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Preparation and *In vitro* Characterization of a Fluconazole Loaded Chitosan Particulate System

Gülsel Yurtdaş Kırımlıoğlu[1*], Evrim Yenilmez[1], Ebru Başaran[1], Yasemin Yazan[1]

1 Anadolu University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 26470 Eskişehir, Türkiye

ABSTRACT

In the present study fluconazole (FZ) was successfully incorporated into cationic chitosan nanoparticles prepared by spray-drying method aiming dermal delivery. Particle size and zeta potential measurements, drug content, morphological, thermal and XRD analyses and FZ quantification by HPLC analyses were performed for characterizing the formulations prepared. Release behavior of FZ from the nanoparticles was determined using a Franz-diffusion cell. Thermal and XRD analyses results indicated that FZ was molecularly dispersed in chitosan nanoparticles. Cationic chitosan nanoparticles released FZ for 180 minutes indicative of the extended release of the drug. *In vitro* characterization results demonstrated that chitosan nanoparticles seem to be promising for enhancement of dermal delivery of FZ and could decrease potential side effects and reduce the potential of drug resistance.

Keywords: Fluconazole, Chitosan, Nanoparticles, Spray-drying, Franz-diffusion cell.

INTRODUCTION

Incorporation techniques of lipophilic drug active ingredients with poor aqueous solubility are used particularly in pharmaceutical technology for drug delivery design. Advantages of encapsulation include enhanced stability of labile drugs, controlled drug release and improved drug bioavailability.[1]

Dermal delivery by topical preparations such as creams, gels and lotions are limited due to the barrier characteristics of *Stratum corneum*. This limitation hinders the drug deposition and leads to relatively poor stability of active

*Corresponding Author: Gülsel Yurtdaş Kırımlıoğlu, e-mail: gyurtdas@anadolu.edu.tr
Gülsel Yurtdaş Kırımlıoğlu ORCID Number: 0000-0001-8897-0885
EvrimYenilmez ORCID Number: 0000-0002-7979-0089
Ebru Başaran ORCID Number: 0000-0003-2104-2069
Yasemin Yazan ORCID Number: 0000-0002-7492-1668
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agents by direct exposure to UV light. [2]

Fluconazole (FZ) is a triazole antifungal agent **(Figure 1)** with poor aqueous solubility administered either orally or intravenously.[3] It is used in the treatment of oropharyngeal and esophageal candidiasis, urinary tract infections, pneumonia, peritonitis and serious systemic candidal infections.[4,5] Adverse effects of FZ were reported to be related to the gastro-intestinal tract including abdominal pain, diarrhea, flatulence, nausea and vomiting. Other side effects associated with FZ are headache, dizziness, leucopenia, thrombocytopenia, hyperlipidemias, and raise in liver enzyme values. Serious hepatotoxicity was also reported while anaphylaxis, angioedema and skin reaction were rarely reported.[5]

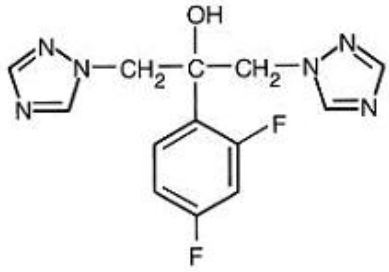

Figure 1. Chemical structure of fluconazole

Several classes of antifungal agents can be used in the treatment of candidiasis. However, among the factors influencing the efficacy of therapy is the immunity of the patient. Clinical failure following therapy may exist in patients with advanced immunodeficiency. In such cases, high doses of drugs or the use of multiple agents may be required thereby increasing the risk of serious side effects. To avoid this complication, entrapment of antifungal agents like FZ in a particulate delivery system can be used with several advantages such as the possibility of targetting the drug to the desired site in a controlled/sustained pattern. In this manner, therapeutic efficacy may be increased while reducing the side effects.[6,7] Some antifungal agents have been entrapped in particulate delivery system and promising results were observed in animal models and clinical therapy. These studies include mainly the polyene drugs, represented by amphotericin B and nystatin.[7,8] There are many studies regarding azole antifungal particulate drug delivery systems. [9,10]

