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Article

Development and validation of a microbial counting method for mebendazole oral suspension

Polyana Araújo de Assis[1], Severino Borba de Andrade[2], Clécia Maria Carvalho de Oliveira[2], Patrícia Menezes de Araújo[2], Severino Grangeiro Júnior[3], Selma Verônica Vieira Ramos[4,*]

[1]Department of Pharmacy, University of Brasília, [2]Laboratory of Immunohematology, Clinical Hospital, Federal University of Pernambuco, [3] Department of Pharmacy, Federal University of Pernambuco, [4]Pharmaceutical Laboratory of Pernambuco State Governor Miguel Arraes, LAFEPE

Mebendazole is an important medicine used to treat helminth infections. These infections affect more than two billion people worldwide. The LAFEPE® (Recife-PE, Brazil) produces the drug mebendazole oral suspension that contains the preservatives methylparaben and propylparaben in its formulation. Drugs that have antimicrobial properties due to preservatives must undergo neutralization of these compounds to allow microbial count testing according to recommendations by the official compendia. In order to obtain a validated method for microbial counting and to ensure its safety and reliability within the pharmaceutical industry, validation of preservative neutralization and of the method for microbial counting was performed according to the USP 30 and PDA Technical Report No. 33. The method used ATCC Gram positive and Gram negative microorganisms, yeasts, most and culture media Tryptic Soy Agar and Sabouraud dextrose agar. The neutralizers were polysorbate 80 and lecithin. Recovery levels of over 70% of the microorganisms used in the test indicated the neutralization of antimicrobial activity and proved the absence of toxicity of neutralizers. The microbial counting method validated proved accurate, precise, robust and linear and can be safely used in routine operations.

Uniterms: Mebendazole/oral suspension. Microbial count.

O mebendazol é um importante medicamento utilizado no tratamento de infecções por helmintos. Essas infecções afetam mais de dois bilhões de pessoas em todo o mundo. O LAFEPE (Recife-PE, Brasil) produz o medicamento mebendazol suspensão oral, que possui em sua formulação os conservantes metilparabeno e propilparabeno. Em medicamentos que possuem propriedades antimicrobianas em decorrência dos conservantes faz-se necessária a neutralização da ação desses compostos para a realização do teste de contagem microbiana segundo preconizado pelos compêndios oficiais. A fim de obter um método de contagem microbiana validado e que garanta sua segurança e reprodutibilidade dentro da indústria farmacêutica foi realizada a validação da neutralização dos antimicrobianos e validação do método de contagem microbiana de acordo com a USP 30 e PDA-Technical Report N° 33. O método desenvolvido utilizou microrganismos ATCC Gram positivos, Gram negativos, leveduras e fungos e meios de cultura Tryptic Soy Agar e Sabouraud-dextrose Agar. Os neutralizantes foram polissorbato 80 e lecitina de soja . Níveis de recuperação superiores a 70% dos microrganismos utilizados no ensaio indicaram neutralização da atividade antimicrobiana e comprovou a ausência de toxicidade dos neutralizantes. O método de contagem microbiana validado revelou-se exato, preciso, robusto e linear podendo ser utilizado com segurança na rotina operacional.

Unitermos: Mebendazol/suspensão oral. Contagem microbiana.

***Correspondence:** S.V.V. Ramos. Laboratório Farmacêutico do Estado de Pernambuco Governador Miguel Arraes. Largo de Dois Irmãos 1117 - Dois Irmãos - 52171-010 - Recife - PE, Brasil. E-mail: selma.vieira@lafepe.pe.gov.br

INTRODUCTION

Scientific and technological advances in recent decades have enabled better development and control of drugs, universally determining the minimum efficacy, safety and quality requirements of medicines (Carvalho *et al.*, 2004; Bomblies, Weiss, Beckmann, 2007). Drug quality is related to the degree of compliance with such requirements and minimum standards determined by official standards. To ensure these requirements are met it is necessary to perform physical, chemical and biological tests (Itah, Udokpoh, Ofum, 2004).

