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Amidated pectin-based wafers for econazole buccal delivery: Formulation optimization and antimicrobial efficacy estimation

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ABSTRACT

Combinations of low-methoxy amidated pectin (LMAP) and carboxymethylcellulose (CMC) were used to develop a lyophilized wafer formulation, aimed to obtain prolonged residence and controlled release of econazole nitrate (ECN) in the oral cavity. Ternary ECN/sulphobutylether-ß-cyclodextrin/citric acid complex, resulted as the most efficient system against selected *Candida* strains, was loaded into this formulation. The final product with the desired and predicted quality was developed by an experimental design strategy. The experimental values of mucoadhesion strength (28.37 ± 0.04 mg/cm^2) and residence time (88.1 ± 0.1 min) obtained for the optimized wafer formulation were very close to the predicted ones, thus demonstrating the actual reliability and usefulness of the assumed model in the preparation of buccal wafers. The optimized formulation provided a constant ECN *in situ* release of 5 mg/h and was efficacious against selected *Candida* strains *in vitro*. This clearly proved its potential as a novel effective delivery system for the therapy of oral candidiasis.

1. Introduction

Candidiasis is one of the most common fungal infections of the oral cavity in humans, caused by yeasts of the genus *Candida.* Its clinical aspects run from relatively trivial conditions, such as acute pseudomembranous forms (oral thrush), to chronic erythematous and hyperplastic forms, up to systemic super-infections in immuno-compromised patients, with mortality of 30–50% (Williams, Kuriyama, Silva, Malic, & Lewis, 2011). Nowadays, oral candidiasis is considered an emerging medical conditions, primarily due to escalation of HIV-infection, where more than 90% of patients are afflicted with such pathology; moreover it is also associated to radiotherapy of head and neck carcinoma, teeth prosthesis use, endocrine disorders such as diabetes mellitus and adverse effects of broad-spectrum antibiotics, immunosuppressive and inhaled corticosteroids therapies (de Freitas et al., 2013; Ellepola & Samaranayake, 2001; Williams & Lewis, 2011).

Despite the availability of several effective antimycotics for the treatment of oral candidiasis, failure of the therapy is not uncommon, due to the unique properties of the oral cavity, where both the saliva flushing effect and the oral musculature cleansing action tend to reduce to sub-therapeutic levels the local drug concentration (Ellepola & Samaranayake, 2000). The design of mucoadhesive formulations able to assure a prolonged residence time at the oral cavity level, together with a drug sustained-release, could be a valid approach to overcome the shortcomings of conventional treatments (Llabot, Manzo, & Allemandi, 2009). In this regard, lyophilised wafers prepared by freeze-drying of different carbohydrate polymer solutions or gels to yield solid porous sponge-like matrices recently emerged as innovative and versatile formulations that can easily be applied to mucosal surfaces (Ayensu, Mitchell, & Boateng, 2012; Boateng et al., 2010). Wafers can provide an effective mean of delivering pharmacological agents to mucosal surfaces for both local and systemic applications, offering several advantages over conventional drug delivery systems. In fact, unlike semi-solid polymeric gels, which flow easily after application, wafers can maintain their swollen gel structure for a longer period, thus ensuring longer *in situ* residence time and more effective and reproducible drug delivery (Boateng, Matthews, Stevens, & Eccleston, 2008). Furthermore, due to their porous nature and

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wide surface area, wafers have higher absorption rate and drug loading capacity compared to the thin and denser solvent cast films (Boateng et al., 2010).

Low methylester amidated pectin (LMAP) can be considered as suitable polymer for the development of wafer formulations, due to its simple and cytocompatible gelling mechanism, high biocompatibility, biodegradability and versatile chemical and physical properties (Espitia, Du, Avena-Bustillos, Soares, & McHugh, 2014; Munarin, Tanzi, & Petrini, 2012; Sriamornsak, 2011). Compared to non-amidated low methylester pectins, LMAP gels at lower calcium levels and the formed gels are thermoreversible (Espitia et al., 2014). Furthermore, LMAP is less sensitive to pH variations and fluctuations in calcium levels than high methylester pectins, which gel only at pH < 3.5 and in the presence of high sucrose levels (Munarin et al., 2012). The presence of amide groups in its structure enhances the mucoadhesive strength of LMAP, especially at the pH range present in the oral cavity (Thirawong, Nunthanid, Puttipipatkhachorn, & Siriamornsak, 2007), thus making it particularly suitable for the development of nasal (Watts & Smith, 2009) and other mucosal drug delivery systems (Jug, Kosalec, Maestrelli, & Mura, 2012; Liu, Fishman, & Hicks, 2007). The unique properties of LMAP could be further improved by blending it with an anionic carbohydrate polymer, which, by electrostatic interactions, could enhance the mechanical and physicochemical properties of the matrices formed and their ability to control the drug release (Jug et al., 2012; Saleem, Azharuddin, Ali, & Patil, 2010). Sodium carboxymethylcellulose (CMC) is anionic cellulose ether commonly used in controlled-release matrix systems (Boateng et al., 2009a,b). Formulations comprising CMC have great potential for delivery of drugs to moist surfaces such as buccal and nasal cavities (Perioli et al., 2004; Ugwoke, Kaufmann, Verbeke, & Kinget, 2000) and wounds (Matthews, Stevens, Eccleston, Auffret, & Humphrey, 2005), due to their bioadhesive nature, pronounced film-forming properties, ease of hydration and subsequent swelling to form a viscous gel able to control the drug release (Boateng et al., 2009a,b). Therefore, CMC represents a good partner for LMAP in the development of buccal wafers.

Among the different antimycotic agents used in the treatment of oral candidiasis, econazole nitrate (ECN) could be particularly useful for a topical therapy, without problems of side effects due to systemic absorption, since its transmucosal and transdermal absorption are not significant (Sweetman, 2009).

On the basis of all the previous considerations, the aim of this work was the development of a lyophilized wafer formulation based on LMAP–CMC mixtures, suitable for application to the mucosal oral surface in order to obtain a prolonged *in situ* residence and a controlled release of ECN. However, before proceeding to the development of such a formulation, the problem of the very low ECN water solubility should be overcome, especially considering the limited volume of saliva, acting as the dissolution medium in the mouth. With this purpose, we recently prepared and characterised different cyclodextrin (CD) complexes with ECN, aimed to improve its aqueous solubility and, consequently, its potential for an efficient delivery to the oral cavity (Jug, Mennini, Kover, & Mura, 2014). The possible synergistic effect of the addition of different suitable additives as third component to the ECN–CD binary systems was also investigated (Loftsson, & Brewster, 2012), and citric acid emerged as the most effective in improving the CD solubilizing and complexing properties towards ECN, indicating the possible formation of a ternary complex between the components.