Chitosan gained an increasing interest as a pharmaceutical excipient.[11] Chitosan is a hydrophilic, biocompatible and biodegradable polymer with low toxicity. It was investigated extensively for pharmaceutical and medical purposes including incorporation of therapeutic agents, proteins, enzymes, DNA and cells, formulation for oral vaccines and stabilization of liposomes.[12,13] Among the pharmaceutical applications of chitosan are the use as a vehicle for directly compressed tablets, as a disintegrant, a binder, a granulating agent in ground mixtures, as a drug carrier for sustained release preparations as well as a co-grinding diluent for the enhancement of dissolution rate and bioavailability of water insoluble drugs.[14] Furthermore, chitosan has mucoadhesive properties due to the molecular attraction formed by electrostatic interactions between positively charged chitosan and negatively charged mucosal surfaces.[15,16]

Spray drying represents a single-step, cheap, continuous and scalable process dedicated for converting liquids into dry, free-flowing powders which enables the production of particles with controlled size and morphological aspects.[17] Spray drying also eliminates the addition of crosslinking agent minimizes the swelling of chitosan based nanoparticles.[12] Therefore, spray drying technology was utilized in this study for the formulation of cationic nanoparticles using advantages of the method.

The objective of this study was to formulate chitosan-based cationic particulate systems containing FZ with high encapsulation efficiency and prolonged effect for reducing side effects and improving its stability. Chitosan nanoparticles were prepared by the spray-drying technique for topical skin fungal infections. Particle size, particle size distribution and thermal behaviour of the polymeric lattice were analyzed. Drug loading and release of the incorporated FZ were analyzed using a validated HPLC method.

METHODOLOGY

Materials

FZ was a kind gift from Bilim İlaç (Türkiye). Chitosan was purchased from Fluka Chemicals (Germany), acetic acid from Sigma-Aldrich (Germany) and ethanol from Carlo Erba (Italy). Sodium chloride, methanol and acetonitrile were the products of Merck (Germany).

Preparation of chitosan nanoparticles

Formulations were prepared using Mini Spray Dryer (B-190, BUCHI, Switzerland). The spray dryer was connected to the Inert Loop B-295 (Buchi Labortechnik AG, Switzerland) due to the organic solvent. Carbon dioxide gas was used at a flow rate of 120 $L.min^{-1}$. The residual oxygen level in the system was controlled below 4%.

When preparing particulate systems by spray-drying method, it has to be kept in the mind that production parameters such as size of nozzle, feeding pump rate, inlet temperature and compressed air flow rate, affect the particle size.[12,18] It was reported that smaller particles are formed with lower feeding pump rate and smaller nozzle size. In addition, smaller particles are formed with greater volume of air input where particle size is not dependent inlet temperature in the range of 120-180°C.[12]

Briefly, FZ and chitosan were dissolved in 96% (v/v) ethanol and 2% (v/v) acetic acid solutions, respectively. The solutions were mixed and homogenized at 1500 rpm for 3 hours. The final clear solution was then spray-dried with an inlet temperature of 145°C ± 1°C and an outlet temperature of 50°C ± 3°C. White dry powders were obtained and kept in tightly closed vials at room temperature until being analyzed. The placebo formulation was prepared as described above without the addition of FZ. These were bare, empty nanoparticles.

Composition of the formulations was kept as simple as possible **(Table 1)**.

Table 1. Compositions of the formulations prepared

CODE	FZ	Chitosan	Acetic acid	Ethanol
Placebo	-	2 g	240 mL (%2 v/v)	240 mL
C-FZ-1	0.2 g	2 g	240 mL (%2 v/v)	240 mL
C-FZ-2	0.5 g	2 g	240 mL (%2 v/v)	240 mL

Characterization of chitosan nanoparticles

Particle size and zeta potential analyses

Particle size, particle size distribution (PDI) and zeta potential measurements of the formulations prepared were performed on freshly prepared samples using Malvern Nano ZS (Zetasizer Nano Series, Worcestershire, UK). Samples of all formulations were dispersed in double distilled water (adjusted to a constant conductivity of 50 μS.cm^{-1} using 0.9% NaCl) just prior to analyses. All analyses were repeated in triplicate at 25°C ± 2°C.

Morphology

The particle shape and surface characteristics of the freshly prepared nanoparticle formulations and FZ were investigated by scanning electron microscope (SEM) (HITACHI TM3030Plus Tabletop Microscope, Japan) at 25°C ± 2°C. Samples were coated with a thin layer gold under argon to avoid charging under the electron beam.