In order to ensure the microbiological quality of mebendazole oral suspension 20mg/mL, microbiological tests should be performed, among them the microbial counting test advocated by both the Brazilian Pharmacopoeia IV edition and the USP 30 (Bou-Chacra, 2003; F.Bras. IV, 1988; USP, 2007).

Methods of microbial counting seek to assess the total number of bacteria, yeasts and most using specific culture media. This method includes a series of validations, such as: neutralization of antimicrobial agents, recovery of test microorganisms and the microbial counting method itself. The USP 30 and PDA Technical Report No. 33 describe which key precision, accuracy, robustness and linearity parameters are to be observed in this validation. In addition, another important factor is that the method implemented is validated for each product analyzed (USP, 2007; Ramos, 2010).

In this study, mebendazole oral suspension 20 mg/mL produced by the LAFEPE Laboratory (Pharmaceutical Laboratory of Pernambuco Governor Miguel Arraes S/A) was investigated. Mebendazole is one of most widely used anthelmintic drugs (Goodman and Gilman, 2006) and classified by the World Health Organization (WHO) as an essential medicine based on its clinical efficacy, low cost and ease of administration (Agatonovic-Küstrin *et al*., 2008). This drug belongs to the class of benzimidazole derivatives which includes albendazole and thiabendazole. It has a broad spectrum of activity, acting on cestodes and nematodes parasites. (Bennett, Guyatt, 2000).

Mebendazole oral suspension 20 mg/mL produced by the LAFEPE laboratory is a non-sterile product containing parabens preservatives in its formulation at the proportions of 0.06% propylparaben (w/v) and 0.16% methylparaben (w/v). The antimicrobial system used in the manufacturing of mebendazole oral suspension is important to ensure the stability and safety of the product with respect to microbiological purity, in addition to protecting the user from any health compromise due to product contamination. This is no substitute however, for the good manufacturing practices inherent in the manufacturing process of pharmaceutical industries (Pinto, Kaneko, Ohara, 2003; Russell, 2003). The conducting of a validation test for antimicrobial agent neutralization and for microbial counting method validation thus become key factors that will prove the method's effectiveness in recovering product contaminants. This is necessary since the presence of preservatives may promote microbial growth inhibition, thereby preventing the determination of total number of colonies present in the sample. Furthermore, immune-compromised patients who make use of oral drugs may experience an aggravated clinical condition if these products are microbiologically contaminated. In addition to the objectives outlined is the review of the scarce scientific publications dealing with the subject and their application in pharmaceutical industries.

MATERIAL AND METHODS

Substances and culture media

To validate the microbial counting method, polysorbate 80 (Oxiteno® - Lot 16 506), soy lecithin (Inlab® - Lot 832490), sodium chloride solution, 0.9% (Fresenius Kabi® - Lot 040 702 059), Soybean casein broth (Difco® - Lot 8150627), casein soy agar (Difco® - Lot 9160882, Merck® -Lot VM803358714 and Oxoid® - Lot 463341), Sabouraud dextrose agar (Difco® - Lot 6319541, Merck® - Lot VM804238723 and Oxoid® - Lot 476969), and Sabouraud dextrose broth (Difco® - Lot 6269292, Merck® - Lot VM462926 and Oxoid® - Lot 455812) were used.

The dosage form used was mebendazole (oral suspension 20 mg/mL – Lot 09120754) produced by the LAFEPE® (Pharmaceutical Laboratory of Pernambuco Governor Miguel Arraes).

Test microorganisms

For microbiological tests, the microorganisms described in the official compendia F. Bras. IV and USP 30 (2007): *Aspergillus niger* ATCC 16404 (Cefar® - Lot CBC295), *Candida albicans* ATCC 10231 (Cefar® - Lot CBH360), *Escherichia coli* ATCC 8739 (Cefar® - Lot CBI370), *Pseudomonas aeruginosa* ATCC 9027 (Cefar® - Lot CBE322), *Staphylococcus aureus* ATCC 6538 (Cefar® - Lot CBJ383) were employed.

