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Validation of Symmetrical Two-Dose Parallel Line Assay Model for Nystatin Potency Determination in Pharmaceutical Product

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ABSTRACT

Objectives: The microbiological antibiotic assay is an important quality control test for the determination of the potency of the antimicrobials whose activity cannot be estimated by the conventional analysis methods (e.g., chemical and HPLC) as raw materials and in the final medicinal dosage form such as tablets, capsules, gels, ointments, creams, powders for reconstitution or any other pharmaceutical preparation. However, biological assay tests must be validated to ensure that the determined potency of the sample is statistically valid. The present study aimed to validate the potency of nystatin in topical pharmaceutical preparations using agar diffusion methodology. **Methods:** The adopted assay module was 2 x 2 Parallel Line Model (PLM) in 8 x 8 large rectangular antibiotic assay plates. The assay model comprises a Latin square design with two treatment (high and low) doses for standard and test preparations. The investigated module is symmetrical with an equal number of replicates in each dose level for both standard and test. The validation methodology parameters cover the evaluation of selectivity, linearity, precision and accuracy (at 50%, 100% and 150%) parameters, in addition to a robustness that includes variation in pH of the antibiotic medium, incubation time and temperature outside the normal range of the regular parameters for the nystatin assay. The selected two-dose concentrations were determined based on the containment within the linear range from the calibration curve. The acceptance criteria for the validation study were established for linearity ($r^2 \geq 0.98$), precision (intraassay variation Relative Standard Deviation (RSD) $\leq 11\%$; inter-assay variation RSD $\leq 10\%$), accuracy, and specificity tests. Antibiotic plates are Incubated at temperatures ranging between 32 and 35°C for a period of 24 hours. **Results:** The assays were calculated statistically by the linear parallel model and regression analysis and verified using analysis of variance (ANOVA). The calibration curve showed $r^2 \approx 0.99$ for the individual and the pooled linearity determination. For accuracy, precision and robustness, none of the RSDs for the individual tests within groups exceeded 7% and the pooled or combined values did not exceed 4%. All validation criteria were met. **Conclusion:** The validation of PLM for nystatin in large 8 x 8 rectangular plates showed an acceptable level of the evaluated parameters and the assay design can be used for the routine quality control (QC) analysis of nystatin activity in medicinal products. Due to the relatively lower number of treatments per preparation on average, an optimum throughput could be achieved with minimal efforts and resources using a 2 x 2 PLM scheme - if compared with 3 x 3 and 5 + 1 designs - with satisfactorily accepted validity for antifungal potency determination of nystatin. Another interesting design that could be investigated in the future is of 3 + 1 type.

Keywords: *Agar diffusion; Accuracy; Linearity; PLM; Precision; Robustness.*

INTRODUCTION

The potency (activity) of the antibiotics can be demonstrated under adequately controlled conditions through their inhibitory characteristic over microbial proliferation[1]. A decline in antimicrobial potency can reveal subtle alterations that are not demonstrable through chemical methods[2,3]. Moreover, microbiological assays do not require specific and sophisticated instruments or highly toxic reagents [4,5]. Agar diffusion is one of the biological methods that are more commonly employed for antibiotic dosage.

The agar diffusion technique relies on the antimicrobial entity dispersion in the matrix of a layer of solidified agar medium, to the extent that completely suppresses the proliferation of the microbial cells in an area or zone around the reservoir containing antibiotic solution[6]. In this type of assay, the diameter (size) of the inhibition zone and the dose of the substance assayed are directly correlated [7]. This is the most widely adopted methodology to determine the activity of antibiotics [1]. The diffusion method employs inoculated solid culture medium, distributed in plates, in a system of single or dual-layered sheets, through which the test-substance diffuses [8.9]. The solution of the test material is applied on the surface of this medium in a confined space, which is usually sterile plates then incubated: the microorganism growth occurs, respecting however the areas in which the antibiotic diffusion has occurred, generating contrast and resulting in the so-called growth inhibition zone [10]. This phenomenon is the basic of the all theory that is the fundamental of the diffusion technique.