Therefore, as a continuation of such study, in the present work we first evaluated the antifungal efficacy of the prepared ECN complexes against *Candida albicans* and *Candida krusei*, chosen as model fungal strains, in order to properly select the most effective system to load into the wafer formulation. Subsequently, for a rational development of such formulation, the design of experiments strategy was used for a systematic study of the influence of the formulation variables on the wafers performance and the obtainment, with the minimal number of experiments, of a final product with the desired and predicted quality (Bragagni et al., 2014; Mennini, Furlanetto, Cirri, & Mura, 2012). The considered formulation variables were the amidation degree of LMAP and the amounts of LMAP and CMC, while mucoadhesive strength, *in situ* residence time and % drug released at a given time were chosen as the responses indicative of the system performance. A screening design was initially used, to rapidly individuate the factors whose variations gave rise to significant changes of the considered responses. A response surface methodology was then applied, in order to investigate in depth the values of the responses for all the possible combinations of the factors selected in the previous screening phase, and to find the optimal conditions. Finally, the antimicrobial properties of the optimized ECN wafer formulation against *C. albicans* and *C. krusei* were tested, in order to evaluate its effective therapeutic potential.

2. Materials and methods

2.1. Materials

Econazole nitrate (ECN) was kindly donated by Italfarmaco (Italy). The cyclodextrin derivatives used in this study were: hydroxypropyl-β-cyclodextrin (HPβCD, MW 1440), hydroxyethyl-β-cyclodextrin (HEβCD, MW 1320), both with an average substitution degree per anhydroglucose unit of 0.6, kindly donated by WackerChemie (Germany); water-soluble β-cyclodextrin-epichlorohydrin polymer (EPIβCD, mean MW 4500) purchased from Cyclolab (Hungary) and sodium salt of sulfobutylether-β-cyclodextrin with a substitution degree of 0.9 (SBEβCD, MW 2163) kindly provided by CyDex, Inc. (USA). Mucoadhesive polymers used were carboxymethylcellulose sodium salt with molecular weight of 90 kDa, degree of polymerization 400 and degree of substitution 0.65–0.90 (CMC; Sigma, Italy), and low methylester amidated citrus pectin (LMAP, Amid CF005 (15% amidation degree and 35% esterification degree) and Amid CF020 (20% amidation degree and 30% esterification degree), kindly gifted by Herbert and Fox KG Pektin-Fabriken, Germany. According the manufacturers' data, both pectins contained between 30 and 35% of glucose in order to standardise their gelling properties and were used as received. Anhydrous citric acid (CA) and HPLC-grade methanol were obtained from Sigma-Aldrich (Italy). Simulated saliva solution was prepared by dissolving 2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, 8.00 g NaCl in 1 L of distilled water and adjusting pH to 6.75 by the use of orthophosphoric acid. All others chemicals and solvents used in this study were of analytical reagent grade.

2.2. Preparation of solid binary and ternary complexes

Solid binary and ternary ECN–CD complexes were prepared by co-grinding equimolar drug/CD or drug/CD/CA mixtures in a high-energy vibration micromill (Retsch, GmbH, Germany) at 24 Hz for 60 min, as described previously (Jug et al., 2014). All samples were kept in desiccator until further use.

2.3. Determination of minimal inhibitory concentration (MIC)

MICs of ECN and co-ground binary and ternary ECN–CD complexes were determined by the two-fold broth microdilution method according to the EUCAST guidelines, with some modifications (Rodriguez-Tudela et al., 2008). *C. albicans* ATTC 90028 and *C. krusei* ATTC 6258, originating from the Collection of Microorganisms at the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, were used as model fungal strains, while RPMI 1640 with L-Glutamine and 25 mM HEPES

(Sigma Aldrich, USA), supplemented with 2% (w/v) glucose solution was used as liquid nutrient medium.

Inocula were prepared by suspending a fresh fungal culture, grown in Sabouraud's 2% (w/v) glucose agar (Merck, Germany) for 18–24 h at 37 °C, in sterile pH 7.4 physiological saline. The density of the inocula was adjusted to 0.5 McFarlands units (5×10^5 CFU/mL) using nephelometer (bioMerioux, France) and then diluted 10 times with the liquid nutrient medium. Stock solutions of ECN and ECN–CD complexes were prepared by dissolving appropriate amounts of the substances in 5% (v/v) dimethyl sulfoxide (DMSO) in order to obtain a final drug concentration of 320 μg/mL. A stock solution in liquid nutrient medium of amphotericin B at the same concentration was prepared as positive control.

Serial two-fold dilutions of 100 μL of the prepared stock solutions in liquid nutrient medium were performed in 96-well flat-bottom microtiter plates (Kartell, Italy). A 100 μL of yeast inoculum was then added to each well of the microdilution trays, in order to obtain final drug concentration ranging from 32 to 0.008 μg/mL and a final inocula of 2.5×10^5 CFU/mL. The inoculated plates were incubated at 35 °C for 24 h. After that, the viability of *C. albicans* and *C. krusei* was analysed using, respectively, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma-Aldrich, USA), and 2,3,5-triphenyl-tetrazolium chloride (TTC, Sigma-Aldrich, USA), following previously described procedures (Ohara & Saito, 1997; Yang et al., 1998). Briefly, MTT test was performed by adding 50 μL of MTT solution (1 mg/mL in PBS) to each well; after 3 h incubation at 37 °C in 5% CO_2, formazan crystals formed by MTT metabolism were dissolved by addition of 50 μL DMSO. Similarly, TTC test was performed by adding 50 μL of TTC solution (50 mg/mL in PBS) to each well of plates cultured with *C. kruzei*, followed by 3 h incubation at 37 °C and subsequent addition of isopropyl alcohol containing 4% HCl (v/v). In all cases, the absorbance was measured at 560 nm using iEMS MF Microplate reader (Thermo Lab Systems, USA). The MIC values of ECN, alone or as CD complex, and of amphotericin B were then calculated as indicated by the EUCAST guidelines (Rodriguez-Tudela et al., 2008).

