Adaptation of the Ph. Eur. test for efficacy of antimicrobial preservation to zinc gelatin: a case study for solid or semi-solid preparations

Favet, Jocelyne Louise; Chappuis, Marie-Louise; Doelker, Eric

Adaptation of the Ph. Eur. Test for Efficacy of Antimicrobial Preservation to Zinc Gelatin

A Case Study for Solid or Semi-Solid Preparations

J. Favet[1], M.-L. Chappuis[1] and E. Doelker[2]*

ABSTRACT

The European Pharmacopoeia test for efficacy of antimicrobial preservation was mainly designed for evaluating liquid dosage forms: eye drops, nose drops, liquids for external use, liquids for oral administration, parenteral solutions. The loading test consists of artificially contaminating the preparation by means of representative types of micro-organisms and monitoring their regression over the course of time. This test, which implies the sampling of homogeneous aliquots over the course of time, is not suitable for dosage forms which are solid or semi-solid at room temperature.

A test method is proposed for zinc gelatin from the Swiss Pharmacopoeia 8, which could be adapted to other non-fluid preparations. A splitting of the preparation is proposed to overcome the problem that the preparation is solid at ambient temperature. In such a case, another change was also implemented consisting of replacing the sample at "time zero" by a neutralised control, in order to take into account the bactericidal effect of the zinc and the physical state of the preparation. Furthermore, in the case of Pseudomonas aeruginosa and Candida albicans, it was suggested that their count on a solid medium be complemented by an enrichment method in a liquid medium, which enables the detection of bacteria which are damaged but nevertheless still viable and which makes it possible to lower the limits of detection when checking whether or not a preparation meets the acceptance criteria.

In view of the overall results, it would appear that the zinc gelatin preparations not containing any preservative (DAB 10 or USP XXI) or containing 0.1 % m/m of methyl parahydroxybenzoate (MPHB) as a preservative (Ph. Helv. 8 or DAB 7) do not meet the acceptance criteria of the European Pharmacopoeia test for efficacy of antimicrobial preservation.

1. INTRODUCTION

The European Pharmacopoeia stipulates that any modification of the preservative system of a preparation be validated in its final container by a test for efficacy of antimicrobial activity during storage and use of the preparation. This test consists of artificially contaminating the preparation with micro-organisms which have been selected in such a manner so as to be able to monitor their regression over the course of time. In practice, this implies that the preparation be contaminated homogeneously and that aliquots be sampled for counting the viable micro-organisms after the homogenisation. However, such a method cannot be applied to a preparation which is a solid at ambient temperature.

The aim of this study was to adapt the method described in the European Pharmacopoeia [1] to the physical properties of zinc gelatin, a formulation found in the Swiss Pharmacopoeia [2], in the German Pharmacopoeia [3] and in former editions of the USP [4], and then to validate the modified method. More generally, the modifications of the test proposed for zinc gelatin could be regarded as useful for other solid preparations.

2. EXPERIMENTAL

2.1. Zinc gelatin preparations

The composition of zinc gelatin was: zinc oxide 10.0 g, gelatin 30.0 g, 85% glycerol 30.09 g, purified water, to 100 g. One batch was prepared without any preservative agent and another with 0.1% *m/m* methyl parahydroxybenzoate (MPHB), as prescribed in Ph. Helv. 8 [2].

2.2. Micro-organisms

Staphylococcus aureus, ATCC 6538P, *Pseudomonas aeruginosa*, ATCC 9027, *Candida albicans*, ATCC 10231 and *Aspergillus niger*, ATCC 16404 were included in the study. The bacteria were cultured at 37 °C for 48 hours, *Candida albicans* at 30 °C for 48 - 72 hours and *Aspergillus niger* at 28 °C for 48 - 72 hours. We also used a strain of *Pseudomonas aeruginosa* which was zinc-resistant and which was isolated in our laboratory (cultured on a rich medium, it grows in the presence of $ZnCl_2$ 20 mM) [5].

(1) Section de biologie, University of Geneva, 3, Place de l'Université, 1211 Geneva 4, Switzerland.
(2) Section de pharmacie, University of Geneva, 30, quai Ernest-Ansermet, 1211 Geneva 4, Switzerland.
* Corresponding author, e-mail: eric.doelker@pharm.unige.ch

2.3. Growth media for bacteria

Tryptone Soja Agar (TSA, Oxoid) was used for bacterial counts and Tryptone Soja Broth (TBS, Oxoid) for culture enrichment; Pseudosel Agar (CET, BBL) and Chapman's medium (CHAP, bioMérieux) were used as confirmation media.

