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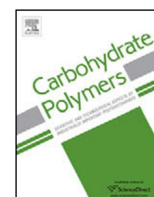

Development of alginate microspheres as nystatin carriers for oral mucosa drug delivery

María J. Martín [a], Ana C. Calpena [b], Francisco Fernández [b], Mireia Mallandrich [b], Patricia Gálvez [a], Beatriz Clares [a,*]

[a] Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Granada, Campus of Cartuja Street, 18071 Granada, Spain
[b] Biopharmaceutical and Pharmacokinetics Unit, School of Pharmacy, University of Barcelona, Joan XXIII Avenue, 08028 Barcelona, Spain

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ABSTRACT

To develop more effective antifungal mucoadhesive systems for the treatment of oral candidiasis, three types of microspheres, alginate (AM1), chitosan coated (CCM) and hydrogel (AM2) containing nystatin (Nys) were successfully elaborated by emulsification/internal gelation method. Physicochemical properties of microspheres resulted in 85–135 μm mean sizes, spherical shaped with narrow distribution. Optimal encapsulation efficiency and negative zeta potentials were observed. AM2 showed a consistent decrease in viscosity with increasing shear rate (Herschel–Bulkley). Optimal mucoadhesive properties and swelling behaviour where evidenced. Nys release from AM1 and CCM followed a concentration gradient pattern, contrary AM2 followed a complex release mechanism. All systems exhibited a marked fungicidal activity against *Candida albicans* strains. *In vivo* studies demonstrated that Nys was not found in systemic circulation assuring the safety of the treatment. Nys amounts retained in the mucosa were more than enough to ensure an effective fungicidal action without tissue damage. Based on the obtained results, AM2 could be proposed as the vehicle with the best properties for the buccal vehiculization of Nys.

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1. Introduction

Nystatin (Nys) is a polyene antifungal antibiotic, one of the oldest antifungal drug, produced by *Streptomyces noursei* strains (Kaur & Kakkar, 2010). Nys monomers selectively interact with ergosterol causing membrane disruption and eventual cell death (Coutinho & Prieto, 2003). Nys possesses a broad spectrum with both antifungal and fungistatic activity (Recamier, Hernandez-Gomez, Gonzalez-Damian, & Ortega-Blake, 2010) being effective against azole-resistant strains of *Candida* and amphotericin B-resistant strains of *Candida albicans* (Ellepola & Samaranayake, 1999). Nys is indicated for treatment of cutaneous and mucocutaneous fungal infections caused by *Candida* species, the main yeast capable of infecting the oral mucosa, being *C. albicans* the most common species isolated (Campos et al., 2012). Oral candidiasis is not a lethal disease in healthy patients. It is mainly caused by antibiotic or corticosteroid treatment and dental prosthesis. However, it must be treated to avoid chronic and systemic invasions of other tissues (Ship, Vissink, & Challacombe, 2007), especially among patients with diabetes mellitus, immunocompromised or under aggressive treatments (*e.g.* chemotherapy) to prevent opportunistic invasive fungal infections (Pemán & Salavert, 2012).

The presence of a large lactone ring with several double bonds renders it an amphiphilic and amphoteric molecule with poor solubility in aqueous media (360 mg/L at 24 °C), which reveals great formulation challenges (Croy & Kwon, 2004). This fact support that an increasingly important area of pharmaceutical research is focused on finding safe and effective methods of solubilizing poorly soluble drugs (Fernandez-Campos, Clares, Lopez, Alonso, & Calpena, 2013). In this way, micellar gels (Croy & Kwon, 2004), mucoadhesive devices for topical use (Sakeer, Al-Zein, Hassan, Desai, & Nokhodchi, 2010), liposomes (Ng, Wasan, & Lopez-Berestein, 2003), nanoemulsions (Fernandez-Campos et al., 2013), intralipids (Marín-Quintero et al., 2013), niosomes (El-Ridy, Abdelbary, Essam, El-Salam, & Kassem, 2011), microparticles (Martín-Villena et al., 2013), and pellets (Pál, Nagy, Bozó, Kocsis, & Dévay, 2013) have been developed for Nys vehiculization.

