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ORIGINAL ARTICLE

Oral sustained release nystatin tablets for the treatment of oral candidiasis: formulation development and validation of UV spectrophotometric analytical methodology for content determination

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Abstract

Objective: In this study, oral sustained release mucoadhesive nystatin tablets were developed to increase nystatin contact time with the oral cavity and mask its unpleasant taste. *Methods*. The best formulation studied included sustained release agents and it was submitted to physical-mechanical characterization, taste assessment and clinical test in twelve patients. The ultraviolet-visible nystatin methodology was also developed and validated in parallel as an alternative to the pharmacopoeial microbiological dosage method. *Results*. The best formulation developed in this study included sustained release agents. The efficacy of this formulation was verified through a clinical assessment, showing that this formulation is more effective (100%) than the commercial oral nystatin suspension used traditionally (50%). Moreover, the UV absorption spectrophotometry method developed to validate the methodology for nystatin content analysis for new oral tablets was shown to be specific, linear, exact and reproducible, as recommended by the ICH regulations. *Conclusion*. The oral nystatin tablets developed showed to present faster therapeutic response than the oral aqueous solution through the preliminary clinical assays. The UV absorption spectrophotometry method showed to be an attractive test for the usual routine in the pharmaceutical industry.

Key words: *Nystatin; candidiasis; oral sustained release tablets; mucoadhesive; clinical efficacy*

Introduction

Fungal infections caused by the genus *Candida* are known as candidiasis, a condition which has *Candida albicans* as its primary etiologic agent[1–3]. Oral candidiasis may affect healthy patients but is more severe in immunocompromized patients[4]. Oral tissue is one of the most common sites for this opportunistic infection[3,5]. Candidiasis does not usually cause complications, but it must be treated to avoid long, chronic, or systemic infection. It might interfere with the patients' nutrition because of loss of appetite, impaired hydration, and food consuption[3,6].

Oral candidiasis usually responds well to topical treatment in imunocompetent patients[5,7]. It also avoids adverse effects caused by systemic treatment and the

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possibility of drug interaction[5,8]. Therapeutic agents for candidiasis include polyenes (nystatin and amphotericin B) and azoles (itraconazole, miconazole, and clotrimazole)[2,5,9]. The topical forms commercialized worldwide for oral treatment are available as rinsing solutions, suspensions, lozenges, tablets, creams, and gels[1,2,5,6,8,10–14].

Nystatin is the first-choice drug for the treatment of oral candidiasis. It is an antifungal drug extracted from *Streptomyces* cultures—found by Hazen and Brown in 1950[8,15–17]. Increased use of nystatin is associated with the growing number of candidiasis cases in patients with cancer, AIDS, and other systemic diseases[6,12,15,18,19]. Nystatin is effective against most infections caused by *Candida* spp. Its pharmacological activity comes from the interaction with ergosterole—a sterol found in the plasma membrane of fungal cells—with subsequent functional disorder of the membrane[8,16,20], resulting in yeast death[8,15,19–21].

For topical treatment of oral, oropharyngeal, and esophageal candidiasis, nystatin is commercialized in Brazil as oral suspension. The tablets for this active are indicated for intravaginal use[8,9]. The oral suspension contains 100,000 IU nystatin for each milliliter and is generally administered four times a day as a mouthwash with subsequent swallowing[8,9]. In other countries, nystatin can come as medicinal lozenges and oral tablets. These forms are usually obtained for rapid dissolution in the mouth with a lower performance than the suspension. Because nystatin efficacy is associated with contact time between drug and oral mucosa, mucoadhesive oral tablet-type pharmaceutical forms might deliver better efficacy for the therapy of oral candidiasis[22]. At the same time, a sustained release of the active from the tablet may represent a technological breakthrough for the treatment of oral candidiasis[23].