One of the most commonly used antifungal compounds that are still tested for their activity using microbiological antibiotic assay is nystatin [11]. Nystatin is a macrolide polyene antifungal antibiotic derived from *Staphylococcus aureus*, *Streptomyces noursei* and other *Streptomyces* species [12, 13]. It is active against a broad spectrum of fungi *in vitro* and *in vivo*, including *Histoplasma capsulatum, Coccidioides immitis, Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus* [14]. However, this antifungal material shows poor absorption from the gastrointestinal tract (GIT) and on the other hand, its parenteral administration results in dose-dependent toxicities and harmful infusion-related reactions which limiting its application through this route.

For this above reason, the medicinal application of nystatin has largely been restricted to the topical use in mucosal and cutaneous forms of candidiasis [17]. The IUPAC name of nystatin is (1S,15S,16R,17R,18S,19E, 21E,25E,27E,29E,31E)-33-[(2S,3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyloxan-2-yl]oxy-1,3,4,7,9,11,17, 37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,25,27,29,31-hexaene-36-carboxylicacid and its molecular formula is $C_{47}H_{75}NO_{17}$.

The most common medicinal forms for this Active Pharmaceutical Ingredient (API) are available in the drug market as oral suspension drops and as topical creams and ointments alone or in combination with other antimicrobial compounds.

This work proposes to examine the two-treatment and four-preparations experimental design for nystatin microbiological assay in 8 x 8 large rectangular plates and validates the method by evaluation of selectivity, linearity, precision and accuracy parameters, in addition to robustness. The study aimed to direct the implementation of a 2 x 2 design based on high and low dose selection according to a previously-verified calibration curve. The aim of the current study was to achieve a simple assay design that accommodates enough samples for regular Quality Control (QC) analysis with reasonable confidence in the potency determinations.

MATERIAL AND METHODS

Chemicals

The nystatin reference substance (assigned purity 99.8%) was supplied by Sigma-Aldrich. The pharmaceutical form semisolid product was commercially obtained and claimed to contain 29.5 mg/g and equivalent to about 4400 IU/mg of nystatin obtained from local market retail [18]. All reagents used were analytical grade [19]. Purified water was used in all experiments [19]. Nystatin solutions must be used freshly prepared and protected from light and heat [20].

Nystatin Reference Solutions

An accurately weighed amount of powder equivalent to 50 mg of nystatin reference standard was transferred to a 25 mL volumetric flask and dissolved in Dimethylformamide (DMF) to obtain a final concentration of 2 µg/mL. Aliquots of this solution were diluted in the n-propanol/phosphate buffer solution to give concentrations of 10 and 20 µg/mL (S1 and S2, respectively), which were used in the bioassay [21].

Preparation of the Sample Solutions

Five grams of the topical semisolid products were squeezed off from the primary packaging aluminium tubes and weighed in 50 mL volumetric flasks. This amount of drug - equivalent to 147.5 mg of nystatin - was dispersed in the volumetric flask with 25-30 mL DMF and shaken for 5 minutes in a mechanical shaker [21]. This step was followed by making up to the final volumes with the same solvent in the volumetric flasks. Aliquots of this solution were further diluted in n-propanol/phosphate buffer solution to obtain the

concentrations of 10and 20µg/mL (T1 and T2, respectively) which were tested against S1 and S2.

