RESEARCH PAPER

Accepted 16 September 2000
Revised 6 July 2000
Received 3 November 1999
Indian J. Pharm. Sci. 2001, 63(1) 10-14

Formulation and Evaluation of Depot Parenteral Preparations for a Combination of Norfloxacin and Tinidazole

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Norfloxacin and tinidazole in combination are used as antibacterial and antidiarrhoeal agents, respectively. In the treatment of bacterial infections, factors of paramount importance are to maintain the therapeutically optimum drug concentration in the plasma and eliminate the need for frequent dose administration. In the present investigation, an attempt was made to design microspheres loaded with norfloxacin and tinidazole intended for parenteral administration. Biodegradable, non-toxic polymers viz., bovine serum albumin (BSA) and chitosan were used to form microspheres. Microspheres were prepared using the chemical cross-linking method. Glutaraldehyde-saturated toluene (GST) was employed as the crosslinking agent. Various process parameters, like drug:polymer ratio, did not effect the mean particle size or particle size distribution to a significant extent and relatively a narrow particle size distribution was obtained. Entrapment efficiency of the albumin microspheres for both the drugs was more than the corresponding chitosan microspheres. Results of *in vitro* release studies indicate a biphasic release pattern characterized by a initial burst-effect followed by a slow release over a period of 12 to 24 h.

Since many years, medical science has recognized the need to control, regulate and target the release of drugs in the body. In general, the aim has been to provide less frequent drug administration, constant and continuous therapeutic levels of drugs in the systems circulation or at a specific target organ site and a reduction in undesirable drug side effects. During the past decade, considerable progress has been made in this regard. A wide variety of drug delivery systems have been designed and evaluated, including drug carriers based on proteins, polysaccharides, synthetic polymers, erythrocytes, DNA and liposomes, to name a few. These drug delivery systems have taken many forms, one of the more popular configurations being that of microspheres. Microspheres as used in drug delivery are discrete, micrometer-sized spherical particles containing an entrapped drug. They can be prepared from a variety of carrier materials[1]. Reconstituted collagen is perhaps the best known example of a natural polymer that undergoes degradation *in vivo*. It is utilized as a bio-absorbable suture in prostheses and as a wound dressing. The rate of absorption can be varied from days to weeks by techniques such as succinylation or crosslinking (with formaldehyde or glutaraldehyde)[2]. The term biodegradation is used in this work for all types of processes in the living organism that lead to the degradation of implants. Processes involving the role of enzymes are referred to as enzymatic degradation.

Combination of norfloxacin and tinidazole is of choice in urinary tract infections and upper respiratory tract infections. Systemic infections may contain a wide diversity of bacteria and there may be no single antibiotic that is effective against all putative pathogens. These mixed infections can include a variety of aerobic, microaerophilic

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and anaerobic bacteria, both gram positive and gram negative. In these instances, it might be necessary to use more than one antibiotic, either serially or in combination.

The aim of the present study was to formulate and evaluate microspheres loaded with norfloxacin and tinidazole, as depot parenteral preparations, using two biodegradable, biocompatible, non-toxic polymers, bovine serum albumin (BSA) and chitosan. The influence of different drug:polymer ratio on the size and size distribution, drug entrapment efficiency and *in vitro* drug release were studied. An attempt also has been made to evaluate the prepared microspheres for antibacterial activity against two organisms. *Staphylococcus aureus* and *Escherichia coli.*

MATERIALS AND METHODS

Norfloxacin and tinidazole were gift samples from Cadila Pharmaceuticals, Ahmedabad. Chitosan was a generous gift from Fisheries Research Institute, Trivandrum. Bovine serum albumin (BSA) was procured from Sigma Chemical Co., USA. All other chemicals and reagents used were of analytical grade.

Formulation of microspheres:

Chitosan microspheres were prepared using the reported methods with slight modifications[3-5]. A 2% w/w chitosan solution was prepared in aqueous (5%) citric acid. Drugs were dispersed in this solution and mixed well with the help of a homogenizer. The suspension was dropped to the liquid paraffin bulk oil phase containing 1% v/v Span 80 to form a water-in-oil (w/o) emulsion. Four millilitres of the crosslinking agent (glutaraldehyde saturated toluene, GST) was added drop-wise and the emulsion was agitated at constant rotation for a period of 4-5 h at ambient temperature. Microspheres formed were separated from the reaction mixture by centrifuging at 2500 rpm, washed several times with n-hexane and air dried for 24 h at room temperature and stored in a dessicator. Microspheres were prepared using three drug:polymer ratios (1:4, 1:2, 1:1).

