviolet (UV) detector (SPD-6A, Shimadzu Co., Japan), an integrator (Waters M-730), a syringe-loading sample injector (U6K), and a reversed-phase μ-Bonda pak C_{18} column (250×2.9 mm i.d.). A 50-mM solution of potassium di-hydrogen phosphate with pH 4.6 was used as a mobile phase.[11] The mobile phase was filtered through a membrane filter (Hvsp 00000) with a pore size of 0.45 μm and degassed. The flow rate of the mobile phase was maintained at 1.5 mL/min with an operating pressure of 3000 psi.

In Vitro Analysis

In vitro release of 5-FU from nanospheres was carried out using the freeze-dried nanoparticles.[12] From batch D the quantity of particles equivalent to 5 mg of drug were suspended in 50 mL of 7.4 phosphate buffer solution and incubated at 37°C under slight agitation (70 cycles/min). Two milliliters of drug-releasing medium was withdrawn at various time intervals of 15 min, 30 min, 45 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, and 24 hr, The samples were centrifuged at 18,000 rpm for 15 min. The volume of release medium was kept constant by adding phosphate buffer solution. One hundred microliters of supernatant were taken, and the 5-FU content was determined by HPLC as described above. The study was done in triplicate.

Freeze Drying of 5-Fluorouracil Nanospheres

To study the difference in the size of nanospheres after freeze drying, 12 batches of drug-loaded nanospheres with various drug concentrations were prepared, as described in the previous stage. Out of these, six batches were subjected to SEM analysis without freeze drying, and another six batches were freeze dried at −40°C using a freeze dryer (Secfroid type, TS 600, Aclens, Switzerland, from Pasteur Institute of India, India) for 48 hr, under vacuum.

Effect of Surfactant in Drug Loading

Among the six batches of 5-FU-loaded nanospheres, the batch loaded with 250 μg/mL (batch D) of drug was selected for further studies on process variables, and in vitro designs based on its satisfactory drug pay load, in vitro release profile, and particle size. To study the effect of sodium lauryl sulfate (SLS) on drug loading, various concentrations of SLS such as 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% of total volume of albumin were selected. Five different batches of nanospheres, with 250 μg/mL of 5-FU and five different concentrations of SLS, were prepared by following the general procedure. The drug loading in each batch was determined by UV spectrophotometry at 266 nm. The increase in percentage drug loading of five batches of nanospheres with SLS was compared with the same batch of nanospheres without SLS. The work was done in triplicate. The test of significance was carried out to know the effect of SLS on drug loading.

Effect of Cryoprotectors in the Prevention of Particle Aggregation

Nanospheres are normally isolated by freeze drying, hence to assure the redispersibility of these colloidal systems, various sugars have been used in preventing the aggregation of nanoparticles during the freeze-drying process. To study the effect of cryoprotectors in the prevention of particle aggregation after freeze drying, two different cryoprotectors, such as glucose and mannitol, were selected at concentrations of 1%, 3%, and 5%, and added to the nanosphere suspension before freeze drying. All the six batches of freeze-dried nanospheres with six different concentrations of cryoprotectors were freeze dried, and then redispersed in normal saline solution by hand shaking, and evaluated for their particle aggregation through SEM.

In Vitro Cytotoxicity Test

To evaluate the efficacy of drug-loaded nanospheres against cancer cells, an in vitro cytotoxicity test[13] was carried out using HEp-2 cell lines where aliquots (1 mL) of cell type HEp-2 (1×10^5/mL) were added to RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum seeded into 24-well microtitre plates, subsequently varying amounts of 5-fluorouracil (50 μg, 100 μg, and 150 μg per well). 5-Fluorouracil-loaded nanospheres equivalent to 50 μg, 100 μg, and 150 μg per well were added along with control and incubated at 37°C for 5 min with gentle agitation, followed by culturing at 37°C under 5% atmospheric CO_2. The number of viable cells remaining in each well was determined by Tryphan blue dye extrusion

technique. From the in vitro cytotoxicity test the percentage viability of 5-fluorouracil nanospheres was compared with that of control and free drug at different concentrations.