2.4. Time-kill assay

The time-kill assay was performed with *C. albicans* ATTC 90028 and *C. krusei* ATTC 6258 as model fungal strains. 1 μL of the inoculum suspension in physiological saline of 2 McFarland units (2×10^7 CFU/mL) was added to 19 mL of a liquid nutrient medium, containing 1 μg/mL of the drug, free or as binary (ECN–CD) or ternary (ECN–CD–CA) complex. In the case of the wafer formulation, it was added to the inoculum suspension in the liquid nutrient medium, while a drug-free inoculated nutrient medium served as a positive control. The culture flasks were incubated at 35 °C. At predetermined time points (0, 1, 3, 6 and 24 h) a 100 μL aliquot was removed from each culture flask, and the viability of *C. albicans* and *C. krusei* was analysed measuring the mitochondrial dehydrogenase activity using TTC and MTT tests, respectively, as described previously. The viability at each time point was calculated according the following equation:

$$\text{viability} = \frac{A_{560 \text{ of the sample}}}{A_{560 \text{ of the drug-free control}}} \times 100$$

2.5. Preparation of the lyophilised wafers

Wafer formulations were prepared by lyophilisation of gels containing LMAP CF005 or LMAP CF020 (1–8% w/w) in different w/w mixtures with CMC (0.5 to 5% w/w). For the gel preparation, a weighed amount of the selected ECN–SBEßCD–CA complex was dissolved in distilled water, to achieve a final drug concentration of 1% w/w; then, the required amounts of polymers were added and dispersed by vigorous stirring in a thermostated bath (90 °C) until a homogenous gel was formed. Aliquots of the obtained gels were then poured into polystyrene blisters, frozen to −18 °C and freeze-dried using a Lyovac GT2 freeze-drier (SRK System Technik GmbH, Germany), to produce porous lyophilised discs (wafers). All wafers prepared were 12 mm in diameter and contained 7 mg of the drug. All samples were stored in a desiccator until further analyses.

2.6. Characterization of the lyophilised wafers

The mucoadhesive strength of the wafers was determined using a tensile test based on a slightly modified version of the two-arms balance method (Nair, Kumria, Harsha Attimarad, Al-Dhubiab, & Alhaider, 2013). Porcine gastric mucosa, used as a model mucoadhesive tissue, was obtained from a local slaughterhouse and treated following a previously described protocol (Jackson & Perkins, 2001). For the tensile test, pieces of the excised tissue were mounted on suitable tissue holders with the mucosal side up. Wafers were then sandwiched between two separate tissue holders and one side of the holder was attached to one of the two-arms balance, while the other one was mounted on a mobile support. The mobile support was then carefully raised to counterbalance the weight of a plastic container attached to the second arm of the balance. After a preset contact time (6 min), water was added to the plastic container at a constant rate of 50 mg/s, by a peristaltic pump. The weight of water required to detach the two surfaces, expressed as mg/cm^2, was recorded and results were presented as mean of at least 5 measurements (*C.V.* < 1%).

The *in situ* residence time of the wafers was measured *in vitro* using a dynamic test as described by Kockisch, Rees, Young, Tsibouklis, and Smart (2003), with some modifications. A piece of isolated porcine gastric mucosa was mounted on a suitable support, inclined at 30°; the wafer was then put on the mucosa by applying a light pressure for 20 s with the fingertip and exposed to a 3 mL/min simulated saliva flow generated by a peristaltic pump. The time necessary for complete wafer detachment/disintegration was recorded results were presented as mean of at least 5 measurements (*C.V.* < 1%).

The structural characterization of the optimized wafer formulation was carried out by using a FEI Quanta200^{TM} environmental scanning electron microscope (ESEM), with a spatial resolution at 30 kV of 3 nm (FEI Company, Oregon, USA).

2.7. In vitro drug release test

The *in vitro* drug release profiles from the wafers were determined using a laboratory flow-through system simulating the conditions present in the mouth (Patel, Liu, & Brown, 2012). The system consisted of a Whatman Grade 1 filter paper (Sigma-Aldrich, Italy) placed on a wire mesh fixed over a 200 mL beaker, thermostated at 37 °C. Different wafer formulations, each containing 7 mg of the drug, were placed on the top of the membrane filter and exposed to a simulated saliva flow of 3 mL/min, generated by a peristaltic pump. The eluate in the beaker was magnetically stirred at 300 rpm. At predetermined time intervals (10, 30, 50 and 70 min), aliquots of the eluate were sampled with a syringe and the drug content was analysed by HPLC as described below.

2.8. HPLC assay of econazole nitrate (ECN)

HPLC assay of ECN was performed using a Merck Hitachi Elite Lachrom apparatus equipped with a L2130 model isocratic pump, a L-2400 UV–vis spectrophotometric detector, a Rheodyne injector fitted with a 20 μL loop and a reversed phase Hibar RT C18 column (5 μm particle size; 150 mm × 4.6 mm). A 80:20 (v/v) mixture of

methanol and 0.01 M aqueous KH_2PO_4 solution was used as mobile phase; the flow rate was 1 mL/min, the oven temperature was 25 °C, and the detection wavelength was set at 230 nm. The retention time of ECN under these experimental conditions was 4.1 min. The working range of the method was between 3.3 and 33.0 μg/mL with a regression coefficient of 0.9992, and the linear regression equation was $y = 144{,}400x - 7779$. The detection (LOD) and quantification (LOQ) limits were 0.05 and 0.17 μg/mL, respectively.

2.9. Software for experimental design

The software NEMRODW was used for the generation and evaluation of the statistical experimental design (Mathieu, Nony, & Phan-Tan-Luu, 2000). Analysis of variance (ANOVA) was applied for testing the significance and validity of the models.

3. Results and discussion

3.1. Evaluation of antifungal efficacy of ECN and its CD complexes

In a previous publication, we have prepared a series of co-ground complexes of ECN with different CD derivatives, aimed to increase the drug solubility and dissolution characteristics in the simulated saliva, thus increasing its potential for an efficient delivery to the oral cavity (Jug et al., 2014). The most effective CDs were HPβCD and SBEβCD, which enabled an increase of ECN dissolution efficiency of 8.3 and 22.13 times, respectively. Moreover, a ternary equimolar complex of ECN with SBEβCD and CA was found to be the most efficient, increasing the drug dissolution efficiency up to 66.62 times (Jug et al., 2014). However, prior the final selection of the best complex to use in the wafer formulation, the antifungal efficacy of the most promising systems against selected *Candida* strains was evaluated in order to individuate the complex with the highest therapeutic potential. As first step, the minimal inhibitory concentration (MIC) of ECN and its different co-ground complexes was determined using the two-fold broth microdilution method. The MTT and TTC assays were used to estimate the fungal viability after exposure to the samples tested (Ohara & Saito, 1997; Yang et al., 1998). The MIC values obtained for pure ECN and amphotericin B (used as positive control) for both *Candida* strains tested (Fig. 1A) are in agreement with literature data obtained by other methods (Canton, Peman, Gobernado, Viudes, & Espinel-Ingroff, 2004; Kokjohn, Bradley, Griffiths, & Ghannoum, 2003; Murat et al., 2013), thus confirming the validity of our approach. The MIC values of binary (ECN–CD) and ternary (ECN–CD–CA) complexes varied somewhat compared to that of the pure drug, but in mayor cases the observed difference was not statistically significant ($p > 0.05$). Exceptions were observed only in case of MIC values for ECN–EPIβCD against *C. albicans* and ECN–SBEβCD–CA against *C. krusei* ($p < 0.01$). However, it should be considered that MICs of azole antimycotics usually do not appear as a well defined singe value, as it is in case of amphotericin B, but rather as a concentration range (Rodriguez-Tudela et al., 2008).