Albumin microspheres were prepared using a technique developed by modification of the emulsion cross-linking method described earlier[6-11]. BSA (200 mg) was dissolved in 1ml of distilled water (pH 7.0) at room temperature and drug was dispersed in it. This dispersion was heated to 60° for 2 min and then homogenized for 5 min. the dispersion was transferred to a 250 ml beaker containing liquid paraffin with 1.0% Span 80. Stirring was maintained at a steady speed and 1 ml of GST was added drop-wise. Agitation was continued at constant temperature until albumin cross-linking was complete (4-5 h). The suspension was centrifuged and microspheres were collected; washed several times with n-hexane until the oil phase was removed completely and finally dried at room temperature in a dessicator. Microspheres were prepared using three drug:polymer ratios (1:4, 1:2 and 1:1).

Physicochemical characterization:

The size of the microspheres was determined by light microscopy using a calibrated eyepiece micrometer. Three hundred microspheres were counted mean diameter and the size distribution characteristics were computed. To determine the entrapment efficiency, known quantity of BSA microspheres were digested in 7.5 ml of 0.25% trypsin solution in phosphate buffer pH 7.5 for 48 h. The resultant solution was diluted suitably and the drug concentration was measured at respective wavelengths for both the drugs and calculated using simultaneous mode of estimation. Similarly, known quantity of chitosan microspheres were digested in 10 ml of 5% acetic acid. The resultant solution was diluted suitably and absorbance was measured at respective wavelengths for both the drugs and concentration was calculated using simultaneous mode of estimation[12-13].

In vitro drug release studies[14]:

Microspheres containing known amount of the drug were taken in a 20 ml vial containing 10 ml of phosphate buffered saline (PBS pH 7.4) maintained at a constant shaking of 60 oscillations per minute and ambient temperature. Samples were withdrawn at periodic time intervals with replacement of equal volume of fresh dissolution medium. The drug concentrations in the samples were measured after suitable dilution using UV-spectrophotometer against a suitable blank.

Antibacterial studies:

A total of seven formulations, including the norfloxacin+tinidazole standard solution, were screened for antibacterial activity. The antibacterial screening was done at the Department of Microbiology, KMC, Manipal. Activity was screened for two organisms. *Staphylococcus aureus* (representing Gram+ve bacteria) and *Escherichia coli* (representing gram-ve bacteria). All the formulations were suspended in phosphate buffer saline

(pH 7.4) Antibacterial activity was tested after 72 h. Zone of inhibition by disc and plate method was recorded for antibacterial screening. Commercially available nutrient agar medium from Hi-media was used as the microbiological medium. The medium consisted of 1% w/v meat extract, 1% w/v peptone, 0.5% w/v sodium chloride, 2% w/v agar. Discs made out to Whatman filter paper were used and were calibrated. One hundred discs absorbed 1 ml of solution (water), i.e., each disc absorbed 10 µl of the solution. The concentrations of the drug solutions from different formulations were also measured. One hundred millilitre capacity petridishes were used for the study. All glasswares were sterilized by dry heat method.

Thirty-seven grams of Hi-media nutrient agar was added to 1000 ml of boiling water to form complete solution. The medium was then transferred to 100 ml conical flasks and plugged with non-absorbent cotton and sterilized by autoclaving at 121° for 20 min. The liquid media, when moderately hot, was transferred into the petridishes and allowed to solidify. Culture broth (0.1 ml) of 24 h was added and spread evenly with the help of a sterile cotton swab. Whatman filter paper discs dipped in different drug solutions were placed on the solidified agar. The standard drugs used for antibacterial screening were norfloxacin and tinidazole at 20 µg/ml and 30 µg/ml concentrations, respectively. Plates were kept at room temperature for 2-h for diffusion and then incubated at 37° for 24 h. The diameter of zone of inhibition was measured after 24 h.

RESULTS AND DISCUSSION

The prepared microspheres exhibited good morphological characteristics and a narrow size distribution. Particles were discrete and most of the particles were spherical. Under the light microscope, no pores were visible in drug-loaded microspheres.

