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Development and Characterization of Chitosan Nanoparticles Containing Erthromycin Estolate

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ABSTRACT:
Chitosan nanoparticles have gained more attention as drug delivery carriers because of their better stability, low toxicity, simple & mild preparation method and providing versatile routes of administration [1]. The aim of the present study was the development and characterization of erythromycin estolate loaded chitosan nanoparticles. Chitosan nanoparticles were prepared with tripolyphosphate (TPP) by ionotropic gelation i.e. crosslinking technique. Nanoparticles were prepared and evaluated for various parameters like particle size analysis using particles size analyzer, surface morphology study by field emission scanning electron microscope (FE-SEM), crystal phase identification using X-ray diffraction (X-RD) and thermo gravimetric analysis using differential scanning calorimeter (DSC). The Particle size analysis of the of nanoparticles showed the particles size distribution in between 700-1000 nm and surface morphology by FE-SEM showed that the nanoparticles had a solid dense cubical or rectangular crystalline structures. The X-RD of the erythromycin estolate loaded chitosan nanoparticles exhibited all characteristic peaks of erythromycin estolate, thus indicating that there was no change in the crystallinity of the pure drug. *In-vitro* assessment of antimicrobial activity of the pure drug and drug nanoparticles were performed and investigated. The antibacterial activity was seen to improve with increasing concentration of the drug nanoparticles in the test sample, confirming no negative impact of the polymer on the drug regarding its antibacterial activity.

Keywords: Chitosan, Nanoparticles, Erythromycin estolate, Ionotropic gelation technique, Tripolyphosphate.

[I] INTRODUCTION

Nanoparticles are made of natural or artificial polymers rangingin size from 10–1000 nm [2]. Nanoparticles displayunique physical and chemical features because of effects such asthe quantum size effect, mini size effect, surface effect and macro-quantum tunnel effect [3].
Chitosan (CS) is the second abundant polysaccharide and a cationic polyelectrolyte

present in nature. CS has shown favorable biocompatibility characteristics [4-6] as well as the ability to increase membrane permeability, both *in vitro* as well as *in-vivo*[7-10], and be degraded by lysozyme in serum. It has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material [11].

Chitosan was selected for nanoparticles because of its recognized mucoadhesivity and ability to enhance the penetration of large molecules across mucosal surface[12]. In this study,erythromycin estolate loaded chitosan nanoparticles were made by ionotropic gelation technique based on the interaction between the negative groups of sodium tripolyphosphate (TPP) and the positively charged amino groups of chitosan.

Non-toxicity and quick gelling ability of TPP arethe important properties that make it a favourablecross-linker for ionic gelation of chitosan. Chitosan nanoparticlesprepared by TPP as an anionic cross-linker arehomogeneous, and possess positive surface chargethat make them suitable for mucosal adhesion applications [13].

[II]MATERIAL AND METHODS

2.1. Materials

Erythromycin Estolate and Chitosan (CS) were kindly received as a gift samples from Mehta Pharmaceutical Industries, Mumbai, and Indian Sea Foods, Thoppumpady, Kochi, India respectively. Lactose, Tween 80, Acetic acid (glacial) and Methanol were purchased from Merck SpecialitiesPvt.Ltd., India.Tripolyphosphate (TPP) was purchased from Merck KGaA Darmstadt, Germany.

2.2. Design of Experiment

A two factor, two-level full factorial design (2^2) was selected to design the experiments(Design Expert-8.1).Total nine batches were designed fully randomized order and experiments were performed according to the randomized run order. Experiment domain ofeach variable are summarized in Table-1.

Run	Factor 1 (A:Chitosan mg/ml)	Factor 2 (B:TPP mg/ml)
1	5	5
2	3	3.5
3	1	5
4	5	2
5	3	5
6	1	3.5
7	3	2
8	1	2
9	5	3.5

Table-1. Randomized run order factorial design batches

2.3. Method of preparation of chitosan nanoparticles

Erythromycin estolate loaded chitosan nanoparticles were prepared by ionotropic gelation technique. A specific quantity of drug and chitosan were dissolved separately in methanol and 1% acetic acid respectively. Subsequently, an emulsion was prepared by dropwise addition of methanol containing drug to the CS solution under magnetic stirrer (Remi, India) followed by high speed homogenization (Omini international, USA) at 20,000 rpm for 2 min. A surfactant (1%, Tween 80) was used as emulsifying agent. In order to prepare the CS nanoparticles, the TTP aqueous solution with various concentrations was added dropwise to above prepared emulsion with mild stirring (600 rpm). Leave it for the overnight at room temperature for well ionic gelation. Nanoparticles were obtained by centrifugation (cooling microfuse, Remi, India) at 12000 rpm for 30 min. followed by lyophilization (scanvaccoolsafe, labogene, Denmark).

