Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, Brasil

Evaluation of preservative systems in opthalmic suspension of polymyxin B and dexamethasone by linear regression

N. A. Bou-Chacra, T. J. A. Pinto, T. M. Kaneko

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Profa. Dra. Terezinha de Jesus Andreoli Pinto, Departamento de Farmácia, Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, Avenida Professor Lineu Prestes, 580, 05508-900, São Paulo, SP, Brasil
ctrabelo@usp.br

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An ophthalmic suspension of polymyxin B and dexametasone with 13 different preservative systems was evaluated by a linear regression method for rapid screening, using D-values. The products were challenged against *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. Since linear regression showed correlation coefficients higher than 0.84 the decimal reduction time (D-value) was then calculated. The comparison of D-values obtained for each microorganism and each product led to the selection of the formula 9 = 10, which was selected to be submitted to the efficacy of antimicrobial preservation according to the European Pharmacopoeia.

1. Introduction

The official methods for the evaluation of preservative systems in pharmaceuticals include a challenge test, which takes at least 28 days. Although the procedures used in these methods are similar, the times at which the number of surviving organisms are determined and the interpretation of test results are different, especially for ophthalmic products when comparing the European and the American compendium. The time required for the conventional test may be considered a limiting factor in the process of developing products.

As a response to this challenge the linear regression method was proposed by Orth (1979) as a faster alternative. In spite of the similarity in some aspects to the technique proposed by the official compendia, a shorter period of time is required to obtain results. The following advantages are claimed for this method: results in a few days, quantification of the rates of death of specific organisms in products, estimation of the time required for complete destruction of any size microbial population and determination of synergy in preservative combinations (Orth and Brueggen 1982; Orth et al. 1987).

Even though advantageous, this technique has been regarded as inappropriate as it is an adaptation from heat or radiation kills that indeed follows first-order rate kinetics since biocide kills follow second-order rate kinetics. The only case where a second-order reaction can approach pseudo-first-order rate kinetics is when the biocide is present in excess (Sutton et al. 1991).

Despite the criticism, this work evaluated the antimicrobial efficacy of 13 formulas containing two different antimicrobials (chlorhexidine digluconate and phenylethanol) and EDTA and their combinations (binary or ternary) and another formula without preservatives or EDTA, using a linear regression method as a preliminary screening of preservative systems.

2. Investigations and results

The samples analyzed complied with the test for sterility. The results obtained revealed the efficacy of the neutralizers used (data not shown). The D-values obtained from linear regression and correlation coefficient for each microorganism are shown in Table 1. Figures 1–3 showed the survivor curve for *Burkholderia cepacia*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* in each formula. The results obtained with *Pseudomonas aeruginosa* revealed extreme sensibility to polymyxin B (no recovery even immediately after inoculation) for all samples tested, with or without preservative.

3. Discussion

The microorganisms used to evaluate the ophthalmic suspensions were those officially recommended, besides *B. cepacia*. This microorganism, also a non-fermentor, similarly to *Pseudomonas aeruginosa*, did not show susceptibility to polymyxin B (Table 2). According to Richards and Mcbride (1974), *Ps. aeruginosa* presented sensivity to concentrations of up to 1 IU/ml of this antibiotic. Thus the inclusion of *B. cepacia* as potencial indicator of preservative efficacy can be justified in this work due its resistance to several antimicrobials. This microorganism has been isolated in cosmetic and pharmaceutical products and has shown resistance to preservative systems (Borovian 1983; Close and Nielsen 1976; Decicco et al. 1982; Khotari et al. 1977; Richards and Richards 1973). As a consequence, in this study, this skin was considered as pathogenic when interpreting the results.

All the tested formulas with *Ps. aeruginosa* revealed total reduction immediately after inoculation even in the control formula. This behavior indicated that the microorganism was highly susceptible to polymyxin B, as stated by Richards and Mcbride (1974).