Preparation of microbial suspension and inoculum standardization

From the stock cultures in casein soy agar, bacteria were transferred with the aid of a platinum loop calibrated

at 10 µL onto 5 mL soybean casein broth liquid medium. These cultures were incubated for 18-24 hours at 32 ± 2 °C. Serial dilutions in 0.9% sodium chloride solution were carried out and the last three were plated and incubated for 24 hours at 32 ± 2 °C and listed in order to obtain a standardized microbial suspension to 10^2 CFU/mL. For yeasts and most standardization, a similar methodology to that for bacteria was used, except for the media employed which were liquid and solid Sabouraud-dextrose. The transfer of *Aspergillus niger* culture was facilitated by suspending spores in 2 mL 0.9% sodium chloride solution added to 0.05% polysorbate 80 solution. These cultures were incubated at 22 ± 2 °C for 48 hours or 96 hours for *Candida albicans* and *Aspergillus niger,* respectively.

Validation of preservative neutralization

Based on the drug formulation, chemical neutralization using 0.4% polysorbate 80 (Tween® 80) (w/v) and 0.5% soy lecithin (w/v) was chosen, as recommended by the official compendia (F. Bras. IV, 1988; USP, 2007) and 1:10 dilution (v/v) was used for the water soluble products.

Determining the efficacy and toxicity of the neutralizer is required to ensure the validation process of antimicrobial agent neutralization.

This is observed through microorganism recovery in different groups for analysis: test (T), peptone (P) and viability (V) (USP, 2007). The comparison between the test group and peptone group demonstrates neutralization effectiveness, whereas the comparison between the peptone group and viability group demonstrates neutralization safety i.e. that it has no toxicity.

These tests were performed in quintuplicate and the criterion used to demonstrate neutralization validation was as recommended by the 2007 USP, which determines the recovery of test microorganisms to be greater than or equal to 70%.

Preparation of analysis groups

The test group (T) and peptone (P) are composed of 90 mL casein soy broth supplemented with 0.4% polysorbate 80 (w/v) and 0.4% soy lecithin (w/v), and 10 mL drug or 10 mL 0.9% sodium chloride, respectively. The viability group (V) comprises casein soy broth only (100 mL).

From the analysis groups, 1.0 mL aliquots were taken and deposited in sterile Petri dishes concomitantly with 0.3 or 1.0 mL of standardized suspension of test microorganisms. Subsequently, about 17 ± 2 mL of solid soy casein or Sabouraud dextrose media was poured and the contents homogenized. Petri dishes remained on a flat surface until complete medium solidification. Subsequently, plates were incubated at 32 ± 2 °C for 48 hours for bacteria and 22 ± 2 °C for 48 hours or 96 hours for *Candida albicans* and *Aspergillus niger,* respectively. The reading was performed after this period by counting the colonies using a digital counter (Quimis ®) and the results expressed in colony forming units/plate.

Development and validation of microbial counting method

The microbial counting method was validated according to the parameters: precision, accuracy, linearity and robustness recommended by the USP-30 and PDA Technical Report No. 33.

Precision was evaluated at two levels: repeatability (intra-run precision, n = 5) and intermediate precision (inter-run precision, n = 5 per analyst). The repeatability and intermediate precision were evaluated in the three groups for analysis (test, peptone and viability) with two levels of microbial incrimination, one with a 10-30 CFU/plate and the other with a 30-300 CFU/plate.