2.4. Particles size determination

Particle size measurement of the erythromycin estolate loaded chitosan nanoparticles was performed by using particle size analyzer (Malvern zetaseizer, Nano ZS). Phase analysis light scattering (PALS), M3-PALS technique was used to determine the particle size. After finishing the gelling process, the nanoparticles were diluted with distilled water and subjected to particle size analysis. Disposable glass cuvette was used to hold the samples. Each experiment was performed in triplicate.

2.5. Surface morphology determination

The morphological study of nanoparticles was performed by Field emission scanning electron microscope (FE-SEM) (Hitachi, Model-S4800 type II). Nanoparticles were dispersed in water (5ml) and sonicated for 3 min. (E- Chom Tech, Taipei, Taiwan). Few drops of the prepared samples were put on double sided adhesive tape fixed on metal stub. After drying, gold coating was performed for 80 second under vacuum.

2.6. X- Ray powder diffraction (X-RD)

In order to confirm the crystalline or amorphous nature of drug in the CS nanoparticles, X-RD pattern of drug, CS and CS nanoparticles containing erythromycin estolate were determined by X-Ray diffractometer (Bruker, D-8 advance) and data collection was performed using Cu anode and the voltage of the monochromator was 40 kV. The diffraction pattern was determined in the area $2^0 < 2\square < 80^0$, using continuous scan.

2.7. Differential Scanning Calorimetry (DSC)

The DSC thermograms of the erythromycin estolate, chitosan and nanoparticles were recorded on DSC calorimeter (DSC-60, Shimadzu Corporation Kyoto, Japan). The accurately weighed samples (±5 mg) were hermetically sealed into aluminum pan (Sample sealer & crimper SSC-30, Shimadzu Corporation, Kyoto, Japan).An empty sealed aluminum pan was used as a reference. The samples were scanned over a temperature range of 27 °C to 400 °C at a heating rate of 10 °C/min.

2.8. Assessment of antimicrobial activity (*In-vitro)*

To prepare assay plates using Petri dishes, 21 mL of medium labeled in Table-2 mixed with 200µg of *B. subtilis* was poured in each of the two plates, and was allowed to harden into a smooth base layer of uniform depth. Sixassay cylinders (wells) were dropped on the inoculated surface from a height of 12 mm, using a mechanical device ensuring even spacing, and the plates were covered to avoidcontamination. The six cylinders on each plate were filled with 50µL of dilutions of erythromycin estolatecontaining thetest levels specified below in Table-3, the plates were incubated at 32˚C to 35˚C, for 16 to 18 hours. Later the plates were removed from incubator, and thediameter of each zone of growth inhibition were observed and recorded.

Sl. No.	Chemicals	Quantity
1.	Peptone	6.0 g
2.	Pancreatic Digest of Casein	4.0 g
3.	Yeast Extract	1.5 g
4.	Beef Extract	1.0 g
5.	Dextrose	1.5 g
6.	Agar	15.0 g
7.	Water	1000 mL

Table-2. Medium for antibacterial test

Samples	Quantity Erythromycin estolate nanoparticles (µg/mL)
Blank	0
Sample 1	5
Sample 2	10
Sample 3	25
Sample 4	150
Sample 5	200

Table-3. Dilution table for erythromycin estolate nanoparticles

[III] RESULTS AND DISCUSSION

Nanoparticles are formed immediately upon mixing of TPP and chitosan solutions as molecular linkages were formed between TPP phosphates and chitosan amino groups. In total nine formulations of chitosan loaded nanoparticles were prepared and evaluated for various parameters such as particle size, morphology, in-vitro antibacterial activity etc.

3.1. Particles size analysis

Particles size distribution of the chitosan nanoparticles are shown in the Fig. 1. It was observed that particles size distribution of the chitosan nanoparticles were in between 700-1000 nm (Fig. 1). Sizes in this range are essential if such particles are intended for the

delivery of pharmaceuticals into lungs. A nanoparticle above 5µm becomes unable to adhere to the mucosal lining of the lungs and gets exhaled easily. But, in our case particles size distribution was found to be well in agreement for the lung delivery. Upon deposition in the lungs and exposure to the humid environment and the lung lining fluid, the matrix of the nanoparticles dissolves and readily releases the drug[14]. As with any biomaterial that is delivered to the body, the safety, toxicology and fate of the nanoparticles, must be deemed suitable for pulmonary drug delivery.

