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Two-Stage Optimization Process for Formulation of Chitosan Microspheres

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ABSTRACT

The objective of the present study was to optimize the concentration of a chitosan solution, stirring speed, and concentration of drugs having different aqueous solubility for the formulation of chitosan microspheres. Chitosan microspheres (unloaded and drug loaded) were prepared by the chemical denaturation method and were subjected to measurement of morphology, mean particle size, particle size distribution, percentage drug entrapment (PDE), drug loading, and drug release (in vitro). Morphology of the microspheres was dependent on the level of independent process parameters. While mean particle size of unloaded microspheres was found to undergo significant change with each increase in concentration of chitosan solution, the stirring rate was found to have a significant effect only at the lower level (ie, 2000 to 3000 rpm). Of importance, spherical unloaded microspheres were also obtained with a chitosan solution of concentration less than 1 mg/mL. Segregated unloaded microspheres with particle size in the range of 7 to 15 µm and mean particle size of 12.68 µm were obtained in the batch prepared by using a chitosan solution of 2 mg/mL concentration and stirring speed of 3000 rpm. The highest drug load (µg drug/mg microspheres) was 50.63 and 13.84 for microspheres containing 5-fluorouracil and methotrexate, respectively. While the release of 5-fluorouracil followed Higuchi's square-root model, methotrexate released more slowly with a combination of first-order kinetics and Higuchi's square-root model. The formation of chitosan microspheres is helped by the use of differential stirring. While an increase in the concentration of water-soluble drug may help to increase PDE and drug load over a large concentration range, the effect is limited in case of water-insoluble drugs.

KEYWORDS: optimization, chitosan microspheres, 5-fluorouracil, methotrexate

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INTRODUCTION

Chitosan is a polysaccharide comprising copolymers of glucosamine and N-acetyl glucosamine. Being biodegradable and biocompatible, chitosan has been used in the formulation of particulate drug delivery systems to achieve controlled drug delivery.[1,2] Chitosan microspheres have been prepared by chemical denaturation,[3,4] ion-induced coagulation,[2,5] and spray-drying methods.[6,7] Of these methods, the most common method used to prepare chitosan microspheres is the chemical denaturation method. Chemical denaturation involves denaturation of chitosan present in the inner phase of water/oil (w/o) emulsion. Denaturation is usually carried out using glutaraldehyde with continuous stirring. Many process parameters affecting characteristics of chitosan microspheres have been identified, and the significance of the effect has been established.[8,9] It has been reported that irrespective of molecular weight, chitosan microspheres are formed only if the concentration of the chitosan solution is at least 1% wt/vol.[10] However, no reason was offered for this observation. This finding has led the workers in this field to restrict the minimum level of chitosan concentration to 1% wt/vol. In addition to concentration of chitosan solution, several other process parameters have been identified and optimized. However, no attempt has been made to study the effect of the physical property of the drug on the attributes of microspheres.

The chemical cross-linking method for preparation of chitosan microspheres involves emulsification followed by cross-linking with a suitable cross-linking agent (eg, glutaraldehyde). The degree of stirring (ie, time and speed of stirring during emulsification) determines the size of dispersed droplets. By varying any one or both of these parameters, the size of droplets can be changed to obtain the product (ie, chitosan microspheres) in the desired size range. However, no further division of quasi-solid or solid particles, formed during the process of cross-linking, should be desired in order to protect the structural integrity of the microspheres. Based on this hypothesis, it was planned to carry out stirring at a higher rate of agitation during initial emulsification and at lower rate during the cross-linking stage.

The present study was carried out with 2 objectives. The first objective was to change the method of preparation based on the above hypothesis and to see if microspheres

could be obtained by the modified method using a chitosan solution of lower concentration (<1% wt/vol). Further, it was planned to compare the pharmaceutical characteristics of the prepared microspheres with the microspheres obtained with a higher concentration (>1% wt/vol) of chitosan solution. The second objective was to study the effect of some drugs having different aqueous solubility on the characteristics of the microspheres. The study was conducted in 2 different stages. In the first stage, optimization of the concentration of chitosan solution was carried out. In the second phase, the effect of the concentration of 2 different drugs having different aqueous solubility was studied. Methotrexate (MTX) was chosen as the drug that is practically insoluble in water, and 5-fluorouracil (5-FU) was chosen as the drug with appreciable water solubility.