Study on Anti-tumor Efficacy

The anti-tumor efficacy of 5-fluorouracil nanospheres was compared with that of free drug and control[14] against Dalton's ascites lymphoma cells in Swiss albino mice. After establishing the tumor in the peritoneal cavity in all the groups of mice,[15] the treatment was started from the second day and continued up to 15 days. An anti-tumor study was carried out using four groups of Swiss albino mice, each containing six healthy animals of the same sex. Among the groups, group I animals were not treated without any drug and were allowed to develop cancer. They served as the control. Group II animals received the solvent Hanks buffer, administered by i.v. route into the tail vein, and served as control. Group III animals received free 5-FU equivalent to 3 mg/kg and the drug was dispersed in Hanks buffer and injected through the tail vein. Group IV animals were treated with 5-FU-loaded nanospheres equivalent to 3 mg/kg in Hanks buffer through the tail vein. After inducing cancer in the latter three groups of mice, treatment was given to the animals through free drug and nanosphere-bound drug till 15 days. Treatment was discontinued on the 16th day, and the animals from all groups were dissected at their peritoneal cavity. After confirming the DLA-induced tumor development in the animals, the tumor was transferred to 5 mL of sterile normal saline solution in each mouse from each group. The centrifugation of tumor cells in 5 mL of sterile normal saline solution resulted in a suspension. It was measured as the packed cell volume. After the measurement of the packed cell volume from each mouse, the samples were washed three times with phosphate buffer solution, and the resultant cell pellets from each sample were resuspended in PBS.

The packed cell volume (PCV) can be determined to find out the total number of Daltons ascites lymphoma cells[16] present in each animal. Subsequently, it is related to the rate of growth of tumor in each animal. The comparative efficacy of nanosphere-bound drug and free drug can be evaluated in terms of percentage tumor inhibition. The formula for the calculation of percentage tumor inhibition is given below:

$$\% \text{ Tumor inhibition} = 100 - x$$

where

$$x = \frac{\text{Test PCV}}{\text{Control PCV}} \times 100$$

RESULTS AND DISCUSSION

It has been reported by a large number of investigators that the nanoparticles produced from natural, hydrophilic polymers show relatively better drug-loading capacity for many drugs than the other hydrophobic synthetic polymers.[17] However, the nanoparticles prepared by the desolvation of the natural macromolecules tend to produce a relatively lower processing yield than the other methods.[18] Since the method adopted in our present study involves the coacervation technique, through a natural macromolecule, it may also be presumed to have a lower yield. The nanospheres produced by pH-coacervation method enabled us to get spherical, discrete spheres with a size ranging from 398 nm to 824 nm. The average particle size was found to be 638±1.2 nm for batch D (Fig. 1), which was selected as the ideal batch and used throughout subsequent studies. The yield of the process was estimated in each batch, and observed to be in the range of 20–23%.

The effect of cryoprotector in preventing particle aggregation during freeze drying was studied through mannitol and glucose at various concentrations, like 1%, 3%, and 5%. When all the batches were redispersed in normal saline after hand shaking and observed through SEM (Fig. 2), only the batch with 5% glucose was found to possess and retain a spherical shape without much clumping. This clearly suggests that the selected cryoprotectors are not effective in preventing particle aggregation at lower concentrations such as 1% and 3%, and that mannitol is not suitable for this system even at a concentration of 5%.

The drug-loading capacity of a nanoparticulate carrier is mainly altered by the nature of the drug incorporated, and the method of production adopted. Hence, the estimation of the drug payload of bovine serum albumin with respect to 5-FU would be an essential parameter to know its

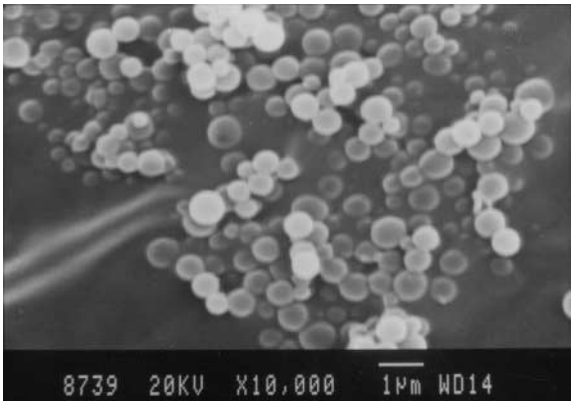

Figure 1. SEM Image of nanospheres suspended in acetone–water mixture.