The criterion used to prove the accuracy of the method at the level of repeatability was as recommended by USP 30, which determines a maximum variation coefficient of 25% or 15% when groups are incriminated with 10-30 CFU/plate or 30-300 CFU/plate, respectively. Data obtained for the intermediate precision were statistically analyzed by ANOVA (Neto, Scarmino, Bruns, 2001). The accuracy was assessed by comparing the values obtained for test microorganism recovery in the test (T), peptone (P) and viability (V) groups. To achieve this, fixed volumes of 0.3 mL and 1.0 mL of microorganism suspension standardized in 10^2 CFU/mL were used to frame the analysis groups, thus achieving incrimination levels of 10-30 CFU/plate and 30 - 300 CFU/plate.

The criterion used to prove the accuracy of the method recommended by USP 30 determines test microorganisms recovery of less than 70%.

The linearity of the method was verified through the correlation among different volumes (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mL) which were taken from the analysis groups (Test, Peptone and Viability) and their corresponding Colony Forming Units. The results were statistically analyzed by calculating the linear regression using the minimum squares method and curves whose linear correlation (R^2) was at least 0.95 (USP, 2007) were considered satisfactory.

The robustness parameter was evaluated through the application of small changes to the microbiological method conditions. This was performed by using casein soy agar and Sabouraud dextrose agar media from three

different laboratories (Difco®, Merck® and Oxoid®) and incubating at different temperatures for yeast and most (22 ± 2 °C and 30 ± 2 °C) and bacteria (30 ± 2 °C and 35 ± 2 °C) and by varying sodium polysorbate 80 neutralizing concentration from 0.4% to 3% in casein soy broth. These tests were performed in triplicate and the results were evaluated by analysis of variance (ANOVA) (Neto, Scarmino, Bruns, 2001).

Microbial count of medicine

After validation of the medicine's microbial counting method and after preservative neutralization, a count of bacteria and most that would possibly grow under aerobic conditions was performed. This was done by incorporating 10.0 mL of mebendazole oral suspension in 90.0 mL soybean casein broth with the addition of neutralizing agents 0.4% sodium polysorbate 80 (w/v) and 0.5% soy lecithin (w/v) (1:10). 1.0 mL aliquots of this preparation were placed in Petri dishes and then 17 ± 2.0 mL of casein soy agar or Sabouraud dextrose agar solid media was poured. The plates were homogenized, their contents solidified and then incubated at 32 ± 2 °C for 48 hours for bacteria and 22 ± 2 °C for 48 hours to 96 hours for yeasts and most. After this period colony counting was performed using a digital counter (Quimis ®) and results expressed in CFU/mL.

Evaluation of microbial growth and sterility of culture media

Liquid and solid casein soy media and Sabouraud-dextrose solid medium were used to evaluate test microorganisms' growth. These media were inoculated with 10^2 CFU/mL and incubated at 32 ± 2 °C for 48 hours for bacteria and 22 ± 2 °C for 48 hours for yeasts and 96 for most. Parallel to this assessment, media sterility and purity of microbial cultures were observed.

RESULTS AND DISCUSSION

Validation of preservative neutralization

Neutralization effectiveness was observed by the high recovery percentage in the test group which ranged from 90.95% to 99.72% for mebendazole oral suspension 20 mg/mL. Along with recovery determination, the non-toxicity of neutralizers was confirmed by the recovery rates of microorganisms in the peptone group which ranged from 93.53% to 103.82% (Table I).

During the validation process of microbiological methods, microorganism variability should be taken into account (USP, 2007). Three sources of variation are commonly present: the distribution error of samples, the cellular morphology and metabolic activity of microorganisms. It is known that microorganism distribution occurs heterogeneously. During laboratory tests, it must be assumed that the product was produced under homogeneous conditions because only then is it possible to extrapolate results (PDA, 2000). From values obtained, analysis groups showed percentage values of test microorganism recovery to lie within pharmacopoeial standards, showing a homogeneous distribution of these cells with 95% confidence of accuracy, whereas Student's t test revealed no difference for the values obtained between test and peptone

