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Preparation and Optimization of Sodium Alginate Nanospheres of Methotrexate.

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Nanoparticles represent a promising drug delivery system of controlled and targeted drug release. They are specially designed to release the drug in the vicinity of target tissue. Nanoparticles made up of natural, hydrophilic carriers like sodium alginate may have the advantage of producing a stearic hindrance in the systemic circulation. Hence, sodium alginate nanospheres containing methotrexate were prepared by controlled gellification method. The average particle size was found to be 452 nm. The carrier capacity of sodium alginate nanospheres with respect to methotrexate was determined by the drug to polymer ratio, and five different batches of drug loaded nanospheres containing various concentrations of drug were subjected to *in vitro* analysis by dialysis method. The study on the drug to polymer ratio showed a linear relationship between the concentration of drug and percentage drug loading. The effect of different concentration of Brij-35 on the drug loading was also checked through the batch with lowest drug loading capacity. The *in vitro* release behaviour from all the drug-loaded batch was found to be pseudo zero order. The ideal batch of nanospheres with highest drug loading and satisfactory *in vitro* release profile was subjected to stability studies for 60 days at 4°. The stability of drug loaded nanospheres was checked in terms of percentage drug leakage into the storage medium. It has been observed that, there was no much drug leakage into the storage media, when the nanospheres were stored for 1 month. The comparative *in vitro* cytotoxicity study between drug loaded nanospheres and free drug was carried out using HEP-2 cell lines. The drug bound to nanospheres produced a comparatively better cytotoxic effects at all concentration than the free drug.

In recent years, there has been a considerable interest in the development of novel drug delivery systems in order to modify and control the pharmacokinetic behaviour of therapeutic agents. By incorporation into a carrier system, it is possible to alter both the therapeutic index and the duration of activity of drugs[1]. Colloidal drug carriers are gaining success by achieving reduced toxicity, enhanced efficacy and site-directed action. The major limitations of current anticancer, antiparasitic and antiinfectious agents are their toxicity and lack of specificity[2]. The ideal dosage form in cancer chemotherapy is the one that provides a specific delivery of anticancer agent to the tumour site in sufficient amount, for a long period of time with no interaction with normal tissue[3]. Colloidal drug delivery systems for targeted distribution of anticancer drugs are of increased interest for research in elevating the therapeutic efficacy of this class of drugs[4].

Nanoparticles containing cytotoxic agents could be useful for the treatment of certain cancer that often show resistance to uptake of free drug. Nanoparticles bound antitumour agents have demonstrated a prolonged drug retention in tumour, and prolonged survival of the tumour bearing animals. Active targeting by using magnetic field focused on the tumour site may improve the therapeutic index of the drugs[5]. Sodium alginate nanospheres containing methotrexate would be a suitable carrier system for site specific delivery. The *in vivo* biofate of particulate system is highly altered by its physicochemical properties. Therefore, before evaluating its *in vivo* targeting ability its

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particle size, drug loading capacity and its *in vitro* characteristics are to be studied. In turn it may have an effect on the *in vivo* behaviour of the system. Hence, in our present study we have made an attempt to optimize and to check the suitability and potentiality of natural carrier such as sodium alginate for anticancer drugs.

MATERIALS AND METHODS

Methotrexate IP was obtained as a gift sample from Biochem, Mumbai. Sodium alginate, Poly-1-lysine, calcium chloride were purchased from S. D. Fine Chemicals, Mumbai. Brij-35 and dialysis bags were purchased from Sigma Chemicals USA. Other reagents like ethanol, mannitol, hydrochloric acid, sodium chloride, acetone, and sodium phosphate were of analytical grade.

Preparation of sodium alginate Nanospheres containing methotrexate:

Alginate nanospheres were obtained by inducing the gellification of sodium alginate solution with calcium chloride[6], where 1.5 ml of calcium chloride (18 mM) solution was added to 28.5 ml of sodium alginate (0.1%w/v). The pH of the solution was adjusted to 9 using 0.05 M NaOH, and the drug, methotrexate (10 mg) was dissolved in the sodium alginate solution. Twelve milliliters of Poly-1-lysine (0.1%) solution was added to get a final suspension of alginate nanoparticles. The suspension was stirred for 1 h using a magnetic stirrer. The suspension was kept for over night and nanospheres were centrifuged at a speed of 40 000 rpm for half an hour. The nanospheres were collected and stored in acetone water mixture and named as batch 'B'. By following the above mentioned procedure, four other batches of sodium alginate nanospheres with varying concentrations of methotrexate such as 5, 15, 20 and 30 mg were also prepared and named as batch A, C, D, and E. For the preparation of various drug loaded batches the concentration of polymer was kept constant, but the concentrations of drug were varied.