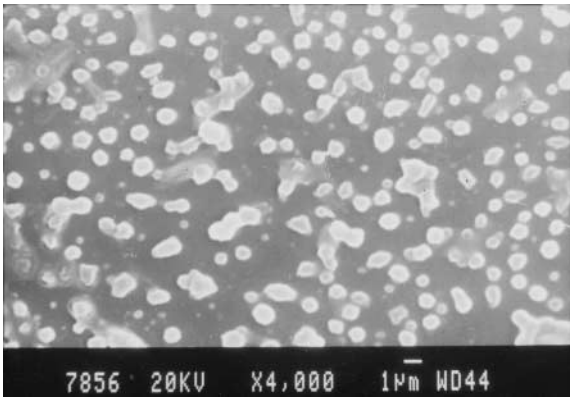

Figure 2. SEM image of freeze-dried nanospheres.

limitations and applications as a drug carrier. A preliminary study on drug-to-albumin ratio was carried out, using various concentrations of 5-FU, i.e., 50 μg/mL, 150 μg/mL, 200 μg/mL, 250 μg/mL, 300 μg/mL, and 500 μg/mL, and they were named batch A, B, C, D, E, and F, is described previously. The drug payload of those batches was determined to be 0.4 μg/mg (0.8%), 15.2 μg/mg (10.2%), 25.3 μg/mg (12.6%), 47 μg/mg (18.8%), 58.2 μg/mg (19.4%), and 92 μg/mg (18.2%), respectively for batches A, B, C, D, E, and F. This clearly indicates that there is a proportional increase in percentage drug incorporation with increase in drug concentration. The linearity between the drug concentration and drug loading is maintained only up to a concentration of 250 μg/mL of drug. Thereafter, there is not much increase in percentage drug incorporation between batches E and F. The results also imply that there is a reduction in percentage drug incorporation after a drug concentration of 300 μg/mL, and the reason could be attributed to saturation of binding sites in the carrier. A similar phenomenon has been observed by other investigators.[19]

All the drug-loaded nanosphere suspensions were subjected to freeze drying only after addition of 5% glucose as cryoprotector. All the batches were evaluated for changes in particle size before lyophilization and after lyophilization through SEM. Table 1 shows the difference in the particle size of nanospheres, before and after freeze drying. From the table it can be seen that there is a slight increase in the particle size of all drug, loaded nanospheres after freeze drying. This is probably due to the dehydration and elongation of spherical structures during freeze drying under vacuum. Figure 2 shows the change in nanosphere size after freeze drying. The lyophilization of all the drug, loaded nanospheres resulted in the formation of a hard, pelleted system, which in turn proved difficulty to re-disperse by hand shaking. A similar instability behavior has been observed for other nanoparticles made up of synthetic polymers.[20]

To check the effect of surfactant in improving the drug payload, the surfactant, sodium lauryl sulfate, was selected and added at a concentration of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% to batch D. The effect of surfactant in drug loading was found to be insignificant ($p > 0.01$). Table 2 shows the effect of surfactant in percentage drug loading. Figure 3 shows the in vitro release profile of 5-FU from three sample batches of D. The pattern of release was found to be bi-phasic, with an initial burst effect within 30 min. The cumulative percentage release was found to be 90 and above, for all the sample batches.

In our attempt to investigate the efficacy of 5-FU-loaded nanospheres against the HEp-2 cancer cell lines, it was evident that the drug bound to nanospheres is more effective than the free drug, in terms of reduction in percentage cell viability, even at a lower concentration. Table 3 shows the percentage cell inhibition by nanosphere-bound drug and free drug. A test of significance was applied to compare the significance of the difference in percentage cell viability between the nanosphere-bound drug and the free drug. It has been found that the decrease in percentage cell viability obtained through the nanosphere-bound drug is significantly different from cell viability through the free drug ($p < 0.05$).

