Journal Pre-proof

Development and optimization of a new tioconazole vaginal mucoadhesive film using an experimental design strategy. Physicochemical and biological characterization

Natalia L. Calvo, Guillermo Tejada, Laura A. Svetaz, Ariel D. Quirog, Vera A. Alvarez, María C. Lamas, Darío Leonardi

PII: S0731-7085(21)00414-3

DOI: https://doi.org/10.1016/j.jpba.2021.114303

Reference: PBA 114303

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received Date: 13 May 2021

Revised Date: 25 July 2021

Accepted Date: 30 July 2021

Please cite this article as: Calvo NL, Tejada G, Svetaz LA, Quirog AD, Alvarez VA, Lamas MC, Leonardi D, Development and optimization of a new tioconazole vaginal mucoadhesive film using an experimental design strategy. Physicochemical and biological characterization, *Journal of Pharmaceutical and Biomedical Analysis* (2021), doi: https://doi.org/10.1016/j.jpba.2021.114303

Development and optimization of a new tioconazole vaginal mucoadhesive film using an experimental design strategy. Physicochemical and biological characterization

Natalia L. Calvo;[a,b,*] Guillermo Tejada;[c] Laura A. Svetaz[d]; Ariel D. Quiroga;[e] Vera A. Alvarez;[f] María C. Lamas;[a,c] Darío Leonardi[a,c,*]

[a]*Instituto de Química Rosario (IQUIR, CONICET-UNR), Suipacha 570, Rosario (S2002LRK), Argentina.*

[b]*Área Análisis de Medicamentos, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario (S2002LRK), Argentina.*

[c]*Área Técnica Farmacéutica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario (S2002LRK), Argentina*

[d]*Área Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario (S2002LRK), Argentina.*

[e]*Instituto de Fisiología Experimental (IFISE, CONICET-UNR), Suipacha 570, Rosario (S2002LRL), Argentina.*

[f]*Instituto de Investigaciones en Ciencia y Tecnología de Materiales (INTEMA, CONICET-UNMdP), Colón 10890, Mar del Plata (7600), Argentina.*

Corresponding authors: Instituto de Química Rosario (IQUIR, CONICET-UNR), Suipacha 570, Rosario S2002LRK, Argentina. Phone: +54-341-4804592; Ext. 200.
E-mail addresses: calvo@iquir-conicet.gov.ar (NLC), leonardi@iquir-conicet.gov.ar (DL).

Highlights

- The technological influence of excipients on characteristics of films was assessed.
- The experimental designs allowed optimize a TCZ vaginal polymeric film formulation.
- The optimal CH:HPMC film eradicated a *Candida* culture at 3 min assay.

Abstract

A new tioconazole (TCZ) mucoadhesive film, based on a biodegradable chitosan/ hydroxypropyl methylcellulose (CH/HPMC) blend, was developed for treatment of vaginal candidiasis. The formulation was optimized through an I-optimal design (minimizing the integral of the prediction variance across the factor space), where the impact of the proportion of the ingredients and processing variables on the quality of the final product was evaluated. Both, the thickness of the film and the swelling index, which affect patients' comfort and compliance, were considered. Mechanical testing, such as load at break, elongation at break, and mucoadhesive strength were also included as dependent variables.

The optimal mucoadhesive film formulation, which should be obtained at a drying temperature of 30 ºC, was found to include the combination of CH and HPMC (forming polymers) at 0.25:0.75 ratio, a mixture of polyethylene glycol 400 and propylene glycol as plasticizers (0.07:0.93, 5% *w/w*), and TCZ loaded at 15% *w/w*.

The optimal preparation was subjected to exhaustive characterization studies, which revealed that the drug was entrapped in the polymeric matrix in an amorphous state and that the film exhibited a smooth and uniform surface, demonstrating excellent component compatibility. *In vitro* tests showed that the formulation has an excellent time to kill value (3 min) and lacks cytotoxicity, suggesting that it should be highly effective and safe.

Keywords: chitosan, experimental design optimization, hydroxypropyl methylcellulose, tioconazole, vaginal film.

1. Introduction

Yeasts of the genus *Candida*, mainly *Candida albicans*, are commensal microorganisms in the gastrointestinal and genitourinary tracts, as well as in the oral and conjunctival mucosae. However, this yeast may cause infections when the host becomes weakened or immunocompromised. Although these infections are often superficial, affecting the skin or mucous membranes, they can also disseminate through the bloodstream, reach internal organs, and endanger patients' life [1].

Vaginal candidiasis is a frequent opportunistic mucosal infection caused by *C. albicans*; this condition, which is one of the most common causes of vaginitis, can be treated with tioconazole (TCZ), a broad-spectrum azole antifungal agent [2]. An ideal pharmaceutical dosage form for local (vaginal) delivery of this drug needs to remain in the infection site as long as possible and release the active compound according to the needs of the treatment while avoiding to produce patient's annoyance.

Unfortunately, conventional vaginal formulations such as creams, gels, pessaries and foams have limited effectivity, due to their poor retention by the self-cleansing action of the vaginal tract [3]; therefore, their use is discouraged. On the other hand, vaginal tablets and ovules have shown acceptable retention abilities; however, they are too rigid and may cause patients' discomfort, and affect their compliance.

Conversely, mucoadhesive films fulfil the requirements for vaginal drug delivery; these dosage forms are suitable to achieve effective drug release for extended periods of time [4,5] and may greatly improve patients' compliance, because they are more flexible than ovules and tablets.

These films have been developed with the aid of several biocompatible polymers, including chitosan (CH) and hydroxypropyl methylcellulose (HPMC). The former is a cationic and physiologically inert natural polymer derivative, with extensive pharmaceutical

applications resulting from its attractive properties of hydrophilicity, biocompatibility and biodegradability [6]. Besides, HPMC is a non-ionic semi-synthetic cellulose derivative, and the most important hydrophilic carrier material used for the preparation of controlled drug delivery systems [7].

Films based on different CH and HPMC mixtures have been prepared using various polymers concentrations, plasticizers and methodologies [8,9]. It was found that the ratio of the polymers have impact on the physicochemical and/or functional properties of the final drug product, the magnitude of which could be product-specific [10].

Current strategies toward the rational design of pharmaceutical drug products require the systematic acquisition of a clear understanding of the impact of formulation variables and their interaction on product quality. This is one of the main goals of the modern "Quality by Design" paradigm [11]; the objective can be reached through an experimental design approach, which entails the assessment of the combined effects of product design, manufacturing process parameters and raw materials quality on product suitability for the intended use, including its quality, safety, and performance [12].

Previously, CH/HPMC-based tioconazole films were prepared and characterized using a trial and error approach, demonstrating that these films may constitute a convenient alternative to currently marketed vaginal ovules for the treatment of vaginal candidiasis [8].

Considering our interest in CH/HPMC tioconazole films for vaginal use, and aiming to fulfill the objectives of the Quality by Design philosophy, herein we disclose the application of a mixture process experimental design strategy coupled to a surface response methodological approach to the development of a new and improved mucoadhesive tioconazole film formulation, with suitable properties to treat vaginal candidiasis. An exhaustive characterization of the optimized film formulation is also reported.

2. Materials and methods

2.1. Chemicals

Pharmaceutical grade TCZ (BP 2002) was acquired from Saporiti (Buenos Aires, Argentina), CH (MW ~ 230 KDa; 80.6% of *N*-deacetylation) was supplied by Aldrich Chemical Co. (Milwaukee, WI, USA), HPMC (MW ~250 kDa, methoxyl content: 19-24%, hydroxypropyl content; 7-12%) was purchased from Eigenman & Veronelli (Milan, Italy), whereas polyethyleneglycol 400 (PEG) and propylene glycol (PPG) were acquired from Anedra (Buenos Aires, Argentina). Double distilled water was used during the experiments. The simulated vaginal fluid (SVF) was prepared according to the literature [8]. All other chemicals used were of analytical grade.

