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IMPROVED BIOPHARMACEUTICAL PERFORMANCE AND ANTIFUNGAL EFFICACY OF MUCOADHESIVE FILMS WITH NYSTATIN

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ABSTRACT

The aim of this study was to improve the biopharmaceutical performance bioadhesive films containing nystatin in terms of swelling ratio, disintegration rate, drug release, adhesion and to evaluate the influence of the excipients on antifungal action. The composition of the films was modified by incorporating Poloxamer 407 (POL407), polymer selected by its known surfactant properties and the ability to limit the swelling exhibited by carbomer. Mixing time and proportion of POL407 were critical parameters affecting the swelling and disintegration of the films. The most satisfactory formulation was film A3 containing carbomer 974P (CB974P), sodium carboxymethylcellulose (NaCMC), POL407 and nystatin (Nys), in the respective proportions (0.25:0.25:0.3:0.2). This film exhibited appropriate swelling ratio and muchoadhesive properties, rapid drug release extended over time and improved antifungal activity as compared with previously developed films.

Keywords: **bioadhesive films, bioassay, *Candida albicans*, Carbomer 974, nystatin, Poloxamer 407**

INTRODUCTION

Oral candidiasis is a common fungal infection in patients with an impaired immune system, such as those undergoing chemotherapy for cancer and patients with AIDS. The incidence is also increased due to factors such as the widespread use of dentures and multiple antibiotic therapies. The majority of infections are due to *Candida albicans* although other species are increasingly isolated. The treatment requires long-term administration of antifungal agents and the options are often limited by toxicity and the emergence of resistance. This has prompted the development of new antifungal agents, as well as the rediscovery and reengineering of known agents [1, 2].

Nys is widely used for treatment of fungal infections caused by the *Candida* species, among other indications. The molecular structure of Nys reveals formulation challenges by being both amphiphilic and amphoteric, which contributes to poor solubility in aqueous media. Nys monomers selectively interact with ergosterol, component of fungal cell membranes, while aggregates of Nys formed due to poor aqueous solubility are non-selective. Consequently, delivering Nys in an unaggregated form may improve its therapeutic index [3].

The development of mucoadhesive drug delivery systems led to sustained release of antifungals while remaining attached to the oral mucosa, reaching drug concentrations in the salivary fluid above the minimum inhibitory concentration for an extended period of time. Adhesive films are preferred between the buccal delivery systems as they are able to cover a large mucosal surface for both drug delivery and physical protection [4].

In previous works we have developed mucoadhesive films with Nys using hydrophilic polymers CB934P and NaCMC, a plasticizer (polyethylenglicol 400, (PEG 400)) and a surfactant (ascorbyl palmitate, (ASC16)). The last two ingredients contributed to improving flexibility and strength of the films and to increase the drug release rate [5, 6]. However, these films exhibited high swelling ratio in water, behavior that could affect patient comfort. That shortcoming is attributed to the presence of CB934P, the main limiting factor for use as matrix for transmucosal drug delivery system.

The aim of this study was to overcome the drawbacks exhibited by the early developed films and try to improve the in vitro biopharmaceutical performance in terms of swelling ratio, dissolution rate and drug release. For these purpose, new films containing Nys were designed by modifying the composition of the films: we changed

CB934P for Carbomer 974P and incorporating POL407, surface-active polymer able to prevent swelling of CB[7]. Moreover, the influence of excipients on the Nys in vitro activity was assessed in new and previously developed films.

MATERIALS AND METHODS

Chemicals.

USP Nys and PEG400 (Parafarm, Buenos Aires, Argentina); NaCMC viscosity grade: 500–2500 mPa s (Fluka AG, Buchs SG, Switzerland); ASC16 (Sigma, Milwaukee, USA). CB934P and Carbomer 974P (CB974P) was kindly provided by BF-Goodrich (Cleveland, OH, USA) and POL407 was kindly provided BASF (Ludwigshafen, Germany).

Formulation and films preparation.