Estimation of amount of drug incorporated in to sodium alginate nanospheres:

A quantity of drug loaded nanospheres from each batch equivalent to 5 mg of drug was incubated with 5 ml of ethanolic hydrochloric acid at 4° for 24 h. After 24 h incubation, the nanospheres were separated by centrifugation at 3000 rpm and the drug content in the supernatant was analyzed by UV spectrometer at 303 nm.

Determination of particle size by scanning electron microscopy analysis[7]:

An aqueous dispersions of the nanospheres was finely spread over a stab and was dried by keeping in a desiccator. The dried film of the nanospheres was given a 25 nm thick gold layer and was observed by SEM. The sizes of minimum of 100 particles were checked for their size distribution to determine the average particle size and size range.

Effect of Surfactants on the drug loading capacity[8]:

The effect of surfactant on drug loading capacity was determined by adding various concentrations of surfactants. The batch of nanospheres with minimum drug loading was selected for this study (Batch A). The surfactant Brij-38 was selected at various concentrations namely, 0.1%, 0.3%, and 0.5% of the total volume of the sodium alginate solution. After the surfactant has been dissolved in the sodium alginate solution at various concentrations, the other steps were followed as it is described in the preparation of the drug loaded nanospheres. Drug loading capacity of the batch A without addition of surfactant was checked and it was then compared with the percentage drug loading after addition of surfactant. Each batch was produced in triplicate and average values of three batches were recorded.

Study on *in vitro* release studies of drug loaded batches by dialysis method[9]:

The *in vitro* release of all drug loaded nanospheres was carried out by employing a diffusion cell. A quantity of nanospheres suspension which is equivalent to 5 mg of drug was taken in a sigma dialysis membrane which was fixed to one end of the apparatus to result a permeation cell. The nanospheres were taken in the cell and the cell was immersed in a beaker containing 50 ml of phosphate buffer (pH 7.4) as receptor compartment. The cell was immersed to a depth of 1 cm below the surface of the receptor solvent. The medium in the receptor compartment was agitated continuously using a magnetic stirrer and a temperature of 37±1° was maintained within the diffusion chambers. Five millilitres of the sample of the receptor compartment were taken at various intervals of time over a period of 24 h and each time fresh buffer was replaced. The sample withdrawn was estimated spectrophotometrically at 303 nm.

Stability study of drug loaded nanospheres:

The stability study was carried out using the batch 'B'. The stability of drug-loaded nanospheres was evaluated in terms of its drug leakage into the storage medium (acetone water mixture at 1:1 ratio) and by the changes in the *in vitro* release characteristics at two different time intervals. To determine the stability of nanospheres system, the

percentage of drug loading and *in vitro* release characteristics have been checked in six sample batches, before starting the stability studies and then those sample batches were stored at 4° for 60 d. During the stability studies, each sample was centrifuged for 10 min, the sediment (nanospheres) was collected, and treated in same manner, as it is described in the estimation procedure. The drug content and *in vitro* release profiles were analyzed at time intervals of 30 d and 60 d. The difference in drug content and changes in the extent of *in vitro* release profiles, before the stability study and after the stability study were considered to be proportional to the drug leakage into the storage medium and also the extent of changes in the stability (*in vitro*) of nanospheres.

***In vitro* cytotoxicity studies by cell culture method[10]:**

Aliquots (1 ml) of cell type HEP_2 (1 × 105 /ml) was added to RPMI 1640 medium supplemented with 10% heat inactivate sheep serum seeded into 24 cell microlitre plates. Subsequently varying amount of methotrexate (50, 100, 150, 200 µg/well). Methotrexate nanospheres (equivalent to 50, 100, 150, 200 mg/ml of methotrexate) were added along with control and incubated at 37° under 5% atmospheric CO_2. Numbers of viable cells remaining in each of the well were determined by Trypan blue dye exclusion technique. Any compound which is cytotoxic to cells, inhibits the cell proliferation and kills the cells. Trypan blue is a dye, which is capable of penetrating in the dead cells, therefore the dead cells take up the blue strain where as the viable cells do not. This method gives an exact number of dead and viable cells.