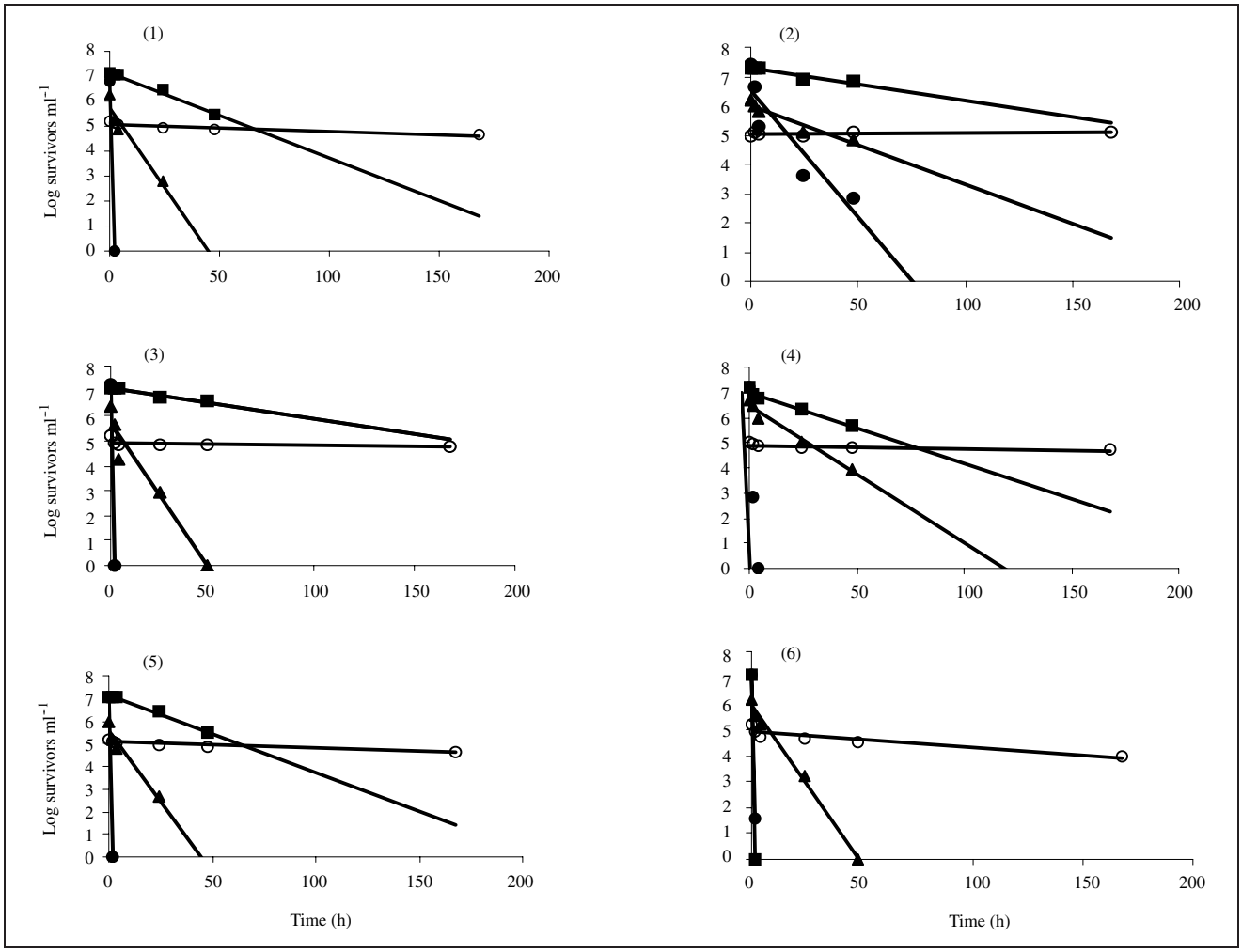

Fig. 1: Survivor curves of the rates of inactivation of (■) *Burkholderia cepacia*; (◆) *Staphylococcus aureus*; (▲) *Candida albicans*; (○) *Aspergillus niger*; in 6 formulas with different preservative systems (formulas 1, 2, 3, 4, 5 and 6).

