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PREPARATION AND *IN VITRO* EVALUATION OF CHITOSAN MICROGRANULES WITH CLOTRIMAZOLE

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Abstract: Mucoadhesive polymers have gained much attention due to the possibility to overcome physiological barriers in long term drug delivery. Chitosan is a biocompatible and non-toxic chitin derivative, which due to its mucoadhesive properties enables to obtain prolonged drug delivery. The aim of this study was to formulate and *in vitro* evaluate chitosan microgranules with clotrimazole. Microgranules were prepared by the wet-granulation method using pentabasic tripolyphosphate (TPP) as an ion cross-linker. It was shown that cross-linked chitosan significantly prolonged the release of clotrimazole. Microgranules in formulation F4 (with chitosan:clotrimazole:TPP ratio 5:1:1) not only maintained regular surface morphology, but also ensured prolonged release of clotrimazole over the period of 6 h. The obtained results indicate that chitosan is a suitable polymer for developing a sustained-release dosage form of clotrimazole for local delivery.

Keywords: chitosan, microgranules, clotrimazole, mucoadhesive polymers

Mucoadhesive microparticles – microspheres, microcapsules and microgranules enable to improve drug bioavailability by promoting the residence time of the dosage form with the mucous membranes (1–3). The increased contact time and localization of the drug due to the strong interaction between the polymer and mucus is also essential for the modification of tissue permeability. Mucoadhesive polymers enhance the adhesion of the administered dosage form to the mucosal tissue and thus may reduce the frequency of application and the amount of the administered drug, which might improve patient compliance and acceptance (4–6).

One of the natural mucoadhesive polymers is chitosan – a polycationic copolymer consisting of glucosamine and N-acetylglucosamine units. It is obtained by deacetylation of chitin derived from exoskeleton of crustaceans. Owing to its cationic properties, chitosan is able to interact with anionic agents and form a water insoluble barrier that participates in a drug release (3, 7). Because of its excellent mucoadhesive properties, biocompatibility, non-toxicity, biodegradation, and antimicrobial activity, chitosan has been used in many biomedical applications, including drug delivery systems (4, 7–9).

Clotrimazole – a safe and locally effective imidazole derivative – is widely used for the treatment of mycotic infections of the genitourinary tract and skin. Clotrimazole is available in several conventional dosage forms, such as solutions, creams, ointments, tablets, and vaginal suppositories. However, these conventional dosage forms do not offer prolonged delivery of clotrimazole due to the relatively short residence time. As a result, the therapeutic efficacy of the drug is impaired and multiple administrations are necessary for treatment. As clotrimazole does not possess an inherent ability to bind to the mucosal tissue itself, the reasonable seems to be using mucoadhesive polymers like chitosan (4, 5, 10).

Therefore, the aim of this study was to design and evaluate chitosan microgranules with clotrimazole, which would provide its prolonged release profile. The microparticles were prepared by modified wet-granulation method using TPP – a non-toxic, multivalent anion – as an ion cross-linker. *In vitro* tests were performed to evaluate the release of clotrimazole from chitosan microgranules at acidic pH, corresponding to the conditions of the vagina (11). Additionally, the effect of the different factors such as chitosan, clotrimazole and TPP ratios, pH of