Ten milligrams of the drug was dissolved in 0.5 ml of sterile dimethyl sulfoxide and volume was made up to 10 ml with minimum essential medium (MEM) to give a drug concentration of 1000 µg/ml. From this further dilution were made in Hanks buffer to get the desired drug concentration. Confluently grown monolayer of HEP-2 cells was trypsinised. Adjusted the cell count 1 lakh per ml in MEM with 10% syrup. Five hundred microlitres of cell suspension was seeded into each well of 24 well microlitre plate so as to get 50000 cells/well. The plates were then incubated for 24 hours at 37° in humidified 5% CO_2 incubator. After 24 h of incubation, the medium was discarded and 0.5 ml of each of the test compounds were added in 3 well and the control wells maintained only with growth medium. The plates were then incubated in a humidified 5% CO_2 incubator for further 72 h. The cells were examined microscopically at an interval of 24 h for visible signs of toxicity such as swelling, shrinkage, granularity, floating cells, vacoularisation and any other morphological changes. After 72 h, each well was treated with 0.05 ml of trypsin versene glucose solution (TPVG) and kept at room temperature for 3 min. To each well, 0.15 ml of maintenance medium was added to stop the action of trypsin, the cells were aspirated and the total number of dead cells and viable cells were counted using trypan blue in a haemocytometer. The total number of viable cells were calculated by using the formula.

Total number of viable cells = Nv×Df×Cf Where, Nv= No. of the viable cells= total no of cells–dead cells. Df=dilution factor, Cf= conversion factor. Percent viability= (Total no of cells – dead cells/total no of cells)×100

RESULTS AND DISCUSSIONS

The sodium alginate nanospheres were prepared by controlled gellification method, yielded spheres with discrete nature. The size of drug loaded nanospheres was found to be 186±12 nm to 544±18 nm. The average size of particle was found to be 386±21 nm. Scanning electron micrograph of sodium alginate nanospheres containing methotrexate is shown in fig. 1. It has been observed that there was a slight increase in the average particle size after drug loading.

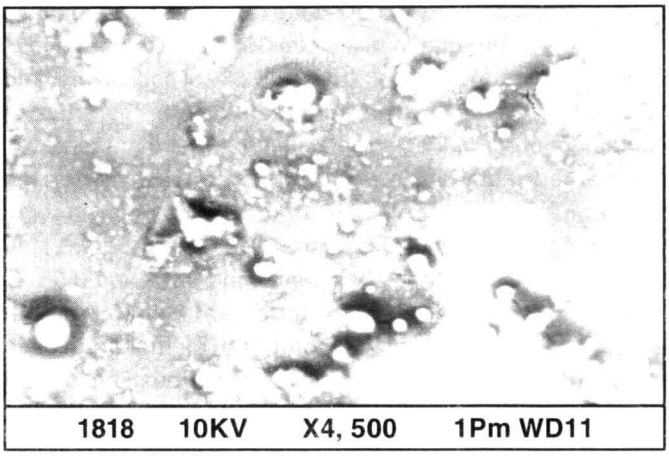

Fig. 1: Shows the scanning electron micrograph of sodium alginate nanospheres containing Methotrexate

The size distribution after drug loading was observed to be in the range of 282±14 nm to 622±18 nm. The average particle size was found to be 452±21 nm. The same phenomenon has been observed by other authors also[11]. The carrier capacity of sodium alginate nanospheres with respect to many hydrophilic drug like doxorubicin was reported to be comparatively higher than other carrier materials. Hence, to evaluate the carrier capacity of sodium alginate nanospheres with respect to the selected lipophillic drug, a study on drug to polymer ratio was carried out, with

TABLE 1: THE DRUG LOADING CAPACITY OF SODIUM ALGINATE NANOSPHERES, PREPARED THROUGH DRUG TO POLYMER RATIO USING METHOTREXATE.