Polymeric films (Serie A) were prepared by casting method using water as solvent, according to the methodology previously used in our laboratory [5] with slight modifications. Briefly, a mixture of CB974P and NaCMC (1:1) was dispersed in water at 60-65 °C and stirred under vacuum. Nys (300 µm particle size) and POL407 were dispersed in water at 60–65 °C and added into CB974P/NaCMC aqueous dispersion under gentle stirring. After the product reached room temperature, the film-forming gels were poured into molds specially designed for thin films (2 mm thickness) and dried in an oven (60-65°C) for 24 h until constant weight.

The films previously reported[5] were designated as Series B and C in this work and used in comparative studies.

The composition of the films is shown in Table 1. In addition, the films were prepared with the same composition as the films A1, B1 and C1 but not containing Nys. These films were designated as film A, B and C, respectively, and were used as controls in the bioassays.

Table 1: composition of films assayed

ingredients	A1	A2	A3	A4	A5	A6	B1	B2	B3	C1
Nys (mg)	200	200	200	200	200	200	200	200	200	200
CB974P (mg)	250	250	250	250	250	250	--	--	--	--
CB934P (mg)	--	--	--	--	--	--	250	250	250	250
NaCMC (mg)	250	250	250	250	250	250	250	250	250	250
POL407 (mg)	100	200	300	400	500	600	--	--	--	--
PEG400 (ml)	--	--	--	--	--	--	1	1	1	--
Asc16 (mg)	--	--	--	--	--	--	--	100	200	--

Swelling ratio and disintegration rate measurement.

The determination of swelling ratio of the films serie A was carried out by adapting the method described by Chun et al. [7]. At predetermined time intervals (from 5 to 120 min), hydrated samples were removed and weighted after blotting the surface water with a filter paper. The swelling ratio was calculated using Ws/Wp equation, where Ws and Wp are wet and dry weights of the films, respectively.

In order to determine the disintegration rate (calculated by (Wp - Ws)/Wp equation), the hydrated films obtained as described above were dried at 60°C until constant weight.

On the other hand, the influence of time mixing time for hydrophilic polymers (CB974P; NaCMC) with POL407 and the POL407:CB974P/NaCMC ratio on the swelling behavior of the films was determined.

In vitro mucoadhesion measurement.

Mucoadhesion was determined as the force required to pull a film out of a mucin gel layer (30%, w/w) using an adapted Jolly Balance as previously reported [6]. The assay was performed for five dosage units (10.8 mm diameter) and then averaged.

In vitro drug release measurement.

Nys release experiments were carried out using a device that simulates oral clearance as described in Spadaro et al. [8]. Briefly, 10 ml of distilled water (37 °C) were placed in a vessel and stirred at 100 rpm. The films (13 mm diameter) were fixed with a metal mesh, and then placed at the bottom of the vessel. Samples were withdrawn using a peristaltic pump Masterflex Dual-Channel Variable-Speed Compact C/L® Pumps (Barrington USA) (1 ml/min). Fractions were analyzed at 306 nm in UV–Vis spectrophotometer (Shimadzu UV 160-A, Shimadzu Corporation, Kyoto, Japan).

Microbiological assay.

An agar diffusion assay was implemented by adapting the methodology described in the Pharmacopoeia guidelines for microbiological testing of antimicrobials [9] in order to evaluate the antifungal activity of films and to determine the effect of the excipients. *C.albicans* ATCC 10231 was used as test organism. Turbidity (transmittance) of a yeasts suspension in sodium chloride 0.9% was adjusted to 25±2 % at 580 nm using a spectrophotometer Shimadzu UV-Visible A-160. Aliquots of 2 ml were added to 25 ml of Nº 19 antibiotic assay medium (Britania, Buenos Aires, Argentina) at 48±2 ºC and poured into glass Petri dishes (90 mm in diameter). Six discs were placed on the solidified surface of the seeded agar following a 3 x 3 design; two different films were evaluated in each plate comparing three dose levels of each film. In this way, discs of 4.8, 8 and 13 mm of each film were

cut with a punch. Several combinations of films were designed. Zone diameters of growth inhibition were measured using a gauge after 24 h incubation at 35ºC. Each comparative assay was performed at least on quadruplicate. Each assay was statistically calculated by the linear parallel model and by means of regression analysis and verified using analysis of variance by adapting guidelines from Argentinean Pharmacopoeia [9].