Table 1: D-values and correlation coefficient of each formula from challenge test against *Burkholderia cepacia*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*

Formulas	*B. cepacia*		*Staph. aureus*		*C. albicans*		*A. niger*	
	D-value	r	D-value	r	D-value	r	D-value	r
1 = 5	29.17	−0.99	0.29	−1.00	7.89	−1.00	346.14	−0.95
2 = 8	90.50	−0.93	11.55	−0.91	35.46	−0.95	–	+0.42
3	81.05	−0.97	0.28	−1.00	7.90	−0.99	–	−0.51
4	35.18	−0.98	0.56	−0.99	17.45	−0.99	–	−0.81
5 = 7	29.38	−0.99	0.28	−1.00	8.16	−1.00	353.86	−0.94
6	0.28	−1.00	0.56	−0.95	7.84	−1.00	165.29	−0.92
7	119.82	−0.98	–	−0.77	13.48	−0.99	–	−0.63
8	90.91	−0.92	11.01	−0.91	28.49	−0.96	–	+0.45
9 = 10	0.29	−1.00	0.28	−1.00	30.03	−0.98	33.78	−1.00
10	0.29	−1.00	0.28	−1.00	31.15	−1.00	33.90	−1.00
11	0.28	−1.00	0.59	−0.97	14.16	−0.98	124.39	−0.96
12	0.29	−1.00	3.99	−0.96	11.96	−0.99	126.01	−0.95
13	0.29	−1.00	0.28	−1.00	12.00	−0.99	–	−0.69
14	0.29	−1.00	0.56	−0.95	9.84	−0.96	124.55	−0.97
15	117.70	−1.00	–	−0.79	13.11	−0.99	–	−0.70
16	23.03	−0.96	0.28	−1.00	8.57	−0.97	–	−0.80
17	3.63	−0.99	0.59	−1.00	10.72	−1.00	–	+0.57
18	–	0.76	22.94	−0.84	–	−0.80	–	+0.69