MATERIALS AND METHODS

Materials

Chitosan, >85% deacetylation, was from the Central Institute of Fisheries Technology, Kochi, India. MTX and 5-FU were both from Biochem Pharmaceutical Ltd, Ahmedabad, India. Light paraffin oil and solvent ether, both laboratory reagent grade, were from Suvidhinath Laboratories, Vadodara, India. Glutaraldehyde, 25% in water, was from Spectrochem Private Ltd, Mumbai, India).

Methods

Experimental Design

The study was conducted in 2 different stages. In the first stage, optimization of the concentration of chitosan solution was carried out with 3 levels of independent parameters (ie, chitosan solution concentration and stirring speed) using 3^2 full factorial design. In the second phase, the effect of the concentration of 2 different drugs having different aqueous solubility was studied at 3 different levels of each drug.

Preparation of Chitosan Microspheres

One hundred milliliters of paraffin oil was placed in a 250-mL plastic beaker. One milliliter of span 80 was mixed with the oil in the beaker by stirring. To this, 3 mL of chitosan solution of different concentration (0.8% wt/vol, 1.4% wt/vol, 2% wt/vol), prepared by dissolving chitosan in 5% acetic acid, was added dropwise using a 22-gauge hypodermic syringe. This addition was accompanied with stirring of paraffin oil at different speeds (2000 rpm, 3000 rpm, and 4000 rpm) with the help of a high-speed stirrer with propellers (RTQ-124A, Remi Motors, Mumbai, India). Stirring was continued for 5 minutes after the complete addition of chitosan solution into oil. Later 0.25 mL of glutaraldehyde was added to the mixture with continuous stirring at the same speed. Stirring was continued at the same speed for next 5 minutes, and then stirring speed was reduced to 500 rpm. Glutaraldehyde (0.25 mL) was added twice to the mixture, once after 1 hour and then after 2 hours, respectively, with continuous stirring. Stirring was stopped after 1 hour of the final addition of glutaraldehyde.

Suspension of chitosan microspheres in paraffin oil thus obtained was allowed to stand for 1 hour to let the microspheres settle down under gravity. Clear supernatant was decanted and microspheres obtained as residue were washed 4 times with solvent ether. After the final wash, microspheres were allowed to dry in air. Dry powder thus obtained was collected and stored in desiccators at room temperature. A total of 9 batches, each in triplicate, were prepared as per the factorial design (3^2).

Preparation of Chitosan Microspheres Containing 5-Fluorouracil

Microspheres were prepared using the same method as described above using chitosan solution (2% wt/vol) containing different concentrations of 5-FU (2.5, 5, and 7.5 mg/mL) and carrying out emulsification at 3000 rpm. A total of 3 batches, each in triplicate, were prepared.

Preparation of Chitosan Microspheres Containing Methotrexate

Microspheres were prepared using the same method as described above using chitosan solution (2% wt/vol) containing different concentrations of MTX (1, 2, and 3 mg/mL) and carrying out emulsification at 3000 rpm. A total of 3 batches, each in triplicate, were prepared.

Determination of Mean Particle Size and Particle Size Distribution

Particle size analysis of unloaded and drug-loaded chitosan microspheres was performed by optical microscopy[11] using a compound microscope (model 1669, Getner, Ambala, India). A small amount of dry microspheres was suspended in purified water (10 mL). The suspension was ultrasonicated for 5 seconds. A small drop of suspension thus obtained was placed on a clean glass slide. The slide containing chitosan microspheres was mounted on the stage of the microscope and Ferret's diameter of at least 300 particles was measured using a calibrated ocular micrometer. The process was repeated for each batch prepared.

Determination of Uniformity Index

Uniformity Index (UI) was determined by the following formula[12]:

$$UI = D_w/D_n \quad (1)$$

where D_w and D_n are weight average diameter and number average diameter, respectively, and are calculated as follows:

$$D_w = \Sigma NiDi^4/\Sigma NiDi^3,\ D_n = \Sigma NiDi/\Sigma Ni \quad (2)$$

where *Ni* is the number of particles with *Di* diameter. As per Shukla et al,[12] values of UI ranging from 1.0 to 1.1 and 1.1 to 1.2 indicate monodisperse and nearly monodisperse particles. In the present case, values higher than 1.2 have been regarded as indicative of particles with broad particle size distribution.

Morphological Study of Microspheres

Photomicrographs of the unloaded chitosan microspheres obtained from various batches were taken using a digital optical microscope (Axioplan microscope, MPM-400 with image analyzer, Zeiss, Oberkochen, Germany). Microspheres of the various batches were characterized in terms of sphericity and clumping of microspheres, as observed from the photomicrograph.