Microorganism and Inoculum Standardization

The strain of *Saccharomyces cerevisiae* ATCC 9763 was used as a test microorganism because of its susceptibility to nystatin and capacity to form largely defined inhibition of growth zones, allowing precision in the measurements. The cultures of *S. cerevisiae* ATCC 9763 were cultivated and maintained on a suitable culture medium. The microorganism standardization was made according to the procedure described in the United States Pharmacopeias [22]. Prior to use, the microorganism was grown on an antibiotic medium No. 19 in a Petri dish or large culture (roux) bottle, which was incubated for 48 h at 30 ± 1 °C. The fully grown culture on agar was harvested in tubes containing sterile saline. Using a spectrophotometer with the wavelength set at 580 nm and a 10 mm absorption cell, saline containing the microorganism was diluted to give a suspension with 22.5 ± 2.5% turbidity (transmittance) with some sterile saline solution as the blank[22]. From this standardized suspension, aliquots of 1-1.5% of the media volume were added to each 250 mL of antibiotic medium No. 19 culture medium of pH 6.1 ± 0.1 (BD Difco™, USA) at 48 °C, and used as the inoculated (seed) layer in the plate over the equivalent volume of plain (base) layer [23].

Calculation of Activity and Method Validation

To calculate the potency of nystatin, the Hewitt equation was adopted for large rectangular plates (8 x 8) for two-doses balanced (symmetrical) parallel line model (PLM) assay (2 x 2) using the agar diffusion method [24]. The design assumes analysis of three unknown preparations or products against a single reference standard of known potency[24]. The assays were calculated statistically by the linear parallel model and regression analysis and verified using analysis of variance (ANOVA) [24-27]. For all preparations, two treatments of high and low doses were prepared. The method was validated by the determination of the following operational characteristics: linearity, precision, accuracy, and robustness [28,29].

***(a) Linearity*—**In order to assess the validity of the assay, two doses of the reference substance were used. The linearity was examined by the linear regression analysis method, which was calculated by the least-squares method. The calibration curve was obtained using five reference standard concentrations (8, 12, 16, 20 and 24 µg/mL), whose assays were performed on four occasions.

***(b) Precision*—**The precision of the analysis method was evaluated by both repeatability and intermediate precision and was expressed as the relative standard deviation (RSD). The repeatability was examined by assaying three samples of nystatin product on the same day (intraday) and under the same experimental conditions against the antifungal (active pharmaceutical ingredient) API reference standard. Intermediate precision was subjected to verification through evaluating the results on two different days.

***(c) Accuracy*—**To determine the accuracy of the proposed method, the test was performed over three concentration levels, 50, 100 and 150%, covering the specified range. Accurate aliquots of nystatin were added to the topical pharmaceutical product to give an equivalent to 65000 IU/g, 130000 IU/g and 195000 IU/g of the final labeled potency claim, respectively.

***(d) Robustness*—**The robustness of the assay method could be verified by analyzing the same sample under a variety of conditions. The factors considered were incubation time, incubation temperature and pH.

***(e) Selectivity*—**the assay is applied for a placebo product (Product without the target analyte) to verify no interference from either other active or any excipients.
The validation method acceptance criteria for linearity ($r^2 \geq 0.98$), precision (intraassay variation ≤ 11%; inter-assay variation ≤ 10%), accuracy, and specificity tests were established according to international pharmacopoeial requirements. Accuracy profiling is calculated through the following equation (1) to determine the percentage error at each concentration [30]:

Percent Error = (Experimental Value – Theoretical Value) / Theoretical Value x 100……. (1)

As a standard control condition of incubation, antibiotic plates for nystatin potency determinations are incubated at temperatures ranging from 32 to 35ºC for a period of 24 hours approximately and the robustness parameters will fall outside these limits.