2.2. Preparation of the films

The films were obtained by the solvent evaporation method. A CH solution (1% *w/v*) was prepared by dispersing the polymer in a lactic acid solution (1% *v/v*). An aqueous HPMC solution (1% *w/v*) was prepared by suspending an accurately weighed amount of HPMC in water, stirring the system overnight and then filtering through Miracloth® (Calbiochem-Novabiochem Corp., San Diego, CA, USA).

In each case, a known amount of TCZ was added to a mixture of PEG and PPG as plasticizers and the dispersion was combined with the CH solution. The resulting solution was dripped over the HPMC solution at 40 °C under magnetic stirring to avoid precipitation. Finally, the mixture was further stirred at 200 rpm for 1 h, cast on a Petri dish (diameter = 10 cm) and dried (30 °C or 40 °C) in an oven. The amounts of the plasticizers and TCZ are informed relative to the forming polymers dry weight. Blank films, devoid of TCZ and the plasticizers were also prepared.

2.3. Characterization of the films

2.3.1. Thickness and folding endurance

Thickness measurements were carried out with a digital micrometer (Schwyz, China). Six determinations were performed for each film sample, one in the center and five equally spaced at the edges. To test the folding endurance of the films, they were folded manually at the same place for 300 times. Compliance was achieved when the films failed to break at the end of the test [13].

2.3.2. Content uniformity

The near infrared (NIR) spectra were determined at room temperature with a NIRS DS2500 spectrophotometer (FOSS, Hillerod, Denmark), in the spectral range 400-2500 nm, with the films placed in a circular quartz cell for solids. All samples were measured in triplicate.

2.3.3. Load at break (LB) and elongation at break (EB)

The mechanical strength of the films was evaluated using an EMIC 2350 universal testing machine (Instron, Norwood, MA, USA) with a 50 N load cell. Each film was conditioned (24 h at 25 °C and 80% RH) and cut into strips (7 × 60 mm). For the test, each strip was held between two grips at a distance of 30 mm and stretched at a crosshead speed of 5.0 mm/min. The LB value was obtained as the peak load at failure from the corresponding stress/strain curve. The EB value was calculated according to Eq. [1], as the percentage of relative film length change at failure, considering the original distance between the grips (Lo) and the length at failure (Lf). Three replicate measurements were performed for each mechanical test.

$$EB = 100 \times (Lf - Lo)/Lo \qquad \text{Eq. [1]}$$

2.3.4. Mucoadhesive strength (MS)

The mucoadhesive strength of the films was evaluated *in vitro* using an EMIC 2350

universal testing machine (Instron, Norwood, MA, USA) with a 50 N load cell, by measuring the force required to detach a standardized disk (diameter = 2.5 cm) of the formulation (fixed to the bottom of a cylindrical probe with double-sided adhesive tape) from a disk of porcine vaginal mucosa. In the test, the mucosal disk was pulled upwards at a constant speed of 5.0 mm/min, and the MS was determined from the resulting force/time plot. The tests were carried out in triplicate.

The vaginal mucosal specimens were obtained from freshly slaughtered pigs at the Matievich slaughterhouse (Carcarañá, Argentina); they were fixed to the upper end of the testing machine probe. Prior to testing, the mucosae were hydrated with SVF (0.5 mL) for 5 min and then placed in contact with the film disk under test for 1 min.

2.3.5. Swelling index (SI)

Swelling measurements were performed by immersing an accurately weighed portion of the films (~ 50 mg) in SVF (0.5 mL) at 37 ºC [8]. At a predetermined time (90 min), each film was carefully removed, the excess of adhering moisture was gently blotted off and the film was weighed. The SI was calculated from the weights of the dried (Wo) and swollen (Ws) films, according to Eq. [2]. The test was carried out in triplicate.

$$SI = (Ws - Wo)/Wo \qquad \text{Eq. [2]}$$

2.3.6. Cytotoxicity of the films

Standardized samples (diameter = 5 mm) were obtained from the films with a biopsy punch and placed in the sidewalls of a 12-well plate. Film extracts were prepared from the films by addition of Dulbecco's modified Eagle's medium (DMEM, 1200 µL) to each well, and incubation of the plate at 37 ºC for 12-16 h.

Human HCC cells Huh7 were obtained from the JCRB Cell Bank (Tokyo, Japan) and kept at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2, in DMEM supplemented

with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

For the MTT assay, the cells were seeded in 96-well plates at a density of 3500 cells/well. After 24 h of attachment, they were treated with the film extracts (200 μL). Control (untreated) cells were incubated without TCZ. After 24 h, MTT was added to the culture medium; then, the cells were lysed by addition of DMSO and the absorbance of the metabolites produced by the viable cells was detected at 540 nm (with a 650 nm reference filter) in a DTX 880 multimode detector (Beckman Coulter Inc., Fullerton, CA, USA). The cell viability (CV) results are expressed as percentage of the absorbance ratio of the treated cells (At) relative to the control cells (Ao), according to Eq. [3].

$$CV = 100 \times (At/Ao) \quad \text{Eq. [3]}$$

2.3.7. Thermogravimetric analysis

Thermogravimetric tests were carried out with a Q500 thermal analyzer (TA Instruments, Hüllhorst, Germany). Samples (around 8 mg) were heated at a constant rate of 10 °C/min from room temperature up to 600 °C, under a nitrogen flow (30 mL/min) in order to avoid thermo oxidative reactions.

2.3.8. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy

The spectra were acquired at 30 °C in a Prestige 21 spectrophotometer (Shimadzu Corp., Kyoto, Japan), fitted with a diamond-based ATR accessory (GladiATR, Pike Technologies, Madison, USA) attached to a Pike temperature control unit. An average of 20 scans was used at a 4 cm^{-1} resolution, over a wavenumber range of 4000-600 cm^{-1}. The pressure exerted during the ATR measurements was standardized. Each sample was scanned three times and the spectra were averaged.

2.3.9. X-ray diffraction analysis

The X-ray diffractograms were obtained with a X'Pert PRO diffractometer (PAN

analytical, Eindhoven, Netherlands) equipped with a monochromatic Cu Kα radiation source (λ = 1.5406 Å) operating at a voltage of 40 kV and a current of 40 mA, at a scanning rate of 0.02 º /sec. The scanning region of the samples was in a 2θ range between 5 and 40 °.

2.3.10. Scanning electron microscopy

The SEM determinations were performed in an AMR 1000 scanning electron microscope (Leitz, Wetzlar, Germany). The samples were mounted on an aluminum support using conductive double-sided adhesive tape, and coated with a fine gold layer for 15 min at 70–80 mTorr, in order to make them conductive before obtaining the SEM micrographs. The samples were examined using an accelerating voltage of 20 kV and magnifications of 1000× and 5000×.

2.3.11. Antifungal activity test

C. albicans ATCC 10231 was cultured in Sabouraud dextrose agar (SDA) for 24 h before testing. The inoculum was prepared by suspending five colonies in sterile distilled water, shaking on a vortex mixer for 15 s and adjusting to 1–5 × 10^7 colony forming units (CFU)/mL [14]. The test samples, including TCZ bulk drug (0.34 mg), the optimum formulation (5-mm disk containing 0.34 mg TCZ) and a blank film (5-mm blank disk), were placed in an aliquot of the inoculum (5 mL).

The suspensions were mixed for 20 s with a vortex mixer; samples (0.05 mL) were taken at pre-determined times, and serially diluted before spreading onto SDA. The plates were incubated for 24 h and the number of viable colonies was evaluated. The time to kill curves were constructed by plotting the CFU/mL surviving at each timepoint as a function of time. The experiments were conducted in triplicate and the mean number of survivors was determined each time.

2.3.12. Graphics and statistical analysis

The graphics and statistical analyses were executed with Origin v. 8.5 (OriginLab Co., Northampton, USA) and DesignExpert v.11.0 (State-Ease Inc., Minneapolis, USA). Differences at $p < 0.05$ were considered significant. A mixture process experimental design strategy coupled to a surface response methodological approach was carried out using an I-optimal design (which chooses runs that minimize the integral of the prediction variance across the factor space). A total of 26 experiments were performed comprising seven parameters, two mixtures (with two mutually dependent components each), two numerical factors, and one categorical factor. The detailed experimental design was described in section 3.4.1.