Determination of Percentage Drug Entrapment

Efficiency of drug entrapment for each batch was calculated in terms of percentage drug entrapment (PDE) as per the following formula:

$$PDE = (\text{practical drug loading}/\text{theoretical drug loading}) \times 100 \quad (3)$$

Theoretical drug loading was determined by calculation assuming that the entire drug present in the chitosan solution used gets entrapped in microspheres and no loss occurs at any stage of preparation of microspheres.

Practical drug loading was determined by taking a weighed quantity of chitosan microspheres (approximately 25 mg) in a 25-mL volumetric flask. Sufficient quantity of methanol was added to make the volume 25 mL. The suspension was shaken vigorously and then left for 24 hours at room temperature with intermittent shaking. Supernatant was collected by centrifugation and drug content in supernatant was determined by UV spectrophotometry at suitable wavelength (257 nm for 5-FU and 304.5 nm for MTX) using a Chemito UV visible spectrophotometer (Chemito, Spectrascan-2200, Nashik, India).

In Vitro Release

One of the 3 batches of drug-loaded chitosan microspheres for each drug was selected on the basis of a predecided protocol. The protocol was in the form of a flowchart based on mean particle size (MPS) (preferred range 7-15 μm), clumping (preferred the "least"), and PDE (preferred the "highest") in that order. The selected batch was subjected to in vitro release test under static condition as per the method used by Nam and Park,[13] with slight modification. In brief, a weighed quantity of microspheres (25 mg of 5-FU-loaded microspheres and 375 mg of MTX-loaded microspheres) was dispersed in 30 mL of n-saline phosphate buffer (pH 7.4) in a conical flask. The mouth of the flask was closed with a cotton plug. The system was kept in an incubator at 37°C. Three milliliters of the dispersion medium was drawn after definite time intervals and was replaced with 3 mL of dissolution media. The drawn sample was filtered using Whatman filter paper (grade 2, Whatman, Kent, UK). The residue was returned to the suspension. The clear filtrate was subjected to UV spectrophotometry (after dilution, wherever required) for determination of drug content.

Scanning Electron Microscopy

Scanning electron photomicrographs of drug-loaded chitosan microspheres were taken. A small amount of microspheres was spread on aluminium stub. Afterwards, the stub containing the sample was placed in the scanning electron microscopy (SEM) chamber. A scanning electron photomicrograph was taken at the acceleration voltage of 30 KV, chamber pressure of 0.6 mm Hg.

Data Analysis

Values of MPS of unloaded microspheres were subjected to 2-way analysis of variance (ANOVA) (between groups) with 3 null hypotheses: (1) there is no significant interaction between the independent parameters; (2) there is no significant effect of change in concentration of chitosan solution; and (3) there is no significant effect of change in stirring speed. Post hoc study using Student-Newman-Keuls test was carried out to determine the exact level of independent parameters where significant change in dependent parameters of microspheres occurs. One-way ANOVA was carried out to study the significance of the effect of change in drug concentration on MPS and PDE of drug-loaded chitosan microspheres. All the statistical studies were performed with the help of SPSS for Windows (Version 9.0).

