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Quality-by-Design Based Development and Characterization of Chitosan Nanoparticles Encapsulating Amphotericin B for Improved Antifungal Efficacy

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Abstract

The rising prevalence and evolving microbiological spectrum of invasive fungal infections necessitate the development of effective and broad-spectrum antifungal therapies. Amphotericin B remains one of the most reliable antifungal agents; however, its conventional formulations are associated with limitations, particularly for topical delivery. The present study aimed to develop and evaluate a chitosan nanoparticle-incorporated gel loaded with amphotericin B for enhanced topical application. Chitosan nanoparticles were prepared using the ionic gelation method, employing sodium tripolyphosphate as a cross-linking agent. The formulation relied on cationic interactions between low molecular weight chitosan and the crosslinker, facilitating nanoparticle formation and stable drug incorporation. The prepared nanoparticles were subsequently incorporated into a gel base, and potential interactions between the drug and polymer were analyzed during formulation development.The developed gel was evaluated for key physicochemical parameters, including viscosity, drug content (% assay), extrudability, spreadability, and in vitro drug release. The formulation exhibited a viscosity of 4520 cps, drug content of 98.96 ± 0.35%, extrudability of 167 g, and spreadability of 11.95 g•cm/sec. In vitro drug release studies, conducted using the Franz diffusion cell method, demonstrated a sustained release profile with 92.23% drug release over 12 hours. In conclusion, the chitosan nanoparticle-based gel of amphotericin B showed favorable physicochemical properties and sustained drug release, indicating its potential as an effective topical delivery system for the treatment of fungal infections.

1. Introduction

Nanoparticles are generally defined as particulate matter with at least one dimension that is less than 100 nm. This definition puts them in a similar size domain as that of ultrafine particles (air borne particulates) and places them as a sub-set of colloidal particles [1]. A considerable fraction of the solid matter on earth can be found in the size range of colloids and nanoparticles. In the last 2 decades scientists have shown that colloids and nanoparticles are present everywhere in the environment [2-3]. By definition nanoparticles constitute a sub-fraction of what is defined as ‘‘colloids’’ by the IUPAC[4]. Since the definition of colloids and nanoparticles is based on a simple spatial dimension of an object, the

variety of colloids and nanoparticles found in the environment is large, and the composition of environmental colloidal systems is complex and heterogeneous. Nanoparticles are aimed to be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. The selection criteria of matrix materials depends on many factors such as: (a) Size of nanoparticles required; (b) Inherent properties of the drug, e.g., aqueous solubility and stability; (c) Surface characteristics such as Charge and Permeability; (d) Degree of biodegradability, biocompatibility and toxicity; (e) Drug release profile desired; and (f) Antigenicity of the final product.

Nanoparticles preparation is most frequently by three methods: (1) Dispersion of preformed polymers; (2) Polymerization of monomers; and (3) Ionic gelation or coacervation of hydrophilic polymers. However, other methods such as supercritical fluid technology 8 and particle replication in non-wetting templates have also been described in the literature for production of nanoparticles. The latter was claimed to have absolute control of particle size, shape and composition, which could set an example for the future mass production of nanoparticles in industry. Chitosan has abundant applications in the field of pharmaceutical and medicine. It is used as a film coating material, as excipients in tablet, like disintegrant, improvement of drug dissolution, and for controlling drug release. It also been used for capsule, microsphere/ microparticles, beads, films and gel. Amphotericin B shows a high order of *in vitro* activity against many species of fungi. *Histoplasma capsulatum, Coccidioides immitis, Candida species, Blastomyces dermatitidis, Rhodotorula, Cryptococcus neoformans, Sporothrix schenckii, Mucor mucedo,* and *Aspergillus fumigatus* are all inhibited by concentrations of amphotericin B ranging from 0.03 to 1.0 mcg/mL *in vitro*. Present investigation aimed to developed chitosan nanoparticle encapsulated gel of Amphotericin B for effective treatment of topical fungal disorder.