TABLE I - Neutralization Evaluation of preservative for Mebendazole Oral Suspension 20 mg/mL by LAFEPE® with three analysis groups: viability (V), peptone (P) and test (T), and respective values for $t_{tabulated}$ and $t_{calculated}$ with 95% accuracy

Microorganisms	UFC/plate	Analysis Groups								
		V(X)	T(X)	%R	t_{tab}	t_{calc}	P(X)	%R	t_{tab}	t_{calc}
Aspergillus niger ATCC 16404	**10-30**	19.00	17.40	93.55		2.35	18.60	97.89		2.44
	30-300	72.20	71.00	99.72		1.41	71.20	98.61		0.13
Candida albicans ATCC 10231	**10-30**	43.0	37.80	92.64		2.52	40.80	94.00		2.02
	30-300	125.60	123.20	99.52		0.79	123.80	98.57		0.26
Escherichia coli ATCC 8739	**10-30**	48.00	46.00	98.71	2.77	0.64	46.60	97.08	2.77	0.36
	30-300	152.80	149.00	97.13		0.36	153.40	100.39		0.95
Pseudomonas aeruginosa ATCC 9027	**10-30**	19.00	18.20	96.81		0.66	18.80	98.94		1.17
	30-300	99.20	96.20	97.96		1.75	98.20	98.99		0.91
Staphylococcus aureus ATCC 6538	**10-30**	40.20	34.20	90.95		1.17	37.60	93.53		1.49
	30-300	130.80	130.40	96.02		1.63	135.80	103.82		1.36

CFU=Colony Forming Units, X= quintuplicate average, %R=recovery percentage

groups, as well as between peptone and viability groups.

Cell morphology is also a point that warrants consideration (USP, 2007). Microorganisms exhibit a variety of morphologies during their formation in colonies that may occur singly or in pairs, tetrads or irregular clusters. Some microorganisms have the ability to form biofilms, which will also affect results. The number of colony forming units formed by plates directly affects the colony morphology and precision of cell counts (PDA, 2000). The USP 30 states that recovery of test microorganisms must be greater than or equal to 70%. Thus, it can be observed that results are in accordance with pharmacopoeial standards.

The nature of contaminant microorganisms and microbiological variability exert a strong effect on the response to the antimicrobial agent and the neutralization required in recovery (USP, 2007). The validation process of neutralization of antimicrobial agents showed adequate recovery of bacteria, most and yeasts with maximum incrimination values of 10^2 CFU/mL, confirming that these microorganisms were not inhibited by the test sample, mebendazole, or by the neutralization system consisting of 0.4% polysorbate 80 and 0.5% soy lecithin (Kampf , Shaffer, Hunte, 2005; Kratzer *et al*., 2006). As shown, the efficacy and toxicity of the neutralizer was confirmed by the recovery rates greater than 70% in the test and peptone groups, respectively, thereby assuring the efficiency of method.

Development and validation of microbial counting method

The microbial counting method proved accurate on both parameters. Accuracy is defined by USP 30 as the degree of data approximation obtained in the analysis. Precision is usually expressed by relative standard deviation (RSD) or coefficient of variation (CV%). In the repeatability parameter, the three analysis groups inoculated with two different microorganism concentrations (10-30 CFU/plate and 30-300 CFU/plate) had lower coefficients of variation than those recommended by USP (Table II). This compendium allows for up to 25% variation in microbial recovery for inoculations of test organisms at 10-30 CFU/plate and 15% for the range of 30-300 CFU/plate. For intermediate precision (Table III), no statistically significant differences were evident between the results of microorganism recovery done by two different analysts. The F values calculated were lower than the tabulated F values, confirming with 95% confidence that the method is accurate.