Therefore, in order to further investigate the therapeutic potential of the different ECN–CD complexes against the selected *Candida* strains, antimycotic activity was monitored as a function of time, using a time-kill assay. This is an indispensable tool for assessing the antimicrobial activity of drugs against bacteria and fungi, allowing the estimation of such activity as a function of time, and therefore offering a more reliable prediction of the therapeutical outputs with respect to the simple determination of the minimal inhibitory concentration (Pappalardo et al., 2009).

The obtained time-kill curves are presented in Fig. 1 B and C. The results showed that the antimycotic effect of the ECN–CD complexes was species-dependent. In fact, the viability of *C. albicans* started to decrease immediately upon exposure to ECN and its CD complexes (Fig. 1B), while in case of *C. krusei* (Fig. 1C), an initial lag-phase was observed during the first 4 h, followed by a decrease in viability during the subsequent hours. Furthermore, the efficacy of ECN alone against *C. albicans* started to decrease after 6 h of the experiment, while ECN–CD complexes were more effective (Fig. 1 B). In case of *C. krusei*, such an effect was not observed and all tested samples showed comparable efficacy (Fig. 1C). The time necessary to obtain the complete eradication of the selected *Candida* strains was calculated by extrapolation of the second straight portion of the time-kill curves and the results obtained for the tested samples are presented in Table 1. Although the values in case of *C. krusei* were somewhat variable, there were no statistically significant differences between the results obtained for ECN alone and its CD complexes ($p > 0.05$). Different results were instead obtained in case of *C. albicans*, where the binary ECN–SBEβCD and even more the ternary ECN–SBEβCD–CA complexes showed significantly higher antimycotic efficacy compared to that of ECN alone ($p < 0.01$ and $p < 0.001$, respectively).

Table 1
Time necessary to achieve the complete eradication of *C. albicans* and *C. krusei*, determined by time-kill assay.

Sample	Time-kill (h)	
	C. albicans	*C. krusei*
ECN	48.97 ± 5.04	34.38 ± 1.30
ECN/HPβCD	43.01 ± 5.27	40.40 ± 5.63
ECN/HEβCD	38.27 ± 1.85	32.73 ± 3.06
ECN/EPIβCD	41.34 ± 4.63	34.88 ± 2.69
ECN/SBEβCD	38.13 ± 3.74[a]	36.02 ± 1.15
ECN/SBEβCD/CA	32.82 ± 0.70[b]	36.28 ± 4.45

[a] Statistically significant difference compared to ECN ($p < 0.01$).
[b] Statistically significant difference compared to ECN ($p < 0.001$).

Taking into account this result, we have selected the ternary ECN–SBEβCD–CA complex as the most effective product to use for further development of the wafer formulation, also considering its superior *in vitro* dissolution properties (Jug et al., 2014).

3.2. Development of lyophilised wafer formulation by use of experimental design

The design of experiments was utilized for wafer formulation development and optimization, in order to systematically evaluate the effect of variations of its composition on its performance and to obtain a product of known and predefined quality (Mennini et al., 2012). With this purpose, two different experimental design strategies have been applied: an initial screening design, aimed to evaluate the influence of level variations of the selected factors on the chosen responses, and thus to eventually reduce the number of factors and/or adjust their variation range, followed by a central composite design, aimed for the formulation optimization.

The critical process variables and the proper responses to evaluate the product quality were initially defined. In the screening design, the % and type of LMAP and the % of CMC were selected as the critical independent variables, while the wafer mucoadhesive strength, *in situ* residence time and the % of drug released after 10 min were chosen as the most important responses for evaluating the performance of the different wafer formulations.

The experimental domain of each of the continuous independent variables selected was set on the basis of preliminary examinations, mainly based on technological factors. In particular, the LMAP and CMC concentration ranges were established, respectively, between 1.0–8.0% w/w and 0.5–5.0% w/w since amounts of the polymers higher than the respective superior limits gave rise to formation of very viscous gels, difficult to be homogeneously poured in the stamps for lyophilisation; on the other hand, amounts