Thermal analysis

Thermal behaviors and the interactions between FZ and chitosan were analyzed using differential scanning calorimetry (DSC) (DSC-60, Shimadzu Scientic Instruments, Columbia, MI, USA). In DSC analyses, the heating rate of 10°C. min^{-1} was employed in the temperature range of 50°C-200°C. Analyses were carried out under nitrogen with a scan rate of 5 $K.min^{-1}$.

X-ray diffractometry analysis

Dry powder X-ray diffractometry (XRD) analyses were performed using RIKAGU D/Max-3C (Japan). The XRD analysis range was 2°C-40°C over 2θ with 2°C min^{-1} scanning rate, with 40 kV voltage and current intensity level of 30 mA.

Pure FZ and placebo formulation were also analyzed and those XRD spectra were used as references in evaluating the chitosan nanoparticles containing FZ.

Determination of FZ content of chitosan nanoparticles

For the quantification of FZ incorporated into FZ formulations, accurately weighed (5 mg) formulations were dissolved in acetic acid (2%, v/v) solution and ethanol (5 mL, 4:1) mixture and agitated at 4000 rpm for 3 min.1 mL of supernatant was collected. Drug content of nanoparticles was determined using the reversed-phase HPLC method equipped with a pump (LC 10-AD), a UV detector (SPD-20A), a data station (Shimadzu, Japan) and C_{18} column (250 mm x 4.6 mm i.d. and 5 μm particle size). The mobile phase consisting of distilled water (tetrabutylammonium hydrogen sulfate): acetonitrile (75:25, v/v) was degassed prior to the analysis. The flow rate was 1 $mL.min^{-1}$ with an injection volume of 25 μL. The oven temperature was adjusted to 30°C ± 1°C and FZ was monitored at 223 nm.

In vitro release studies of FZ from chitosan nanoparticles

In vitro release studies were performed using Franz diffusion cells.[2] The diffusion cells were thermoregulated with a water jacket at 32° C. Polypropylene membrane was placed on a Franz diffusion cell after keeping it 20 minutes in the donor compartment. The receptor chamber was filled with distilled water. 1000 μg of formulation was applied to the donor compartment. 0.5 mL aliquots were withdrawn from the receptor compartment at specific time intervals. The amounts withdrawn were replaced by the fresh distilled water. The amount of FZ in aliquots was analyzed by a validated HPLC method and release profiles were obtained.

RESULTS AND DISCUSSION

Preparation of chitosan nanoparticles

In this study, spray-drying method was successfully used to prepare chitosan nanoparticles since it does not involve toilsome procedures and avoids the use of harsh cross-linking agents and organic solvents which might possibly trigger chemical reactions with the active agent.[19]

Characterization of chitosan nanoparticles

Particle size and zeta potential measurements

Particle size is one of the important physical properties of colloidal systems. Particle size distribution of the formulation is especially significant in the physical stability and activity of colloidal systems.[20] It was also found that the size of nanoparticles play an important role in their adhesion to and interaction with the biological cells.[21] Particle sizes of formulations were found to be 552.50 ± 10.30 nm and 648.25 ± 8.81 nm for C-FZ-1 and C-FZ-2, respectively (**Table 2**). It was found that decrease in the amount of FZ in formulations was in parallel with the relative decrease in average particle size.

Table 2. Mean particle size, PDI and zeta potential values of formulations prepared (SE: Standard error) (n=3)

Code	Particle size (nm) ± SE	PDI ± SE	Zeta Potential (mV) ± SE
Placebo	410.00 ± 11.41	0.480 ± 0.018	7.0 ± 4.1
C-FZ-1	552.50 ± 10.30	0.450 ± 0.083	38.4 ± 0.2
C-FZ-2	648.25 ± 8.81	0.313 ± 0.051	40.4 ± 2.3

The acceptable value for PDI is 0.05-0.7; values greater than 0.7 indicate very broad size distribution and probably no suitability for dynamic light scattering technique.[22] As shown in **Table 2**, acceptable PDI values were obtained for all batches. Also, PDI data showed that the homogeneity increased with the addition of FZ into the formulations.[23]