RESULTS AND DISCUSSION

The official compendia and pharmacopeias include antibiotic microbiological dosage assays. However, there is no consensus regarding the experimental designs[31]. The balanced designs 2 x 2 are adopted along with 3 x 3 by the Brazilian (Farmacopéia Brasileira, 1988) and British (British Pharmacopoeia, 2004) pharmacopeias, whereas the American (United States Pharmacopeia, 2006) pharmacopeia presents 5 x 1 design for antibiotic dosage [32]. The use of an adequate experimental design in relation to the criteria of linearity, precision and accuracy of the analytical results is fundamental[1,32]. It is highly recommended to adopt a planned experiment that, without extensive efforts,

Table 1. Diameters of growth inhibition zones for nystatin reference substance solutions obtained for standard curve

Concentration, µg/mL	Range of zone radius, mm[a]	Mean diameters of growth inhibition zones, mm[b]	RSD, %
8.00	8.55 – 8.85	17.41	1.42
12.01	9.15 – 9.90	19.01	2.79
16.01	9.90 – 10.40	20.30	1.77
20.01	10.15 – 10.75	21.00	1.87
24.01	10.50 - 11.15	21.75	1.89

[a] *Digital caliper resolution is extended to two decimal digits of mm*
[b] *Mean of eight groups with large rectangular plates.*

Table 2. Regression analysis of the inhibition zone (mm) - as responses - versus potency (IU/ml) of standard nystatin – as continuous predictors - for the pooled calibration curve

Analysis of Variance					
Source	**DF[a]**	**Adj SS[b]**	**Adj MS[c]**	**F-Value**	**P-Value**
Regression	1	11.7469	11.7469	1695.34	0.000
Log_{10}Potency (IU/ml)	1	11.7469	11.7469	1695.34	
Error	3	0.0208	0.0069		
Total	4	11.7677			
Model Summary	S	R^2	R^2(adjusted)	R^2(predicted)	
	0.0832403	99.82%	99.76%	99.65%	
	Term	SE Coef.	T-Value	P-Value	VIF
Coefficients	Constant	0.482	0.25	0.816	
	Log_{10} Potency (IU/ml)	0.221	41.17	0.000	1.00

[a] *Degree of freedom;* [b] *Adjusted sums of squares*[c] *Adjusted mean squares*

Table 3. Parameters of calibration curve (intercept – *b* and slope –*a*) for nystatin within the range 8.00 to 24.01 µg/ml

Linearity Curves	***a*** **± SD[a]**	***b*** **± SD[a]**	***r*[b]**	***r*²[c]**
Calibration 1	8.86 ± 0.74	9.46 ± 0.08	0.998	0.996
Calibration 2	9.02 ± 0.10	9.30 ± 0.66	0.999	0.999
Calibration 3	9.23 ± 0.22	9.10 ± 0.51	0.999	0.998
Calibration 4	9.27 ± 0.23	9.02 ± 0.38	0.998	0.996

[a] *Standard Deviation;* [b] *coefficient of correlation;* [c] *coefficient of determination*

yields better results and enables an indication respecting the assay validity. The number of replicates and the sample nature, as well as the level of confidence, are among the most crucial factors to be taken into account, in the selection of a design [32]. The 2 x 2 assay, known as a symmetrical or balanced experiment, is a simple yet efficient design that employs two dose levels of the standard and two other of the sample, with an identical ratio between the doses in both preparations. In each dish, all four doses must be included, in such a way that the number of replicas equals that of dishes [33-35]. The present study covers all doses for the preparations in a single large 8 x 8 rectangular plate.

The association of the positive characteristics of 2 x2 PLM design would probably be of considerable interest, as it could improve validation parameters, generating safer and more reliable results [36]. To calculate the activity of nystatin, the Hewitt equation was used [37].

The assays were calculated statistically by the linear parallel model and regression analysis and verified using analysis of variance (ANOVA) [38]. All assay plates showed an acceptable level for the suitability of the analysis method in terms of parallelism and regression.