In this context, there is a need for the development of oral pharmaceutical forms that allow longer contact between nystatin and the oral mucosa, which may result in a more effective treatment of oral candidiasis[2,12]. Thus, the purpose of this study is to develop sustained release oral mucoadhesive nystatin tablets for the treatment of oral candidiasis. The ultraviolet (UV)-visible nystatin methodology was also developed and validated in parallel as an alternative to the pharmacopoeial microbiological dosage method[24], which is slower, more expensive, and has lower reproducibility.

Materials and methods

Materials

Carboxymethylcellulose (Galena); carrageenan (FMC Corporation, Sandvika, Norway); vanilla essence (Farmos, Rio de Janeiro, Brazil); magnesium stearate (Farmos); hydroxypropylmethylcellulose 50cps (Chitosan Sigma-Aldrich, St. Louis, MO, USA); maltose (Farmos); menthol (Farmos); nystatin (Genix and Neo Quimica, Rio de Janeiro, Brazil); Noveon AA1 (Nuveon BFGoodrich Company, Cleveland, OH, USA); chitosan, 400 to 2000 Da (Sigma-Aldrich); sodium saccharin (Farmos); crystalline sorbitol (Merck Darmstad, Darmstadt, Germany); glacial acetic acid P.A. (Vetec, Rio de Janeiro, Brazil); agar (Peptone Oxoid, Hampshire, UK); sodium chloride (Vetec); dextrose (Merck); dimethylformamide P.A. (Vetec); meat extract (Merck); yeast extract (Merck); potassium phosphate monobasic P.A. (Merck); potassium phosphate dibasic P.A. (Merck); methanol P.A. (Vetec); peptone (Oxoid); *Saccharomyces cerevisae* ATCC 2601 (Brazilian pharmacopoeia standard); sorbitol aqueous solution, 70% (Galena).

Nystatin assay

Nystatin content analyses, both in raw-material and in pharmaceutical forms prepared during validation studies for the newly developed methodology, were carried out by means of microbiological assay in agar diffusion in accordance with the US Pharmacopoeia (USP)[24]. Raw-material complementary analyses, such as identification and pH, also followed the descriptions in the USP. The following procedure was used for assay: nystatin-sensitive microorganism *S. cerevisae* ATCC 2601[25]; inoculation in *Petri* dishes $8 \times 6 \times 10$ mm (inner diameter, outer diameter, and length); stainless steel cylinders were placed in the dishes where both standard nystatin and raw-material nystatin solutions were added. Inhibition zones formed by nystatin test solution were measured and compared with standard nystatin solution standard curve[24].

Development of the UV spectrophotometry nystatin assay

Considering the long time needed for analysis and the high cost of the microbiological technique recommended by the USP[24], the use of UV absorption spectrophotometry was proposed not only to determine nystatin tablet content but also its percent dissolution (dissolution test). Thus, a solution was prepared by transferring approximately 50 mg nystatin to a 100.0-mL volumetric flask to which 25.0 mL methanol and 5.0 mL glacial acetic acid were added for total solute solubilization. Methanol was used to adjust the volume of this solution. A 2.0-mL aliquot was removed from this solution and transferred to a 100.0-mL methanol-adjusted volumetric flask. Final solution concentration was 10 μg/mL. Solution absorbance was measured at 279 nm.

Validation of UV spectrophotometry quantitative determination of nystatin

To validate the analytical method proposed, the parameters investigated were linearity, accuracy, precision, and specificity, according to the International Conference on Harmonization recommendations[26].

Linearity was tested at a concentration range of 60%, 80%, 100%, 120%, and 140%, with 100% corresponding to 91 mg nystatin in a 100.0-mL solution. A calibration curve was built and the method linearity was evaluated by its correlation coefficient and interception value, calculated in the corresponding statistic study (ANOVA) ($p < 0.05$).

Accuracy was evaluated by analyzing three spiked samples in three levels 80%, 100%, and 120% of working concentration, using three preparations (A, B, C) for all levels, which were tested three times.