RESULTS

Swelling rate of the film was influenced by mixing time (Figure 1) and proportion of POL407 in the mixture with hydrophilic polymers (Figure 2).

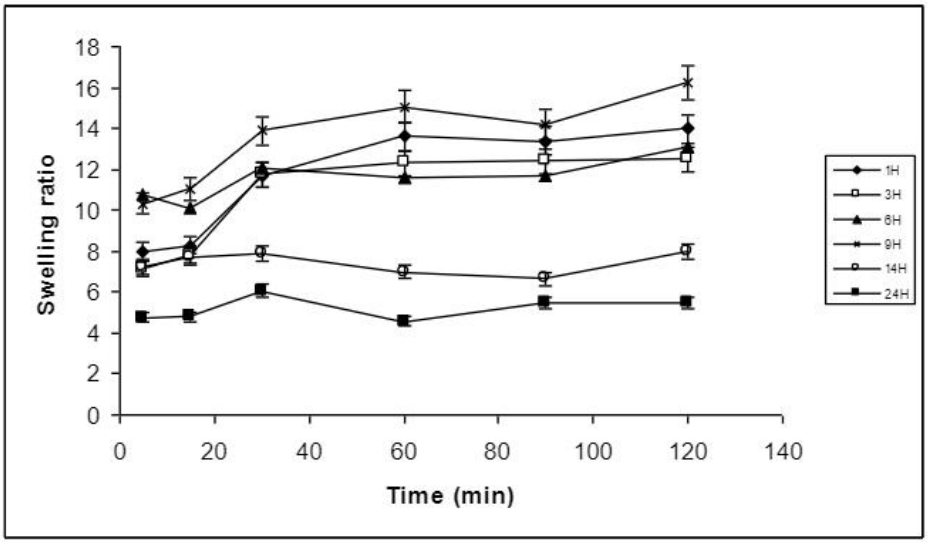

Figure 1. Effect of mixing time between POL407 and hydrophilic polymers mixture on water uptake of the films.

Lowest swelling ratio was exhibited by films prepared by mixing for a period ≥ 14 h (Fig. 1). Small variations in swelling rate were observed by prolonging the mixing time up to 24 h. Therefore, 14 h was selected as the optimal mixing time for obtaining the films in order to decreasing the total preparation time.

As shown in Figure 2, the highest swelling ratio was exhibited by the film containing the lowest proportion of POL407 in the mixture with hydrophilic polymers (Table 1, film A1) with a high swelling ratio in the first 5 min and rapidly reaching a ratio of about 70-fold.

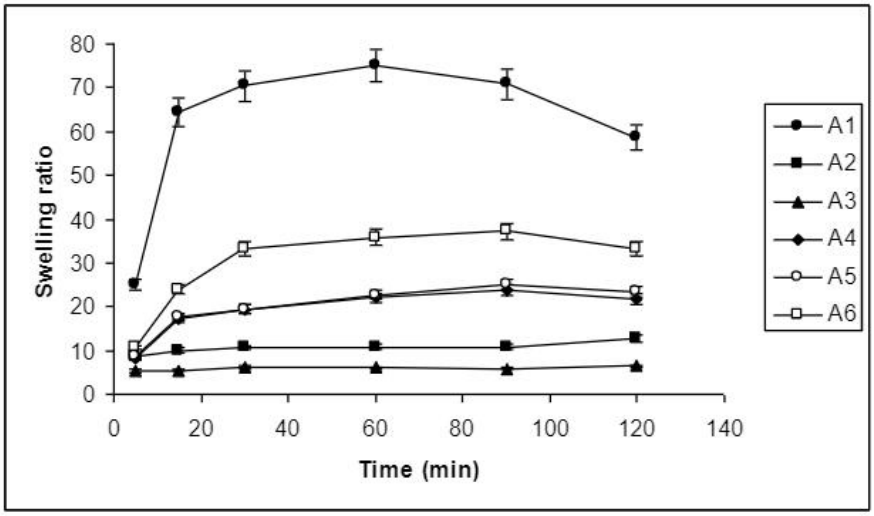

Figure 2. Swelling ratio of polymeric films according to the mixing ratio

This parameter decreases as the proportion of POL407 in the mixture increases until a critical condition (film A3) after which the swelling rate increases again (films A4 - A6). As expected, the proportions of POL407 to CB974P/NaCMC mixture in serie A films influenced the disintegration rate (Figure 3) with the same trend as exhibited in the water uptake assay (Fig. 2).