2.4. Growth media for yeasts

Sabouraud Dextrose Agar (SDA, Oxoid) with neutralising agents was used for yeast counts and Potato Dextrose Broth (PDB, Difco) for culture enrichment.

2.5. Growth media for fungi

Malt agar (Fluka) containing 2 % *m/V* malt and 1.5 % *m/V* agar was normally used for fungus counts, but without agar for culture enrichment.

2.6. Neutralising fluids

An aqueous solution of 0.07 % *m/V* lecithin, 0.5 % *m/V* polysorbate 80 (Tween® 80) and 0.05 % *m/V* L-histidine was used. All the media used for carrying out counts or culture enrichment contained neutralising agents at the same concentration as the neutralising solution.

3. PARTICULARITIES OF THE ZINC GELATIN PREPARATION

The efficacy test for antimicrobial preservation of the European Pharmacopoeia (*5.1.3.*) consists of homogeneously contaminating the preparation with one of the micro-organisms selected and sampling an aliquot to carry out a count of the initial number of germs. Then, the contaminated preparation is stored at 25 °C until the next sampling. The preparation is mixed before taking a new sample for counting the viable germs.

This method presents no difficulty in the case of fluid preparations, but cannot be applied to solid preparations. Zinc gelatin has at least two particularities: it is solid and before being used it is made fluid by heating in a water-bath. Since it is changed from a solid state to a liquid state, we cannot consider a surface contamination. It is necessary to uniformly contaminate the bulk of the preparation. Accordingly, the entire sample will solidify and it will be necessary to heat it up again to take aliquots and release the bacteria in view of their counting. The heat could cause the death of certain micro-organisms and activate the preservative agents in an unpredictable manner.

Our purpose here was to develop a test for efficacy of antimicrobial preservation (which should not be confused with the so-called "in-use" tests where the method of use is taken into account). A repeated heating in a water-bath should hence be excluded. Therefore, we propose to split the preparation into aliquots and use one aliquot at each sampling. All the aliquots are contaminated at the same time and they are stored at 30 °C (instead of 25 °C), a temperature at which the preparation is solid while still remaining soft.

Though this method makes it possible to avoid an increase in the temperature for liquefying the solid gelatin, it has the disadvantage of reducing the quality of results. One can expect that the standard deviation from the average will be higher in the case of the different aliquots being prepared in parallel. In the present case, there were no parallel determinations, the different aliquots were taken at intervals of time and the results showed a strong reduction in the number of surviving germs. The analysis of the results obtained through the application of this method should enable the validation, or conversely the invalidation of this procedure.

Another problem which can arise is that the neutralising agents used for the preservative agents will not prevent any antimicrobial activity exhibited by zinc. Actually, this metal has an inhibitory action at low concentration on many micro-organisms [6,7]. The actual biological effect is difficult to predict, because zinc oxide is practically insoluble. To improve confidence, a control was added to the "time zero" (T0) sample, where the preparation was neutralised before the addition of the micro-organisms, which will hence not come directly into contact with the preparation as such. Furthermore, in addition to the conventional counts, a quantitative enrichment method was used (see section *2.6.13* of the European Pharmacopoeia relating to the testing of preparations which need not necessarily be sterile), since we had previously found that it can prove difficult for damaged bacteria to form colonies on a solid medium. This is the case, in particular, for *Pseudomonas aeruginosa*.

4. PROPOSED METHOD

The European Pharmacopoeia proposes that the micro-organisms *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger* be used for testing and that the plate counts be carried out on plates prepared at T0 and days 2, 7, 14 and 28. In our experimental design, at each sampling time, one sample was used for each micro-organism tested. Accordingly and taking into account the controls, the total number of samples was 24 for each preservative system tested.

Preparation of the samples: The preparation was liquefied using a water-bath and split amongst 24 brown glass vials (volume: 25 ml) provided with a threaded stopper, by aliquots of 5.2 g per vial. The samples were placed temporarily in a water-bath at 50 °C.

Preparation of inoculum: In the case of bacteria, a suspension was made of a 24-hour pre-culture on TSA, in 5 ml of a physiological saline solution (the density corresponded to MacFarland's standard No. 2 in the case of *Pseudomonas aeruginosa* and to standard No. 3 in the case of *Staphylococcus aureus*. In the case of the yeast, a 3-day pre-culture on SDA was used and the density of the suspension corresponded to MacFarland's standard No. 4. As for the fungus, a 7 day pre-culture was used, which had abundantly sporulated. A suspension of the spores was prepared in 5 ml of a physiological saline

solution to which 0.1 % *m/V* of polysorbate 80 had been added. The suspension was adjusted to 107 spores/ml using a Zeiss-Thoma cell.