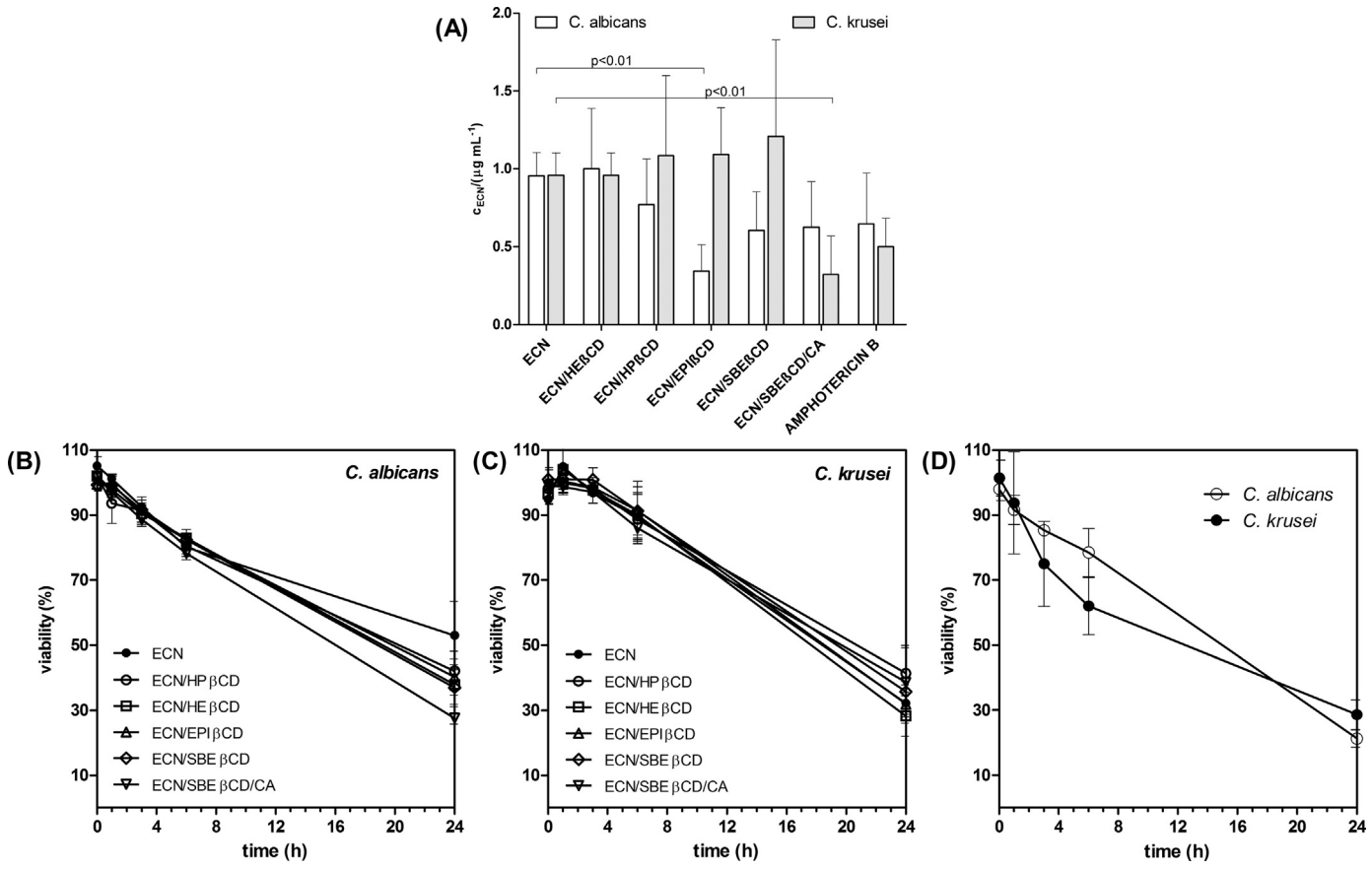

Fig. 1. Antifungal activity of the tested samples against *C. albicans* and *C krusei*: (A) minimal inhibitory concentrations of econazole nitrate (ECN), its equimolar co-ground binary and ternary complexes with cyclodextrins (CDs) and citric acid (CA); (B and C) related time-kill curves and (D) time-kill curves of *C. albicans* (open symbols) and *C. krusei* (closed symbols) treated with the optimized ECN wafer formulation developed (mean ± S.D. $n = 9$). Amphotericin B served as positive control.

of the polymers lower than the respective inferior limits did not allow the gel formation. As for the categorical factor, *i.e.* the LMAP type, since various kinds of LMAP, with different reactivity and setting rate, are available on the market so it was considered worthy of interest to evaluate and compare the effect of two different LMAPs (LMAP CF005 or CF020) at different amidation and esterification degrees. It is important to stress out that those commercially available pectin types have standardised and constant gelling properties, obtained through addition of glucose. The continuous factors were instead studied at four levels. Thus, the independent variables and their respective levels were:

U_1 = LMAP CF005 or LMAP CF020;

U_2 = % LMAP (1.0, 3.0, 6.0, 8.0% w/w);

U_3 = % CMC (0.5, 2.0, 3.5, 5.0% w/w)

The following Free–Wilson model was postulated among responses and factors under study, that contained one constant plus, for each factor, a number of terms equal to its number of levels minus one (Broudiscou, Leardi, & PhanTanLuu, 1996):

$$Y = b_0 + b_{1A}(X_{1A}) + b_{2A}(X_{2A}) + b_{2B}(X_{2B}) + b_{2C}(X_{2C}) + b_{3A}(X_{3A}) + b_{3B}(X_{3B}) + b_{3C}(X_{3C})$$

where Y represents the response; b_0 is the constant term, b_1A is the coefficient relative to the effect on the response of the level changes of the factor U_1, whereas b_2A, b_2B, b_2C and b_3A, b_3B, b_3C are the coefficients relative to the changes of level of the factors U_2 and U_3, respectively.

The coefficients of the model were estimated by a 16 run-screening asymmetric matrix, and the corresponding experimental plan is reported in Table 2. In compliance with this experimental plan, 16 different wafer formulations were then prepared and evaluated in a random order for both mucoadhesive strength, *in situ* residence time and % drug released after 10 min (Table 2). It is important to stress out that the addition of an equimolar CA amount to the binary ECN/SBEβCD complex resulted in the formation of a ternary ECN/SBEβCD/CA complex with superior solubility compared to the drug alone or its binary complexes tested (Jug et al., 2014). This allowed the complete dissolution of the required drug dose in the LMAP/CMC gels prior lyophilisation, thereby avoiding problems of poor drug dosage uniformity and homogeneity and/or unsatisfactory reproducibility in the drug release profile, which on the contrary might easily occur if the suspension of the drug or the binary complex was used.

Statistical evaluation of the experimental results by ANOVA (applied using a 1% significance level) showed that the postulated regression model was valid and significant for all the three responses, thus explaining the response variation due to the change in level factors (please see the Supplement, Table S1).

The graphic analysis of the effects was used to identify the factors whose variations determined significant effects on the responses (Furlanetto, Cirri, Maestrelli, Corti, & Mura, 2006). The results of the graphic analysis are shown in Fig. 2. The coefficients *A*, *B*, and *C* indicate the effects on each response of the change of level of the factors U_1, U_2, and U_3, respectively. In the series of graphs at the top of Fig. 2, the difference of effects between the considered levels for each factor is highlighted, which is directly related to the length of the bars. The differences which exceeded the reference

Table 2
Experimental plan and observed responses (mucoadhesive strength, % of drug released at 10 min and *in situ* residence time) for wafer formulations containing different percentages of low methoxy amidated pectin (LMAP) type CF020 or CF 005 and carboxymethylcellulose (CMC).

batch	variable U_1 LMAP type	variable U_2% LMAP	variable U_3% CMC	Y_1 mucoadhesive strength (g/cm^2)	Y_2% drug released at 10 min	Y_3 *in situ* residence time (min)
1	CF 020	1.0	0.5	16.03	61.46	28.01
2	CF 020	3.0	2.0	23.40	30.75	46.52
3	CF 020	6.0	3.5	26.06	22.61	70.30
4	CF 020	8	5.0	30.72	9.95	87.40
5	CF 005	1.0	2.0	17.05	34.12	37.22
6	CF 005	3.0	0.5	20.26	60.67	35.61
7	CF 005	6.0	5.0	27.24	10.17	80.34
8	CF 005	8.0	3.5	26.30	16.50	74.03
9	CF 020	1.0	3.5	19.90	24.96	51.44
10	CF 020	3.0	5.0	26.92	12.69	73.50
11	CF 020	6.0	0.5	22.90	56.45	44.35
12	CF 020	8.0	2.0	25.50	25.72	59.38
13	CF 005	1.0	5.0	22.11	15.53	60.51
14	CF 005	3.0	3.5	24.10	23.46	62.36
15	CF 005	6.0	2.0	23.97	27.33	53.24
16	CF 005	8.0	0.5	22.40	37.95	50.09