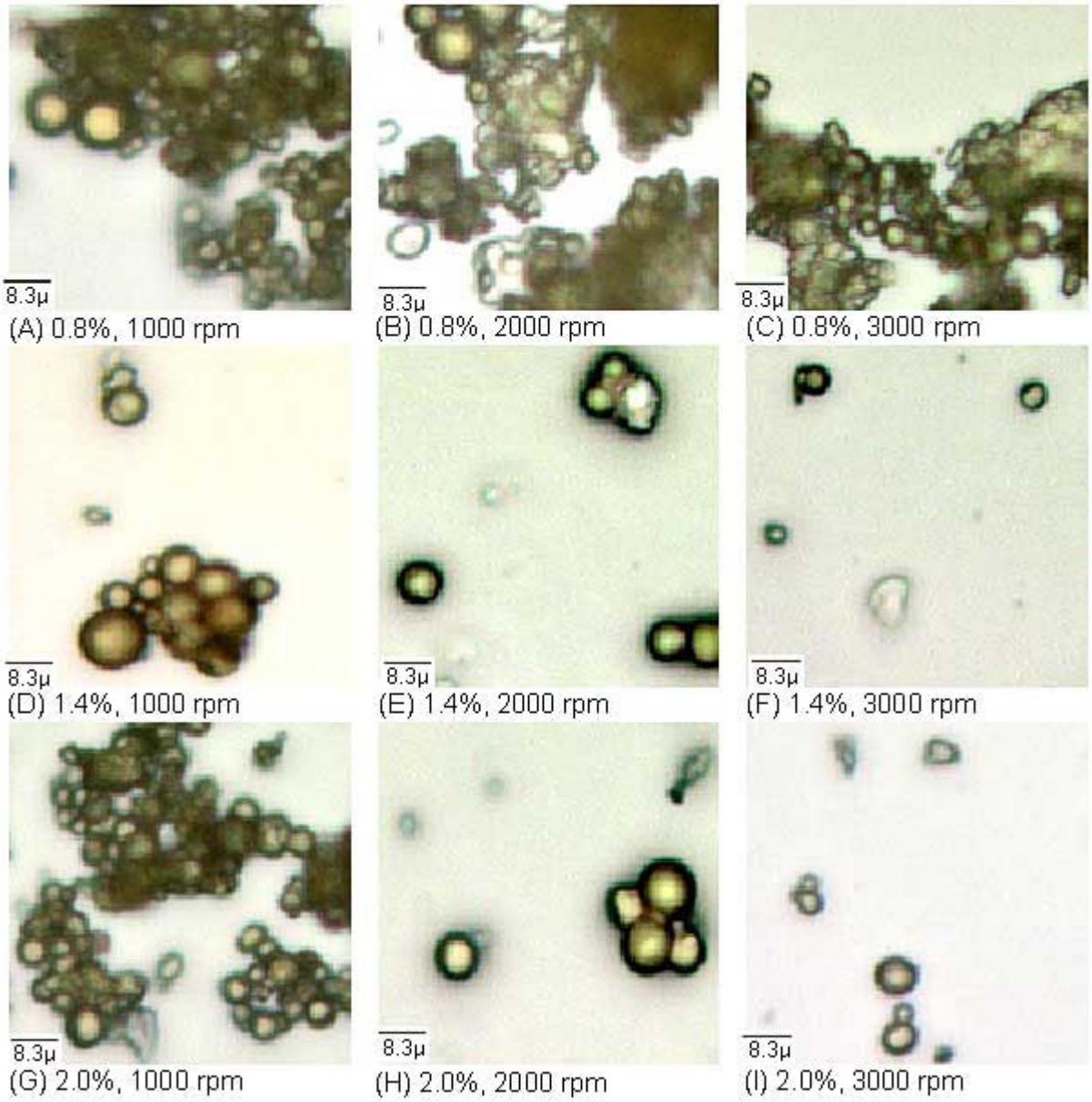

Figure 1. Photomicrographs of unloaded chitosan microspheres prepared using chitosan solution of different concentration (mg/mL chitosan solution) at different stirring speed (rpm) (original magnification × 1200).

RESULTS AND DISCUSSION

Effect of Chitosan Solution Concentration and Stirring Speed on Morphology, Mean Particle Size, and Uniformity Index of Unloaded Chitosan Microspheres

Although spherical particles were obtained in all the batches (Figure 1), clumping of the microspheres obtained in various batches varied with the variation in process parameters. It is important to note that in our experiment, microspheres were obtained from chitosan solution having a concentration of less than 1% wt/vol (shown in Figure 1, A-C). This was contrary to an earlier report that a minimum 1% wt/vol concentration of chitosan solution was needed to prepare chitosan microspheres (irrespective of molecular weight).[10] The presence of microspheres in batches prepared with a lower concentration of chitosan solution, in contradiction to the earlier results, may be due to the difference in the method of preparation. In our experiment, stirring was carried out at a higher level (2000/3000/4000 rpm) for first 20 minutes only (including 5 minutes after addition of glutaraldehyde, which initiates cross-linking), and then at 500 rpm for the remaining period of preparation of microspheres on the basis of our hypotheses as explained earlier. The formation of micro-

Table 1. Effect of Process Parameters on the Mean Particle Size and Uniformity Index of Unloaded Chitosan Microspheres

Chitosan Solution Concentration (% wt/wt)	Stirring Speed (rpm)	Mean Particle Size (Average ± SD) (μm)	Clumping*	Uniformity Index (Average ± SD)
0.8	2000	11.94 ± 6.16	+++	1.33 ± 0.02
1.4	2000	12.83 ± 6.59	+++	1.61 ± 0.04
2.0	2000	13.97 ± 7.2	+++	1.36 ± 0.02
0.8	3000	10.22 ± 5.05	++	1.45 ± 0.06
1.4	3000	10.66 ± 5.45	+	1.33 ± 0.1
2.0	3000	12.68 ± 6.53	–	1.4 ± 0.14
0.8	4000	10.19 ± 5.26	+++	1.42 ± 0.04
1.4	4000	10.49 ± 5.42	+	1.4 ± 0.02
2.0	4000	12.28 ± 6.34	+	1.52 ± 0.08