3. Results and discussion

3.1. Preparation of the films

An array of mucoadhesive films based on CH and HPMC (Mixture 1) with different polymer proportions was prepared (Table 1), covering a conveniently wide experimental domain (CH:HPMC from 1.00:0.00 to 0.25:0.75) for their compositional optimization. Films based on 100% HPMC have been developed previously [15]; however, they presented a high swelling index and disintegrated within 60 minutes. Therefore, they were considered unsuitable for the required long time drug release purposes; hence, were excluded from this study.

The load of TCZ in the films ranged between 1.0 and 15% *w/w*, in agreement with that found in the commercial formulations of the drug. Aiming to obtain easy to handle films, a mixture of PPG and PEG (Mixture 2) was added as internal and external plasticizers, respectively, and evaluated in the 5-40% *w/w* concentration range [8]. Additionally, three blank films (B1-B3) containing solely the polymer forming agents were formulated at different CH:HPMC ratios (0.25:0.75, 1.00:0.00 and 0:1.00, respectively).

Table 1. Composition and properties of the different TCZ vaginal films.

Run N°	Std N°	Batch code	Factors[a]							Responses[b]					
			Mixture 1		Mixture 2		Process								
			CH	HPMC	PEG	PPG	P (%*w*/*w*)	TCZ (%*w*/*w*)	T (ºC)	EB (%)	LB (N)	MS (N)	TH (mm)	SI	CV (%)
1	9	F1	0.25	0.75	1.00	0.00	5.00	15.00	40	22.8	9.9	3.8	0.197	7.6	91.9
2	20	F2	0.53	0.47	0.50	0.50	39.83	7.09	30	48.8	9.9	4.0	0.249	8.3	79.0
3	21	F3*	0.53	0.47	0.50	0.50	39.83	7.09	30	54.3	8.0	4.1	0.225	7.7	74.1
4	17	F4	0.48	0.53	0.57	0.43	5.00	9.80	30	16.3	13.7	4.0	0.176	9.9	67.8
5	2	F5*	0.48	0.53	0.57	0.43	5.00	9.80	30	18.6	10.9	4.1	0.221	10.6	67.2
6	19	F6	1.00	0.00	0.28	0.72	20.58	7.09	30	85.5	8.8	3.5	0.278	13.6	48.6
7	15	F7*	1.00	0.00	0.28	0.72	20.58	7.09	30	88.1	8.8	3.1	0.282	13.8	51.1
8	1	F8	0.25	0.75	1.00	0.00	5.00	1.00	30	23.8	10.0	4.1	0.148	8.2	94.4
9	8	F9	1.00	0.00	0.00	1.00	5.00	1.00	40	42.9	10.9	4.0	0.224	11.8	65.5
10	22	F10	1.00	0.00	1.00	0.00	5.00	15.00	40	45.7	8.7	4.6	0.263	13.1	56.7
11	7	F11	1.00	0.00	0.00	1.00	5.00	15.00	30	83.8	7.6	4.5	0.238	14.2	60.8
12	26	F12	0.63	0.37	1.00	0.00	26.35	10.54	40	56.4	9.3	3.9	0.243	8.2	76.7
13	16	F13*	0.63	0.37	1.00	0.00	26.35	10.54	40	50.3	12.8	3.5	0.256	7.9	76.8
14	25	F14	0.25	0.75	0.00	1.00	40.00	15.00	30	15.5	8.0	4.1	0.179	11.4	90.8
15	6	F15	0.25	0.75	0.00	1.00	5.00	1.00	30	10.9	6.2	4.6	0.170	12.7	95.6
16	14	F16	0.25	0.75	1.00	0.00	40.00	15.00	30	35.8	9.4	4.1	0.249	4.7	95.2
17	18	F17	0.44	0.56	0.06	0.94	18.62	6.44	40	17.6	14.3	5.0	0.219	9.2	85.4
18	11	F18*	0.44	0.56	0.06	0.94	18.62	6.44	40	25.0	13.8	4.6	0.215	11.2	87.3
19	5	F19	1.00	0.00	1.00	0.00	5.00	1.00	30	93.8	8.1	4.1	0.256	10.1	65.9
20	4	F20	1.00	0.00	0.00	1.00	40.00	1.00	30	44.4	3.9	4.0	0.273	12.1	64.8
21	24	F21	0.25	0.75	0.00	1.00	40.00	1.00	40	9.6	12.2	4.3	0.211	14.0	84.3
22	3	F22	0.25	0.75	1.00	0.00	40.00	1.00	40	43.5	10.9	4.1	0.192	4.7	98.0
23	12	F23	1.00	0.00	1.00	0.00	40.00	1.00	40	60.9	15.0	4.4	0.304	7.4	64.5
24	13	F24	1.00	0.00	0.00	1.00	40.00	15.00	40	42.8	12.0	4.5	0.293	17.4	58.9
25	10	F25	0.25	0.75	0.00	1.00	5.00	15.00	40	10.9	17.5	4.3	0.193	5.8	89.1
26	23	F26	1.00	0.00	1.00	0.00	40.00	15.00	30	102.7	12.4	3.6	0.297	7.9	65.7

* Replicate.

[a] P: Plasticizer, T: temperature.

[b] EB: Elongation at break, LB: Load at break, MS: Mucoadhesive strength, TH: Thickness, SI: Swelling index at 90 min; CV: Cellular viability. Data are presented as mean (n = 3).

3.2. Physical characterization of the films. Visual appearance, thickness, folding endurance

Prior to the optimization stage, the films were visually examined, observing that they exhibited a smooth and uniform texture, which demonstrated compatibility among the components [16]. In addition, their flexibility was assessed to guarantee their secure application. All films complied with the folding endurance test, not evidencing signs of breakage after being folded 300 times [13]. This indicates that the films have a high mechanical strength and could be able to avoid breaks during handling and administration. On the other hand, the film thickness was found to be in the range from 0.148 ± 0.036 to 0.304 ± 0.065 mm.

3.3. Compositional uniformity

In order to confirm the uniformity of the composition of the films, three NIR spectra were acquired from different parts of each film and statistically compared pairwise in the full spectral region (SI, **Table S1**). The corresponding Pearson correlation coefficients [17] were calculated and a cut-off value was arbitrarily set at r = 0.990 for the tests. The comparisons gave r values better than 0.998, unequivocally suggesting that the composition of the films is uniform.

3.4. Experimental design with response surface methodological optimization

3.4.1. Experimental setup and model selection

The optimization of the film formulations was performed with a response surface methodology approach, using an I-optimal design (Table 1). Seven factors (SI, **Table S2**) involved two mixtures [the forming polymers (CH:HPMC, Mixture 1) and the plasticizers (PEG:PPG, Mixture 2)], two numerical factors, namely the loads of the plasticizers (P) and TCZ, and the drying temperature (T) as a categorical variable. All

experiments were performed in random order to minimize the effects of uncontrolled factors that may introduce a bias in the measurements.

Since all films presented *in vitro* activity against *C. albicans* and met the folding endurance test and the visual appearance examination, it was deemed not relevant to optimize these responses. On the other hand, the film thickness property was included among the optimization goals, with the aim to reduce discomfort in the patient by preparation of the thinnest possible formulation.

Hence, six dependent variables were considered for the optimization (Table 1), including elongation at break (EB), load at break (LB), mucoadhesive strength (MS), film thickness (TH), swelling index at 90 min (SI) and cell viability (CV). Each response was recorded in triplicate and the average was used for optimization purposes.

The factors that significantly influenced the behavior of each response, according to the respective ANOVA results ($p < 0.05$), were selected for the elaboration of the corresponding predictive statistical model (SI, **Table S3**). Combinations of these variables were fitted in polynomic models up to the third order, employing stepwise backward regression, in order to finally optimize their values. The Box–Cox transformation on the response data was performed for EB and SI in order to comply with model assumptions [18,19]. It was observed that the resulting models (p-Model < 0.05) were significant (*i.e.*, not due to noise), the lack-of-fit for all responses was not significant (p-LOF > 0.05) relative to the pure error [20], and displayed acceptable standard deviation (SD) values [21]. They also had adequate correlation coefficients (R^2) and the differences between predicted and adjusted R^2 values were satisfactory ($R^2_{pred} - R^2_{adj} < 0.2$) [21].