Table 1

Particle Size Variation of Drug-Loaded Nanospheres Before and After Freeze Drying

Batch	Average Particle Size of Nanospheres Before Freeze Drying	Average Particle Size of Nanospheres After Freeze Drying
A	428 nm (1.2)	494 nm (0.8)
B	466 nm (0.6)	523 nm (1.4)
C	574 nm (0.8)	594 nm (0.4)
D	638 nm (1.2)	676 nm (2.0)
E	658 nm (1.4)	692 nm (1.6)
F	724 nm (2.4)	786 nm (1.8)

Each value represents the mean. SD shown in parentheses.

Table 2

Effect of Surfactant on Percentage Drug Loading

Batch D (loaded with 250 μg/mL of drug)	Percentage Drug Loading
Without SLS	28.6% (1.4)
0.1% SLS	29.3% (2.1)
0.2% SLS	30.5% (0.9)
0.3% SLS	30.8% (0.6)
0.4% SLS	31.6% (0.4)
0.5% SLS	31.9% (1.6)

Each value represents the mean of three determinations. SD shown in parentheses. The data are statistically insignificant at $p > 0.01$.

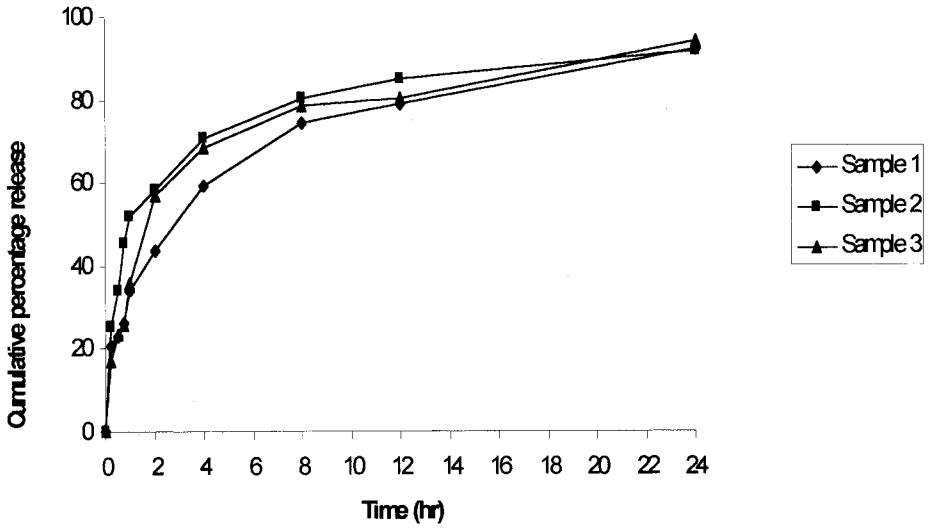

Figure 3. In vitro release profile of 5-fluorouracil from different samples of batch D.

Table 3

Percentage Cell Inhibition by Nanosphere-Bound-5-FU and Free Drug

Group (μg/well)	Viable Count ($\times 10^4$)	Viability (%)
Control	14.5	100
5-FU (50)	8.2	76.7
5-FU (100)	5	52.6
5-FU (150)	3.1	42.4
5-FU nanosphere (50)	5.8	56.3[a]
5-FU nanosphere (100)	2.9	40.8[a]
5-FU nanosphere (150)	2.2	32.4[a]

[a]Nanosphere-bound drug, data statistically significant at $p < 0.05$.

Table 4

Percentage Tumor Inhibition Produced by Nanosphere-Bound Drug, and Free Drug in DLA-Induced Mice

Group	Total Volume (mL)	Packed Cell Count	Tumor Inhibition(%)
Control	17.2 (1.2)	11.2 (2.1)	0
5-FU	11.8 (1.8)	7.8 (2.1)	30.1 (2.2)
5-FU nanospheres	8.2 (2.4)	4.3 (0.8)	60.6 (2.6)

Each value represents the mean of 3 determinations. SD shown in parentheses.

Table 4 shows the percentage tumor inhibition produced by different groups. The data obtained from the study on anti-tumor activity in DLA-induced mice indicates that there is twofold increase in percentage tumor inhibition through the nanosphere-bound drug compared to the free drug.

In conclusion, the investigations of bovine serum albumin nanospheres containing 5-FU, through preliminary studies on the carrier capacity, in vitro release, anti-tumor efficacy, and in vitro cytotoxicity, reveal its suitability as a delivery system, for the reduction of the dose of cytostatic agents through their enhanced efficacy against cancer cells.

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