2. Material and method

2.1 Materials

Amphotericin B was given by Abbott India, Ltd. Chitosan purchased from Himedia, Mumbai.. All other chemical were purchased from Himedia, Mumbai (India) and Sigma Aldrich. Sodium tripolyphosphate and glacial acetic acids were purchased from Merck Specialities pvt. Ltd., Mumbai, All other chemicals and reagent used were of analytical grade. Ultrapure water was used throughout the study.

2.1 Preparation of chitosan nanoparticles of Amphotericin B

Chitosan nanoparticles were prepared by ionotropic gelation method [5].

Preparation I: Chitosan stock solution (1% w/v) was prepared by dissolving chitosan in acetic acid (1% v/v) at room temperature.

Preparation II: The drug (2-5 mg) was dissolved in chitosan solution.

Preparation III: 1% Sodium tripolyphosphate solution was prepared in water.

Preparation IV: Sodium tripolyphosphate solution was added drop wise with a syringe to chitosan solution while stirring. The solution was magnetically stirred for half an hour followed by filtration and rinsing with distilled water. Nanoparticles were obtained which was air dried for twenty four hours followed by oven drying for six hours at 40˚C.

Table 1: Formulations optimization of chitosan nanoparticles

Sr. No	Formulation Code	Amphotericin B (mg)	Chitosan (mg)	STPP (mg)
1.	F1	10	250	500

2.	F2	10	250	750
3.	F3	10	250	1000
4.	F4	10	500	500
5.	F5	10	500	750
6.	F6	10	500	1000

Preparation of Gel Base

Carbopol 934 (1 % w/v) was accurately weighed and dispersed into double distilled water (80ml) in a beaker. This solution was stirred continuously at 800 rpm for 1 hour and then 10ml of propylene glycol was added to this solution. Volume of gel was adjusted to 100 ml and then sonicated for 10 min on bath sonicator to remove air bubbles. Final pH of the gel base was adjusted to 6.5. Nanoparticles gel preparation corresponding to 2 % w/w of Amphotericin B was incorporated into the gel base to get the desired concentration of drug in gel base.

2.2 Evaluation of nanoparticles

2.2.1 Percentage Yield

The prepared nanoparticles F1-F6 were collected and weighed from each formulation. The percentage yield (%) was calculated using formula given below:

% Yield = Actual weight of product Total weight of drug and polymerx 100

2.2.2 Entrapment Efficiency

Amount of Amphotericin B in each formulation was calculated according to procedure given below [6]: 10 mg of chitosan nanoparticles from each batch were accurately weighed. The powder of chitosan nanoparticles were dissolved in 10 ml 7.4 pH phosphate buffer and centrifuge at 1000 rpm. This supernatant solution is than filtered through whatmann filter paper No. 44. After filtration, from this solution 0.1 ml was taken out and diluted up to 10 ml with 7.4 pH Phosphate Buffer. The supernant was analyzed for drug content by measuring the absorbance at 284 nm.

2.2.3 Measurement of mean particle size

The mean particle size of the nanoparticle was determined by Photo Correlation Spectroscopy (PCS) on a submicron particle size analyzer (Malvern particle size analyser**)** at a scattering angle of 90°. A sample (0.5mg) of the nanoparticle suspended in 5 ml of distilled water was used for the measurement [7].

2.3.4 Determination of zeta potential

The zeta potential of the drug-loaded nanoparticless was measured on a zeta sizer (Malvern particle size analyser) by determining the electrophoretic mobility in a micro electrophoresis flow cell [8]. All the samples were measured in water at 25°C in triplicate.

2.4 Characterization of chitosan nanoparticles containing Gel

2.4.1 Measurement of Viscosity

Viscosity measurements of prepared topical chitosan nanoparticles based gel were measured by Brookfield viscometer using spindle no. 63 with the optimum speed of 10rpm; viscosity [9].

2.4.2 pH measurements

pH of selected optimized formulations was determined with the help of digital pH meter. Before each measurement of pH, pH meter should be calibrated with the help of buffer solution of pH 4, pH 7 and

pH 9.2. After calibration, the electrode was dipped into the vesicles as long as covered by the vesicles. Then pH of selected formulation was measured and readings shown on display were noted.