*As determined from photomicrographs (+++ indicates very large; ++, large; +, less; and –, almost absent).

spheres in batches prepared by using chitosan solution containing 0.8% wt/vol chitosan may be caused by the low shear force produced during stirring at 500 rpm that may not be high enough to deform the quasi-solid dispersed phase that may have escaped the rupture during initial stirring at higher speed. This also means that the dispersed phase that escapes the rupture during the initial stirring decreases with an increase in speed of stirring. This finding is confirmed by the observation that with an increase in stirring speed, the number of spherical particles started to decline, and very few microspheres were obtained when stirring speed was increased to 4000 rpm (Figure 1). This is in agreement with our hypothesis that higher stirring speed leads to a breakdown of spherical particles. Although microspheres prepared from chitosan solution having a concentration higher than 1% wt/vol (ie, 1.4% wt/vol and 2% wt/vol) were perfectly spherical irrespective of stirring speed, large clumping was found at the lower stirring speed. Microspheres became more discrete with increase in stirring speed.

As shown in Table 1 and Figure 1, an increase in concentration of chitosan solution resulted in an increase in MPS of chitosan microspheres. From the result of 2-way ANOVA, significant interaction between the concentration of chitosan solution and the stirring rate was found ($P < .05$). Further, each of the 2 factors was found to have a significant effect on the MPS of the microspheres. From the post hoc studies, separate homogeneous subsets were produced by each level of the 2 parameters, depicting that each level of each parameter produced a significantly different effect than other levels.

This significant increase may be because of the increase in viscosity of the droplets (due to the increase in concentration of chitosan solution). This increase is high enough to result in difficult dispersion and subdivision of droplets.[14] Increase in mean particle size due to increased viscosity of the polymer solution has also been reported by Jeyanthi et al[15] for peptide containing polylactic-co-glycolic acid (PLGA) microspheres.

The observed effect of stirring rate on MPS may be because of the effect of stirring rate on the size of globules during emulsification. As reported by Denkbas et al,[16] stirring during addition of chitosan solution and emulsification produces energy for dispersion of chitosan solution into droplets. Increase in stirring speed produces higher energy, which leads to a further decrease in droplet size. As the stirring speed increases, the size of dispersed droplets decreases, and small droplets are produced that undergo cross-linking on addition of glutaraldehyde, thus producing smaller microspheres.

It was found that UI is affected by concentration of chitosan solution as well as stirring speed. However, no exact correlation could be established. The values of uniformity index for all the batches were more than 1.2 indicating that the microspheres have broad particle size distribution.

Effect of Drug Concentration in Chitosan Solution on Characteristics of Drug-Loaded Chitosan Microspheres

Mean Particle Size, Particle Size Distribution, and Morphology

Increase in drug concentration of 5-FU at lower (ie, 2.5-5 mg/mL) as well as higher levels (5-7.5 mg/mL) resulted in increase in MPS of microspheres (Table 2). From the result of 1-way ANOVA, chitosan solution concentration was found to have significant effect on MPS. However, only 2 different homogeneous subsets were obtained from the post hoc studies. The first subset was for 2.5 mg/mL of 5-FU and the second was for 5 and 7.5 mg/mL 5-FU. These results indicate that the concentration of 5-FU affects MPS significantly only at lower concentration. Similar results were ob-

Table 2. Effect of Drug Concentration on Mean Particle Size and Uniformity Index of Chitosan Microspheres*

Characteristics of Microspheres	5-FU Concentration (mg/mL Chitosan Solution)			MTX Concentration (mg/mL Chitosan Solution)		
	2.5	5	7.5	1	2	3
MPS (μm) ± SD	11.14 ± 0.16	14.45 ± 0.33	14.87 ± 0.19	10.42 ± 0.24	11.67 ± 0.09	11.75 ± 0.26
UI ± SD	2.01 ± 0.06	1.81 ± 0.13	1.92 ± 0.02	1.54 ± 0.02	1.81 ± 0.13	1.92 ± 0.02

*MPS indicates mean particle size; and MTX, methotrexate.