The response surface plot for each dependent variable as a function of 2 factors was constructed by setting fixed values to the rest of the parameters; these values were

chosen based on a better observation of the factors that influence the performance of more than one response.

3.4.2. Effects on the elongation at break

The most significant factors affecting the EB response were the composition of both polymer and plasticizer (SI, **Table S4**). As shown in Fig. 1A, at P = 5% *w/w*, TCZ = 15% *w/w* and T = 30 °C, the formulations exhibited maximum elongation when the content of CH was at its maximum level and the plasticizers mixture contained much more PEG than PPG. This is reminiscent of the results obtained by Tejada et al. [15], who studied miconazole buccal films based on chitosan and observed that the highest elongation values were achieved by the formulations containing the maximum amount of the polymer.

3.4.3. Effects on the mucoadhesive strength

Fig. 1B suggests that the MS of the films mostly depends on the proportions of the plasticizers (PEG and PPG) and the polymers (CH and HPMC) employed to build the film. In the case of the former, increasing the level of PEG in the plasticizers mixture reduced the MS, to a minimum at PEG:PPG = 0.28:0.72 (Table 1, F7), when CH:HPMC = 1.00:0. On the other hand, the MS increased up to a certain amount of HPMC, and then decreased. In the experimental domain, the MS acquired its maximum value with CH:HPMC = 0.44:0.56 and PEG:PPG = 0.06:0.94 (Table 1, F17).

These results are in agreement with those of Rajput et al., who studied the effect of the loads of hydroxypropyl cellulose and PEG 400 on the MS of glipizide films. The authors observed that MS improved with an increase in the proportions of hydroxypropyl cellulose up to an optimum value and then it began to decrease. It was also informed that increasing concentrations of PEG 400 in the films diminished their

mucoadhesive strength [22].

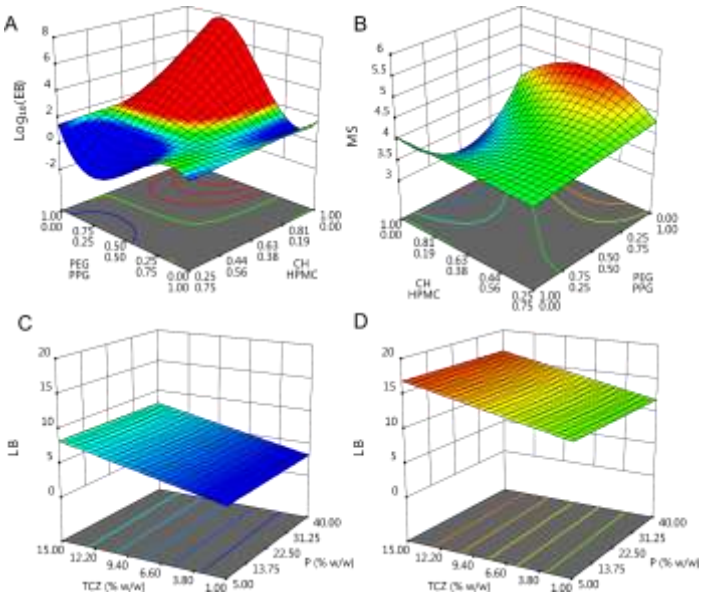

Figure 1. Response surface plot for mechanical dependent variables. A) Elongation at break as a function of the CH:HPMC ratio and the PEG:PPG ratio (P = 5% *w/w*, TCZ = 15% *w/w*, T = 30 ºC). B) Mucoadhesive strength as a function of the CH:HPMC and PEG:PPG ratios (P = 5% *w/w*, TCZ = 15% *w/w*, T = 30 ºC). C, D) Load at break as a function of the amounts of TCZ and plasticizers at 30 and 40 ºC respectively, when PPG is at its maximum value (PEG:PPG = 0:1.00) and CH is at its minimum level.

3.4.4. Effects on the load at break

The amount of the internal plasticizer (PPG) in Mixture 2 and the load of TCZ, as well as the drying temperature (T), proved to be the factors that have the greatest influence on LB (SI, **Table S4**). According to Figs. 1C and 1D, this property significantly increases with the drying temperature and the concentration of TCZ, when PPG is at its maximum value (PEG:PPG = 0:1.00) and CH is at its minimum level. This result is in agreement with the findings of Real et al. [23], who informed that films containing sorbitol as an internal plasticizer exhibited increased tensile strength, at the lowest level of chitosan.

Furthermore, LB increased directly with the drying temperature at a constant

relative humidity, in agreement with the reported by Chinma et al. [24]. This behavior could be attributed to the moisture content in the films. According to Labuza [25], the amount of water absorbed by food materials at constant relative humidity decreases with the increase in temperature. This results in a less weakened film structure, which is reflected in improved LB values.

3.4.5. Effects on the film thickness

As stemmed from the response surface plot (Fig. 2A) and the corresponding coefficient values (SI, **Table S4**), TH increases with the proportion of CH in the film forming polymers mixture (Table 1, F1 vs. F10; F16 vs. F26, and F14 vs. F24) and with the amount of the plasticizers (Table 1, F11 vs. F24). The latter results are similar to those obtained by Thakhiew et al. [26] during their study of the effect of plasticizer concentration on the physical properties of edible CH films. In this work, the authors concluded that the films became thicker with the increase of glycerol concentration.

3.4.6. Effects on the swelling index

Swelling of the film causes patient discomfort; therefore, it should be minimized by adjusting the variables affecting this phenomenon. After a series of trial and error experiments, it was observed that, in general, at 90 min the films reached their maximum SI. Therefore, the mentioned timepoint was chosen to determine the swelling index and to examine its variability under different conditions.

Four factors were found to significantly affect the SI response, including the CH:HPMC and PEG:PPG proportions, the amount of P and the load of TCZ (SI, **Table S4**). The lowest SI values (Fig. 2B) were obtained with the films containing the highest amounts of HPMC and PEG in their corresponding mixtures and the maximum load of TCZ (15% *w/w*).

Regarding the effect of the content of plasticizers (P), it was detected that when P is increased, the highest SI value is achieved at the maximum level of PPG (40% *w/w*, Table 1 F24), in agreement with the report by Bamigbola et al. [27]. On the other hand, it was also observed that an increase in P causes a reduction in the SI values at the maximum amount of PEG in the PEG:PPG mixture, confirming our previous results [8].

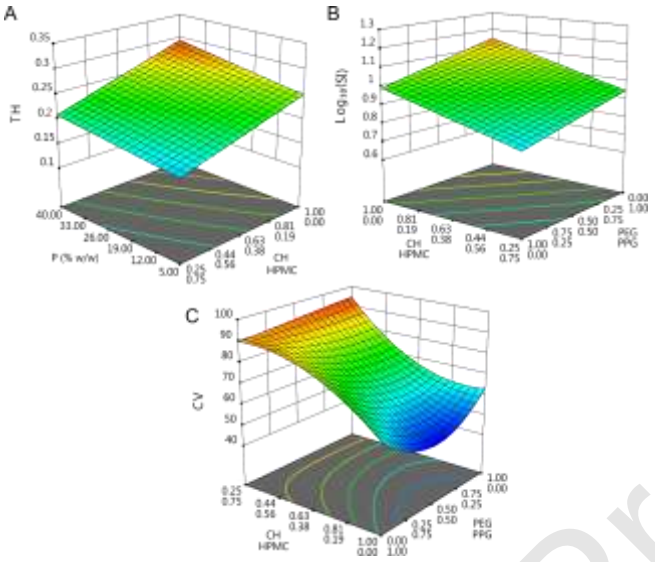

Figure 2. Response surface plots of the influence of some factors on the characteristics of the films. A) Thickness as a function of the CH:HPMC ratio and the amount of plasticizers (TCZ = 15% *w/w*, T = 30 ºC). B) Swelling index as a function of the CH:HPMC and PEG:PPG ratios (P = 5% *w/w*, TCZ = 15% *w/w*, T = 30 ºC). C) Cell viability as a function of the CH:HPMC and PEG:PPG ratios (P = 5% *w/w*, TCZ = 15% *w/w*, T = 30 ºC).