The method precision was tested at two levels including repeatability and intermediate precision. The repeatability was tested using six replicated samples at a concentration of 100% (0.91 mg/mL) of the regular analytical working concentration. Intermediate precision expresses the variations among laboratories and was assessed nine times by using different equipment, analysts, and days to analyze three samples[27].

Samples containing 100% drug (91 mg nystatin) and 100% matrix placebo spiked with drug, using the best formulation developed in this study, were prepared to assess microbiological and UV method selectivity. The results observed with each solution were compared and variations below 2% were accepted. The inhibition zone and UV absorption spectra were also determined using the placebo in order to observe some matrix formulation interference in the analyses.

A solution containing work concentration of nystatin was exposed to sunlight for 5hours and to 50°C for another 5hours to ensure the solution stability.

Oral nystatin tablet formulations

In all nystatin tablets that were developed, the proposed 500,000 IU/tablet dosage was followed—this dosage is used in oral candidiasis pharmaceutical suspensions[8,27]. This concentration led to 91.0 mg of active mass per tablet (Table 1). Direct compression and wet granulation techniques were assessed as preparation techniques for the tablets to be developed. Sorbitol, maltose, and isomalt DC were investigated as excipients for these tablets given their high palatability and fast and total dissolution in water. Moreover, a knowingly efficient mucoadhesive excipient, chitosan, was added to increase adherence to nystatin tablets[28].

When using wet granulation, purified water was utilized as the granulation liquid. After that the formulation taste as well as disintegration and nystatin dissolution rate were adjusted. In this way, sweeteners and flavors were added to the external phase of the tablets simultaneously with the addition of a sustained release matrix (Table 2).

Table 1. Base formulation used to choose optimal nystatin tablet preparation technique.

	Ingredients	%	Quantity
Internal phase	Nystatin	9.1	91 mg
	Mucoadhesive	0.5	5.0 mg
	Polyol	89.5	0.895 g
External phase	Magnesium stearate	0.9	9.0 mg
Granulation liquid	Distilled water	—	7.6 mL

Mucoadhesive: chitosan; Polyol: sorbitol, maltose, or isomalt DC.

Table 2. Base formulation used for taste adjustment and physical-chemical properties of nystatin tablets.

	Excipients	%	Quantity
Internal phase	Nystatin	15.5	91.0 mg
	Crystalline sorbitol	77.0	452.5 mg
	Saccharine	0.46	2.7 mg
	Menthol	0.46	2.7 mg
	Vanilla essence	0.46	2.7 mg
External phase	Magnesium stearate	0.92	5.4 mg
	HPMC 50 cps	2.6	15.0 mg
	CMC	2.6	15.0 mg
Granulation liquid	Distilled water	—	4.9 mL

Binding solution: distilled water;
Retarding agent: HPMC 50 cps and carboxymethylcellulose.

Physical-mechanical tablets characterization

The physical-mechanical characterization of the tablets was conducted in accordance with the USP description for tablets considering their hardness, friability, mean weight, and disintegration time[24]. Tablet assay was determined by microbiological methodology as described in USP and the newly developed and validated UV spectroscopy methodology[24]. Dissolution experiments were conducted using apparatus II (50 rpm), at 37°C with 400.0 mL as dissolution medium, which in this case was the enzyme-free artificial saliva. In 15-, 45-, and 60-minute intervals 10.0-mL aliquots were collected with no replacement. Aliquots containg 5.0 mL were removed from these solutions and then transferred to a 50.0-mL flask and diluted with artificial saliva. These new solutions were then filtered in a 0.22-μm millex and subsequently taken to the 279-nm spectrophotometer for absorbance reading. The proposed tests were based on previous examples of the literature for oral tablets[29–31]. The artificial saliva

formulation used has the following composition: 0.274 g potassium phosphate monobasic, 24.0 g aqueous sorbitol solution 70% (w/v) and purified water to complete the amount of 500.0 mL.