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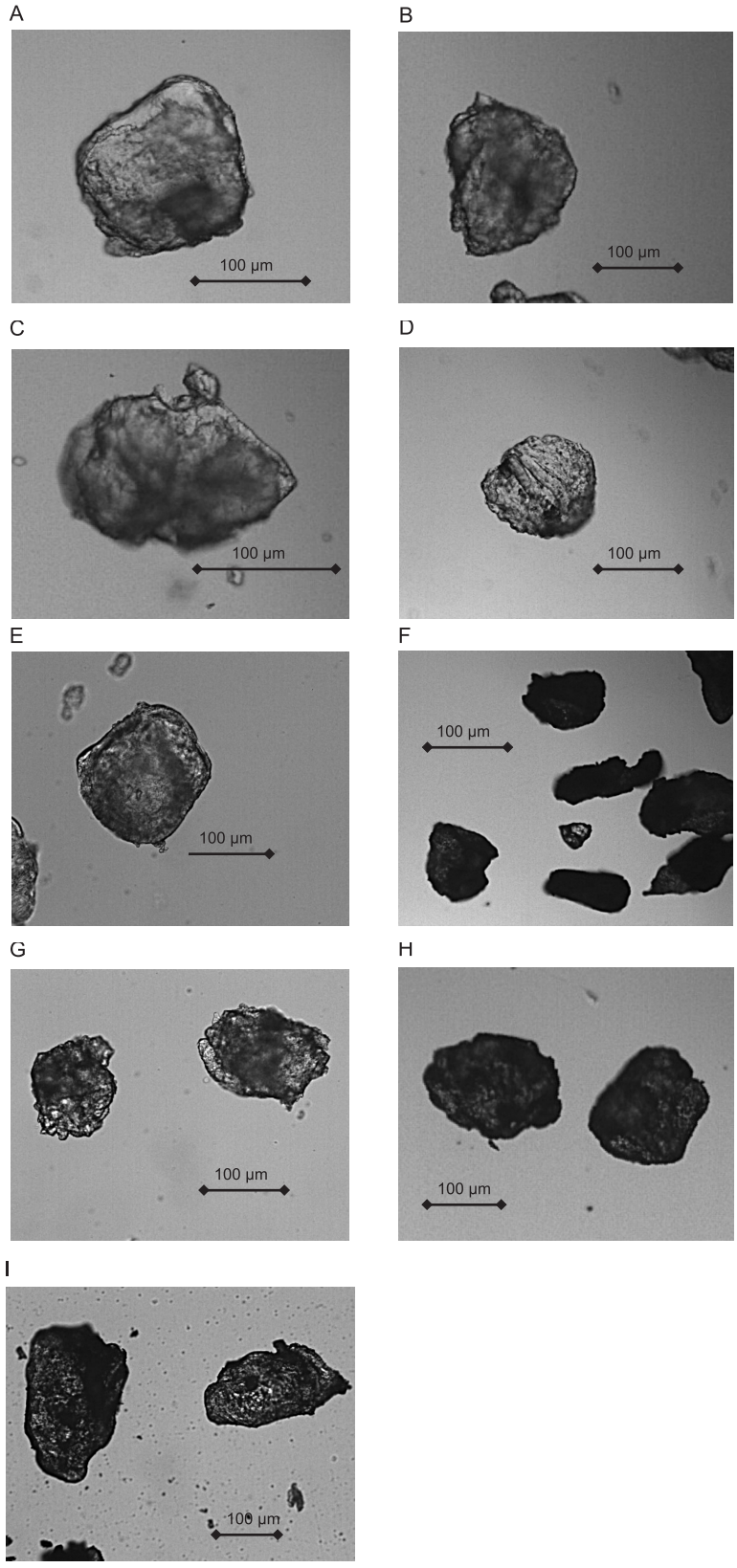

Figure 1. Microscopic images of chitosan microgranules: formulation F1 (**A**), formulation F2 (**B**), formulation F3 (**C**), formulation F4 (**D**), formulation F5 (**E**), formulation F6 (**F**)**,** formulation F7 (**G**), formulation F8 (**H**) and formulation F9 (**I**)

TPP solution and presence of the surfactant on microgranules formation and release profile of clotrimazole was investigated.

EXPERIMENTAL

Materials

Clotrimazole was a gift from Aflofarm (Pabianice, Poland). Chitosan (medium molecular weight, with 75–85% of deacetylation degree, a viscosity of 1% solution in 1% acetic acid: 200 cP) and TPP were purchased from Sigma Aldrich (Steinheim, Germany). Acetic acid (80%), sodium acetate, potassium dihydrogen phosphate, sodium hydroxide and disodium hydrogen phosphate were obtained from Chempur (Piekary Śląskie, Poland). Sodium dodecyl sulfate (SDS) was purchased from POCH (Gliwice, Poland). Methanol was HPLC grade and purchased from Merck (Darmstadt, Germany). Water for HPLC was distilled and passed through a reverse osmosis system Milli-Q Reagent Water System (Billerica, USA).

Microgranules preparation

Microgranules were prepared in at least 3 batches in a ceramic mortar by the modified wet granulation technique (12). Chitosan and clotrimazole were ground to fine powder, blended and mixed thoroughly. Then, the proper amount of 2% acetic acid was gradually added to moisten the powders (Table 1). After the formation of wet-mass, an aqueous solution of TPP at a concentration of 10% was slowly dropped to the mortar with simultaneous rubbing and grinding of the mixture. The wet granules were dried (at a temperature of 35 ± 0.5°C) for 20 h. Next, the obtained granules were sieved through the set of sieves. For further investigations microgranules of 100–200 μm diameters were chosen, because that size of particles is unlikely to cause local irritation (12).

Microgranules size and shape analysis

The microgranules were observed (under magnification 10×) using the optical microscope equipped with a camera (Motic BA400, Wetzlar, Germany). Microscopic images of obtained microgranules are shown in Figure 1.