2.4.3 Drug Content

Accurately weighed equivalent to 100 mg of topical chitosan nanoparticles gel was taken in beaker and added 20 ml of methanol. This solution was mixed thoroughly and filtered using Whatman filter paper no.1. Then 1.0 mL of filtered solution was taken in 10 mL capacity of volumetric flask and volume was made upto 10 mL with methanol. This solution was analyzed using UV-Spectroscope at λ_{max} 284nm [10].

2.4.4 Extrudability study

Extrudability was based upon the quantity of the gel extruded from collapsible tube on application of certain load. More the quantity of gel extruded shows better extrudability. It was determine by applying the weight on gel filled collapsible tube and recorded the weight on which gel was extruded from tube [11].

2.4.5 Spreadibility

Spreadibility of formulation is necessary to provide sufficient dose available to absorb from skin to get good therapeutic response[12]. An apparatus in which a slide fixed on wooded block and upper slide has movable and one end of movable slide tied with weight pan. To determine spreadibility, placing 2-5 g of gel between two slide and gradually weight was increased by adding it on the weight pan and time required by the top plate to cover a distance of 6cm upon adding 20g of weight was noted. Good spreadibility show lesser time to spread.

2.5 *In vitro* drug diffusion study

The *In-vitro* diffusion study is carried by using Franz Diffusion Cell. Egg membrane is taken as semi permeable membrane for diffusion [13]. The Franz diffusion cell has receptor compartment with an effective volume approximately 60 mL and effective surface area of permeation 3.14 sq.cms. The egg membrane is mounted between the donor and the receptor compartment. A two cm^2 size patch taken and weighed then placed on one side of membrane facing donor compartment. The receptor medium is phosphate buffer pH 7.4. The receptor compartment is surrounded by water jacket so as to maintain the temperature at 32 ± 0.5°C. Heat is provided using a thermostatic hot plate with a magnetic stirrer. The receptor fluid is stirred by Teflon coated magnetic bead which is placed in the diffusion cell.

During each sampling interval, samples are withdrawn and replaced by equal volumes of fresh receptor fluid on each sampling. The samples withdrawn are analyzed spectrophotometrically at wavelength of drug 284 nm.

2.6 Antifungal activity of chitosan nanoparticles encapsulated gel

The fungal cultures used in the study were obtained in lyophilized form. With the help aseptic techniques the lyophilized cultures are inoculated in sterile potato dextrose broth than incubated for 24 hours at 37ºC[14]. After incubation the growth is observed in the form of turbidity. These broth cultures were further inoculated on to the potato dextrose agar plates with loop full of fungus and further incubated for next 48 hours at 37ºC to obtain the pure culture and stored as stocks that are to be used in further research work.

The well diffusion method was used to determine the antifungal activity of the chitosan nanoparticles gel prepared from the Amphotericin B using standard procedure. There were 3 concentration used which are 30, 20 and 10 µg/ml for antibiogram studies. The plates were incubated at 37ºC for 24 hr. and then

examined for clear zones of inhibition around the wells with particular concentration of drug. Results of the experiment are being concluded in the Table 7.9-7.10, which clearly shows the antifungal activity of chitosan nanoparticles gel against *Candida albicans.*

2.7 Stability studies for optimized formulation

Stability study data was revealed that the optimized nanoparticle formulation (F4) stable after 3 month of storage at 4ºC while at 25-28±2°C, the formulation was found unstable [15]. Stability of formulation was observed on the basis of % EE, average particle size and physical appearance.

3. Results and Discussion

Chitosan nanoparticles were prepared by ionotropic gelation method and evaluated for Percentage yield, drug entrapment, zeta Potential and Particle Size determination. Percentage yield of different formulation was determined by weighing the nanoparticles after drying. The percentage yield of different formulation was in range of 63.23±0.32–70.12±0.47%. The drug entrapment of different formulations was in range of 62.23±0.74-78.98±0.56% w/w Table 2. This is due to the mucoadhesion characteristics of chitosan that could facilitate the diffusion of part of entrapped drug to surrounding medium during preparation of Amphotericin B nanoparticles. The maximum percentage yield and entrapment efficiency was found formulation F4. The optimized formulation among other batches subjected to further studies. The mean size of the nanoparticles was determined by photo correlation spectroscopy (PCS) on a submicron particle size analyzer (Particle Size Analyzer from Malvern) at a scattering angle of 90°C. A sample (0.5mg) of the nanoparticles suspended in 5 ml of distilled water was used for the measurement. The results of measurement of mean particle size of optimized formulation F4 nanoparticles were found 123.45 nm respectively figure 1.