Name of the Batches	Drug to polymer ratio Drug (g): polymer ratio (ml)	Drug loading efficiency (%)
Batch A	0.17 :1	48.1±1.2
Batch B	0.33:1	60.1±0.8
Batch C	0.50:1	62.2±0.4
Batch D	0.67:1	76±1.1
Batch E	1:1	79±1.4

varying volume of polymer. Table 1 represents the drug loading capacity of sodium alginate nanospheres, prepared through drug to polymer ratio using methotrexate. From the drug to polymer ratio study, the drug loading capacity of all 5 drug loaded nanospheres was evaluated and it was found to be 48.12, 60.18, 62.20, 76.0 and 79%, respectively for the batches A, B, C, D and E Table 1. It can be understood that, there is a linear relationship between the concentration of drug and drug loading capacity. It is also evident that, the sodium alginate has a better drug loading capacity even for the lipophillic drug like methotrexate. To study the effect of surfactant, on drug loading, Brij-35, was used at various concentrations like 0.1%, 0.3% and 0.5% of sodium alginate solution. The data derived from the study on the effect of surfactant on drug loading revealed the fact that there was no marked increase in drug loading capacity after the addition of surfactant. The comparative increase in percentage of drug loading was observed to be 1% and 1.2% respectively for batches containing 0.3% and 0.5% of Brij-35. Hence, the selected surfactant Brij-35 was not effective in improving the solubility of the drug. The investigations of Zimmer *et al*[12], describes the use of polysorbate 80 and Tween 80 as solubilizing agents to improve the solubility of hydrocortisone in the preparation of hydrocortisone loaded albumin nanoparticles for ocular delivery. Figure 2 shows the *In vitro* release profile of Methotrexate from different drug loaded nanospheres. It can be observed that, all the drug loaded nanospheres exhibit a bi-phasic release. The initial burst effect occurs within 1 h from all the drug loaded batches. The burst effect could be due to the release of drug loaded on the surface of the nanospheres, and the remaining part of release may be due to the slow diffusion of the drug from the nanospheres matrix, by erosion. The cumulative percentage of drug release was found to be 90% and above for all the batches. A similar finding and release profile has been reported by Heussler *et al*[13] where they have reported about the *in vitro*

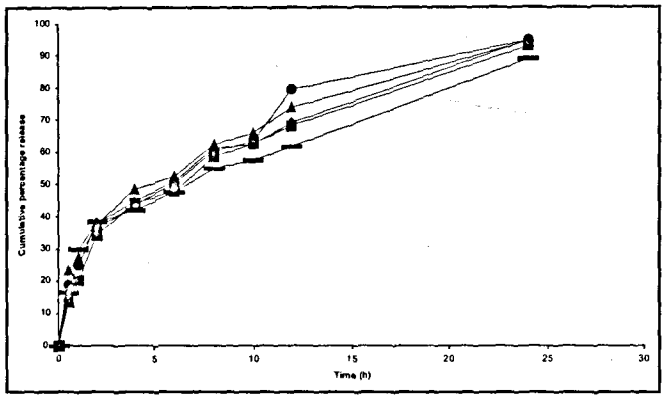

Fig. 2: in vitro release patterns of sodium alginate nanospheres containing methotrexate

Methotrexate loaded nanospheres Batch A (●), Batch B (▲), Batch C (◆), Batch D (□) and Batch E (□□) were incubated in PBS at 37°. Samples withdrawn at different time intervals and methotrexate were determined spectrophotometrically.

release of betaxolol chlorhydrate from isobutyl cyanoacrylate nanoparticles, and it was found to exhibit a zero order release. The nanoparticles prepared from natural polymers like sodium alginate are obtained as aqueous gels, they are stabilized and obtained as dried films only after lyophillisation. Before lyophillisation the nanospheres are stored in different storage media like acetone water mixture. To evaluate the stability of sodium alginate nanospheres in the storage medium at 4° the stability study was carried out in terms of percentage drug leakage into the storage medium for a period of 60 d at 4°. The results of stability studies on drug loaded nanospheres clearly indicates that the percentage leakage of drug into the storage medium was comparatively higher when the nanospheres were stored for 60 d, than storing the nanospheres for 30 d. The mean percentage decrease in drug loading up to 30 d of the storage was found to be

TABLE 2: COMPARATIVE CHANGES IN THE IN VITRO RELEASE CHARACTERS OF DRUG LOADED NANOSPHERS BEFORE AND AFTER STORAGE PERIOD.