In vitro release

The *in vitro* release of clotrimazole from chitosan microgranules was studied using as the dissolution medium 50 mL of acetate buffer (pH 5.2) with 1% SDS to maintain the sink condition. In each study, the amount of microgranules equivalent to 5 mg of clotrimazole was analyzed. The *in vitro* release assay was performed in glass beakers tightly covered with foil "Parafilm" (Pechiney Plastic Packaging, Menasha, USA) and placed in the water bath at 37 ± 0.5°C with a rotation speed of 150 rpm. Samples (1 mL) were withdrawn at the predetermined time intervals (after 1, 2, 3, 4, 6 and 24 h) and replaced with fresh medium. The samples were diluted with methanol (1:5), filtered through 0.45 μm CA membrane filter paper (Witko, Łódź, Poland) and analyzed by HPLC method. The *in vitro* release test was conducted at least two times for three different batches.

HPLC analysis of clotrimazole

The concentration of clotrimazole in the dissolution medium was determined by the HPLC system Agilent Technologies 1200 equipped with a

Table 1. Composition of various chitosan microgranules formulations (F1–F9).

COMPOUND/ FORMULATION	F1	F2	F3	F4	F5	F6	F7	F8	F9
Chitosan (g)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Clotrimazole (g)	0.4	1.0	0.4	0.8	0.8	0.8	0.8	0.4	0.8
2% CH_3COOH (mL)	16.0	15.0	16.0	17.0	16.0	18.0	16.0	16.0	17.0
10% TPP solution (w/v)	+	+	+	+	+	–	+	+	+
pH of 10% TPP solution	9.1	8.8	6.55	8.8	6.55**	–	6.55	6.55	6.55
Volume of 10% TPP solution (mL)	8.0/-	4.5/3.5*	4.0/2.0*	8.0/-	5.0/4.0*	–	5.0/4.0*	8.0*/–	8.0/–
Weight ratio Chitosan: Clotrimazole:TPP	10:1:2	10:2.5:2	10:1:1.5	5:1:1	5:1:1.125	5:1:–	5:1:1.125	10:1:2	5:1:1

*) in some formulations, cross-linking solution of 10% TPP was divided into two parts – after adding the first one granules were dried (at a temperature of 35°C ± 0.5°C for 30 min.), and next the remaining volume of TPP solution was dropped. **) solution of 10% TPP with 0.5% SDS

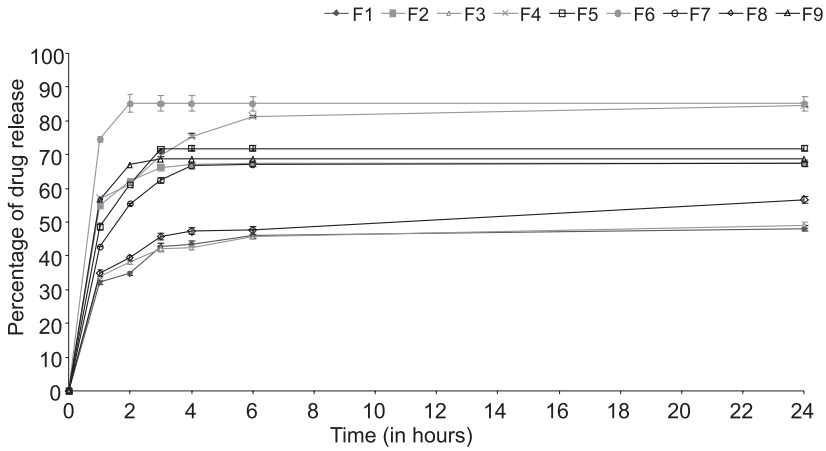

Figure 2. *In vitro* clotrimazole release profiles from various chitosan microgranules formulations (F1 – F9) (mean ± SD)

G1312A binary pump, a G1316A thermostat, a G1379B degasser and a G1315B diode array detector (Agilent, Waldbronn, Germany). Data collection and analysis were performed using Chemstation 6.0 software. Isocratic separation was achieved on a Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 µm column (Agilent, Waldbronn, Germany). Mobile phase was methanol – phosphate buffer pH 7.4 (4:1, v/v), the flow rate was 1.0 mL/min and UV detection was performed at a wavelength of 210 nm (13, 14). The column temperature was maintained at 25°C. For injection into the HPLC system 20 µL of sample was used. All reagents used for analysis were HPLC grade. The retention time of clotrimazole was 5.2 min. Standard calibration curve was linear over the range of 1–100 µg/mL.