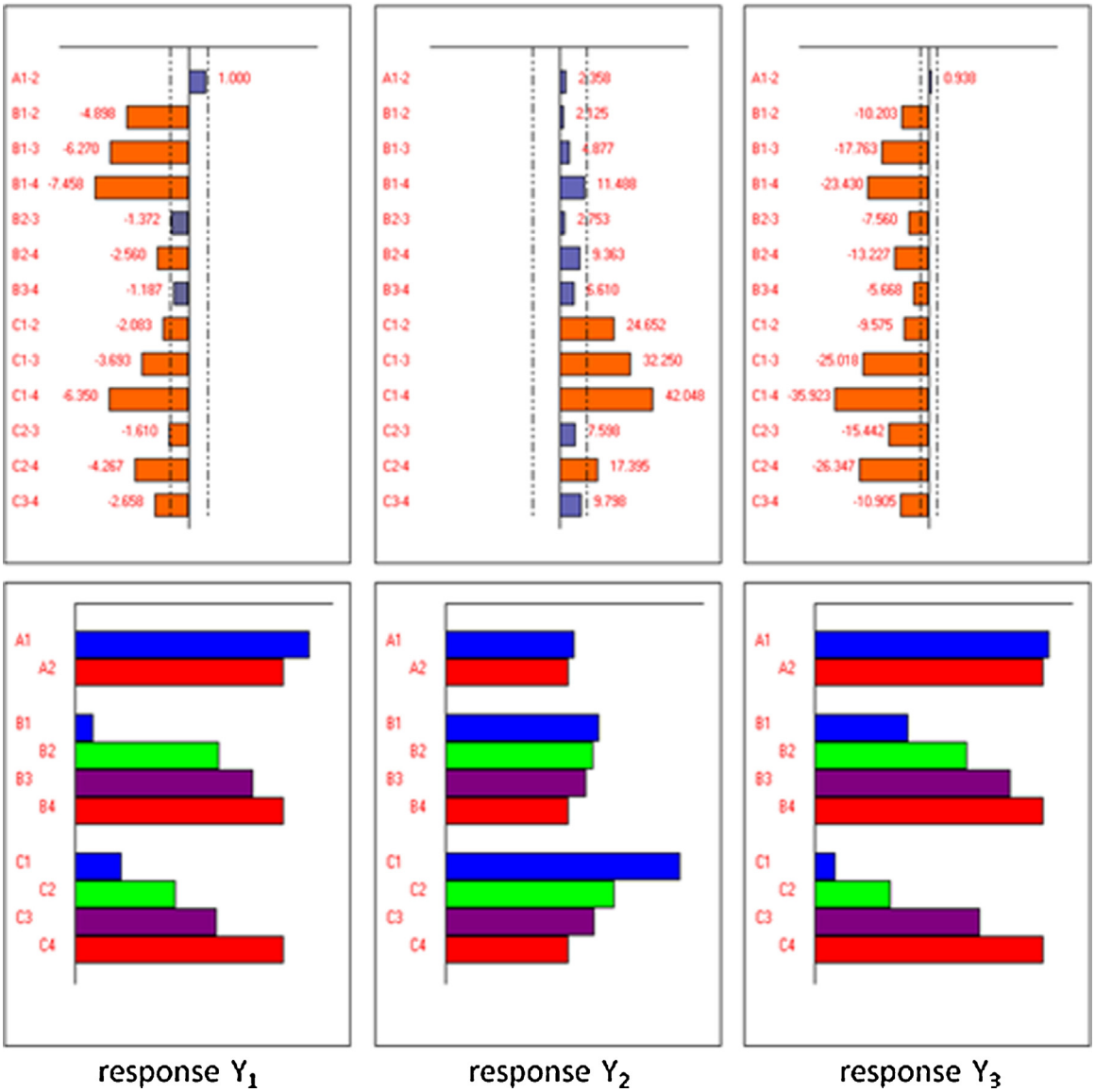

Fig. 2. Different weights of the different levels (top) and graphic analysis of the effects of the levels (bottom) of the examined factors LMAP type (A1–2); LMAP % (B1–4) and CMC % (C1–4) on the considered responses: Y_1 mucoadhesive strength (g/cm^2); Y_2% drug released at 10 min; Y_3 residence time (min).

dashed lines, (which define a 95% confidence interval), were significant on the selected responses. The series of graphs at the bottom of Fig. 2 shows instead the effect of the different levels of each factor: the longest bar corresponds to the level leading to the maximization of the response.

The obtained results showed that the LMAP type variation (CF020 or CF005) did not significantly influence the response Y_1, *i.e.* the mucoadhesive strength of the wafer formulations; on the contrary, it significantly increased with increasing the content of both LMAP and CMC. A very similar behaviour was observed when considering the response Y_3, *i.e.* the *in situ* residence time. On the other hand, changes of the LMAP type in the wafer formulation did not cause a significant variation of the response Y_2, *i.e.* the % of drug released at 10 min; a slight progressive decrease in drug release rate was instead observed with increasing the LMAP content, and this same effect was clearly more marked with increasing the CMC content.

Therefore, based on the results of the graphic analysis of the effects, it was deduced that the factor U_1 (LMAP type) can be excluded in the following phase of formulation optimization, since its variation did not significantly influence any of the considered responses. LMAP CF020 was then selected for the following part of the study, due to its slightly better mucoadhesive strength than LMAP CF005.

Moreover, we considered that the response Y_2 (% drug released at 10 min) can be neglected in the subsequent step of formulation optimization. In fact, even if it significantly decreased with increasing the LMAP and particularly the CMC content in the wafer formulation, the lowest value obtained for the % drug released at 10 min, given by the formulation with the highest levels of both polymers (8% LMAP CF020 and 5% CMC), was around to 10% of the drug wafer content (Table 2); this value corresponded to a concentration of more than 20 μg/mL, well above the MIC value of ECN found in microbiologic studies (Table 1). Furthermore, results of release studies indicated that all the examined formulations allowed a complete drug release within at maximum about 70 min, which can be considered a suitable time, compatible with the desired *in situ* residence time of the wafer.

Finally, since maximization of both mucoadhesive strength and residence time requested high levels of both factors U_2 (LMAP %) and U_3 (CMC %), it was considered opportune to investigate in greater detail the effects of these factors, limiting their variation range around their higher levels, *i.e.* between 4.5–8.5% and 2.5–5.5% w/w, respectively.

For the subsequent step of formulation optimization, a central composite design has been applied, using a 2-levels factorial design, according to the following model:

$$Y = b_0 + b_1(X_1) + b_2(X_2) + b_{11}(X_1 \times X_1) + b_{22}(X_2 \times X_2) + b_{12}(X_1 \times X_2)$$

where Y represents the response; b_1 and b_2 are the coefficients relative to the effects on the response of the level changes of the factors U_1, (% LMAP CF020) and U_2, (% CMC), respectively, whereas b_{11}, b_{22}, are the quadratic effects and b_{12} is the coefficient relative to the interactions among the variables.