Oral cavity for drug delivery has attracted particular attention. It is composed of stratified squamous epithelium (outermost layer), the basement membrane, the lamina propria and the submucosa (innermost layer). Its permeability is estimated to be 4–4000 times greater than skin depending on the region (Galey, Lonsdale, &

* Corresponding author. Tel.: +34 958 243904; fax: +34 958 248958.
E-mail address: beatrizclares@ugr.es (B. Clares).

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Nacht, 1976). Taking into account the special characteristics of the buccal mucosa, the general features expected of delivery systems for local drug delivery to the oral mucosa include biocompatibility, mucoadhesiveness, stability, non-immunogenicity and capability of sustained drug release to maintain therapeutic levels over an extended period of time (Aduba et al., 2013). The use of mucoadhesive microspheres would meet all those requirements as strategy to the buccal Nys delivery. Most of microspheres are based on natural or synthetic polymeric materials. The natural polymer alginate, an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid, has been widely used due to its biodegradable nature with low toxicity, low cost and compatibility with the encapsulation of a wide range of drugs, with minimal use of organic solvents (Martins, Sarmento, Souto, & Ferreira, 2007). Alginate also exhibits optimal mucoadhesive properties to increase the contact time with absorptive sites enhancing the uptake of the encapsulated drug. On the other hand, chitosan, a cationic polysaccharide, produced by the deacetylation of chitin has been utilized as a membrane coating material to improve encapsulation efficiency and enhance stability with also good mucoadhesion capacity (Xiao & Sun, 2013).

Thus based on these considerations, and considering that no studies have been published about buccal Nys microspheres for the treatment of oral candidiasis, the major goal of this study was to get a more effective antifungal mucoadhesive system for the treatment of oral candidiasis. This system will provide a high spectrum of activity and a fungicidal activity against *C. albicans*. In this respect, in the present work we elaborated three types of Nys loaded microspheres. Their physicochemical properties, antimicrobiological effectiveness; *in vitro* release behaviour, and *in vivo* absorption were analysed.

2. Experimental

2.1. Materials

Nys and sodium alginate (65–75% guluronic and 25–35% mannuronic acid, ≈220 kDa) were provided by Fagron Iberica (Terrassa, Spain). Low molecular weight chitosan (≈50 kDa, 75–85% deacetylated) was from Sigma-Aldrich (Madrid, Spain). Calcium carbonate, calcium chloride, acetic acid, HPLC-grade methanol, *N*-dimethylformamide (DMF), acetonitrile and dimethilsulfoxide (DMSO) were obtained from Panreac (Barcelona, Spain). Soya oil, corn starch and Span® 80 were purchased from Guinama S.L.U. (Alboraya, Spain). Double distilled water was obtained from a Milli-Q® Gradinet A10 system apparatus (Millipore Iberica S.A.U.; Madrid, Spain). Polysulphone membranes were purchased from Iberlabo S.A. (Madrid, Spain). Sabouraud dextrose medium from Scharlab S.L. (Barcelona, Spain). Commercially available Nys, Mycostatin® oral suspension (Bristol-Myers Squibb Pharmaceutical Limited) was obtained from a local pharmacy. Phosphate buffered solutions (PBS) were elaborated according to European Pharmacopoeia.