Contamination of the samples: To each of the preparations, maintained at 50 °C and which were still fluid, 0.1 ml of a suspension of a selected micro-organism was added. A glass rod was used to mix the samples. The samples were then stored at 30 °C in the dark.

T0: The preparation contaminated as described above was maintained at 50 °C during the addition, in 2 or 3 steps, of 45 ml of neutralising fluid (also at 50 °C). The sample was then mixed using a glass rod. The duration of the operation did not exceed 5 minutes. The 10-fold diluted sample was incubated for 10 minutes at 30 °C, then the micro-organisms were plated in duplicate for counting.

Control: The sample was first neutralised as described above. The micro-organisms were added, the sample was then diluted 10-fold and the micro-organisms plated for counting.

Sampling in the course of time: The sample was first immersed into a water-bath at 50 °C for 2 minutes to ensure it was fully liquefied before the addition of the neutralising solution. After 10 minutes of incubation at 30 °C, the micro-organisms were plated for counting.

Enrichment: After dilution and plating on a solid medium for counting the viable micro-organisms, an enrichment procedure was carried out. 5 ml of a 10-fold concentrate of an appropriate liquid medium was added to the sample diluted 10-fold with the neutralising solution. Then, repeated 10-fold dilutions were carried out in duplicate, using the same liquid medium containing the neutralising agents (in the case of *Aspergillus niger*, we used an Erlenmeyer flask containing 20 ml of medium, to provide a larger surface for the fungi). The duration of the incubation was 48 hours in the case of bacteria, 3 days in the case of the yeast and 5 days in the case of the fungus. When the solutions become cloudy, they were plated on a solid medium to verify that the micro-organisms corresponded to those used (with the exception of *Aspergillus niger*, which was easily recognised by the whitish down which formed on the surface of the liquid medium). In case of doubt concerning the bacteria, the colonies were transferred onto a suitable selective medium. The number of micro-organisms per millilitre was evaluated according to Table 1.

5. VALIDATION OF THE METHOD

5.1. Growth of micro-organisms in the presence of neutralising agents

The micro-organisms were prepared as described above and 0.1 ml of each suspension were added to 50 ml of neutralising solution. After dilution, the micro-organisms were plated on growth media with and without the neutralising agents (see Table 2). There was no significant difference between the two series. At the concentrations used, the neutralising agents did not hinder the growth of the micro-organisms.

Table 1 — *Interpretation of the results obtained using the quantitative enrichment method (+ : growth, - : no growth)*

10^{-1} dilution *	10^{-2} dilution	10^{-3} dilution	CFU/ml or /g
+	+ +	+ +	$x > 10^3$
+	+ +	+ -	$x \equiv 10^3$
+	+ +	- -	$10^2 < x < 10^3$
+	+ -	- -	$x \equiv 10^2$
+	- -	- -	$10^1 \leq x < 10^2$
-	- -	- -	$x < 10^1$

* Only one flask was prepared at the 10^{-1} concentration, from which the dilutions were prepared in duplicate.

Table 2 — *Growth in the absence and in the presence of the neutralising agent (in CFU/ml)*

Micro-organism	Medium without the neutralising agent	Medium with the neutralising agent
Staphylococcus aureus	$3.3 \cdot 10^7$	$4.6 \cdot 10^7$
Pseudomonas aeruginosa	$3.5 \cdot 10^6$	$2.9 \cdot 10^6$
Candida albicans	$1\text{-}2 \cdot 10^5$	$1\text{-}2 \cdot 10^4$
Aspergillus niger	$1.7 \cdot 10^4$	$1.9 \cdot 10^4$

5.2. Efficacy of the neutralising solution

In order to verify the neutralisation of MPHB, we compared the results of three experimental conditions on two preparations (with and without the preservative) :

1) Reference (without any preparation): The micro-organisms were added to 50 ml of a neutralising solution heated to 50 °C. After an incubation of 10 minutes at 30 °C, they were plated for counting;

2) Control: A sample of gelatin was liquefied and neutralised as described above. The micro-organisms were then added and plated for counting;

3) T0: A sample of gelatin was liquefied, contaminated and then neutralised. After an incubation of 10 minutes at 30 °C, the micro-organisms were plated for counting.