The accuracy of the method was demonstrated through two levels of incrimination, 10-30 CFU/plate and 30-300 CFU/plate. The test accuracy was attained by comparing the CFU/plate of the test and peptone groups with the viability group (control group). There was no significant difference in the recovery of test microorganisms between peptone and test groups when compared to viability ($p \leq 0.05$). The recovery percentages for the two levels of microbial incrimination were greater than 70% (Table IV). These data are consistent with the limits set forth by the USP 30, establishing that the microbiological recovery must be less than 70%. The method was therefore accurate, as confirmed by Student's t test, since no significant differences were found between obtained (t cal) and expected (t tab) values ($p \leq 0.05$).

TABLE II - Repeatability values obtained of microbial counting method for mebendazole oral suspension 20 mg/mL LAFEPE® with two levels of incrimination among three analysis groups: viability (V), peptone (P) and test (T)

Microorganisms	Levels of Incrimination	Analysis groups (CV%)		
	CFU/plate	V	P	T
Aspergillus niger ATCC	10-30	14.89	14.43	15.02
	30-300	9.76	11.56	9.50
Candida albicans ATCC	10-30	16.66	13.80	6.59
	30-300	1.83	6.99	2.88
Escherichia coli ATCC	10-30	10.10	15.52	9.22
	30-300	3.96	2.24	5.69
Pseudomonas aeruginosa ATCC	10-30	13.99	15.25	11.53
	30-300	6.55	5.26	2.80
Staphylococcus aureus ATCC	10-30	14.88	4.34	21.36
	30-300	2.72	4.37	4.49

CFU=Colony Forming Units, CV%=Coefficient of Variation

TABLE III – Values obtained on Determination of Intermediate Precision of microbial counting method done by two different analysts for Mebendazole Oral Suspension 20 mg/mL LAFEPE® with two levels of incrimination among three analysis groups: viability (V), peptone (P) and test (T) and respective values for $F_{tabulated}$ and $F_{calculated}$ with 95% accuracy

Microorganisms	Analyst	Levels of Incrimination									
		10 - 30 CFU/plate (X±SD)					30 - 300 CFU/plate (X±SD)				
		V	P	T	F_{tab}	F_{cal}	V	P	T	F^*_{tab}	F^*_{cal}
Aspergillus niger ATCC 16404	1	20.20 ± 3.35	20.40 ± 2.97	20.60 ± 3.44	2.62	0.78	79.00 ± 1.58	78.80 ± 3.96	77.80 ± 2.77	2.62	2.43
	2	19.00 ± 2.83	17.40 ± 2.51	18.60 ± 2.79			72.20 ± 7.50	71.20 ± 8.23	71.00 ± 6.75		
Candida albicans ATCC 10231	1	33.40 ± 7.23	25.00 ± 4.66	38.40 ± 5.22		1.71	131.20 ± 7.19	128.00 ± 6.71	128.40 ± 9.40		1.01
	2	43.40 ± 7.23	40.80 ± 5.63	37.80 ± 2.49			125.60 ± 2.30	123.20 ± 8.61	123.80 ± 3.56		
Escherichia coli ATCC 8739	1	52.08 ± 6.38	51.80 ± 6.53	44.20 ± 2.77		1.86	160.80 ± 8.98	151.80 ± 7.33	147.40 ± 8.14		2.02
	2	48.00 ± 4.85	46.60 ± 7.23	46.00 ± 4.24			152.80 ± 6.06	153.40 ± 3.44	149.00 ± 8.49		
Pseudomonas aeruginosa ATCC 9027	1	19.60 ± 4.39	20.40 ± 3.51	16.20 ± 1.79		1.21	89.60 ± 13.16	97.00 ± 3.67	96.20 ± 3.11		0.84
	2	17.80 ± 2.49	18.20 ± 2.77	18.80 ± 2.17			96.20 ± 6.30	98.20 ± 5.17	99.20 ± 2.77		
Staphylococcus aureus ATCC 6538	1	37.20 ± 4.87	36.40 ± 3.58	45.40 ± 9.48		1.95	138.20 ± 6.61	138.80 ± 6.76	137.40 ± 7.89		1.78
	2	37.60 ± 5.59	34.20 ± 1.48	40.20 ± 8.58			130.80 ± 3.56	135.80 ± 5.93	130.40 ± 5.86		