Calibration curve and linearity

The calibration curve for nystatin was constructed by plotting zone diameter (mm) versus the logarithm of concentrations (µg/mL) and showed good linearity in the 8–24µg/mL range. The representative linear equation for nystatin was $y = 9.0945 \text{ Log}_{10}(x) + 9.2205$ ($n = 5$, $r = 0.9991$), where x is the dose and y is zone diameter (**Figure 1**) and the plot of residuals was reasonably normal. The experimental values obtained for the determination of nystatin in samples are presented in Table 1. The least-square regression analysis method revealed the following regression equation 2 with an estimation of the variance inflation factor (VIF) of 1.00. Other regression analysis parameters are shown in **Table 2**:

$$\text{Inhibition Zone Diameter (mm)} = 0.123 + 9.097 \text{ Log}_{10} \text{ Potency (IU/ml)} \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Knowing the activity of the standard per unit weight, the potency of the test sample could be estimated accordingly. The original four calibration curves for nystatin were previously constructed (**Table 3**) from which the pooled standard curve in **Figure 1** was charted. All presented a coefficient of correlation (r) greater than 0.98 according to the Brazilian reference (2003) and FDA (2001) and when compared, no significant difference was found between the intercepts and slopes[39]. This will ensure that the high and low doses - viz 10 and 20 µg/ml - will fall within linear ranges covering overage or decline in the target concentration.

Accuracy

Specifically, this test is conducted to determine how close is the measured value compared to the true or target value. The relative Error for accuracy ($RE_{accuracy}$) could be estimated if the actual "true" measurement is known. The accuracy was proved by a recovery test that is experimental designs to determine the agreement between the values found of the analyte and the real value that analyses [40]. The accuracy of the bioassay proposed was accessed by the addition of a known amount of nystatin [41]. The results obtained showed a good recovery of the drug added to the sample products (**Table 4**). The mean recovery value was 100.31% and RSD 1.59%, which confirms the suitability of the analytical conditions employed and ensures the ability of the method to quantify nystatin concentrations within the range of 50 to 150% of the target value.

Taking into consideration that the upper and lower acceptance criteria of the finished pharmaceutical product is set to be±10% of the target value [42]. **Figure 2** illustrates the assay results at each concentration level with upper and lower 95% confidence limits are shown. Since the results of the assays and the confidence intervals (CIs) are confined between ±2-3% of RE (%), the result of the accuracy verification is highly satisfactory. In a reported previous collaborative study of assays of erythromycin, using *Bacillus pumilis* as the test organism Fraser (1996) achieved confidence limits of ±2.0 to ±3.0% ($p = 0.95$) for an assay in which one unknown was compared with the standard on a 64-zone large plate[24]. Although this study was not designed to evaluate the zone reading system, it is obvious that to achieve such excellent results, a high precision must have been attained in the reading of the zone sizes.

Precision

The precision of the assay was determined by repeatability and intermediate precision which results were expressed as the relative standard deviation (RSD) of a series of measurements in the same day and on different days, respectively [43, 44]. In the microbiological assay, the number of replications per dose must be sufficient to ensure the required precision [45]. Furthermore, the assay may be repeated and the results combined statistically to obtain the required precision [46]. The repeatability was studied by the determination of the samples in three assays, at the same concentration, during the same day under the same experimental conditions. The result obtained shows RSD of 3.85 indicating good intra-assay precision. Inter-assay variability was calculated from assays on two days and shows an RSD of 2.92. The overall RSD for the average assay potency of 120186 IU/g is 3.24%.

Table 4. Accuracy of the microbiological assay of nystatin

Run	Theoretical amount added (IU/g)	Actual amount recovered (IU/g)	Recovery, [a]%	RSD, %	Combined RSD, %
50%	65000	66381	102.12	0.30	1.59
100%	130000	128816	99.09	0.88	
150%	195000	194479	99.73	0.77	

[a] *Mean of duplicate large plate assays*

Table 5. Results of potency determinations of different assays of nystatin determined by agar diffusion method to study precision

Precision parameters	2x2 Design			
	Replicates	Mean potency (IU/g)	RSD, %	Combined RSD, %
Repeatability (intra-assay)	A	121429	3.17	3.85
	B	125479	0.85	
	C	116195	3.19	
Intermediate(inter-assays)	I	121368	3.17	2.92
	II	116461	2.83	