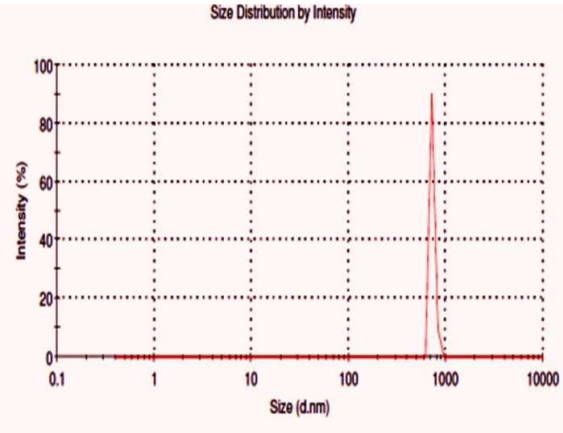

Fig. 1. Size distribution intensity of the nanoparticles

3.2. Surface morphology

The morphological characteristics of erythromycin loaded chitosan nanoparticles are shown in Fig. 2.The FE-SEM of the erythromycin estolate loaded chitosan nanoparticles showed that the nanoparticles have a solid dense cubical or rectangular structure and not aggregated. The spheres have mean diameters around 800 nm. The nanoparticles dry powder consists of individual nanoparticles, which touch each other, but retain their original size and shape. The size variation may be related to different conditions of sample preparation for FE-SEM. Tween 80, a non-ionic surfactant, was applied in the formulation. The addition of Tween 80 to the chitosan solution altered the balance of surface-to-viscous forces so as to promote a smooth particle surface. Addition of surfactant improved the sphericity of the particle.

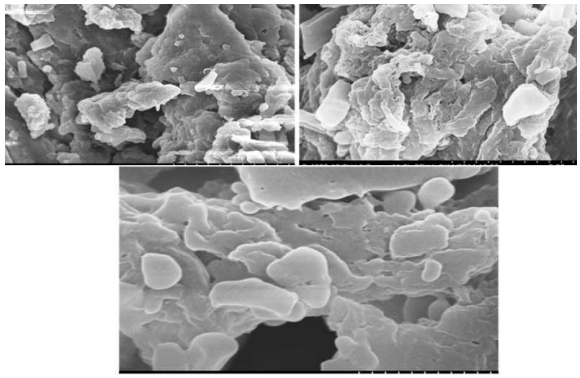

Fig. 2. SEM image of chitosan nanoparticles loaded with erythromycin estolate

3.3. X- Ray powder diffraction (X-RD)

The crystal phase identification of the studied samples was carried out using X-ray diffraction (X-RD). It is a non-destructive technique widely used for the characterization of crystalline materials.The X-RD of the drug, CS and CS nanoparticles containing erythromycin estolate were determined (Fig. 3). XRD patterns of pure samples showed sharp peaks at 2θ-scattered angles of 17, 20 and 21 (Fig. 3 A); these peaks were indicating the crystalline nature of drug. The drug crystalline peak was increased in the erythromycin estolatenano formulation (Fig. 3 C). This indicated slight increase in crystalline nature of the drug form. Intensities of drug peaks were also increased in the nano formulation. This increased intensity indicated the increased crystallinity of drug in nanoparticle form.

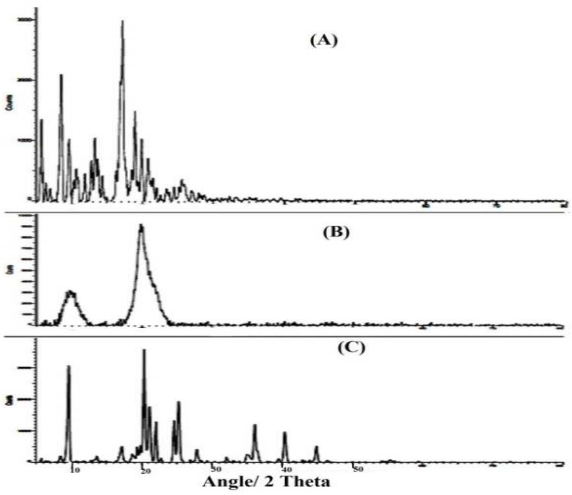

Fig. 3. XRD of (A) Drug (B) Chitosan (C) Nanoparticles

3.4. Differential Scanning Calorimetry (DSC)

The Differential Scanning Calorimeter (DSC) is a primary technique for measuring the thermal properties of materials to establish a connection between temperature and specific physical properties of substances and is the only method for the direct determination of the enthalpy associated with the process of interest. It can also be used to study the oxidative stability of samples and optimum storage conditions. The DSCthermogram of drug, chitosan and chitosan nanoparticles is shown in the Fig. 4. The DSC curve of pure erythromycin estolate showed endothermic peak at nearly 165ºC corresponding to its melting point. However, the characteristic peak of erythromycin estolate shifted to nearly 170 ºC (Fig. 4(C)). The thermogram of erythromycin estolate loaded chitosan nanoparticles exhibited all characteristic peaks of erythromycin estolate, thus indicating that there was no interaction between drug and polymer.