CFU = Colony Forming Units; X = quintuplicate average, SD = standard deviation

TABLE IV - Values obtained for Accuracy of microbial counting method for Mebendazole Oral Suspension 20 mg/mL LAFEPE® with two levels of incrimination among three analysis groups: viability (V), peptone (P) and test (T), and respective values for $t_{tabulated}$ and $t_{calculated}$ with 95% accuracy

Microorganisms		Levels of Incrimination					
		10 - 30 CFU/plate			30 - 300 CFU/plate		
		%R	t_{tab}	t_{cal}	%R	t_{tab}	t_{cal}
Aspergillus niger ATCC 16404	V	100.00			100.00		
	P	91.58		2.35	98.61		1.41
	T	97.89		1.63	98.34		1.17
Candida albicans ATCC 10231	V	100.00			100.00		
	P	94.01		2.52	98.09		0.79
	T	87.10		2.48	98.57		1.72
Escherichia coli ATCC 8739	V	100.00			100.00		
	P	98.11	2.77	0.64	100.39	2.77	0.36
	T	83.71		1.36	97.51		0.63
Pseudomonas aeruginosa ATCC 9027	V	100.00			100.00		
	P	102.25		0.66	102.08		1.75
	T	105.62		1.11	103.12		1.76
Staphylococcus aureus ATCC 6538	V	100.00			100.00		
	P	90.96		1.17	103.82		1.63
	T	106.91		1.51	99.69		0.30

CFU = Colony Forming Units, % R = percentage recovery on comparison of test microorganisms

The microbial count is influenced by metabolic activity, genotype and microorganism distribution for growth. During the analysis, microorganisms may be stressed due to environmental conditions or inhibitory components of the formulation itself, thus reducing the method accuracy. It may be noted that peptone recovery values of over 100% were found, where this may be due to microbial variability. Regarding the values over 100% in the test group, these

TABLE V - Linearity values obtained of microbial counting method for Mebendazole Oral Suspension 20 mg/mL LAFEPE® among the analysis groups: viability (V), peptone (P) and test (T), and respective values for $F_{tabulated}$ and $F_{calculated}$ with 95% accuracy

Microorganisms		Linear Coefficient ± SD	R^2	F_{tab}	F_{cal}
Aspergillus niger ATCC 16404	V	87.85 ± 0.49	0.9872		0.87
	P	73.66 ± 2.00	0.9784		
	T	128.57 ± 4.64	0.9854		
Candida albicans ATCC 10231	V	109.47 ± 3.03	0.9694		2.23
	P	102.57 ± 5.82	0.9846		
	T	116.62 ± 2.76	0.9932		
Escherichia coli ATCC 8739	V	77.53 ± 9.83	0.9799		0.10
	P	102.52 ± 8.83	0.9766	5.14	
	T	90.76 ± 4.28	0.9806		
Pseudomonas aeruginosa ATCC 9027	V	30.94 ± 2.30	0.9788		0.21
	P	43.52 ± 7.03	0.9710		
	T	51.38 ± 4.65	0.9544		
Staphylococcus aureus ATCC 6538	V	34.79 ± 3.18	0.9773		0.55
	P	39.04 ± 2.50	0.9759		
	T	45.38 ± 3.38	0.9853		

SD = standard deviation, R^2 = correlation coefficient

may be due to the use of neutralizing agents that can inactivate some products that cause stress to cells. Similarly, the mebendazole may contain sufficient nutrients to ensure the survival of microorganisms or maintenance of their growth rates.

The method's linearity was demonstrated by the proportional relationship between the number of colony forming units detected in the three analysis groups and their volumes taken (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mL). The values of correlation coefficients (R^2) ranged from 0.9694 to 0.9932 for the oral suspension of mebendazole (Table V). These values are consistent with values established by the USP 30, which determines $R^2 > 0.95$. No statistically significant correlation of the linear coefficients obtained for the three analysis groups was found ($p \leq 0.05$).