Zeta potential of nanoparticles is commonly used to characterize the surface property of nanoparticles. Results showed that zeta potentials measured were 38.4 ± 0.2 mV and 40.4 ± 2.3 mV for C-FZ-1 and C-FZ-2, respectively, which may be attributed to the positive charges on polymeric matrices indicating adequate physical stability. Cationic chitosan nanoparticles with mucoadhesive properties may interact with the negatively charged of skin and open up the tight junctions of epithelial cells to allow the paracellular transport pathway resulting in an increase in bioavailability of the active agents.[16,21]

Morphology

SEM images of pure FZ and formulations were demonstrated in **Figure 2**. SEM images showed that all formulations prepared were nearly in spherical shape while some of the spheres reminded the collapsed balloons with smooth surfaces. Crystalline structure of FZ was not observed in the formulations indicating successful incorporation of FZ into the polymeric matrices.[24]

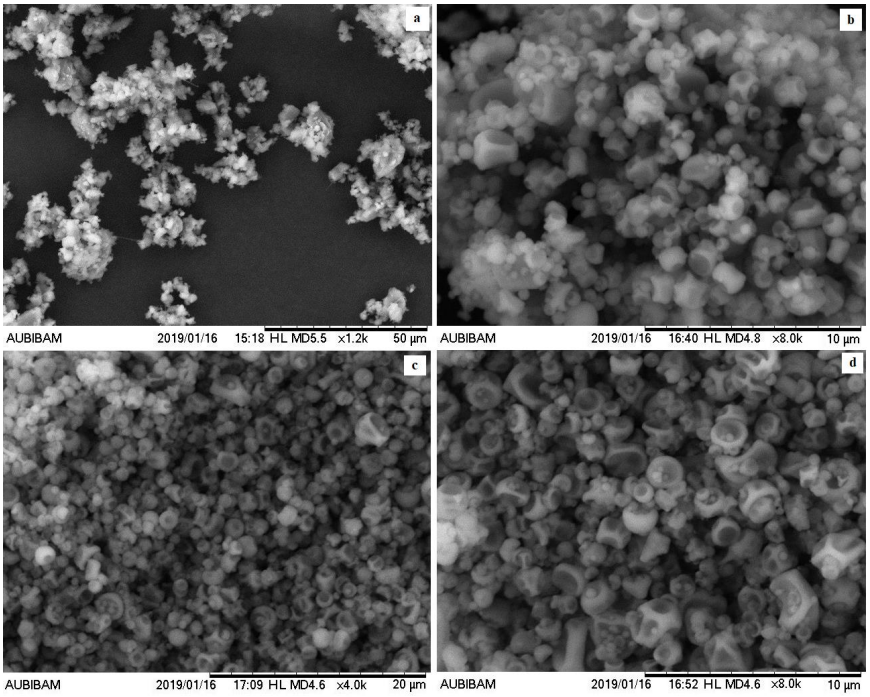

Figure 2. SEM images of pure fluconazole and chitosan nanoparticles prepared (a: FZ, b: placebo formulation, c: C-FZ-1, d: C-FZ-2).

Thermal analysis

DSC was in order to determine the incorporation of FZ into the polymeric network and also the status of polymer and active agent after spray-drying process.[11]

The thermogram of FZ shows the simple thermal behaviour of the drug **(Figure 3a)**. The sharp endothermal peak the observed at 142.66°C is the first order solid-liquid phase transition corresponding to the melting of the drug.[3] Placebo formulation **(Figure 3b)** was characterized by its amorphous state since no endothermic peak was observed. In the DSC thermogram of formula-

tions, sharp peak belonging to FZ was not observed **(Figure 3c)**. There may be two explanations of this peak disappearance. First explanation is the molecular incorporation of FZ into chitosan nanoparticles. When FZ was molecularly dispersed within the polymeric matrix resulting in a solid solution, the endothermal peak of FZ has disappeared.[11,12,25] Second explanation is the dilution effect of the polymer network. When the ratio of drug:polymer is so small, the massive amount of polymer shades the endothermal peak of the drug.[26]