3.4.7. Effects on cell viability

The top priority of this assay was to measure the cell damage produced by the drug in its association with the polymers and plasticizers contained in the formulation. Allen et al. reported that some cell lines tend to exhibit stability and/or viability problems, suggesting that the use of a cell line able to overcome these limitations, such as one derived from other tissues, is recommended as a feasible alternative, especially

regarding new potential applications in drug testing therapy [28]. Therefore, the use of a cell line other than a vaginal-derived one was considered as a proper system.

The contents of TCZ and P, as well as the drying temperature (T), showed no significant influence on cell viability, which proved to depend on the proportion of CH and PEG in the samples (SI, **Table S4**). It has been shown that CH-based materials could potentially alter cell growth and that CH inhibits cell proliferation [29], becoming cytostatic towards fibroblasts. Films based on CH alone induced a 35–54% reduction in CV after 24 h incubation when compared to the control (CV = 97 ± 3%), whereas specimens containing equal amounts of PEG and PPG exhibited the highest cytotoxic effect (Fig. 2C). In contrast, the films with increased proportions of PEG and HPMC exhibited less cytotoxic effects. This is in agreement with Shahabeddin et al., who concluded that the cytocompatibility of CH can be improved through association with other materials [30].

3.4.8. Global desirability (D) function

The optimization approach developed by Derringer and Suich [31] was used, due to its suitability for the simultaneous analysis of several responses, by defining the areas in the design space where the process is likely to give desirable results. The method involves the generation of a partial desirability function (d_i) for each response as a means to obtain the global desirability function (D), defined as in Eq. [4],

$$D = \left[\prod_{i=1}^{N} (d_i)^{W_i}\right]^{(\sum_{i=1}^{N} W_i)^{-1}} \quad \text{Eq. [4]}$$

where d_i corresponds to the individual desirability function for each response being optimized, (W_i) is the importance attached to each response, and (N) is the number of responses.

The values of D range from 0 for a fully undesirable response to 1 for a fully desirable response. The optimal formulation was obtained after setting the conditions for each response (Table 2). Minimum values of TH and SI were sought, in order to reduce discomfort during and after application, whereas CV was maximized to ensure formulation safety. On the other hand, MS was maximized for improved local action. Finally, EB was maximized between 10 and 30% in order to guarantee the obtention of a manageable film, while LB was kept in the range, to produce resistant films.

Table 2. Parameters used to generate the *D* function.

Dependent Variable[b]	Unit	Goal	Lower Limit (W^a)	Upper Limit (W^a)
Elongation at break (EB)	%	Maximum	9.6 (1)	30 (1)
Load at break (LB)	N	In range	3.9 (1)	17.5 (1)
Mucoadhesive strength (MS)	N	Maximum	3.1 (1)	5.0 (1)
Thickness (TH)	mm	Minimum	0.148 (1)	0.304 (2)
Swelling index (SI)	-	Minimum	4.7 (1)	17.4 (1)
Cellular viability (CV)	%	Maximum	48.6 (1)	97.9 (1)

[a]*W*: Weight used for defining the d_i function associated with the response.

[b]The relative importance assigned to the d_i function associated with the responses, and used for generating the *D* function was set to 3.

The overall results showed that the optimal formulation (D = 0.769) should be prepared with CH:HPMC = 0.25:0.75, PEG:PPG = 0.07:0.93, P = 5% *w/w*, TCZ = 15% *w/w* and T = 30 °C. Fig. 3 shows the surface of D as a function of selected pairs of factors.

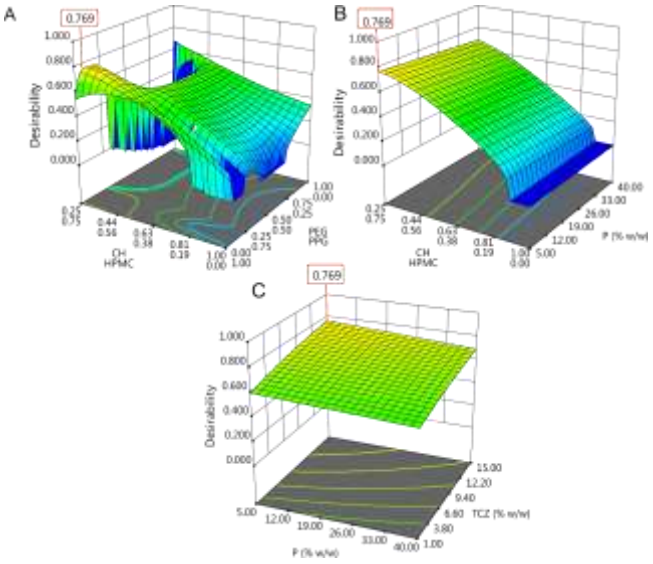

Figure 3. Response surface plots corresponding to the *D* function. A) CH:HPMC ratio vs. PEG:PPG ratio. B) CH:HPMC ratio vs. amount of plasticizer (P, % *w/w*). C) Amount of plasticizer (P, % *w/w*) vs. load of TCZ (% *w/w*).

3.5. Verification of the predicted characteristics of the optimal film formulation

The optimal formulation (OF) was prepared in triplicate and its predicted characteristics were verified experimentally. The results (Table 3) showed that no significant differences were found between predicted and observed values, indicating that the mathematical models obtained from the design were well fitted.

Table 3. Comparison between predicted and experimental values of the responses obtained under the optimized conditions.

Response	Predicted	Experimental[a]	*p*-value[b]
Elongation at break (%)	30.1	31.2 ± 4.6	0.719
Mucoadhesive strength (N)	4.3	3.8 ± 0.3	0.102
Load at break (N)	9.3	11.2 ± 2.6	0.333
Thickness (mm)	0.179	0.189 ± 0.037	0.686
Swelling index	6.06	6.54 ± 0.28	0.097
Cellular viability (%)	90.2	92.5 ± 5.9	0.569

[a]The experiments were run on three independent samples.

[b]Differences are considered statistically significant when $p < 0.05$.

3.6. Characterization of the optimal formulation

3.6.1. Thermogravimetric analysis

The DTG curve of OF (Fig. 4A) presented 4 stages, where the first 2 broad bands (centered at 78.9 °C and 200.8 °C, respectively) were related to the loss of adsorbed and bound water [8], together with dehydration of PPG and the elimination of possible trace amounts of lactic acid. On the other hand, the remaining bands relate to dehydration of the saccharide rings of the polymers; the third signal (centered at 281 °C) was associated to the concomitant degradation of the drug and CH, whereas the last peak (centered at 335 °C) was attributed to the decomposition of HPMC [8].

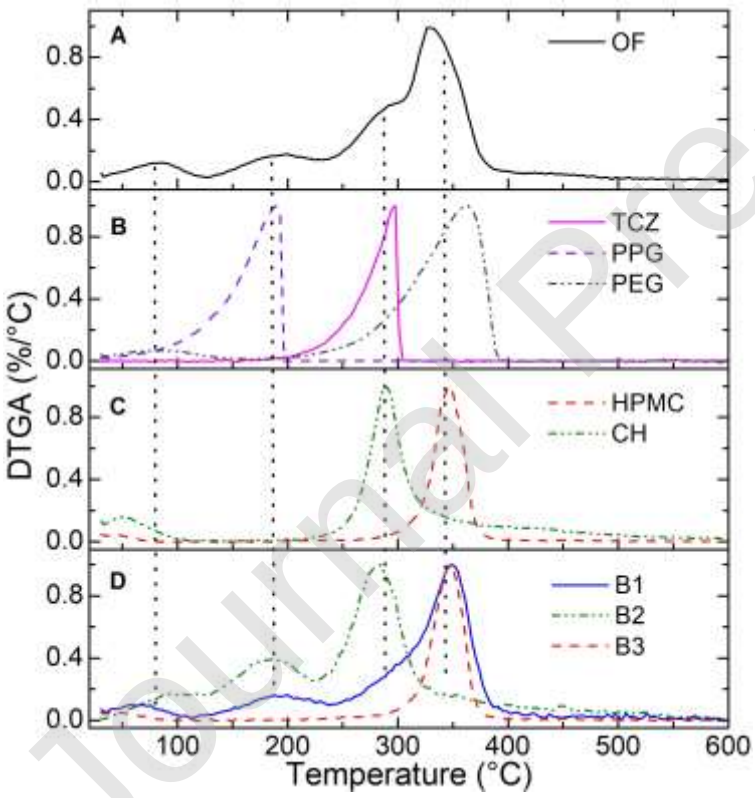

Figure 4. DTG curves of A) the optimum formulation (OF); B) TCZ bulk drug and the plasticizers (PEG and PPG); C) the forming polymers (CH and HPMC) and D) Blank films B1-B3.