2.2. Synthesis of alginate microspheres and coating procedure

The formulation of the Nys loaded microspheres was based on the emulsification/internal gelation method with modification (Martín-Villena et al., 2013). The W/O emulsion was performed with a sodium alginate aqueous solution, $CaCO_3$ and Nys as the internal phase and vegetable oil as the external phase. Briefly, 0.2 g of $CaCO_3$ were added to 40 mL of 5% (w/v) sodium alginate solution containing 474.55 ± 1.00 mg of Nys, and the resulted system stirred for 15 min at 450 rpm. After homogenization, the suspension was dispersed in 100 mL of vegetable oil (continuous phase) containing 2% (w/v) Span® 80. The mixture was stirred at 700 rpm for 10 min to form W/O emulsion. Under continuous stirring, 20 mL of vegetable oil containing 0.850 mL of glacial acetic acid were added to the W/O emulsion, to get the $CaCO_3$ solubilization. After 10 min under stirring, pregelified microspheres were separated from the oil dispersion by mixing with $CaCl_2$ solution 5% (w/v). The supernatant was discarded. The alginate microspheres (AM1) were centrifuged using an Eppendorf Centrifuge 5804 (Hamburg, Germany) at 1150 × *g* for 10 min, collected and washed using 100 mL distilled water by vacuum filtration and stored at 4 °C in Petri dishes.

Low molecular weight chitosan was selected as coating material for the microspheres at different concentrations. Coating procedures followed the method that had already been previously reported (Liu, Rauth, & Wu, 2007).

Then, 10 g uncoated microspheres (AM1) were immersed in a bath containing 100 mL 0.5% (w/v) chitosan acetic acid solution (1% v/v), and stirred at 300 rpm for 30 min on an orbital shaker for coating. The resulting chitosan-coated microspheres (CCM) were washed with 100 mL distilled water by vacuum filtration and kept at 4 °C. Finally a type of hydrogel microspheres (AM2) was prepared using a modification of the technique above described. 0.12 g of $CaCO_3$ were added to 40 mL sodium alginate solution 1.5% (w/v) containing 474.8 ± 0.30 mg of Nys. The suspension was dispersed in 100 mL vegetable oil containing Span 80® 3% (w/v), 0.4% (v/v) acetic acid under stirring at 760 rpm at room temperature for 10 min. Subsequently, pregelified microspheres were separated from the oil dispersion by mixing with $CaCl_2$ solution 0.08% (w/v). The supernatant was removed and the microspheres were centrifuged at 1150 × *g* for 10 min, collected and washed in 100 mL distilled water. Finally resulting AM2 were stored at 4 °C in Petri dishes.

Each type of microsphere without Nys (unloaded microspheres) was similarly elaborated for comparative studies.

2.3. Physicochemical characterization

2.3.1. Particle size and morphological analysis

Laser diffractometry (LD) was performed using the LS 13 320 laser particle counter (Beckman Coulter Inc., Brea, CA, USA), 0.02–2000 μm size range, yielding the volume distribution of the unloaded and Nys-loaded microspheres. Characterization parameters were the diameters $LD_{0.1}$, $LD_{0.5}$ and $LD_{0.9}$. A diameter $LD_{0.5}$ of 1 μm means that 50% of all particles possess a diameter of 1 μm or less. Equally, mean diameter over the volume distribution ($LD_{4.3}$) and polydispersity expressed as the Span factor were also calculated [Span = $LD_{0.9} - LD_{0.1}/LD_{0.5}$]. All measurements were repeated 24 h after preparation (t_0) and after 1–3 months stored at 4 °C. The shape and surface morphology were also examined by scanning electron microscopy (SEM). The samples were mounted on metal stubs, using double sided adhesive tape, gold coated under vacuum and then examined using a S510 electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

2.3.2. Zeta potential

Zeta potential measurements of diluted microspheres in PBS at 25 °C with a detection angle of 90° with a ZetaSizer® 2000 (Malvern Instruments Ltd., Malvern, UK). Samples were slowly injected through the cell, checking that all air bubbles under the sample port were removed. Nine measurements were done on the same sample at pH 5.5 and 7.5 after elaboration and each storage period at 4 °C.

2.3.3. Determination of percentage yield, loading content and encapsulation efficiency

The percentage yield (PY) was calculated as [PY = (Mass of microspheres/Mass of drug fed + Mass of polymer fed) × 100].

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