r: correlation coefficient (−): not calculated

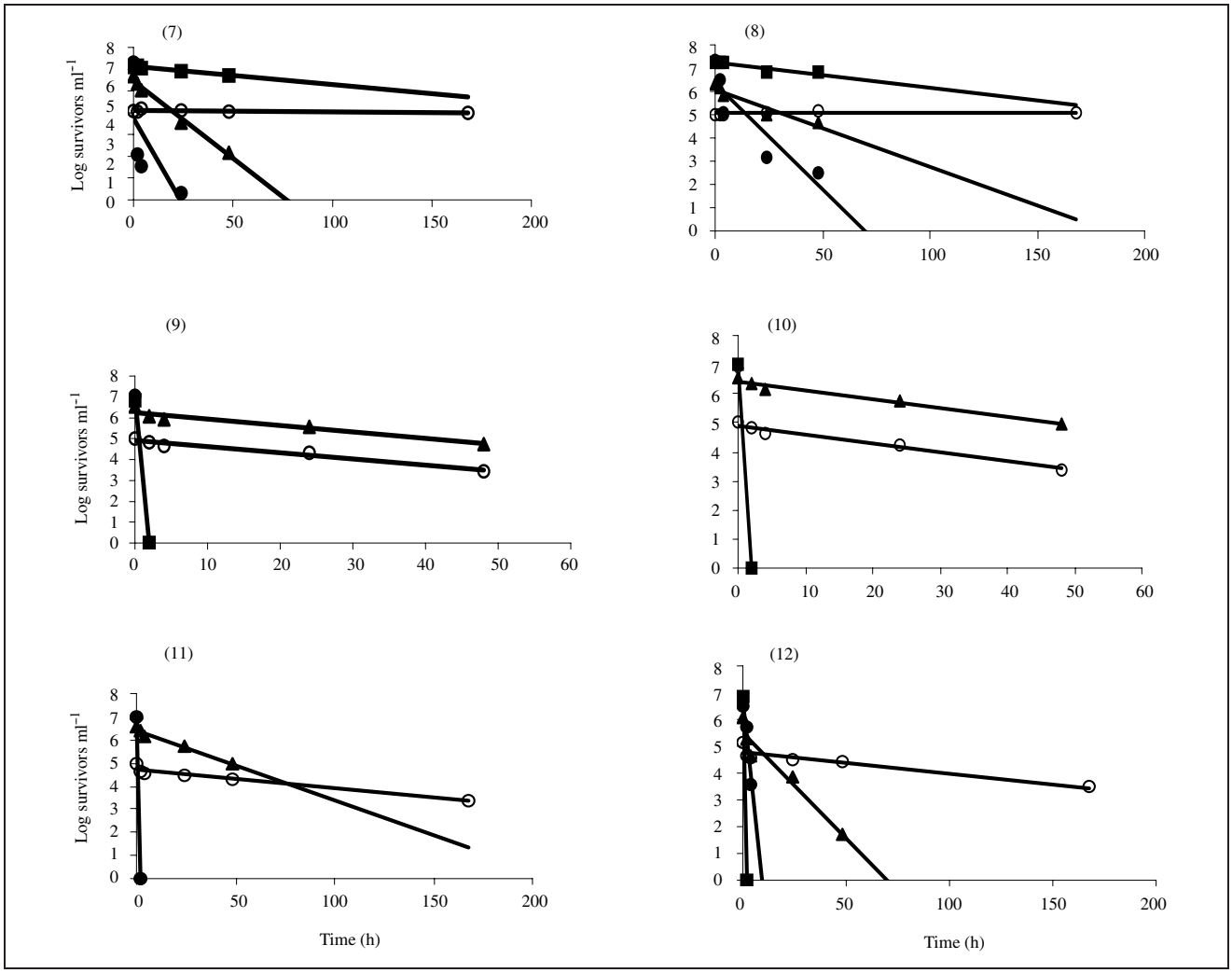

Fig. 2: Survivor curves of the rates of inactivation of (■) *Burkholderia cepacia*; (◆) *Staphylococcus aureus*; (▲) *Candida albicans*; (○) *Aspergillus niger*; in 6 formulas with different preservative systems (formulas 7, 8, 9, 10, 11 and 12).

Table 2: Experimental runs of the X_1 (0.010% w/v chlorhexidine digluconate), X_2 (0.500% w/v phenylethanol) and X_3 (0.100% w/v EDTA) mixture design.

Formulas	X_1		X_2		X_3	
	Fraction	%	Fraction	%	Fraction	%
1 = 5	1	1	0	0	0	0
2 = 8	0	0	0	0	1	1
3	2/3	0.667	0	0	1/3	0.333
4	1/6	0.167	1/6	0.167	4/6	0.667
5	1	1	0	0	0	0
6	2/3	0.667	1/3	0.333	0	0
7 = 15	1/3	0.333	0	0	2/3	0.667
8	0	0	0	0	1	1
9 = 10	0	0	1	1	0	0
10	0	0	1	1	0	0
11	1/6	0.167	4/6	0.667	1/6	0.167
12	1/3	0.333	2/3	0.667	0	0
13	1/3	0.333	1/3	0.333	1/3	0.333
14	0	0	2/3	0.667	1/3	0.333
15	1/3	0.333	0	0	2/3	0.667
16	4/6	0.667	1/6	0.167	1/6	0.167
17	0	0	1/3	0.333	2/3	0.667
18	0	0	0	0	0	0