For all the micro-organisms, the values obtained for the control corresponded to those of the reference (Table 3). Therefore, the preservative was correctly neutralised. However, a comparison of the values obtained for the control and for T0 indicated a bactericidal action in the case of T0, where the bacteria were added directly to the preparation. This effect could be ascribed to the zinc, but it was surprising that it was stronger in the case of the preparation without the preservative.

The method is valid, but it is preferable to use a control (a preparation which is first neutralised then contaminated) rather than T0 (a preparation which is contaminated and then neutralised), to estimate the initial micro-organism population.

5.3. Determination of the lower limit stipulated in the acceptance criteria

The logarithmic reduction of the germ population required by the European Pharmacopoeia for topical preparations is 3 in 7 days for bacteria and 2 in 14 days for fungi. The initial concentrations were 10^5 to 10^6 CFU/ml for the bacteria and 10^4 to 10^5 CFU/ml for the fungi. The lower limits were hence situated at 10^2 CFU/ml.

The suspensions of the germs were diluted in the neutralising solution in such a manner as to have approximately 10^4, 10^2 and 10^1 CFU/ml. The numbers of bacteria were determined after a bulk inoculation and a quantitative enrichment, the theoretical limit of detection being 10. It is to be noted that the two bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, behave differently (Tables 4 and 5). The conventional count method is sufficient for *Staphylococcus aureus*, but does not enable the detection of *Pseudomonas aeruginosa* when the number of bacteria is equal to or less than 10^2 CFU/ml. In this case, the use of the enrichment method is a necessity.

The fungi were counted after plating, with the theoretical limit of detection being 10^2 CFU/ml. In the case of *Candida albicans*, the enrichment method is more sensitive than the method of counting on a solid medium (see Table 6). The counting of mould colonies is always inaccurate and is only possible for high dilutions (maximum of 40 colonies per dish). The enrichment method makes it possible to confirm the results obtained by counting. In all cases, the limit of detection was lower than 10^2 for both methods (plating + enrichment).

5.4. Effects of zinc

The fact that T0 was significantly low led us to believe that zinc can exert a fast microbicidal action, in particular on *Pseudomonas aeruginosa*. Accordingly, two strains were compared: the officially recommended strain and a strain resistant to zinc. The test was carried out in accordance with the procedure described for the evaluation of the efficacy of the neutralising solution (5.2) and is applicable only to the preparation without any preservative (Table 7). Although at T0, a reduction corresponding to one logarithmic unit was found for the test population relative to the reference, the number of bacteria was 10^5 times higher for the mutant than for the officially recommended strain.

The problems relating to zinc are complex. On the one hand, there is a natural ingress route for this metal (zinc

Table 3 — *Efficacy of the neutralisation (in CFU/ml)*

Test	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Candida albicans*	*Aspergillus niger*
Reference	$1.3 \cdot 10^7$	$2.9 \cdot 10^6$	$4.0 \cdot 10^5$	$1.9 \cdot 10^4$
Without preservative				
• Control	$2.6 \cdot 10^7$	$6.6 \cdot 10^6$	$5.7 \cdot 10^5$	$2.0 \cdot 10^4$
• T0	$6.8 \cdot 10^6$	$9.0 \cdot 10^2$	$4.0 \cdot 10^5$	$1.5 \cdot 10^4$
With MPHB				
• Control	$1.5 \cdot 10^7$	$2.5 \cdot 10^6$	$4.9 \cdot 10^5$	$1.8 \cdot 10^4$
• T0	$1.7 \cdot 10^7$	$4.6 \cdot 10^4$	$3.6 \cdot 10^5$	$1.0 \cdot 10^4$

Table 4 — *Limit of detection of Staphylococcus aureus (colonies/Petri dish, results in CFU/g)*

	Count in the bulk				Enrichment method			
Susp*	**10^{-1}**	**10^{-2}**	**10^{-3}**	**result**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**result**
	> 300	204	16	$2.0 \cdot 10^4$		+	+	
"10^{-4}"					+			x>10^3
	> 300	182	30	$1.9 \cdot 10^4$		+	+	
	7	5	1	$5.0 \cdot 10^2$		-	+	
"10^{-2}"					-			x≡10^3
	12	24	5	$2.4 \cdot 10^3$		-	-	
	1	1	/	≤10		-	-	
"10^{-1}"					-			x<10
	0	0	/	<10		-	-	