Taste assessment and clinical test on nystatin tablet

A clinical trial comparing nystatin tablet efficacy with commercial oral aqueous suspension is being carried out. The protocol was approved by the Institutional Review Board *of Hospital Universitário Clementino Fraga Filho* (Committee on Ethics and Research at the University Hospital *Clementino Fraga Filho*) at UFRJ under protocol 163/05 CEP. A pilot study has analyzed 12 patients who presented oral candidiasis. Patients were randomly assigned to two groups as follows: Group 1 was treated with nystatin suspension and Group 2 with nystatin oral tablet. Patients in Group 1 were told to rinse the mouth three times a day using 5.0 mL nystatin aqueous solution with a dosage of 100,000 IU/mL. Patients in Group 2 were told to slowly dissolve oral tablets in the mouth three times a day. Both groups received treatment for 1 week. After this period, they returned to the clinic to check the need for continuation of the treatment for one more week. The study finished at the end of a 14-day period.

Taste adjustment followed Lubbers and Guichard test[32]. It consisted of 12 healthy volunteers selected to distinguish perception and masking of the unpleasant taste in nystatin oral tablets for the newly developed formulation. Each volunteer received one oral tablet of a different formulation per day to be dissolved in saliva until total dissolution. The feedback on the taste ranking was registered in a questionnaire as sweet, acid, bittersweet, bad, very bad or repugnant, and as weak or strong[32].

Results and discussion

Method development and optimization

Nystatin raw-material assays were initially done using the microbiological method described in USP so as to guarantee that raw-material content used in developing formulations and validating analytical UV methodology would be determined by an established method using standard nystatin (batch 4980, Neo Quimica, Rio de Janeiro, Brazil). As observed, raw-material content was 101.57% with a positive identification test and pH value of 7.5, within the specified value. After that, raw material was UV-dosed at 279 nm with and without placebo addition. No interference was observed with the registered absorption spectrum (data not shown).

Method validation

Characteristic parameters for the regression equation of the UV method obtained from the least squares treatment of the results confirmed the good linearity of the method developed in the concentration range tested (Table 3). To test the apparent dispersion of the estimated experimental value variances, a Cochran homocedasticity test was performed. According to this statistical test, when G_{exp} is smaller than $G_{critical}$, homogeneity of the variances (i.e., homocedasticity) is admitted. In this study, homocedasticity was obtained, because G_{exp} was 0.344 whereas $G_{critical}$ was 0.684 (Table 3).

Accuracy was evaluated by the recovery of nystatin using three preparations (A, B, C) for all levels, which were tested three times. The individual recovery of nystatin ranged from 100.05% to 101.57%. The mean recovery data for each level (80%, 100%, and 120%) were within accepted values as presented in Table 4. These results indicated good accuracy of the method to determine nystatin.

Data presented in Table 5 show an RSD of 0.45, which indicated repeatability. Intermediate precision, which expresses the variations within laboratories, was

Table 3. Results of regression analysis of data for quantification of nystatin by UV methods.

Features	UV method
Range (ug/mL)	10–25
Regression equation[a]	$y = 0.0254x + 0.0142$
Correlation coefficient (r^2)	0.9995
Homocedasticity ($G = 0.684$)	0.344

[a] $y = bx + a$, where x is the concentration in μg/mL, y is the absorbance, a is the intercept, and b is the slope. Confidence limit of the intercept and slope (P = 0.05). Homocedasticity is confirmed because G_{exp} is smaller than $G_{critical}$.

Table 4. Results for accuracy test.

Level (%)	Concentration (μg/mL)	Recovery ± RSD (%)
80	14.80	101.51 ± 0.155
100	18.51	101.57 ± 1.39
120	21.85	100.05 ± 1.85

Table 5. Results for the 100% repeatability test.