The Doehlert design was selected for an accurate estimation of the coefficients of the model and for a precise, in depth description of the investigated problem through the study of the relative response surfaces. The number of experiments required by a Doehlert design is $k^2 + k + n$, where k is the number of factors and n the number of replicates at the centre of the experimental domain. The experiments needed in this case where 12, including four replicates (Table 3). In compliance with this plan, nine distinct wafer formulations were prepared and examined in a random order for both mucoadhesive strength and residence time properties. The experimental plan and the obtained responses are reported in Table 3. Analysis of variance (ANOVA), applied using a 1% significance level (Supplement, Table S2) showed that the postulated regression model was significant and valid for both the examined responses (Lewis, Mathieu, & Phan-Tan-Luu, 1999), meaning that the found relationship was actually able to describe the response variation in function of factors variations. Statistic analysis of the coefficients indicated that both the responses were significantly influenced by varying the levels of the variables ($p < 0.01$), while no significant interactions between the factors were evidenced.

Three-dimensional response surface plots were then generated, using the statistical model obtained from multiple regression analysis, in order to investigate in depth the effects of changing the independent variables on the considered responses (Fig. 3). The mucoadhesive strength increased with increasing the % of LMAP and CMC, confirming the results of the preliminary screening study; however, satisfying values were obtained within the entire explored experimental domain, ranging from a minimum of 25.32 up to a maximum of 28.37 mg/cm^2. Also the *in situ* residence time increased with increasing the % of the two polymers, but this response was more sensitive to variations of the factors, varying from a minimum of 63.79 to a maximum of 88.01 min.

A desirability function was finally applied in order to find the best values for the variables so that to simultaneously maximize both mucoadhesive strength and residence time. With this aim, each response was associated with its partial desirability function, which varied from 0 to 1 as a function of its closeness to its desired value (Lewis et al., 1999); the partial desirability functions were subsequently combined together as geometrical mean to obtain the overall desirability function. Moreover, it was decided to assign a double weight to the response "residence time" with respect to the "mucoadhesive strength", considering its basic importance for the formulation effectiveness. As for the partial desirability functions, 28–30 mg/cm^2 and 80–100 min were selected as the ranges of desired values for the mucoadhesive strength and the residence time, respectively. The values to give to the variables for optimizing at the same time both the responses, as calculated by the overall desirability function, were LMAP 7.3% w/w and CMC 5.2% w/w, and the predicted values of the responses were a mucoadhesive strength of 28.39 mg/cm^2 and a residence time of 88.2 min.

In order to validate the predictive ability of the model, the agreement between predicted and experimentally measured responses was verified. A wafer formulation was then prepared according to the optimized conditions and characterized for mucoadhesive strength and residence time. The obtained experimental values (mean of three replicates) were 28.37 ± 0.04 mg/cm^2 for the mucoadhesive strength and 88.1 ± 0.1 min for the *in situ* residence time. The confidence interval for each predicted response, at a probability level of 95%, was calculated using mean and standard deviation obtained from the replicates carried out with the predicted optimum conditions, and were 28.39 ± 0.03 mg/cm^2 for the mucoadhesive strength and of 88.2 ± 0.1 min for the residence time. As it is evident, in both cases the experimental values were inside the confidence interval of each predicted response, thus indicating statistical equivalence between the experimental data and the predicted ones, and confirming the validity of the applied model.

Furthermore, the combination of the polymers in the optimised ratio clearly improved the mucoadhesive properties of wafers prepared. In fact, the mucoadhesive strength and *in situ* residence time of control wafers prepared with pure LMAP CF 020 were 22.62 ± 0.15 g/cm^2 and 52.5 ± 0.1 min, while for control wafers prepared with pure CMC, values of 24.84 ± 0.56 g/cm^2 and 73.6 ± 0.2 min were obtained. When compared with the values of the corresponding parameters obtained for the optimised wafer formulation, a significant increase in mucoadhesion can be

Table 3
Experimental plan of Doehlert design and observed responses (mucoadhesive strength and *in situ* residence time) for the new series of the wafer formulations.

batch	variable U_1% LMAP	variable U_2% CMC	Y_1 mucoadhesive strength (g/cm^2)	Y_2 *in situ* residence time (min)
1	5.4	3.0	25.63	64.43
2	7.6	3.0	26.20	70.08
3	5.4	5.0	27.38	81.72
4	7.6	5.0	28.30	87.30
5	4.9	4.0	26.54	74.36
6	8.1	4.0	27.46	80.01
7	6.5	2.6	25.32	63.79
8	6.5	5.4	28.27	88.01
9	6.5	4.0	26.79	75.90
10	6.5	4.0	26.85	77.00
11	6.5	4.0	26.68	76.20
12	6.5	4.0	26.75	76.00

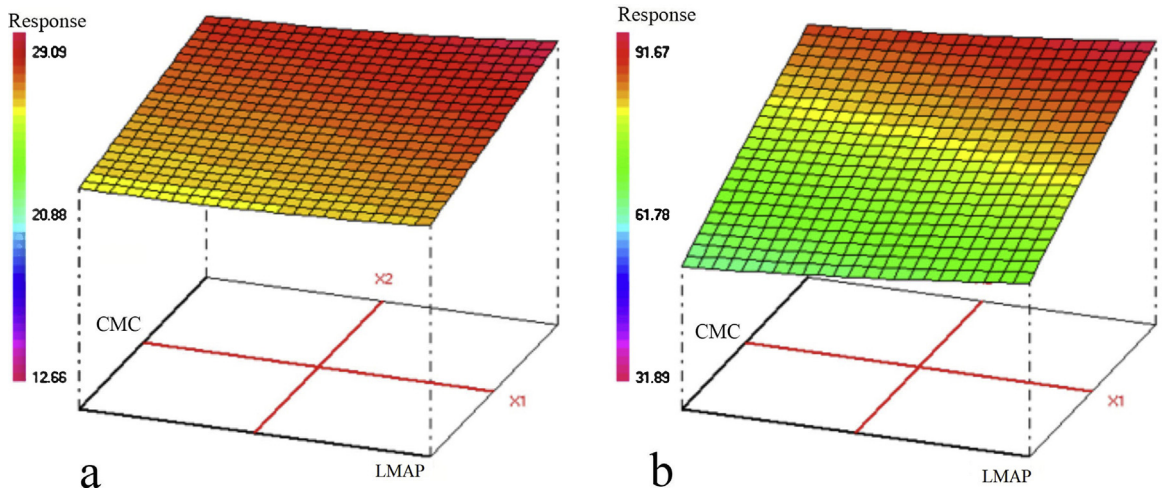

Fig. 3. Response surface plots of the effect of variations of % LMAP and % CMC on the (a) mucoadhesive strength (g/cm^2) and (b) *in situ* residence time (min) of the wafer formulations.

observed ($p < 0.05$), probably due to inter-polymers complex formation (Jug et al., 2012).