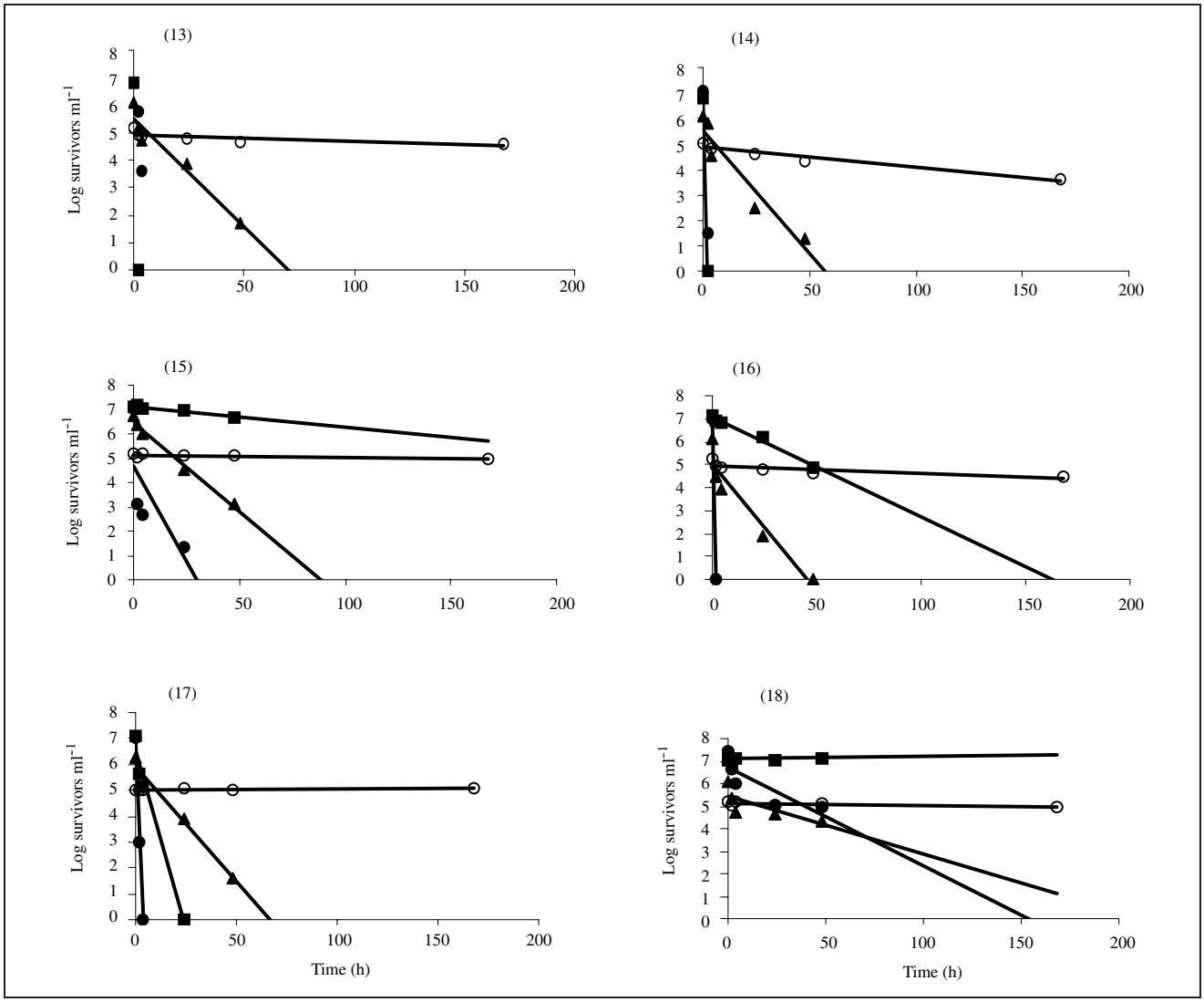

Fig. 3: Survivor curves of the rates of inactivation of (■) *Burkholderia cepacia*; (◆) *Staphylococcus aureus*; (▲) *Candida albicans*; (○) *Aspergillus niger*; in 6 formulas with different preservative systems (formulas 13, 14, 15, 16, 17 and 18).

The D-value of the remaining microorganisms was calculated when the correlation coefficient of linear regression was superior to 0.84. This criterion was adopted according to previous experiments (Tran and Collier 1992), which revealed that these values might indicate behavior of pseudo-first order rate kinetics reaction.

The formulas challenged with *B. cepacia* containing a phenylethanol concentration equal or superior to 0.167% (w/v) led to D-values equal to: 0.29 h (formulas 6, 9 = 10, 11, 12, 13 and 14) and 3.63 h (formula 17). Therefore, these were the formulas that showed the shortest decimal reduction time observed in this assay. All the remaining suspensions presented D-values between 29.03 and 119.82 h (Table 1). The distinct behaviours showed in Fig. 1–3 clearly indicate the advantages of the use of the following binary associations: EDTA + phenylethanol, and chlorhexidine digluconate + phenylethanol, and ternary association of chlorhexidine digluconate + phenylethanol + EDTA, besides the formula containing only phenylethanol.