Sample	Absorbance	C (μg/mL)	Purity (%)	Purity ± RSD (%)
1	0.458	17.47	98.00	
2	0.465	17.75	98.52	
3	0.468	17.87	98.17	98.56 ± 0.450
4	0.47	17.94	98.60	
5	0.471	17.98	98.81	
6	0.473	18.06	99.25	

Table 6. Results for the 100% intermediate precision test with other analysts and equipment.

Analysts and equipment	Sample concentration (μg/mL)	Purity (%)	Intra-day found ± RSD (%)	Inter-day found ± RSD (%)
1	16.65	98.46	98.62 ± 0.15	
	16.88	98.76		
	16.75	98.65		
2	16.96	99.19	99.62 ± 0.38	99.18 ± 0.52
	17.08	99.84		
	17.08	99.84		
3	17.12	99.06	99.29 ± 0.36	
	17.24	99.70		
	17.12	99.10		

Table 7. Tablet characterization for sorbitol and maltose formulations obtained by wet granulation.

Characterization		Sorbitol formulation	Maltose formulation
Weight (g)		1.024 ± 0.01142	1.005 ± 0.01537
Hardness (kgf)		10.60 ± 2.413	13.22 ± 3.008
Friability (%)		0.148	0.263
Disintegration (min)		11	11
Dissolution (%)	15 min	15.12 ± 2.041	15.10 ± 2.202
	45 min	18.10 ± 2.194	15.78 ± 2.426
	60 min	18.63 ± 2.047	17.68 ± 2.163

Mean ± RSD.

assessed nine times by using different equipment, analysts, and days. Accordingly, the results also indicated good intermediate precision for the method (Table 6).

Samples containing nystatin were analyzed using microbiological method and showed 101.57%. Samples containing nystatin and placebo matrix showed an increase of 0.62%. The same procedure was carried out using UV spectrophotometric methodology. After three determinations, 98.87 ± 0.23% of the drug was found. The sample with placebo presented an increase of UV absorbance of 0.82% with an RSD of 0.21%. The values are within the acceptable limits of variation. In both methods the matrix placebo does not present interference when the assay was determined without the drug. The solution with 100% of the active was exposed to sunlight for 5hours and heated to 50°C for another 5hours. UV-measured nystatin content was 101.2% with an RSD of 0.32 for the light exposure test and 100.9% with an RSD of 0.23 for the heating test, indicating solution stability. No interference was observed in all UV spectra obtained for the different experiments conducted.

Development of oral nystatin tablets

The dissolution conditions for nystatin tablets were established in an attempt to develop a new formulation. For this part of the study, we selected artificial saliva as the dissolution medium by using a 400-mL volume, apparatus II (paddle) at 50 rpm at 37°C, and collecting aliquots at 15, 45, and 60 minutes so as to most effectively reproduce in vivo conditions. Even with an increased medium volume of 900 mL, sink conditions would not be observed and this would not justify changing medium volume, especially when considering that 400 mL is the volume typically used for oral tablets[33].

Regardless of the polyol that was used, the direct compression technique did not produce tablets with hardness greater than 4.5 kg force and friability smaller than 1.5%. Thus, this technique was discarded for the production of new oral tablets. When wet granulation was used, maltose and sorbitol produced appropriate tablets. With isomalt DC it was not possible to obtain tablets that would meet pharmacopoeial hardness and friability parameters (Table 7).

When undergoing the taste assessment test for the formulation, maltose tablets crumbled in the mouth and produced a strong unpleasant nystatin-like taste, whereas sorbitol tablets managed to obtain sustained dissolution, but still delivered an unpleasant taste; sweetener and flavors were added to the external phase (Table 2). To investigate the possibility of adding another mucoadhesive to the formulation, the addition of a 1:1 CMC Na and HPMC 50 cps mixture was proposed in the external phase as an attempt to increase nystatin residence time in the mouth, because HPMC is a retarding agent and CMC is a mucoadhesive (Table 2).