Statistical analysis

The analysis was performed using the analysis of variance (ANOVA) and multiple comparisons were made to check statistical significance. The statistical significance between means was verified by Sheffe's comparison test accepting $p < 0.05$ as significant.

RESULTS AND DISCUSSION

The chitosan microgranules with clotrimazole were prepared by the wet-granulation method in the presence of ion cross-linker – TPP. TPP is carrying five negative charges which effect electrostatic interactions with positively charged amine groups of chitosan. As the charge numbers of TPP and chitosan are related to acidic pH, ionic cross-linked chitosan formulations could be useful for a specific vaginal drug delivery (2). The most appropriate shape and surface of obtained microgranules were observed in formulations F4 (Fig. 1D), whereas microgranules in formulation F6 were fragile and with irregular shape (Figure 1F). This observation indicates that the presence of TPP is crucial for the morphology of the obtained microgranules. Moreover, in order to receive spherical microparticles, the cross-linking time ought to be long enough (at least 30 min) to ensure the entire diffusion of TPP molecules into microgranules (2, 8, 9).

The *in vitro* release profiles of clotrimazole from different microgranules formulations are shown in Figure 2. It was noticed that cross-linking in the presence of TPP significantly prolonged the release of clotrimazole (up to 6 h in formulation F4), whereas from formulation F6 (comprised of the polymer and clotrimazole only) almost 75% of the drug was released during the first hour. After 6 h of the study, the range of released clotrimazole from chitosan-TPP microgranules was from about 48% in formulations F1, F3 and F8, to over 82% in formulation F4.

Additionally, in order to appoint the optimal formulation, various factors that influence the preparation of microgranules and drug release profile were also investigated. Too low volume of 10% TPP solution was responsible for obtaining fragile and irregular microgranules in formulation F3 compared to F8. This observation may indicate that the most appropriate chitosan:TPP ratio was 5:1.

The chitosan:clotrimazole ratio was another important parameter which affected the properties of microgranules. It was noticed that higher amounts of chitosan (in formulations F1, F3 and F8) contributed

to much slower release of the drug (Fig. 2). It can be explained by higher viscosity of the swelling chitosan, which is responsible for slower dissolution rate and in consequence slower diffusion of the drug through the gel layer (4).

To investigate the effect of the cross-linking agent pH on microgranules formation, pH of TPP solution was set at 6.55, 8.8 and 9.1. The cationic nature of chitosan is pH-dependent and interactions between chitosan and TPP are possible in a wide range of pH from 1.9 to 7.5 (8, 15). When pH of TPP solution was higher than 9.0, only small amount of chitosan amine groups was ionized, so that a thick coacervation layer was formed, which could hinder the counter anion groups of TPP from diffusing into the chitosan droplets. That thick layer could also make the release of clotrimazole impossible. However, under slightly acidic conditions (at pH ~ 6.55), even though the amount of amine groups was higher, the concentration of phosphate ions was too low, there was less interactions between cations and anions and eventually obtained microgranules were irregular (formulation F9). It was found out, that the most appropriate pH of TPP solution was 8.8 – sufficient for keeping the medium-high concentration of both amine groups and TPP counter anions. As a result, reaction between cations and anions appeared to sufficient extent and spherical microgranules were formed (formulation F4).

A surfactant plays an important role in microparticles preparation by affecting not only microparticle morphology, drug encapsulation efficiency but also delivery properties (2). SDS is a great solubilizer which significantly increases clotrimazole solubility (16). However, it was observed that SDS only slightly increased the total amount of released drug but helped to obtain more spherical microgranules (formulation F5 and F7).

The next purpose of this study was to state the optimal amount of clotrimazole in chitosan microgranules. When the amount of drug increased from 0.4 g (in formulations F1, F3 and F8) to 0.8 g (in formulations F4, F5 and F9), the significant increase in the percentage of clotrimazole release appeared. However, higher amounts of drug (up to 1.0 g in formulation F2) had a negative effect on its release profile (Figure 2). These results indicate that microgranules loading capacity influences clotrimazole release profile and reaches the optimal value when the chitosan:clotrimazole ratio is 5:1.

To summarize, the obtained results suggest that chitosan can be used to formulate microgranules for the prolonged delivery of clotrimazole. Microgranules in formulation F4 (with chitosan: clotrimazole:TPP ratio 5:1:1) not only maintained regular surface morphology, but also ensured prolonged release of drug up to 6 h. In view of the biodegradable nature of chitosan, these microgranules might be used as useful carriers for local mucosal drug delivery of clotrimazole but further study is needed.

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