* Suspension of bacteria diluted to 10^4, 10^2 or 10^1
/ Not tested

Table 5 — *Limit of detection of Pseudomonas aeruginosa (colonies/Petri dish, results in CFU/g)*

	Count in the bulk				Enrichment method			
Susp*	**10^{-1}**	**10^{-2}**	**10^{-3}**	**result**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**result**
	> 300	172	18	$1.8 \cdot 10^4$		+	+	
"10^{-4}"					+			x>10^3
	> 300	200	25	$2.0 \cdot 10^4$		+	+	
	50	0	0	$5.0 \cdot 10^2$		+	+	
"10^{-2}"					+			x≡10^3
	6	0	0	$6.0 \cdot 10^1$		+	-	
	0	0	/	<10		+	-	
"10^{-1}"					+			x≡10^2
	0	0	/	<10		-	-	

* Suspension of bacteria diluted to 10^4, 10^2 or 10^1
/ Not tested

Table 6 — *Limit of detection of Candida albicans (CFU/g)*

Count after surface plating	Enrichment method
$6.2 \cdot 10^4$	< 10^3
$1.9 \cdot 10^3$	< 10^3
Approx. 10^2	< 10^3
< 10^2	10^2 < x < 10^3
< 10^2	< 10

Table 7 — *Effect of zinc in an unpreserved preparation on Pseudomona aeruginosa (in CFU/g)*

Test	Officially recommended strain	Zinc-resistant strain
Reference	$2.9 \cdot 10^6$	$6.0 \cdot 10^7$
Without preservative		
• control	$6.6 \cdot 10^2$	$1.4 \cdot 10^8$
• T0	$9.0 \cdot 10^2$	$1.3 \cdot 10^7$

is a trace element which the bacteria need). On the other hand, various bacteria are known for their ability to adsorb this metal on their wall [8-10]. The mechanism of resistance can vary, but it mainly relies on a precipitation inside the cell or on an ATP-dependent efflux transport pump [11].

Actually, only dissolved zinc is capable of acting on bacteria. Its concentration in the liquid fraction (water + glycerol) of zinc gelatin was estimated by atomic absorption spectrometry to be 13 µg/ml (0.2 mM). Prior tests with *Pseudomonas aeruginosa* in a minimal liquid medium (without prior induction) gave maximum tolerable concentrations (MTC) of respectively 0.3 mM for Bergey's reference strain (NCTC 10332) and 5 mM for the mutant used here [5]. It therefore appears likely that the zinc fraction which is soluble can already exert an action which adds to the unfavourable environment in the preparation, and explains the fast bactericidal action found on the ATCC strain and the survival of the mutant.

6. EVALUATION OF THE EFFICACY OF ANTIMICROBIAL PRESERVATION USING THE MODIFIED METHOD

When comparing zinc gelatin without any preservative and that containing 0.1 % *m/m* MPHB (Tables 8 to 11), it appears that the preparation without any preservative exerts per se a sterilising action against the two bacteria, which cannot be detected any more after only 2 days of exposure. As we had already noted during the validation tests, the addition of MPHB hinders this bactericidal action. However, in both cases, the acceptance criteria of a reduction by 2 logarithmic units after 2 days and 3 logarithmic units after 7 days are satisfied.

Fungi proved to be much less sensitive. The preparation without any preservative does not satisfy the acceptance criteria (reduction by 2 logarithmic units after 14 days). The same holds true in the case of MPHB, which exerts an acceptable microbicidal action against yeasts, but has

Table 8 — *Results for Staphylococcus aureus (expressed in CFU/g)*

Sample	Preparation without preservative		Preparation with MPHB	
	Plating	**Enrichment**	**Plating**	**Enrichment**
Control	$4.7 \cdot 10^6$	/	$6.4 \cdot 10^6$	/
Day 0	$2.2 \cdot 10^6$	/	$4.1 \cdot 10^6$	/
Day 2	< 10	< 10	$4.0 \cdot 10^3$	> 10^3
Day 7	< 10	< 10	< 10	< 10
Day 14	< 10	< 10	< 10	< 10^3
Day 28	< 10	< 10	< 10	< 10
Compliance with criteria of the Ph. Eur.		Yes		Yes