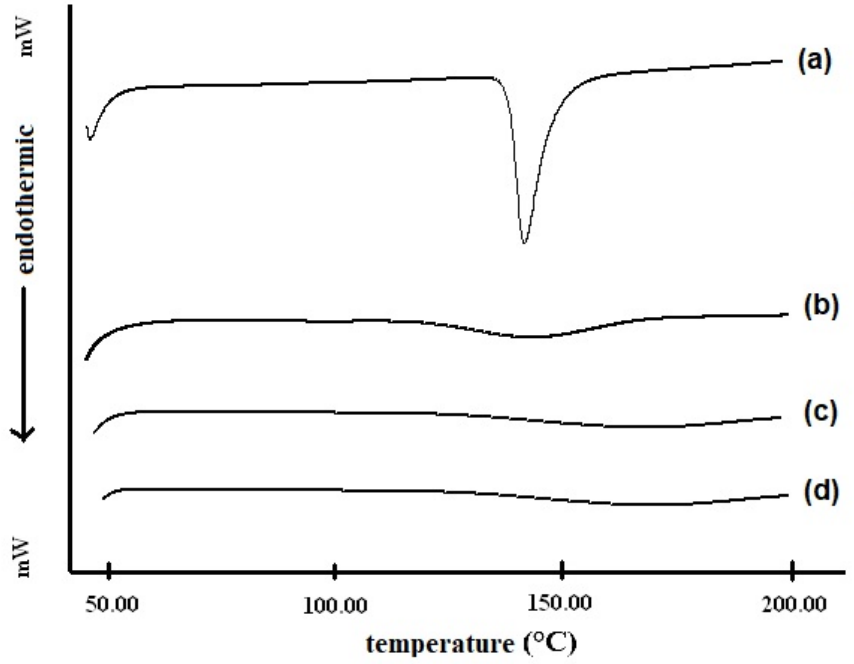

Figure 3. DSC thermograms of pure fluconazole and chitosan nanoparticles prepared (a: FZ, b: placebo formulation, c: C-FZ-1, d: C-FZ-2).

X-ray diffractometry analysis

DSC and XRD play a prominent role in the characterization of polymeric matrices because they are able to provide structural information on the dispersed particles.[27] Therefore, in this study for better evaluation of the crystalline polymeric structure of polymeric particles DSC and XRD analyses were performed simultaneously.

Dry powder XRD analyses of nanoparticles confirmed the DSC results showing the amorphous state of the polymeric network. XRD patterns of pure FZ, placebo and formulations were demonstrated in **Figure 4**. The FZ spectrum

shows several sharp diffraction peaks typical of its crystalline state **(Figure 4a)** while placebo and FZ-containing formulations were of amorphous state with no sharp XRD peaks in the spectra **(Figure 4b and Figure 4c)**. In the patterns of the FZ loaded formulations, peaks corresponding to FZ disappeared, indicating that dispersion in an amorphous form.[13,26]

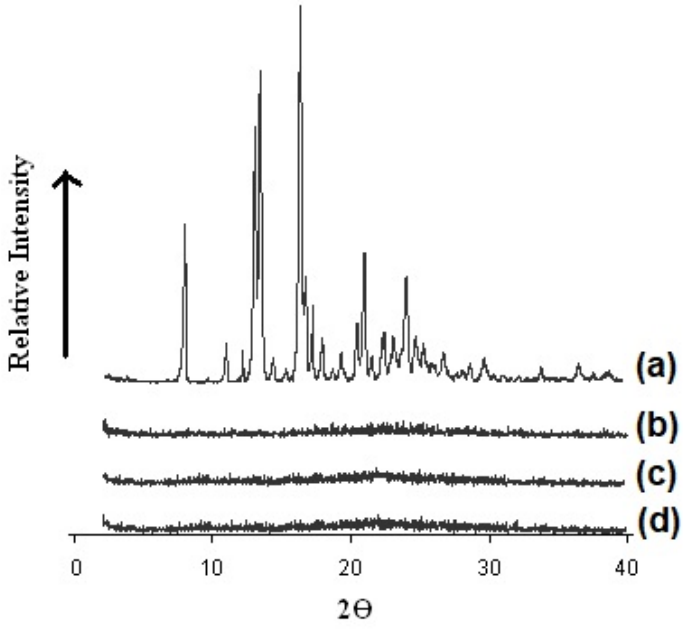

Figure 4. X-ray diffraction of patterns of pure fluconazole and chitosan nanoparticles prepared (a: FZ, b: placebo formulation, c: C-FZ-1, d: C-FZ-2).