Tablet disintegration time increased against base formulation, and dissolution was maintained at around 20% after 60 minutes (Table 8). Tablets were produced with the appropriate physical-mechanical properties, and nystatin taste was effectively masked according to volunteers' feedback, proving this formulation to be the most suitable one for in vivo assessment. The result for the taste assessment performed with volunteers is described in Table 9.

The efficacy of nystatin oral tablets against nystatin oral suspension was tested in a pilot study with 12 volunteers

Table 8. Characterization of nystatin oral tablets with sustained release matrix.

Characterization		Results
Weight (g)		0.5649 ± 0.01385
Hardness (kgf)		17.48 ± 2.975
Friability (%)		0.449
Disintegration (min)		29
Dissolution (%)	15 min	7.31 ± 0.00551
	45 min	18.86 ± 0.01991
	60 min	24.63 ± 0.03067

Mean ± RSD; $n = 3$.

Table 9. Final nystatin formulation for taste assessment.

Requisites		Answer options	Answers of volunteers
Appearance		Bad	12 excellent
		Good	
		Excellent	
Color		Yellow	12 yellow
		Light brown	
		Dark brown	
Texture	Oral cavity	Weak	02 strong
	adhesion	Strong	10 excellent
		Excellent	
	Tablet	Weak	12 excellent
	cohesion	Strong	
		Excellent	
Odor	Menthol	Weak	12 strong
	Slightly sweet	Strong	12 strong
	Vanilla		12 strong
Taste	Sweet		12 strong
	Acid	Weak	11 weak/ 01 strong
	Bittersweet		12 weak
	Bad	Strong	12 weak
	Very bad		12 weak
	Repugnant	Yes	12 no
		No	

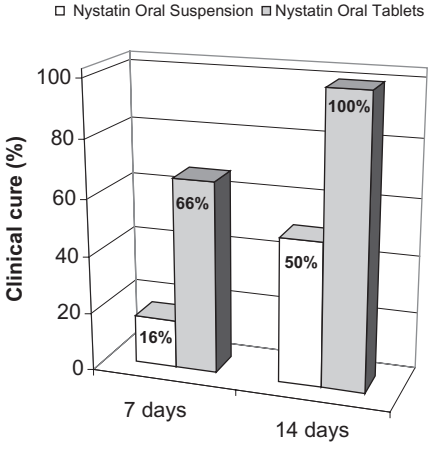

Figure 1. Clinical efficacy of nystatin oral suspension versus nystatin oral tablets (n = 12 patients). Gray bars represent the percentage of clinical cure obtained with new nystatin oral tablets; White bars represent the percentage of clinical cure obtained with traditional nystatin oral suspension.

over a period of 14 days (Figure 1). Our results showed that after 7 days of nystatin oral suspension therapy only one patient (16%) presented clinical cure, whereas four patients (66%) treated with nystatin oral tablets obtained the same clinical results. After 14 days of treatment, the efficacy obtained by nystatin oral suspension rose to three patients (50%), whereas a 100% efficacy was induced by nystatin oral tablets (Figure 1). Therefore, mucoadhesive and delaying formulation was shown to have greater efficacy in controlling candidiasis when compared with the oral suspension.

Conclusion

The successful production of nystatin oral tablets was possible by meeting the required quality requisites for clinical use. The unpleasant taste of nystatin was masked. A sustained dissolution of tablets in the mouth for uniform drug release was achieved. An increased contact time between nystatin and the oral mucosa was also made possible. Oral nystatin tablets also showed to present faster therapeutic response than the oral aqueous solution through the preliminary clinical assays.

The UV absorption spectrophotometry method developed to validate the methodology for nystatin content analysis for new oral tablets was shown to be specific, linear, exact, and reproducible, as recommended by the ICH regulations. Therefore, it might be used for the analysis of nystatin raw-material and nystatin tablets, because it is a faster and cheaper method compared with the microbiological pharmacopoeial method. The UV absorption spectrophotometry method showed to be an attractive test for the usual routine in the pharmaceutical industry.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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