All films of serie A hold their shape during the 2 hours of assay. However, the best performance was exhibited by the film A3.

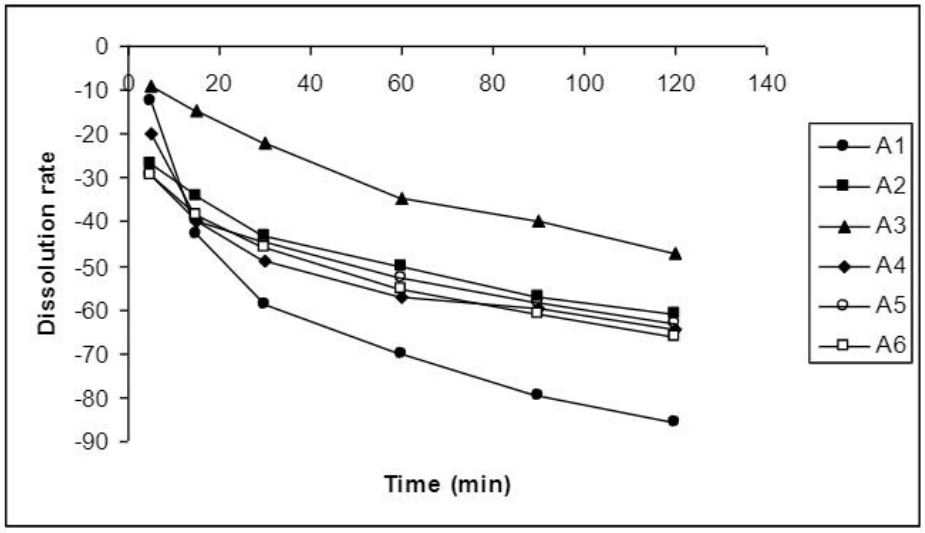

Figure 3. Influence of POL407 concentration on dissolution rate of a set of serie A films.

Based on these results, A3 film was selected for further study.

Figure 4 show the swelling behavior exhibited by film A3 compared to previously developed films B3 and C1.

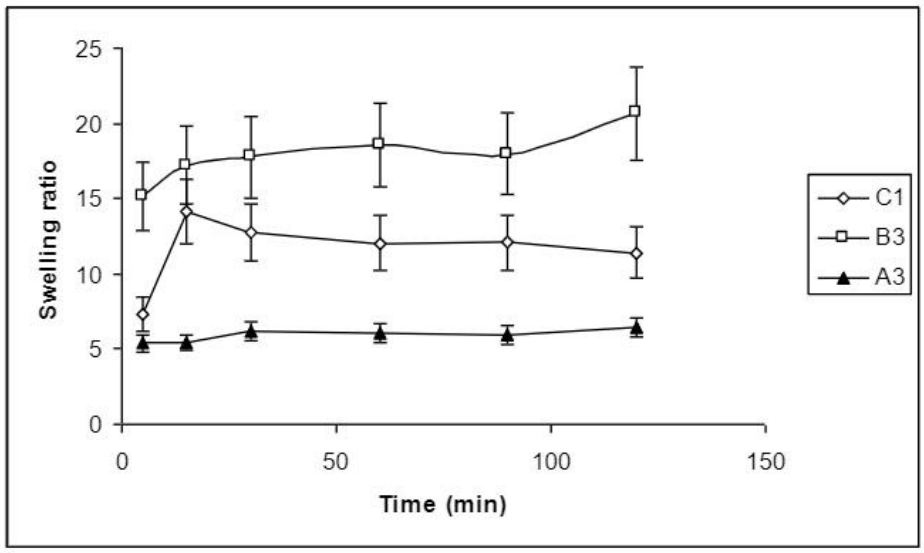

Figure 4. Comparative swelling behavior of selected films. Each experiment was performed on triplicate.