These assignments are in agreement with the DTG curve of TCZ (Fig. 4B), which

exhibited a single degradation step centered at 297.8 °C [8], and with the DTG plots of CH, HPMC (Fig. 4C) and the plasticizers (Fig. 4B). Interestingly, the decomposition curves of the blank films B1-B3 (Fig. 4D) were in agreement with their respective composition. The specimens containing CH (B1 and B2) exhibited the water loss-related bands around 80 °C and 200 °C, whereas the graphic of the film based on 100% HPMC (B3), displayed high similarity with that of its precursor at the solid state (Fig. 4C).

3.6.2. Spectroscopic data

Fig. 5A shows the FTIR-ATR spectra of OF, B1 and TCZ. The spectra of the first two are highly similar, exhibiting as main characteristics a broad band between 3600-2800 cm^{-1} which is typical of both polymers. It is related to the O–H stretching of the hydroxyl groups, either free, or attached via intra- and inter-molecular H-bonds. This band overlaps with the N–H stretching band of CH and the stretching vibration of the C–H bonds of the sample.

Other signals attributable to CH were detected at 1643 cm^{-1} (amide I) and 1595 cm^{-1} (NH_2 bending) [32], while the bands around 1420 cm^{-1} ($\nu_{(C–O)}$) may be assigned to both polymers. Signals of the polymers were also observed in the 1450-1350 cm^{-1} region ($\nu_{(C-C)}$ and $\delta_{(C-H)}$ of the methyl groups) [33], whereas the peak at 944 cm^{-1} represents the in-phase vibrations of the ether linkages.

Besides, the characteristic peaks of TCZ, corresponding to $\nu_{(C=N)}$ of the imidazole group (1562 cm^{-1}), $\delta_{(C–H)}$ (1464 cm^{-1}), $\nu_{(C=C)}$ (1433 cm^{-1}), $\nu_{(C–N)}$ (1279 cm^{-1}), $\nu_{(C–O–C)}$ (1119 cm^{-1}), $\nu_{(C–S)}$ (733 cm^{-1}) and $\nu_{(C–Cl)}$ (627 cm^{-1}) were clearly visible in the spectrum of the bulk drug; contrastingly, except perhaps for the band at 733 cm^{-1}, they were almost completely obscured by the presence of the polymers and the plasticizers in OF [8].

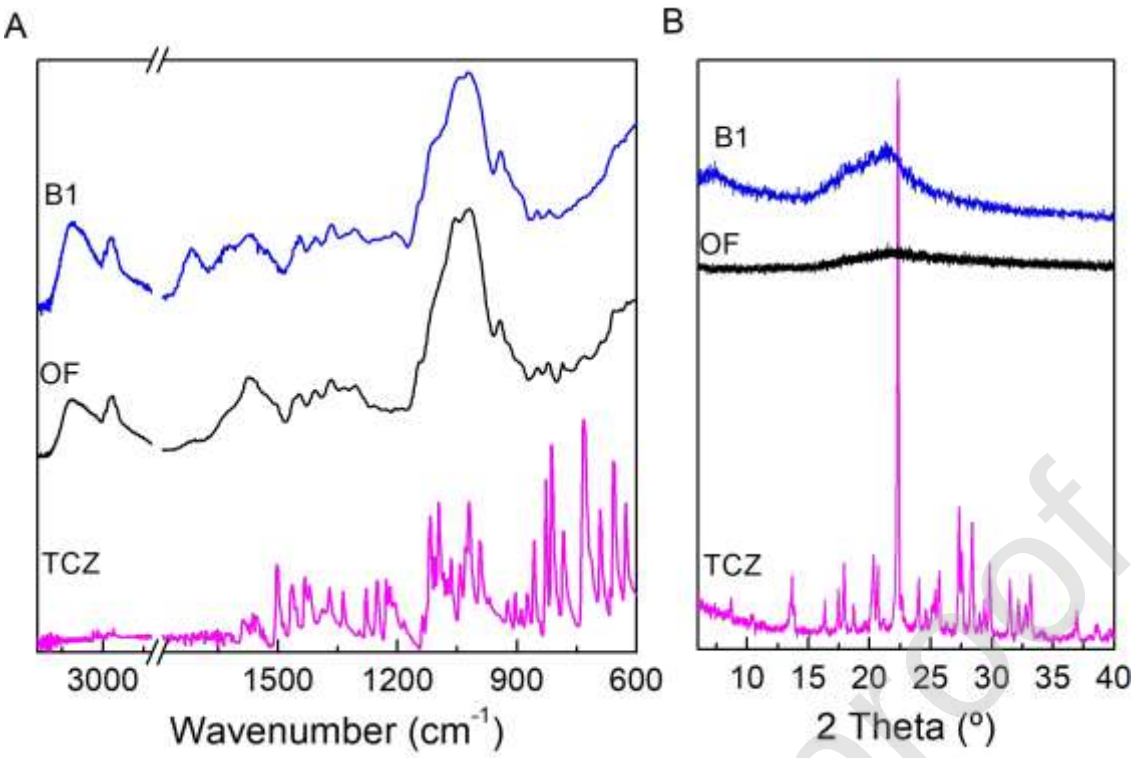

Figure 5. A) FTIR-ATR spectra B) X-ray diffraction patterns of TCZ, OF and its respective blank (B1).

3.6.3. X-ray diffraction analysis

The X-ray study was carried out to confirm the results of the thermal analyses and to complete the characterization of the films. The X-ray patterns of TCZ, the optimal film, and its blank (B1) in the 2θ range 5-40° are shown in Fig. 5B.

TCZ showed sharp and narrow peaks at diffraction angles, which were fully coherent with the literature [34]. Furthermore, the X-ray spectra of the optimal film did not present any peak corresponding to crystalline TCZ.

3.6.4. Scanning electron microscopy

SEM surface and transversal section studies were undertaken to gain detailed information on the morphology of the OF. The micrographs showed a smooth and uniform surface without pores or cracks and a compact structure (Fig. 6A-B) which

could be attributed to a uniform mixing, and to high structural integrity and excellent compatibility among the drug, the plasticizers, and the polymer forming agents [16]. Such homogeneous film matrix is a good indicator of structural integrity and, therefore, of good mechanical properties, as experimentally verified. The examination of the transversal cut (Fig. 6C) showed lattice porosity. It is known that interconnected pores allow water to be absorbed in a very fast rate, swelling the film and affecting the diffusion of the solute [35].