Table 6. Results of potency determinations of different assays of nystatin determined by agar diffusion method to study robustness

Robustness	Mean potency (IU/g)	Relative deviation, %	RSD, %	Combined RSD, %
Control	116813	0.00	3.84	2.76
pH	120059	2.78	2.07	
Incubation time	117474	0.57	6.26	
Incubation temperature	113386	-2.93	0.16	

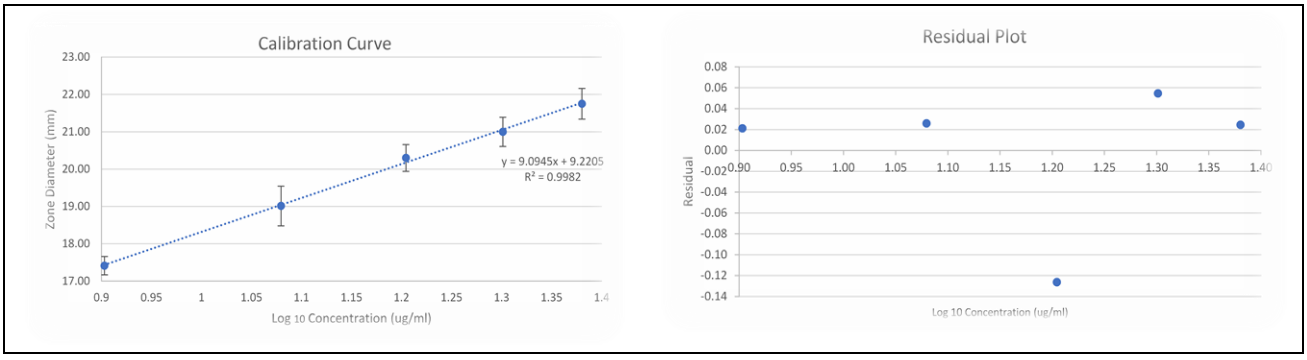

Figure 1. Linearity of Nystatin showing calibration curve (with standard deviation bars) and residuals plot.

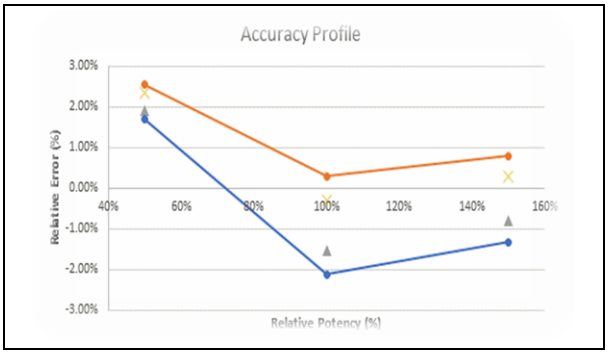

Figure 2. Accuracy profile obtained for method of microbiological dosage of nystatin using 2 x 2 and four preparations designs. Solid lines represent 95% tolerance interval reached. When toleranceintervals are included in acceptance limits, the assay can be quantified accurately.

Robustness

The robustness is the capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication for reliability during normal activity and test processing [47-49]. Table 6 provides the results after a change in parameters (change in incubation temperature., pH of medium, incubation period) for nystatin. Deviation did not exceed 3.00% from the potency of the control.

CONCLUSION

In the world of an ever-increasing population with a weak and defective immune system, antimicrobial medications should be delivered with a high level of confidence in their dosing accuracy. Thus, appropriate

validation of the biological analysis technique is essential to ensure the validity of the potency determination reporting. It is an integral part of the product efficacy and safety issue to confirm its reliability in the treatment of infected patients.

In our study, the investigated microbiological antibiotic assay 8 x 8 design showed acceptable validation criteria. Accordingly, this robust PLM herein could be used in routine QC testing of nystatin as raw material and in finished product formulation with reasonable accuracy and precision.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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