Film A3 showed the lowest swelling ratio (only 5-fold) while the film B3 and C1 reached a swelling ratio of almost 20- and 15-fold, respectively, after 15 minutes. In addition, film C1 began to disintegrate after 30 minutes of hydration.

Figure 5 shows a comparison of the disintegration rate profiles of film A3 and films without POL407 (B3 and C1). The films B3 and C1 showed high disintegration rate leading to the total disintegration in the period evaluated, while film A3 exhibited less than 50% after 2 h testing.

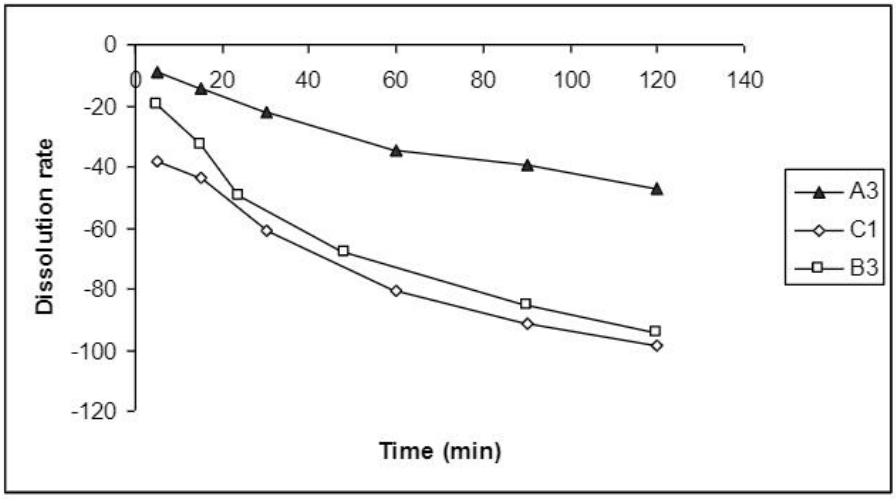

Figure 5. Dissolution rate of selected films in distilled water. Each experiment was performed on triplicate.

The in vitro mucoadhesion of film A3 did not differ significantly from that exhibited by the previously developed films B3 and C1 (Figure 6), indicating that POL407 not interfere with this property attributed to the hydrophilic polymers.

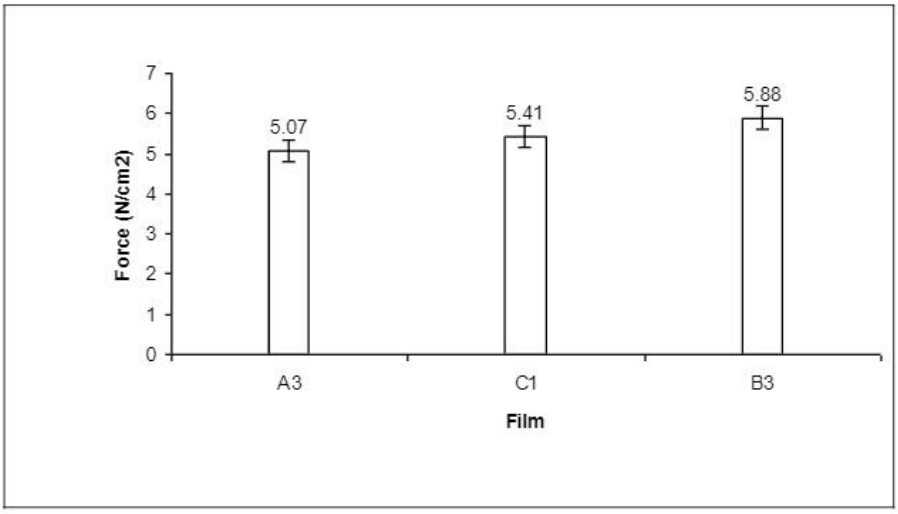

Figure 6. Comparative bioadhesive force of selected polymeric films.

Nys release profiles from selected films are shown in Figure 7. The percentage of drug released after 6 h were 71.8 ± 3.4, 60.4 ± 4.2 and 21.1 ± 1.1 for films A3, B3 and C1, respectively. The improved drug release exhibited by films A3 and B3 with a rapid initial release is attributed to the surfactant included in the formulation. However, film B3 reached a plateau after 5 h while drug release from film A3 was prolonged, without reaching a plateau throughout the period studied.