The particle size range for albumin microspheres was between 3 to 93 µm with mean particle size of 28.51 µm whereas the particle size range for chitosan microspheres was between 6 to 99 µm with mean particle size of 33 µm. Different drug to polymer ratios did not affect the mean particle size or particle size distribution to a significant extent and relatively a narrow particle size distribution was obtained. The entrapment efficiency of BSA microspheres for both the drugs, i.e., norfloxacin and tinidazole, was more compared to chitosan microspheres (Table 1). The reason for better encapsulation into BSA microspheres could be because the total polymer available for encapsulation was much more for BSA microspheres (20% w/v) compared to chitosan formulation (1% w/v). It could also be assumed that, albumin having many reactive groups distributed throughout the surface, gets involved in weak hydrophobic interactions with the drugs, leading to a higher degree of encapsulation.

TABLE 1: PHYSICOCHEMICAL CHARACTERIZATION OF NORFLOXACIN AND TINIDAZOLE MICROSPHERES

Formulation Code	D:P Ratio	Mean particle size (µm)	Norfloxacin		Tinidazole	
			Drug Content in 100 mg of Microspheres	Entrapment Efficiency (%)	Drug Content in 100 mg of Microspheres	Entrapment Efficiency (%)
A_1	1:1	20.32	18.22	91.11	24.35	81.17
A_2	1:2	21.84	13.16	98.76	19.52	97.81
A_4	1:4	25.27	6.14	76.77	11.32	94.37
C_1	1:1	24.38	9.43	47.17	5.65	18.86
C_2	1:2	24.68	4.56	34.27	2.13	10.67
C_4	1:4	27.06	2.39	29.94	1.02	8.52

Mean particle size, drug content and entrapment efficiency of BSA (A) and Chitosan (C) microspheres containing norfloxacin and tinidazole in 2:3 ratio. D:P ratio indicates drug to polymer ratio.

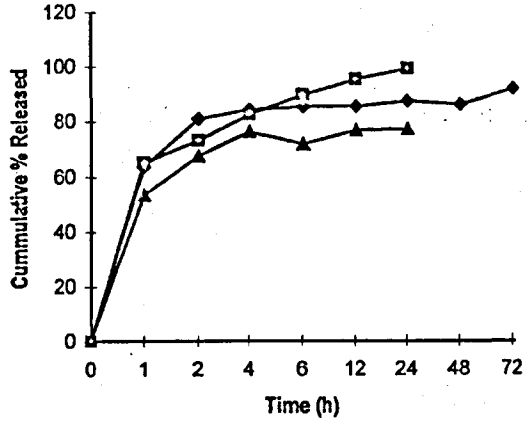

Fig. 1: Percentage cumulative release of norfloxacin

Release of norfloxacin from BSA microspheres containing drug:polymer ratio of 1:1 (-◆-), 1:2 (-□-) and 1:4 (-▲-)

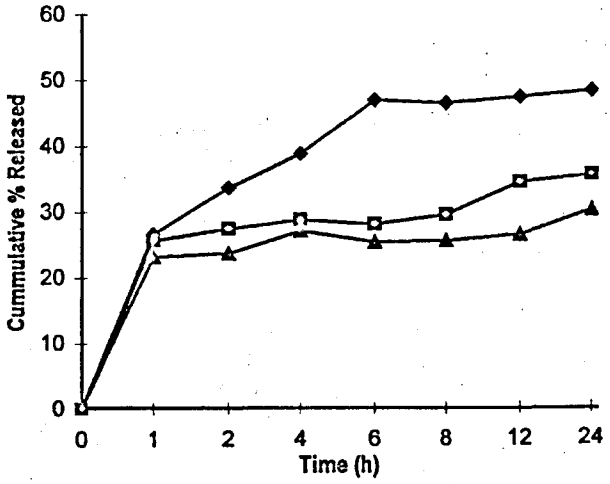

Fig. 2: Percentage cumulative release of norfloxacin

Release of norfloxacin from chitosan microspheres containing drug:polymer ratio of 1:1 (-◆-), 1:2 (-□-) and 1:4 (-▲-)