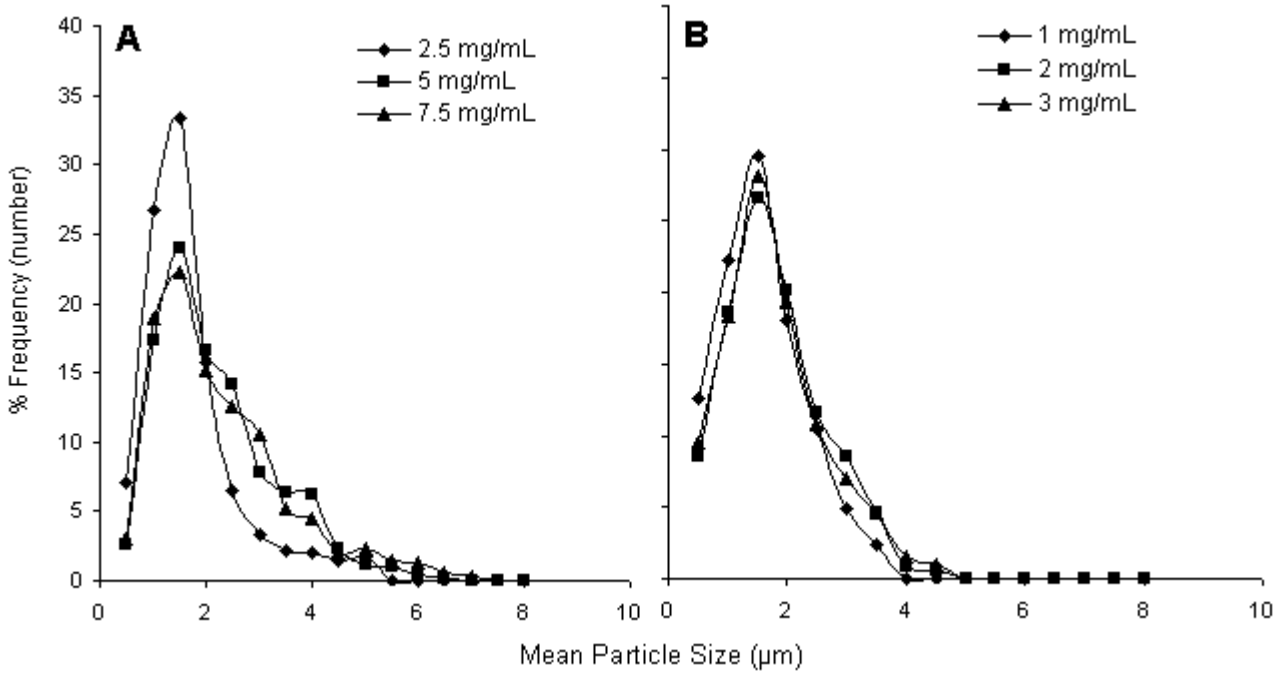

Figure 2. Effect of drug concentration on PSD of drug-loaded chitosan microspheres. (A) effect of concentration of 5-F and (B) effect of concentration of MTX.

tained for chitosan microspheres containing MTX. A sharp increase in MPS of the microspheres with increase in concentration of 5-FU and MTX at lower level is also shown in Table 2.

Figure 2 shows the frequency polygon representing the particle size distribution (PSD) in various batches. From the figures, it can be inferred that the PSD of microspheres changes differently at different levels of change in drug concentration. While the PSD increased when the 5-FU concentration increased from 2.5 to 5 mg/mL (Figure 2A), it remained unchanged on further increase in drug concentration to 7.5 mg/mL. In the case of MTX-loaded microspheres (Figure 2B), the effect was much subdued with almost the same PSD at all the levels of drug concentration. From the figure, it is evident that the distribution of size was skewed with a larger number of particles in the lower size range. However, the distribution of particles in all the batches was broad, as evident from the values (more than 1.2) of UI shown in Table 2. Increase in MPS and PSD may be because of the increase in viscosity of the droplets present in the internal phase caused by the increase in drug concentration as explained by Denkbas et al.[16] However, it appears that this applies only at the lower level of concentration for both 5-FU and MTX. Although further increase in drug concentration might be increasing the viscosity of the droplet, it does not result in significant change in MPS or PSD.

As shown in Figure 3, spherical microspheres were obtained in both chitosan microspheres containing 5-FU and that containing MTX. Further, from the figure, it appears that clumping is present in the case of chitosan microspheres containing MTX, while chitosan microspheres containing 5-FU are more segregated.