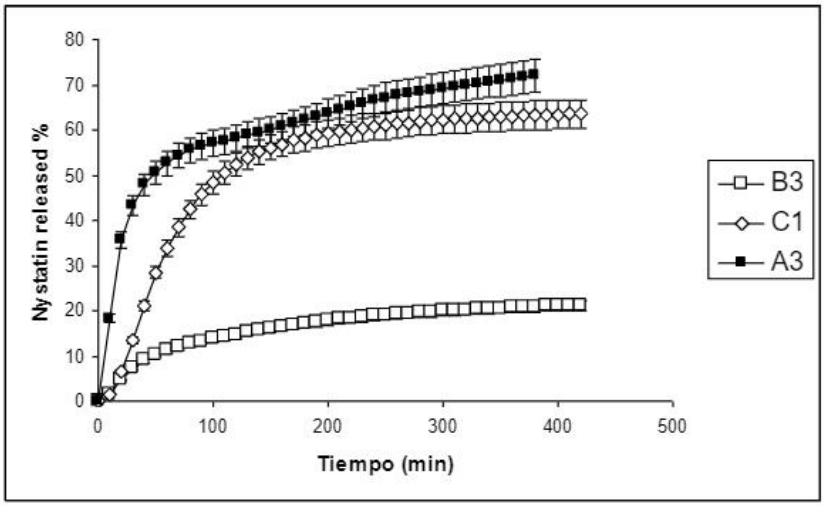

Figure 7. Release profiles of Nys from films in distilled water at 37 °C. Each experiment was performed on triplicate.

The bioassay results were represented by plotting log concentration versus Nys inhibition zone diameter (mm) as shown in Figure 8 and statistically calculated. Polymeric films without Nys used as control (A, B and C) did not exhibit growth inhibition zone (mm) whereas, as expected, all films containing nystatin exhibited inhibition zones which were proportional to drug concentration.

Figure 8 presents comparative results of the antifungal activity exhibited by selected films. Film A3 exhibited higher antifungal potency against *C. albicans* than the film B3. The activity of B3 represents only 45% of that of A3.

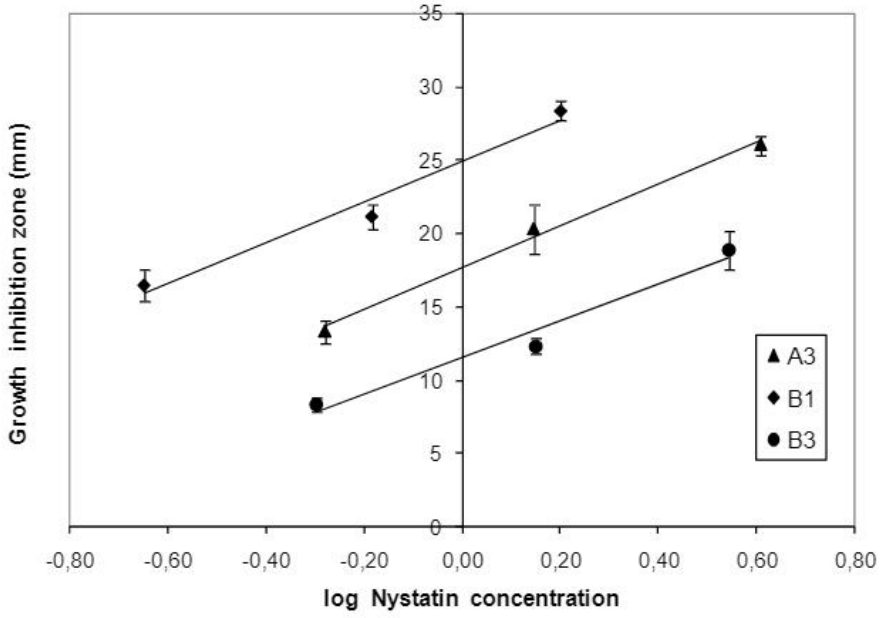

Figure 8. Comparison of inhibitory activities of selected films against *Candida albicans* ATCC 10231 by agar diffusion bioassay

On the other hand, film B1 exhibited the greatest potency antifungal among all films evaluated in this study. Additional tests were conducted comparing the performance of all films of serie B in order to explain the reduced activity exhibited by film B3. The results indicated that the potency of B2 was equivalent to 38% of that exhibited by film B1 and those film B3 represents only 15% of B1.