A method is considered robust when it has the ability to provide unchanged results even when subjected to changes. Robustness is the parameter that most closely matches the laboratory reality since it evaluates the results under different conditions of analysis showing the values that can be acceptable in the face of changing conditions of analysis.

Given the change of parameters such as temperature, concentration of sodium polysorbate 80 and culture media, the results of microbial counts were not statistically different from those obtained under conditions defined as standard ($p \leq 0.05$) (Table VI).

Microbial count of medicine

The microbial count test was performed for Mebendazole oral suspension 20 mg/mL - LAFEPE® and no viable cells were recovered.

Test of microbial growth promotion and sterility of culture media

The culture media used in this experiment proved its ability to promote microbial growth under the test conditions. The sterility test showed absence of viable microorganisms.

CONCLUSION

In the routine microbiological analyses of drugs, analytical methods should be validated to allow reliable

TABLE VI – Values obtained on Robustness Determination of methodology for Mebendazole Oral Suspension 20mg/ml LAFEPE®, based on average CFU from triplicates with variation in temperature, polysorbate 80 and culture media, and respective values for $F_{tabulated}$ and $F_{calculated}$ with 95% accuracy

Parameters	Microorganisms 30 - 300 CFU/plate				
	AN (X ± SD)	CA (X ± SD)	EC (X ± SD)	PA (X ± SD)	SA (X ± SD)
Temperature (°C)					
22 ± 2	129.33 ± 2.84	120.88 ± 2.52	NA	NA	NA
30 ± 2	119.77 ± 0.50	122.33 ± 5.50	72.22 ± 3.28	43.11 ± 5.16	31.66 ± 4.72
35 ± 2	NA	NA	69.88 ± 2.77	44.00 ± 9.86	34.44 ± 4.22
F_{tab}	3.10				
F_{cal}	1.67	0.55	0.91	1.66	2.74
Polysorbate 80					
0.4% (p/v)	75.33 ± 1.17	126.13 ± 5.52	82.40 ± 3.90	232.26 ± 13.32	38.73 ± 3.05
3.0% (p/v)	72.26 ± 4.74	130.73 ± 2.71	82.26 ± 1.52	239.73 ± 16.02	38.53 ± 4.27
F_{tab}	2.62				
F_{cal}	1.39	0.65	0.50	0.29	2.31
Culture medium (TSA or SDA)					
Merck	142.78 ± 3.56	123.33 ± 2.67	69.89 ± 2.78	39.22 ± 2.71	31.78 ± 3.47
Difco	138.44 ± 13.04	122.22 ± 1.84	77.11 ± 10.33	38.33 ± 4.37	33.56 ± 1.64
Oxoid	129.33 ± 2.85	123.33 ± 5.51	83.56 ± 2.78	44.00 ± 9.87	34.44 ± 4.22
F_{tab}	2.51				
F_{cal}	1.06	0.40	2.41	2.28	1.40

CFU = Colony Forming Units; AN = *Aspergillus niger* ATCC 16 404, CA = *Candida albicans* ATCC 10231, EC = *Escherichia coli* ATCC 8739, PA = *Pseudomonas aeruginosa* ATCC 9027; SA = *Staphylococcus aureus* ATCC 6538; NA = not assessed; X = triplicate average, SD = standard deviation; TSA = casein soy agar, SDA = Sabouraud dextrose agar

results which can be interpreted satisfactorily given the wide variability of microorganisms involved in microbial analysis.

The aim of this study was to validate the microbial counting method in mebendazole oral suspension. The method was in accordance with the Good Laboratory Practice, and proved to be accurate, robust while exhibiting good linearity. Thus, the method was in accordance with official standards established for the validation of microbiological methods and for providing reproducibility within the pharmaceutical industry.

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