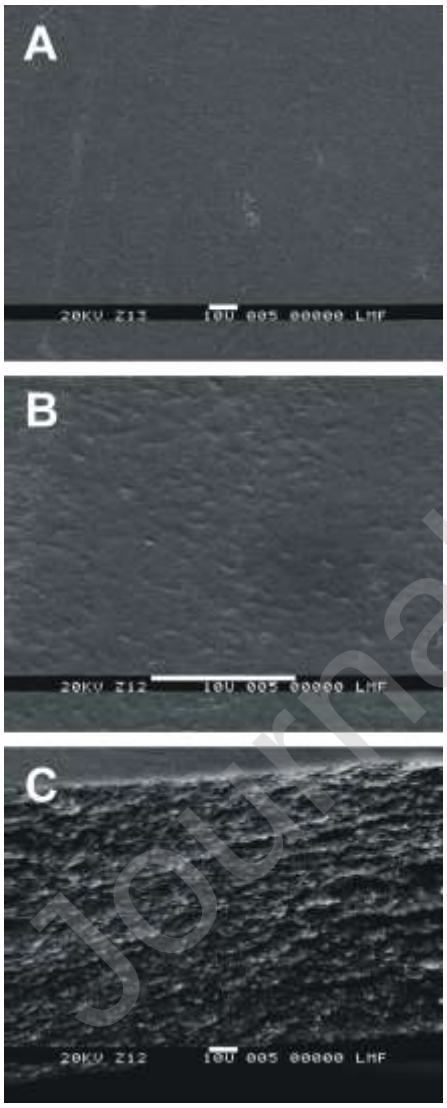

Figure 6. Scanning electron microscopy. Micrographs with different magnifications and orientation. Film surface at A) 1000×; B) 5000×; C) Transversal section at 1000×.

3.6.5. Time to kill

A time to kill study was performed to assess the exposure time required to kill a standardized Candida inoculum. Fig. 7A shows the profiles (CFU/mL vs. time) of the blank film B1, TCZ, and OF, with samples taken every 15 min. As expected, the B1 control film, which lacks TCZ and the plasticizers found in OF, was inactive against *C. albicans*, in agreement with previous work using similar blank films [8].

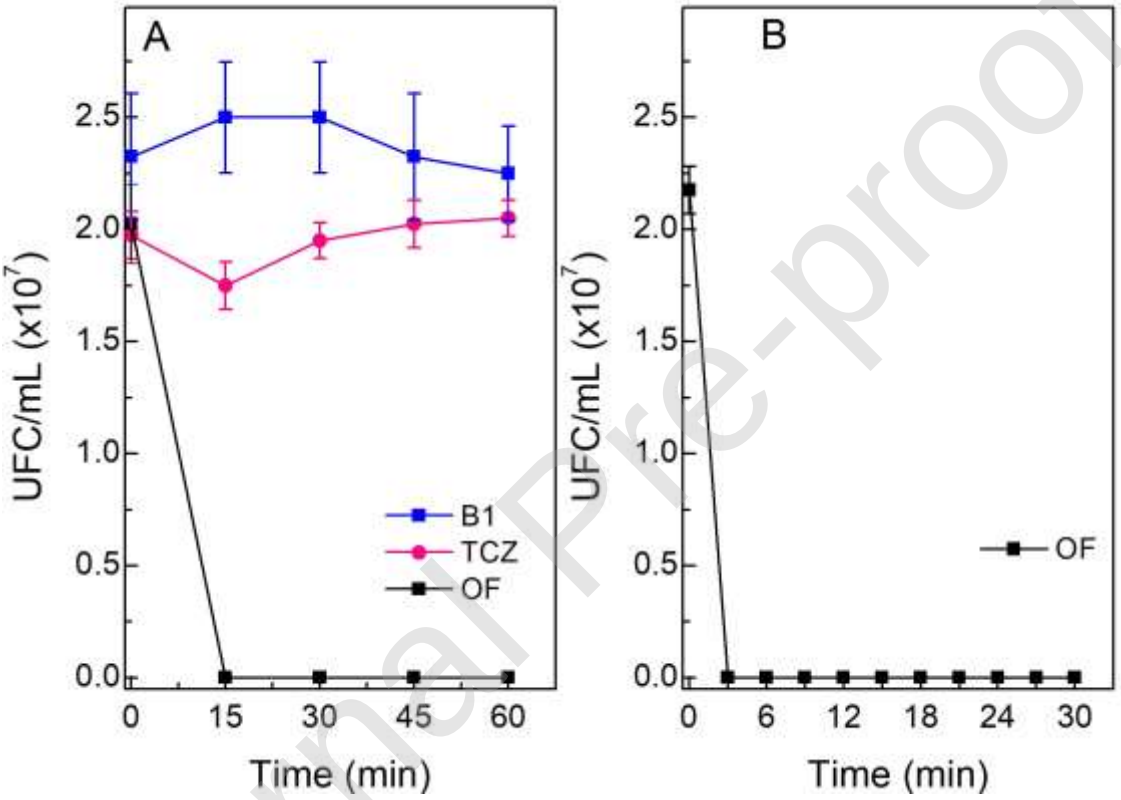

Figure 7. A) Time-to-kill curves of TCZ, OF and its respective blank, B1; B) Time-to-kill curve of OF at 3 min intervals.

On the other hand, it was observed that TCZ just showed slight activity even after 60 minutes and the number of CFU/mL remained almost constant. Presumably, this is a result of the scarce aqueous solubility and slow dissolution rate of the pure drug, which proves its poor competency to kill the yeasts in the inoculum. Contrastingly, the optimal formulation proved to be fast and effective, affording the complete eradication of the

inoculum at the 15 min time point. This result is probably related to the interconnected pores observed by scanning electron microscopy which allow a fast water absorption releasing the drug by diffusion.

For a more accurate determination of the efficiency of OF, a second time to kill test was performed, sampling the inoculum at 3 min intervals. As depicted in Fig. 7B, the OF required only 3 min to produce a 99.9% reduction in the number of CFU/mL. The X-ray diffraction studies proved that TCZ is in the film in an amorphous state; this could explain the improved drug release from the film and its resulting outstanding performance.

It is worth mentioning that previously it has been prepared two CH-based TCZ films which displayed a time to kill of 15 min but proved to be slightly cytotoxic [8]. In addition, a CH/HPMC film was designed, which contained 5% *w/w* PEG 400 as plasticizer (S1) and exhibited a slightly longer time to kill, of 30 min [8]. Hence, the rationally developed new formulation exhibits not only optimized values in some of its physical characteristics but also an improved degree of fungicidal efficiency. The latter can be attributed to the increase in microporosity with respect to the previous published film S1 without producing an increase in thickness or the swelling index at 90 min that could produce discomfort in the patient.

4. Conclusions

A safe and effective tioconazole mucoadhesive film formulation was rationally developed under the modern Quality by Design paradigm. This was achieved through an I-optimal experimental design coupled with statistical optimization techniques and a sound analysis of the parameters which significantly affect the key physicochemical and functional properties of the product.

The overall strategy involved only four key phases, including a) construction of

response surface models from mixture-process experiments; b) judicious search of the optimal formulation conditions; c) experimental verification of the predicted properties of the optimized formulation, and d) characterization of the final product.

This methodology proved to be very efficient for improving the desirable properties of the films, including many critical aspects such as their mucoadhesiveness and antifungal activity, while at the same time minimizing undesirable aspects, such as their swelling ability and cytotoxicity.

The optimal film formulation, where tioconazole was found in an amorphous state, exhibited a smooth and uniform surface, lacked cytotoxicity, and showed satisfactory antifungal activity in the time to kill test.

5. Conflicts of the Interest

All authors declare that they have no conflict of interest

6. Acknowledgements

The authors gratefully acknowledge Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and Universidad Nacional de Rosario (UNR) for financial support. NLC thanks CONICET for her postdoctoral fellowship.

7. Statement of human and animal rights.

This article does not contain any studies with human participants or animals performed by any of the authors.

Author Statement

Natalia L. Calvo: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing

Guillermo Tejada: Investigation

Laura A. Svetaz: Investigation, Formal analysis

Ariel D. Quiroga: Investigation, Formal analysis

Vera A. Alvarez: Writing - review & editing

María C. Lamas: Writing - review & editing

Darío Leonardi: Funding acquisition, Writing - review & editing,

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] C. Spampinato, D. Leonardi, Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents, BioMed Res. Int. 2013 (2013) 204237.

[2] S.C. Sweetman, Martindale: The complete Drug Reference, 38th Ed., London, UK: The Pharmaceutical Press, London, UK, 2014.