Percentage Drug Entrapment and Drug Loading

As shown in Figure 4, increase in drug concentration resulted in increase in PDE of the drug irrespective of the na-

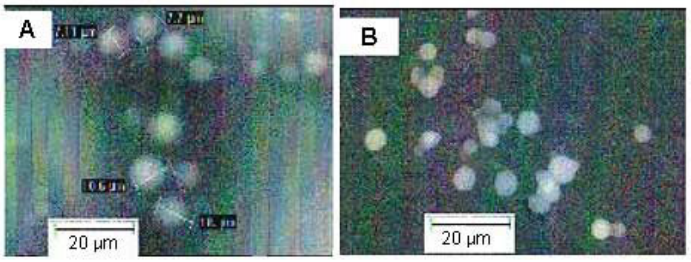

Figure 3. Scanning electron photomicrograph of chitosan microsphreres containing (A) 5-FU and (B) MTX. The parameters of scanning electron microscopy were : Acceleration voltage-30.0 kV; Spot- 4.0; Magnification-original × 2000; Working Distance : 9.6; Vacuum: 0.6 Torr.

ture of the drug. Highest PDE was 29.05 and 7.55 for 5-FU- and MTX-loaded microspheres, respectively. From the result of 1-way ANOVA, drug concentration was found to have significant effect on the PDE. From post hoc studies, 3 different homogeneous subsets were obtained for each level of drug concentration for 5-FU as well as MTX. This finding shows that the effect was significant at both lower and higher levels of change in drug concentration. From Figure 4, it may be inferred that in the case of 5-FU-loaded chitosan microspheres, there is a more proportionate increase in percentage drug encapsulation efficiency for every increase in drug concentration within the range used in our experiment.

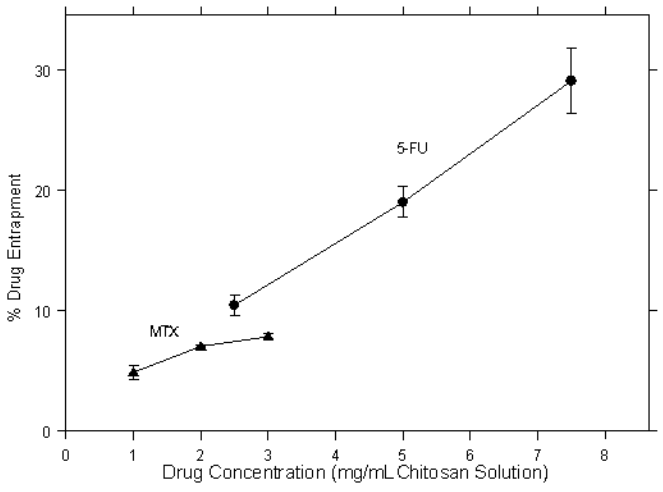

Figure 4. Effect of drug concentration on PDE in chitosan microspheres.

As shown in Figure 5, the effect of change in concentration of 5-FU and MTX on the drug loading in microspheres was very similar to that in the case of PDE. Moreover, the result of 1-way ANOVA and post hoc studies indicated that change

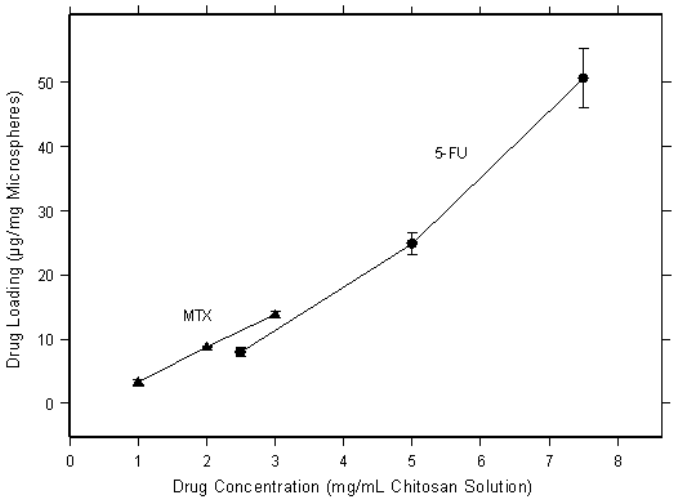

Figure 5. Effect of drug concentration on drug loading in chitosan microspheres.