Representative ESEM images of the optimized wafer formulation loaded with ECN as ternary complex with citric acid and SBEβCD are shown in Fig. 4. ESEM analysis, being a non-invasive technique not needing any pre-treatment of the sample, allowed a careful examination of the actual morphology of the wafer, maintaining unchanged its structural properties. Fig. 4A–C represents a vertical section of the wafer formulation observed at different magnifications. The images show a uniform, highly porous, sponge-like network, with several elongated pores responsible for the high rate of water access within the structure. No crystals of the drug were detectable, also at the highest magnification, confirming that it maintained its amorphous structure and was homogeneously dispersed within the polymeric network structure. This might be attributed to the high stability and solubility of the ternary ECN/SBEβCD/CA complex, which dissolved completely in the LMAP/CMC gel during the wafer preparation, and remained stable over time. Addition of a single drop of simulated saliva to the wafer (Fig. 4D) immediately changed its aspect, giving rise to an evident swelling phenomenon, confirming the high hydration capacity of the highly porous interconnecting polymeric network.

The optimized formulation showed a zero-order release profile ($r^2 = 0.9971$; Fig. 5), assuring a constant release of drug at a rate of 5.14 ± 0.16 mg/h, during all the *in situ* residence time of the wafer, and then enabling the maintenance of therapeutic ECN levels during the whole wafer application time. A linear drug release was already observed in case of buccal patches based on *in situ* cross-linked matrices containing LMAP, Carbopol® and triclosan as inclusion complex with a polymeric β-cyclodextrin (Jug et al.,

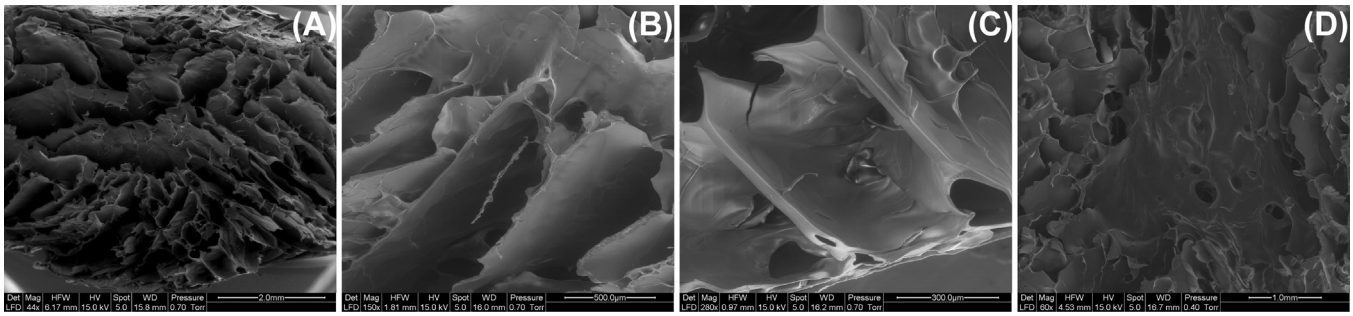

Fig. 4. ESEM images of the optimized wafer formulation loaded with ECN (as coground system with SBEßCD and citric acid). The images A (44×), B (150×) and C (280×) represent three different magnifications of a vertical section of the wafer; the image D (60×) shows the effect of addition of a drop of simulated saliva.

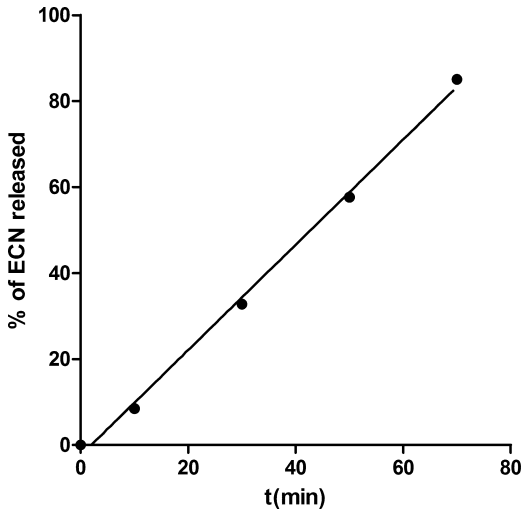

Fig. 5. Release profile of ECN (as coground system with SBEßCD and citric acid) from the optimized wafer formulation (mean ± S.D., $n = 3$).

2012). It was assumed that the high molecular mass of the complex formed restrained its diffusion through the swollen matrix; therefore, the overall drug release kinetic in such system was controlled by the complex dissociation, resulting in a zero-order release profile. Taking that into account, it might be presumed that, in the present case, electrostatic interactions between positively charged LMAP and negatively charged SBEβCD/CA in the ternary complex, restrained the diffusion of this highly soluble complex through the swollen LMAP/CMC matrix: as a consequence, the dissociation of the complex also controlled the overall ECN release rate, thus resulting in a zero-order release rate.

3.3. Evaluation of the antifungal efficacy of the optimized wafer formulation

The time-kill assay was used in order to assess the potential therapeutic efficacy of the optimised wafer formulation loaded with the ternary ECN–SBEβCD–CA complex. The obtained time-kill curve was practically superimposable to that of the pure ECN–SBEβCD–CA complex (Fig. 1D), and no significant differences ($p > 0.05$) were observed for the time necessary to obtain the complete eradication of the selected fungal strains (30.99 ± 1.23 h for *C. albicans* and 33.01 ± 4.01 h for *C. krusei*) compared to that of pure ternary system (see Table 1). These results proved that incorporation of the ternary ECN–SBEβCD–CA complex into the wafer formulation did not affect its antimycotic activity, which remained significantly higher ($p < 0.001$) with respect to that of pure ECN.

4. Conclusions

Preliminary antimicrobial efficacy studies allowed identification of the ternary system ECN–SBEßCD–CA as the most therapeutically effective, and then it was selected for incorporation into the mucoadhesive buccal delivery system.

Experimental design was successfully used in the development of a freeze-dried buccal wafer formulation of ECN, endowed with predictable mucoadhesive and *in situ* residence properties. In particular, graphic analysis of the effects enabled identification of the formulation factors affecting the considered responses, and determination of their best levels for the response optimization. Moreover, the subsequent response-surface study allowed the simultaneous evaluation of the effects of the selected variables on the responses. Finally, the use of the desirability function allowed for finding the values of the variables enabling the simultaneous maximization of both mucoadhesive strength and in situ residence time. The experimental values of the responses obtained from the optimized wafer formulation were very close to the predicted ones, thus demonstrating the actual reliability and usefulness of the assumed model in the preparation of buccal wafers with optimized and predictable properties, suitable for assuring a prolonged permanence *in situ* of the device and a controlled drug release at level of the oral cavity.

Furthermore, the results obtained by the time-kill assay of the optimized wafer formulation demonstrated its therapeutic efficacy, thus confirming its good potential as a novel effective topical delivery system able to improve the existing therapy of oral candidiasis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.11.065.

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