In summary, the films can be ordered according to the *in vitro* potency as follows: B1> A3> B2> B3> C1.

DISCUSSION.

Poloxamers, also known as Pluronics®, are safe and commercially available nonionic polyoxyethylene-polyoxypropylene (PEO-

PPO) copolymers, used in pharmaceutical formulations mainly as emulsifying or solubilizing agents [10]. Later was used to reduce the aqueous solubility of polyacrylic acid preventing the swelling [11]. In this work POL407 was included into films serie A containing Nys in order to improve the performance of films previously developed.

The strong influence of mixing time of the polymers mixture and the proportion of POL407 in POL407:CB974P/NaCMC mixture on swelling behavior of the films showed in Figures 1 and 2, respectively, can be explained as the result of hydrophilic and hydrophobic interactions between POL407 and CB974P after water intake, which may form stable complexes [12]. Briefly, polypropilene oxide groups (PPO) of POL407 interact with the aliphatic side chains of polyacrylic acids of Carbomer and hydrophilic groups of POL407 (ethylene oxide, PEO) are able to interact with [-COOH] groups of Carbomer through hydrogen bondings. Such interactions are dependent on the poloxamer/polyacrilic acid ratio. The excess PPO and PEO oxygen atoms saturated the poly-acrylic acid molecules causing less cross-linkages between polymers and hence the formation of smaller complexes. Continuing the addition of POL407, the complexes formed become very small and eventually dissolve causing increased swelling of the film again.

The aqueous solubility of the drug and adhesive force are two important requirements in developing of bioadhesive systems. It is well known that surfactants can facilitate the dispersion of hydrophobic compounds in aqueous media (i.e. Nys) by increasing the wetability of these substances. In addition, some properties of the films such as mechanical strength, homogeneity and drug release can be modified according to the process by which the drug is incorporated in the system. In this way, the initial rapid release showed by both A3 and B3 films is attributed to the surfactant properties of POL407 and ASC16, respectively, included in the formulations. The difference in drug release profiles observed later could be explained by lower dissolution of film A3 due to interactions between the poper proportions of POL407 and CB974P. Consequently, the film retains shape and modulates the drug release while it remains attached to the oral mucosa. By contrast, the large swelling exhibited by film B3 leads to disintegration of the film and diffusion of the drug.

The bioassay results indicate that the polymers, surfactants and plasticizers used in the films do not exhibit antifungal activity. The drug concentration-dependant activity exhibited by Nys-containing films indicates that the drug loaded on the developed films is available to exert the action against yeast.

However, differences were observed between some films depending on their composition.

Increase in vitro efficacy observed with film A3 as compared to film B3 could be attributed to the antioxidant properties of ASC16 [13] . Several reports have described an additional mechanism of action for Amphotericin B, polyene macrolide structurally and mechanistically similar to Nys, through oxidative damage induced by reactive oxygen species [14-16]. Consequently, the reduction in the antifungal activity exhibited by film B3 may be attributed to the antagonism between ASC16 and Nys. This hypothesis is confirmed by the results indicating an inverse relationship between in vitro antifungal efficacy and ASC16 concentration in the films of the series B. Although these results of in vitro activity against *C.albicans* shows that the film B1exhibited the highest antifungal activity, but its biopharmaceutical performance previously reported was not the most favorable (less than 20% drug released after 8 h) [6] .

In summary, the most satisfactory formulation is film A3, which allows overcoming the deficiencies above described. The inclusion of POL407 in adequate proportion provided the required flexibility by these mucoadhesive systems, reduced swelling, and consequently lower disintegration of the film, and the release behavior should be appropriate to provide an attack dose after administration of the film which is prolonged in the time. In addition, this film exhibited greater antifungal activity than films containing ASC16. So, film A3 is the most suitable formulation and will be object of further in vivo studies.

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