The zeta potential of the drug-loaded nanoparticles was measured on a zeta sizer (Malvern Instruments) by determining the electrophoretic mobility in a micro electrophoresis flow cell. All the samples were measured in water at 25°C in triplicate. Results of zeta potential of optimized formulation F4 nanoparticles were found to be -32.5 mV respectively figure 2**.**

Prepared gel was prepared and evaluate for viscosity, % assay, extrudability, spreadability and drug release study. It was found that viscosity of prepared gel was 4520cps, % assay was 98.96±0.35, Extrudability was 167g and Spreadibility (g.cm/sec) was found that 11.95(g.cm/sec) respectively table 3. In vitro drug release from chitosan nanoparticles gel was carried out using Franz diffusion cell method and found 92.23% in 12 hr. In first 30 min it was 23.36% drug release which slightly high. It was due to the release of free drug present in bag after leaching from chitosan nanoparticles. Drug release from chitosan nanoparticles formulation was found in very sustained and controlled manner table 4.

Results of Stability studies showed the average particle size of nanoparticle was found 110.23±0.23, 118.56±0.36 and 135.65±0.32 nm after 1, 2 and 3 month of storage at 4.0 ±0.2°C while at 25-28±2°C the average vesicle size was found 125.32±0.45, 145.65±0.45 and 186.65±0.54 nm after 1, 2 and 3 month of storage. % EE in nanoparticle formulation was 73.23±0.23, 71.45±0.54 and 69.98±0.45 % after 1, 2 and 3 month of storage at 4.0 ±0.2°C while there were no significant changes in % EE and physical appearance in nanoparticle formulation was observed after 3 month of storage at 4ºC. The antifungal activity of chitosan nanoparticles against *Candida albicans* was evaluated by well diffusion method. Results show that chitosan nanoparticles could inhibit the growth of *Candida albicans* tested table 5.

Table 2: Percentage yield for different formulation

Formulation	Percentage Yield	Percentage entrapment efficiency
F1	65.56±0.65	63.32±0.45
F2	68.56±0.42	65.56±0.32
F3	63.23±0.32	62.23±0.74
F4	70.12±0.47	78.98±0.56
F5	62.23±058	70.23±0.41
F6	69.98±0.93	71.23±0.56

Figure 1: Measurement of mean particle size
Figure 2: Graph of zeta potential

Table 3: Characterization of prepared Gel formulation

Optimized Gel formulation	Viscosity (cps)	% Assay	Extrudability (g)	Spreadability (g.cm/sec)
	4520±15	98.96±0.35	167±3	11.95±0.45

Table 4: *In vitro* drug release study of Optimized Gel formulation

S. No.	Time (hr)	% Cumulative Drug Release
1	0.5	23.36±0.65
2	1	39.98±0.32
3	2	46.65±0.47
4	4	55.52±0.85
5	6	69.98±0.45
6	8	73.32±0.65
7	12	92.23±0.52

Table 5: Antifungal activity of standard drug against *Candida albicans and*

S. No.	Name of drug	Microbes	Zone of inhibition		
			10 µg/ml	20 µg/ml	30 µg/ml
1.	Amphotericin B	*Candida albicans*	14±0.5	17±0.57	23±0.74
2.	Chitosan nanoparticles gel		15±0.57	18±0.94	25±0.86

Conclusion

Chitosan nanoparticles were prepared by ionotropic gelation method and evaluated for Percentage yield, drug entrapment, zeta Potential and Particle Size determination. Percentage yield of different formulation was determined by weighing the nanoparticles after drying. The prepared chitosan nanoparticles further incorporated into gel base and evaluated for evaluate for viscosity, % assay, extrudability, spreadability and drug release study. The antifungal activity of chitosan nanoparticles against *Candida albicans* was evaluated by well diffusion method. Results show that chitosan nanoparticles could inhibit the growth of *Candida albicans* tested.

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