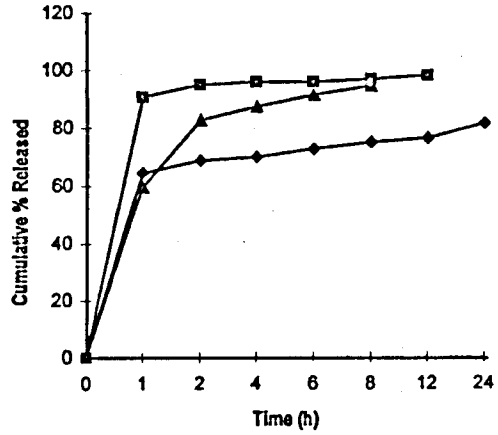

Fig. 3: Percentage cumulative release of tinidazole

Release of tinidazole from BSA microspheres containing drug:polymer ratio of 1:1 (-◆-), 1:2 (-□-) and 1:4 (-▲-)

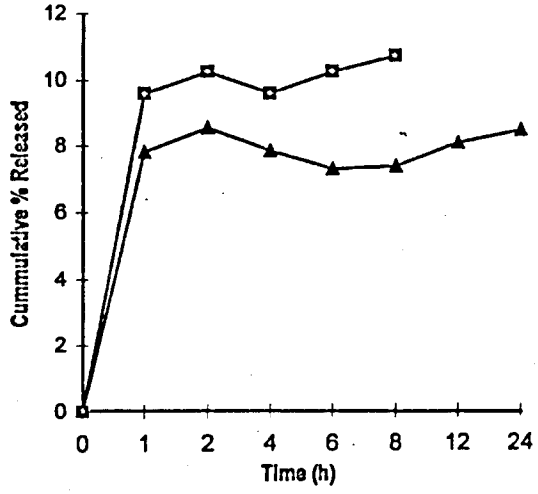

Fig. 4: Percentage cumulative release of tinidazole

Release of tinidazole from microspheres containing drug:polymer ratio of 1:2 (-□-) and 1:4 (-▲-)

The release pattern of both the drugs from the microspheres was observed to follow a biphasic pattern (figs. 1 to 4), characterized by an initial burst effect followed by a slow release over a period of 12 to 14 h. The initial burst effect corresponds to the release of the drug located on or near the surface of the microspheres or release of poorly entrapped drug. The slow release period may be due to the medium being diffused into the polymer matrix, whereby degradation occurs and the drug diffuses out of the microspheres. The burst release of drugs obtained in the initial period could be useful in destroying the microorganisms in their proliferative phase. The later slow release of the drugs can prevent the further growth phase of the microorganisms.

The drug:polymer ratio has an influence on the rate of drug release. The rate of drug release increased with decrease in the polymer ratio in the formulation. Chitosan formulations released drug payload much slower than the BSA counterparts because of higher molecular weight of the former and also higher degree of crosslinking density (6.66% w/v for chitosan against 1.66% v/v for BSA).

The results of microbiological studies are shown in Table 2. Albumin microspheres were evaluated against

TABLE 2: ANTIBACTERIAL ACTIVITY OF MICROSPHERES

Formulation	Zone of inhibition		Amount of drug present in the disc (µg)	
	E. coli	*S. aureus*	Norfloxacin	Tinidazole
A_1	27	20	4.13	5.76
A_2	26	19	2.9	4.65
A_4	24	R*	1.37	2.64
S	27	14	4	6
C_1	25	15	4.41	2.64
C_2	25	14	2.05	0.97
C_4	21	R*	0.82	0.47

*R = Resistant

Antibacterial activity of norfloxacin and tinidazole incorporated in BSA (A) and chitosan (C) microspheres against *Escherichia coli* and *Staphylococcus aureus*

E. coli. As the amount of drug released in the dissolution medium gradually increased, the zone of inhibition also increased. Similar results were obtained with chitosan microspheres when evaluated against *S. aureus*. No such correlation could be established in case of chitosan microspheres and albumin formulations when evaluated against *E. coli* and *S. aureus*, respectively. Point of sampling was at the end of release study. Most of the drug was depleted from the formulation, leaving behind only a small fraction. The initial burst effect, as observed with *in vitro* release studies, would provide the initial kill of bacterial flora, followed by the maintenance dose, released in a controlled fashion, preventing further spread of the infection.

The study shows that it might be possible to manufacture biodegradable microspheres using natural polymers such as chitosan and bovine serum albumin (BSA) loaded with antiinfective agents for use in the treatment of mixed infections.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. P.G. Shivananda, Professor and Head, Department of Microbiology, K.M.C., Manipal for providing the necessary facilities and guidance during the microbiological studies.

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