The advantages of associating phenylethanol and/or EDTA to others preservatives have been intensively described (Richards 1971; Richards and Mcbride 1971; Richards and Hardie 1972; Richards and Mcbride 1972, 1973a, 1973b, 1973c; Richards and Richards 1973; Richards and Mcbride 1974; Richards 1978).

The authors proposed a ternary association including chlorocresol or benzalkonium chloride combined with phenylethanol and EDTA, especially against resistant *Ps. aeruginosa*. The antimicrobial efficacy of the ternary association proposed by Richards (1971) was confirmed in this work concerning the *B. cepacia* in 11 and 13 suspensions as shown on Table 1. The positive correlation coefficient found for this microorganism in the formula 18, without preservatives, demonstrated the low sensivity of *B. cepacia* to polymyxin B (Fig. 3).

As for the gram-positive representative, the results demonstrated its susceptibility to the control formula, probably due to its antimicrobial activity. This preservative-free formula revealed antimicrobial activity through the reduction of 2.5 logarithmic cycles in 48 hours and D-value equal to 22.94 h, according to Table 2. When 0.1% (w/v) of EDTA was added to the control formula there was a reduction of about 50% in the D-value (average of 11.28 h). Such a behaviour may be the result of the association of this agent with the antibiotic. The remaining formulas presented a rapid reduction of the *Staph. aureus* population, being the obtained D-values from 0.29 to 3.99 (Table 1). This microorganism revealed high susceptibility in the studied formulas. The 7 = 15 formula D-value was not calculated, as the correlation coefficient

presented a value under 0.84 (Table 1). However, the microorganism profile of this formula can be clearly observed in Figs. 1, 2 and 3.

Similarly to the microorganism previously discussed, the yeast revealed sensivity to formula 18. A reduction of 1.7 logarithmic cycles in the time interval equal to 48 h was observed. Such a result can be the consequence of the polymyxin B presence, as described above for the gram-positive representative. The formulas containing only phenylethanol (9 = 10) and EDTA (2 = 8) presented less antimicrobial efficacy when compared to the others. The average D-values observed were 31.98 and 30.59 to formula (2 = 8) and (9 = 10) respectively. The remaining ones showed D-values between 7.84 and 17.45 h, as in Table 2. The formulas (1 = 5, 3, 6 and 16) containing 0.01% (w/v) and 0.007% (w/v) of chlorhexidine digluconate associated to phenylethanol and/or EDTA revealed higher antimicrobial efficacy (D-values between 7.84 and 8.57 h). Thus, *C. albicans* demonstrated higher sensibility to chlorhexidine digluconate, either when it was isolated or associated to phenylethanol and/or EDTA, according to Figs. 1, 2 and 3. The suspensions challenged with *A. niger* showed high resistance to the preservatives studied. Part a from the average D-value calculated for the 9 = 10 formula, containing 0.5% (w/v) of phenylethanol (average D-value equal to 33.84 h), the remaining values were situated between 124.39 and 353.86 h and, therefore, 4 to 10 times higher, according to Table 2. The suspensions 2 = 8, 17 and 18 presented positive correlation coefficient, that indicated the possibility of microbial growth in these formulas (Figs. 1, 2 and 3).

In spite of the arguments of Sutton et al. (1991), the linear regression method permitted the selection of the formulas with the most efficient. The following formulas attained the criteria adopted for the linear regression method (Orth 1980) (D-value $\leq$ 4 h for pathogenic bacteria and D-value $\leq$ 28 h for non-pathogenic bacteria and fungi): 6, 9 = 10, 11, 12, 13, 14, and 17 for *B. cepacia*; 1 = 5, 3, 4, 6, 9 = 10, 11, 12, 13, 14, 16 and 17 for *Staph. aureus*; 1 = 5, 3, 4, 6, 7 = 15, 11, 12, 13, 14, 16 and 17, for *C. albicans*; none of the tested formulas attained the adopted criteria for *A. niger*. Comparing the results, in the studied time intervals, to the European Pharmacopoeia criteria (1997) (reduction of 2 logarithmic cycles in 6 h and 3 cycles in 24 h besides the absence of any recovered organism in the time interval equal to 28 days, for bacteria and reduction of 2 logarithmic cycles in the time interval equal to 7 days with no increase after that period during the 28 days of the test, for fungi) the present study permitted the previous selection of the formula 9 = 10 among the 13 different suspensions. Only this formula accomplished the criterion (A criterion, European Pharmacopoeia 1997) related to the first week of the official test, for bacteria and fungi, what enables it to be submitted to the remaining periods officially required. The utility of the linear regression method as a preliminary screening of preservative systems is therefore evident.