[3] M.A. Alam, F.J. Ahmad, Z.I. Khan, R.K. Khar, M. Ali, Development and evaluation of acid-buffering bioadhesive vaginal tablet for mixed vaginal infections, AAPS PharmSciTech. 8 (2007) 229.

[4] M.P. Cautela, H. Moshe, A. Sosnik, B. Sarmento, J. das Neves, Composite films for vaginal delivery of tenofovir disoproxil fumarate and emtricitabine, Eur. J. Pharm. Biopharm. 138 (2019) 3–10.

[5] P. Srinivasan, J. Zhang, A. Martin, K. Kelley, J.M. McNicholl, R.W. Buckheit, J.M. Smith, A.S. Ham, Safety and pharmacokinetics of quick-dissolving polymeric vaginal films delivering the antiretroviral IQP-0528 for preexposure prophylaxis, Antimicrob. Agents Chemother. 60 (2016) 4140–50.

[6] A. Fini, I. Orienti, The role of chitosan in drug delivery, Am. J. Drug Deliv. 1 (2003) 43–59.

[7] S. Kamel, N. Ali, K. Jahangir, S.M. Shah, A.A. El-Gendy, Pharmaceutical significance of cellulose: A review, Express Polym. Lett. 2 (2008) 758–778.

[8] N.L. Calvo, L.A. Svetaz, V.A. Alvarez, A.D. Quiroga, M.C. Lamas, D. Leonardi, Chitosan-hydroxypropyl methylcellulose tioconazole films: A promising alternative dosage form for the treatment of vaginal candidiasis, Int. J. Pharm. 556 (2019) 181–191.

[9] S.S. Timur, S. Yüksel, G. Akca, S. Şenel, Localized drug delivery with mono and bilayered mucoadhesive films and wafers for oral mucosal infections, Int. J.

Pharm. 559 (2019) 102–112.

[10] A.B. Nair, R. Kumria, S. Harsha, M. Attimarad, B.E. Al-Dhubiab, I.A. Alhaider, In vitro techniques to evaluate buccal films, J. Control. Release. 166 (2013) 10–21.

[11] L.X. Yu, G. Amidon, M.A. Khan, S.W. Hoag, J. Polli, G.K. Raju, J. Woodcock, Understanding pharmaceutical quality by design, AAPS J. 16 (2014) 771–783.

[12] D. Leonardi, M.C. Lamas, A.C. Olivieri, Multiresponse optimization of the properties of albendazole-chitosan microparticles, J. Pharm. Biomed. Anal. 48 (2008) 802–807.

[13] A.M. Avachat, K.N. Gujar, K. V. Wagh, Development and evaluation of tamarind seed xyloglucan-based mucoadhesive buccal films of rizatriptan benzoate, Carbohydr. Polym. 91 (2013) 537–542.

[14] CLSI, Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard document M27, 4th ed., Pennsylvania, USA, 2017.

[15] G. Tejada, M.G. Barrera, G.N. Piccirilli, M. Sortino, A. Frattini, C.J. Salomón, M.C. Lamas, D. Leonardi, Development and evaluation of buccal films based on chitosan for the potential treatment of oral candidiasis, AAPS PharmSciTech. 18 (2017) 936–946.

[16] R.A. Espinel Villacrés, S.K. Flores, L.N. Gerschenson, Biopolymeric antimicrobial films: Study of the influence of hydroxypropyl methylcellulose, tapioca starch and glycerol contents on physical properties, Mater. Sci. Eng. C. 36 (2014) 108–117.

[17] N.L. Calvo, R.M. Maggio, T.S. Kaufman, A dynamic thermal ATR-FTIR/chemometric approach to the analysis of polymorphic interconversions. Cimetidine as a model drug, J. Pharm. Biomed. Anal. 92 (2014) 90–97.

[18] P. Vasandani, Z.-H. Mao, W. Jia, M. Sun, Design of simulation experiments to predict triboelectric generator output using structural parameters, Simul. Model. Pract. Theory. 68 (2016) 95–107.

[19] Z. Jeirani, B. Mohamed Jan, B. Si Ali, I. Mohd. Noor, S. Chun Hwa, W. Saphanuchart, The optimal mixture design of experiments: Alternative method in optimizing the aqueous phase composition of a microemulsion, Chemom. Intell. Lab. Syst. 112 (2012) 1–7.

[20] C. Didier, M. Etcheverrigaray, R. Kratje, H.C. Goicoechea, Crossed mixture design and multiple response analysis for developing complex culture media used in recombinant protein production, Chemom. Intell. Lab. Syst. 86 (2007) 1–9.

[21] K. Bharti, P. Mittal, B. Mishra, Formulation and characterization of fast dissolving oral films containing buspirone hydrochloride nanoparticles using design of experiment, J. Drug Deliv. Sci. Technol. 49 (2019) 420–432.

[22] G. Rajput, F. Majmudar, J. Patel, Formulation and evaluation of mucoadhesive glipizide films, Acta Pharm. 61 (2011) 203–216.

[23] D.A. Real, M. V. Martinez, A. Frattini, M. Soazo, A.G. Luque, M.S. Biasoli, C.J. Salomon, A.C. Olivieri, D. Leonardi, Design, characterization, and in vitro evaluation of antifungal polymeric films, AAPS PharmSciTech. 14 (2013) 64–73.

[24] C.E. Chinma, C.C. Ariahu, J.S. Alakali, Effect of temperature and relative humidity on the water vapour permeability and mechanical properties of cassava starch and soy protein concentrate based edible films, J. Food Sci. Technol. 52 (2015) 2380–2386.

[25] T.P. Labuza, Moisture Sorption: Practical aspects of isotherm measurement and use, Food / Nahrung. 29 (1984) 92–92.

[26] W. Thakhiew, S. Devahastin, S. Soponronnarit, Effects of drying methods and plasticizer concentration on some physical and mechanical properties of edible chitosan films, J. Food Eng. 99 (2010) 216–224.

[27] E.A. Bamigbola, A.A. Attama, P.C. Ogeh, Evaluation of physico-mechanical and mucoadhesive properties of biopolymer films from Cola Acuminata gum, Nig. J. Pharm. Res. 14 (2018) 1–13.

[28] D.D. Allen, R. Caviedes, A.M. Cárdenas, T. Shimahara, J. Segura-Aguilar, P.A. Caviedes, Cell lines as in vitro models for drug screening and toxicity studies, Drug Dev. Ind. Pharm. 31 (2005) 757–768.

[29] M. Jiang, H. Ouyang, P. Ruan, H. Zhao, Z. Pi, S. Huang, P. Yi, M. Crepin, Chitosan derivatives inhibit cell proliferation and induce apoptosis in breast cancer cells, Anticancer Res. 31 (2011) 1321–1328.

[30] L. Shahabeddin, O. Damour, F. Berthod, P. Rousselle, G. Saintigny, C. Collombel, Reconstructed skin from co-cultured human keratinocytes and fibroblasts on a chitosane cross-linked collagen-GAG matrix, J. Mater. Sci. Mater. Med. 2 (1991) 222–226.

[31] G. Derringer, R. Suich, Simultaneous optimization of several response variables, J. Qual. Technol. 12 (1980) 214–219.

[32] G. Tejada, G.N. Piccirilli, M. Sortino, C.J. Salomón, M.C. Lamas, D. Leonardi, Formulation and in-vitro efficacy of antifungal mucoadhesive polymeric matrices for the delivery of miconazole nitrate, Mater. Sci. Eng. C. 79 (2017) 140–150.

[33] L. Wang, W. Dong, Y. Xu, Synthesis and characterization of hydroxypropyl methylcellulose and ethyl acrylate graft copolymers, Carbohydr. Polym. 68 (2007) 626–636.

[34] N.L. Calvo, V.A. Alvarez, M.C. Lamas, D. Leonardi, New approaches to

identification and characterization of tioconazole in raw material and in pharmaceutical dosage forms, J. Pharm. Anal. 9 (2019) 40–48.

[35] V. Thakur, M. Thakur, Hydrogels: Recent Advances, Springer, Singapore, 2018.