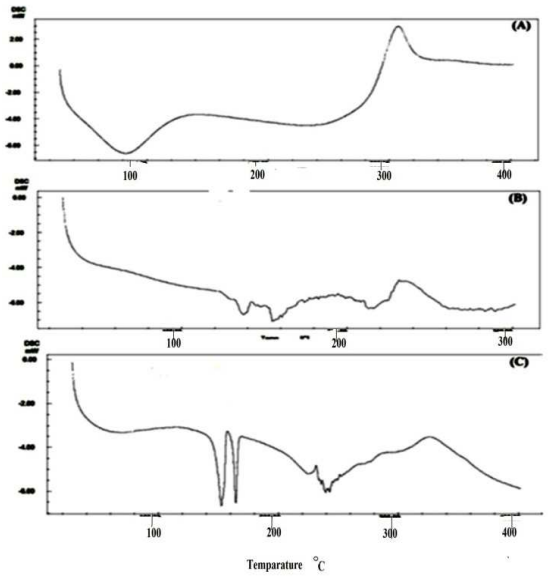

Fig. 4. DCS thermogram of(A) Chitosan, (B) Erythromycin estolate(C) Nanoparticles

3.5. Assessment of antimicrobial activity (*In-vitro)*

The antibacterial properties of the pure drug and drug nanoparticles were studied. The activity of pure drug and drug nanoparticles were assessed under controlled conditions by comparing the inhibition of growth of the microorganism by known concentrations of that antibiotic, producing meaningful results by well characterized method. Fig. 5 showedthe effect of different concentration of drug nanoparticles on the inhibition of *B. subtilis*under investigation. The antibacterial activity was seen to improve with increasing concentration of the drug nanoparticles. Also drug and drug nanoparticles were observed to have identical antibacterial properties confirming no negative impact of the polymer on the drug regarding its antibacterial activity. Appropriate sized drug nanoparticles can easily bind and absorb to mucosal lining of lungs. At the site of infection, the positively charged chitosan can bind to bacterial cell surface which is negatively charged and disrupt the normal functions of the membrane, e.g. by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells.

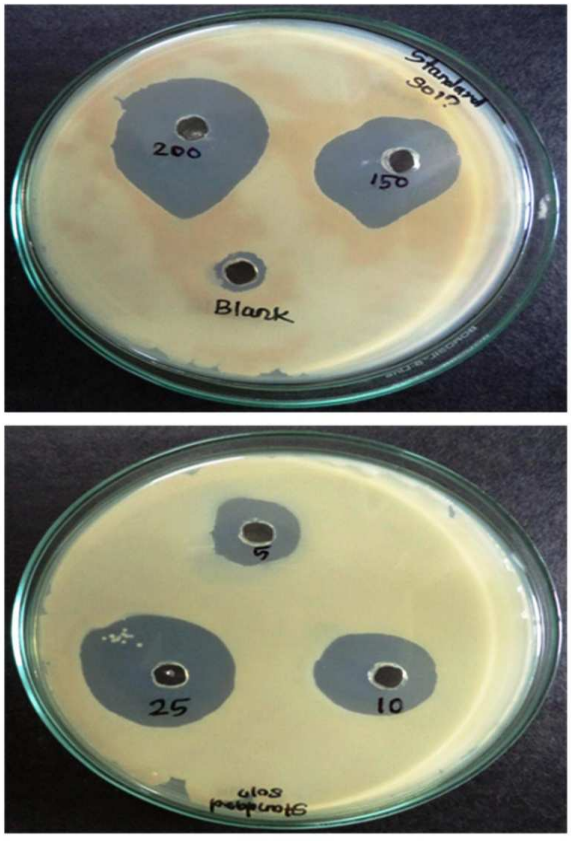

Fig. 5. Antibacterial activity of drug nanoparticles

[V] CONCLUSION

In this work, it was observed that the erythromycin estolate nanoparticles with ionotropic gelation method showed the following conclusive results:

- Particles size was found in nano range which was 700-100 nm.
- Erythromycin estolate nanoparticles were found to be suitable for pulmonary route of delivery.
- Nanoparticles were solid, dense, cubical or rectangular structure which was characterized by FE-SEM.
- Nanoparticles were found to be crystalline in nature confirmed by X-ray diffraction (XRD).
- Better stability of nanoparticles was observed by differential scanning calorimeter (DSC).
- Antituberculant property (in-vitro) was confirmed with agar plate well method.

On the basis of these observations and studies, finally it can be concluded that nanoparticles with erythromycin estolate and chitosan have better results in particle size, solubility, crystallinity, bioavalaibility, stability.

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