4. Experimental

4.1. Material

The test samples included 18 (four repetitions) ophthalmic suspension of dexametasone and polymyxin B obtained from a simplex-lattice statistical project (Cornell 1995). These formulas were different from each other due to the concentration of chlorhexidine digluconate, phenylethanol and EDTA. The statistical project included their binary and ternary combinations besides one other without these substances or their combinations, presented in Table 2.

4.2. Methods

4.2.1. Sterility Test

The samples were submitted to enzymatic digest of cellulose (cellulase solution 0.05% w/v) before the sterility test was performed using the membrane filtration technique, according to the European Pharmacopoeia (1997). Appropriate negative controls and validation test were performed. The proportion used was 1 (enzymatic solution) to 2 (sample) for 3 min at 45 °C.

4.3. Challenge test by linear regression

4.3.1. Organisms

The American Type Culture Collection (ATCC) organisms used in this study consisted of *Pseudomonas aeruginosa* (ATCC 9027), *Burkholderia cepacia* (ATCC 17759), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404).

4.3.2. Preparation and standardization of the inoculum

The strains were obtained from the harvest of microbial growth of a 24 h-culture incubated at 32.5 °C, on the surface of Triptic Soy agar (Difco®) for bacteria, a 48 h-culture incubated at 22.5 °C and a 7 day-culture, incubated at 22.5 °C for the yeast and for the mold, respectively on the surface of Sabouraud dextrose agar, Difco®. The microbial growth was recovered using 5 ml of sodium chloride solution (0.85% w/v) for bacteria and the yeast and 5 ml of the same solution with 0.05% (w/v) polysorbate 80 for the mold.

The number of colony forming units (cfu ml^{-1}) of each suspension was determined by the pour-plate count method using the media and the temperature mentioned above. The period of incubation was 48 h for bacteria and yeast and 7 days for the mould. The concentration of microorganisms in the suspensions should be suitable to provide 10^5–10^6 cfu ml^{-1} in the test immediately after the inoculation. The standardized suspensions were used to inoculate the samples.

4.3.3. Test procedure

Five portions of 50 ml samples were transferred to sterile tubes and individually inoculated with 0.1 ml of the standardized microbial suspensions (single-strain challenge). The inoculated tubes were stirred and each test preparation submitted to the determination of the number of viable organisms using the membrane filtration method (undiluted sample) and the pour-plate method (diluted sample). The dilutions were made with a solution composed of 0.85% (w/v) of sodium chloride, 2% (w/v) of polysorbate 20 and 0.2% (w/v) of soy lecithin. The media were Triptic Soy agar with 0.7% (w/v) of polysorbate 20 and 0.1% (w/v) of soy lecithin for bacteria and Sabouraud dextrose agar with the same added substances mentioned, for fungi.

The number of surviving organisms was determined immediately after inoculation and after 2, 4, 24 and 48 h for bacteria and immediately after inoculation and after 4, 8, 24, 48 h and 7 days for fungi. The D-values were calculated when the correlation coefficient was higher than 0.84. The decimal reduction time (D-value) was calculated for each assay by taking the negative reciprocal of the slope of the line, obtained by linear regression (the plot of the log number of surviving organisms as a function of the time after inoculation into the sample).

4.4. Validation test

The evaluation of the preservative systems neutralization was performed, in both techniques for the estimation of the number of viable microorganisms used in these experiments (pour-plate and filtration method) according to the official guideline (European Pharmacopoeia 1997).

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