in drug concentration resulted in significant increase in drug loading in microspheres (overall as well as at each level). The result was partially in accordance with the results reported by Denkbas et al,[16] where only a marginal increase in drug loading (as opposed to significant increase in our case) was found on an increase in concentration of 5-FU from 2.5 mg/mL to 5.0 mg/mL. However, it was also reported[16] that the effect of a further increase in drug concentration from 5 to 10 mg/mL solution led to a sharp increase in drug loading. This finding was in line with the observation in our experiment. The aberration in the results at the lower level of change in drug concentration may be because of the difference in the method of preparation. The external phase in our experiment was only liquid paraffin containing 1% span 80, whereas it was a mixture of paraffin oil and petroleum ether (60:40) containing 1% Tween 80 in the method used by Denkbas et al.[16] Use of Tween 80 may increase the solubility of 5-FU in oil leading to less entrapment at lower concentration. Also, the method of washing microspheres was slightly different where aqueous media was used to wash microspheres. This method may have resulted in loss of some drug from the microspheres. The difference in the PDE and drug loading between the microspheres loaded with 5-FU and MTX may be because of the difference in the distribution pattern of the drug in the matrix. It has been reported that MTX, which is practically insoluble in water, may be getting precipitated and thus embedded near the periphery in the matrix. On the contrary, 5-FU, being soluble in water, remains in dissolved state for a longer time, resulting in wider distribution in the matrix.[17] Lower entrapment in the case of MTX may be because of more concentration of the drug at periphery. The higher drug concentration of the drug near the periphery in case of MTX-loaded microspheres leads to further removal of drug from the matrix.

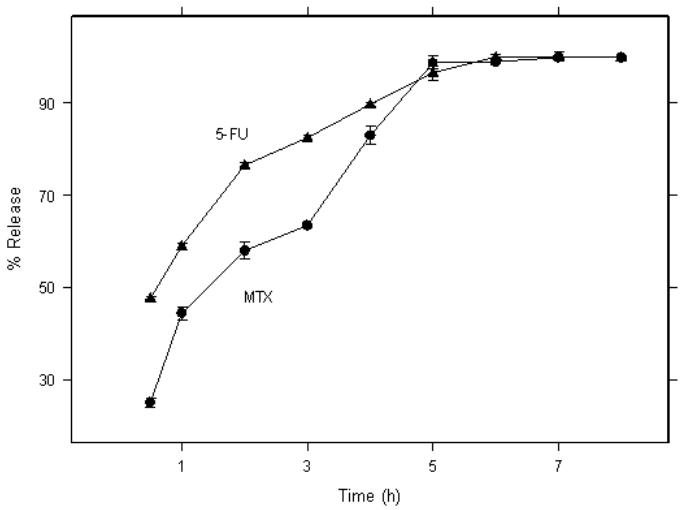

Figure 6. Drug release (in vitro) from chitosan microspheres containing 5-FU (0.1 mg/mg microspheres with MPS 14.87 μm) and MTX (0.029 mg/mg microspheres with MPS 10.42 μm).

In Vitro Release

As shown in Figure 6, release of the drug from the matrix of chitosan microspheres was found to be higher for drug having more aqueous solubility (ie, 5-FU). The release of the drug in the present study performed in duplicates (ie, 2 test runs) was quite reproducible, as indicated by the error bars in Figure 6. Although biphasic release of drug from chitosan microspheres, as explained by Tomlinson,[18] was seen in the case of chitosan microspheres containing 5-FU as well as MTX, the effect was prominent in case of microspheres containing MTX. A different pattern of release for drugs with different water solubility has also been reported by Sato et al.[19] To determine the mode of release of the drug from the microspheres, 3 graphs were plotted: (1) drug remaining in the matrix vs time, (2) log (drug remaining in the matrix) vs time, and (3) percentage drug released vs square root of time. The curves obtained were regressed. The values of "R^2 Square" for the 3 plots were 0.84, 0.81, and 0.94, respectively, for 5-FU-loaded chitosan microspheres and 0.88, 0.92, and 0.94, respectively, for MTX-loaded microspheres. From the values, it can be inferred that the release of the 5-FU from the matrix of the microspheres follows square root of time kinetics, while release of MTX occurs by a combination of first order kinetics and square root of time kinetics. The initial fast release may be because of the release of the drug located at the outer layer as explained by Denkbas et al.[16] Being more soluble, 5-FU is released faster than MTX, thus giving higher release (>50%) than MTX (<50%) in the first 1 hour. The sharp increase in the release of the MTX in second phase may be because of the erosion of the matrix leading to sudden efflux of the drug from the matrix.

CONCLUSION

Use of differential stirring speed during the preparation of chitosan microspheres by the chemical cross-linking method may help to obtain chitosan microspheres using a chitosan solution of less than 1% wt/vol concentration. The pharmaceutical attributes of microspheres thus obtained are significantly affected by stirring speed and chitosan concentration as well as their interaction. Effect of change in drug concentration on the pharmaceutical characteristics of drug-loaded chitosan microspheres is more prominent for water-soluble drug.

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