Determination of FZ content of formulations

A validated HPLC method used for the determination of FZ demonstrated that the incorporation efficiency of FZ in formulations were found to be 8.99 ± 0.54 % and 12.04 ± 2.05 % (mean ± SE), for C-FZ-1 and C-FZ-2, respectively. It is evident from the data that incorporation efficiency was affected by the drug: polymer ratio. The results revealed that the incorporation efficiency of formulations was increased with increasing concentration of the active agent in the formulations.[21] Relatively low incorporation efficiency of nanoparticles prepared may be attributed to the spray-drying parameters such as nozzle diameter, spraying rate or viscosity of the spraying solution.

In vitro drug release

In vitro dissolution test plays an important role in drug formulation development and quality control. It can be used not only primary tool to observe the consistency and stability of drug products but also as a relatively rapid and inexpensive process to estimate *in vivo* absorption of a drug formulation.[11]

The *in vitro* release profile of FZ from formulations were shown in **Figure 5**. Franz diffusion cell analyses results showed that the release of FZ from formulations were 26.12 % and 29.52 % at the end of 180 minutes for C-FZ-1 and C-FZ-2, respectively. Flux (*J*) and permeation coefficient (k_p) values were determined using the slope of the steady-state portion of the amount of the drug permeated and divided by time. The steady-state flux (J_s) of FZ from the formulations were calculated to be $9.7x10^{-2}$±0.001 μg.cm^{-2}h^{-1} and $1.2x10^{-1}$±0.0001 μg.cm^{-2}h^{-1} while permeability coefficient (k_p) was determined to be $3.10x10^{-3}$ ±0.001 cm.h^{-1} and $4.0565x\ 10^{-4}$ for the C-FZ-1 and C-FZ-2, respectively. The results indicated that the release of FZ from the formulations varied depending on time.[2]

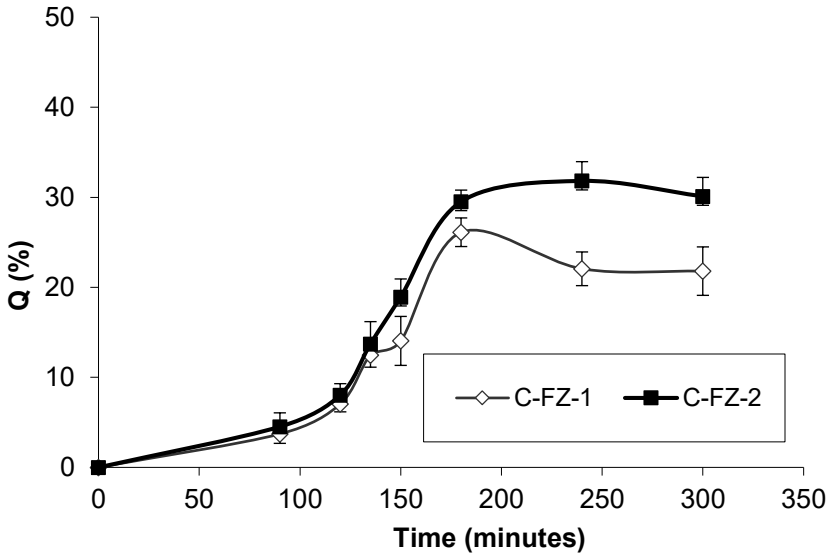

Figure 5. In vitro release profile of FZ from FZ loaded chitosan nanoparticles.

FZ was successfully incorporated into chitosan nanoparticles using a spray-drying method. *In vitro* characteristics of nanoparticles prepared were analyzed to confirm incorporation of FZ into the polymeric structure. When submicron sized with homogenous size distribution and nearly spherical nanoparticles

were prepared. DSC and XRD assays confirmed decrease in FZ crystallinity in cationic nanoparticles. Chitosan nanoparticles released FZ for 180 minutes indicative of the extended release of the drug, which will reduce the side effects in treating the infections induced by *Candida albicans*. With the cationic and mocuadhesive properties of chitosan-based system, nanoparticles will ensure longer residence at the infection site, providing a favorable release profile for the FZ for the dermal delivery.

Conclusively, chitosan nanoparticles may be good alternative for delivery of FZ, which need to be, investigated further using *in vivo* tests before final decision.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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