Time (h)	Mean cumulative percentage release before storage.	Mean cumulative percentage release after 30 d of storage.	Mean cumulative percentage release after 60 d of storage.
0.5	14.2±0.01	12.6±0.11*	8.2±0.02
1	26.6±0.02	21.8±0.14*	17.2±0.08
2	30.2±0.01	38.2±0.08	27.2±0.09
4	43.8±0.04	46.8±0.14	46.4±0.12
6	49.7±0.03	51.2±0.13	48.6±0.18
8	57.5±0.02	62.6±0.06	58.3±0.2
10	63.3±0.02	69.4±0.16	66.2±0.18
12	76.5±0.01	78.2±0.18	79.4±0.21
18	84.2±0.1	82.4±0.02	82.1±0.06
24	96.7±0.08	93±0.07	92.8±1.2

±SD n=6 *Mean cumulative percentage, data statistically significant at $p<0.05$.

0.76±0.8, which is considered to be very minimum and negligible. Similarly, the mean percentage of decrease in drug loading up to 60 d of storage was found to be 2.06±0.6. The loss of drug to storage medium from nanospheres after 60 d of storage was found to be insignificant ($p>0.05$) when data was statistically analysed. Hence, the formulated sodium alginate nanospheres were found to be stable in the storage medium up to 30 d before lyophillisation. The stability of gliadin nanoparticles was studied in phosphate buffer saline and it was reported to be stable in that storage medium without any esterases[14]. To compare the changes in the *in vitro* release characters of drug loaded nanospheres before and after the storage period, the release profile of drug, from drug loaded nanospheres was carried out by dialysis method for 24h. Table 2 shows the comparative changes in the *in vitro* release characters of drug loaded nanospheres before and after storage period. It can be observed that, there is no significant change in the rate and extent of initial burst release and total cumulative percentage release, from nanospheres stored up to 30 d. Whereas there is a significant decrease in the extent of initial burst release from nanospheres stored up to 60 d. The probable reason for the significant decrease in the extent of initial burst release (from 14.2% to 8.2%) and (from 26.6% to 17.2%) may be attributed to the loss of drug from the surface of nanospheres to the storage medium when stored for 60 d. There was no significant difference in the total cumulative percentage release, even after 60 d of storage and it may be due to the slow diffusion of drug from the inner matrix structure of nanospheres, which has not leached the drug during storage period. The findings of Zhang *et al*[15], reveals the stability of poly butyl cyano acrylate nanoparticles for 90 d at 25°. They have reported that there were no marked changes in their morphology and *in vitro* release characters after storage period. The *in vitro* cytotoxicity studies done using HEP-2 cell lines showed a comparatively better efficacy through the drug loaded nanospheres, than the free drug. Table 3. The percentage viability of drug loaded nanospheres was determined to be 75.9%, 60.7%, 45.8% and 37.5% at concentrations of 50, 100, 150 and 200 µg/ml, respectively. Where as the percentage viability through free drug was observed to be 87.8, 70, 57, and 46.15 at concentrations of 50, 100, 150 and 200 µg/ml, respectively. The results derived through *in vitro* cytotoxicity study reveals the enhanced cell killing efficacy of nanospheres bound drug over the free drug. It could be due to the enhanced endocytic activity of nanospheres by the selected cell lines. Similarly, the cytotoxic activity of small sized chitosan gel nanospheres containing 5-flurouracil was found to be better than the free drug against HLE human hepatoma cells[16].

The formulated sodium alginate nanospheres containing Methotrexate is found to be a suitable and

TABLE 3: COMPARATIVE *IN VITRO* CYTOTOXICITY STUDY OF DRUG LOADED NANOSPHERES USING HEP-2 CELLS.

Group	Viable count C X 10^4	% Viability
Control	22	100
MTX-50 (Free)	17	87.8
MTX-100 (Free)	10.5	70
MTX-150 (Free)	8	57
MTX-200 (Free)	6	46.2
MTX-Nano-50	14	75.9
MTX-Nano-100	8.5	60.7
MTX-Nano-150	5.5	45.8*
MTX-Nano-200	3.75	37.5*

*Nanospheres bound drug, data statistically significant at $p<0.05$.

potential natural carrier interms of their particle size, drug loading capacity, *in vitro* release characteristics, physical stability and *in vitro* cytotoxic activities. Hence, it may be used as an alternative and cheaper carrier in site specific delivery of anticancer drug. In turn it may be useful in reducing the total cost of the therapy.

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