/ Not tested

Table 9 - *Results for Pseudomonas aeruginosa (expressed in CFU/g)*

Sample	Preparation without preservative		Preparation with MPHB	
	Plating	**Enrichment**	**Plating**	**Enrichment**
Control	$3.0 \cdot 10^6$	/	$4.1 \cdot 10^6$	/
Day 0	< 10	> 10^3	$7.3 \cdot 10^3$	> 10^3
Day 2	< 10	< 10	< 10	< 10
Day 7	< 10	< 10	< 10	< 10
Day 14	< 10	< 10	< 10	< 10
Day 28	< 10	< 10	< 10	< 10
Compliance with criteria of the Ph. Eur.		Yes		Yes

/ Not tested

Table 10 — *Results for Candida albicans (expressed in CFU/g)*

Sample	Preparation without preservative		Preparation with MPHB	
	Plating	Enrichment	Plating	Enrichment
Control	$6.6 \cdot 10^5$	/	$6.0 \cdot 10^5$	/
Day 0	$3.7 \cdot 10^5$	$> 10^3$	$5.2 \cdot 10^5$	$> 10^3$
Day 2	$2.6 \cdot 10^5$	$> 10^3$	$2.7 \cdot 10^5$	$> 10^3$
Day 7	$9.8 \cdot 10^4$	$> 10^3$	$6.2 \cdot 10^4$	$> 10^3$
Day 14	$1.6 \cdot 10^4$	$> 10^3$	approx. 10^2	$> 10^3$
Day 28	$< 10^2$	$10^2 < x < 10^3$	$< 10^2$	< 10
Compliance with criteria of the Ph. Eur.		No		No

/ Not tested

Table 11 — *Results for Aspergillus niger (expressed in CFU/g)*

Sample	Preparation without preservative		Preparation with MPHB	
	Plating	Enrichment	Plating	Enrichment
Control	$1.6 \cdot 10^5$	/	$1.0 \cdot 10^5$	/
Day 0	$3.0 \cdot 10^5$	$> 10^3$	$2.1 \cdot 10^5$	$> 10^3$
Day 2	$2.1 \cdot 10^5$	$> 10^3$	$1.5 \cdot 10^5$	$> 10^3$
Day 7	$2.8 \cdot 10^5$	$> 10^3$	$2.4 \cdot 10^4$	$> 10^3$
Day 14	$2.5 \cdot 10^5$	$> 10^3$	$2.2 \cdot 10^2$	$> 10^3$
Day 28	$2.8 \cdot 10^5$	$> 10^3$	$1.6 \cdot 10^5$	$> 10^3$
Compliance with criteria of the Ph. Eur.		No		No

/ Not tested

only a fungistatic action against fungal spores. Accordingly, the acceptance criteria are not satisfied.

In view of the overall results, the two systems, with and without preservative, do not satisfy the criteria of efficacy of antimicrobial preservation of the European Pharmacopoeia. Furthermore, we have validated the different technical aspects which could alter the results (growth of the micro-organisms, neutralisation, limit of detection, effect of zinc). It remains to be elucidated whether the fact of using different samples inoculated in parallel introduces an excessive variability in the results. To this end, a regression curve needs to be established, in order to check the alignment of the points.

Unfortunately, the results do not yield many regression curves which can be usefully exploited. The bactericidal action on bacteria was too fast to produce a sufficient number of points; in the case of *aspergillus niger*, the effect was only a fungistatic one (no decrease in the number of spores). Only those results obtained with *Candida albicans* can be analysed. The correlation coefficient, r, of the regression curves was – 0.9954 for the preparation without any preservative and – 0.9885 for the preparation containing MPHB, i.e. it was close to unity, which indicates a good correlation between the points and the curve. One can conclude from this that the error range was negligible as the population regressed in the course of time, since even in the case of the preparation without any preservative and which does not satisfy the Pharmacopoeia criteria, the coefficient, r, remained close to unity.

7. CONCLUSIONS

Our purpose here was to adapt the method designed for the evaluation of the efficacy of antimicrobial preservation to the particular conditions of zinc gelatin which is solid at ambient temperature. The main modifications which we introduced were, on the one hand, the splitting of the preparation into aliquots and, on the other hand, the introduction of a control in which the preservative

was neutralised at the inoculation and of a quantitative enrichment method, in complement to the conventional plate counts.

The splitting of the preparation was suggested to solve the problem of the preparation's solid state. It can cause an increase in the error range, since each sample was contaminated separately. However, the acceptance criteria require a reduction of the population in the course of time which is sufficiently important (slope of the regression curves of - 0.38 for bacteria, - 0.14 for fungi) for this drawback not to have any influence on the interpretation of the results.

Other modifications were introduced to take into account the rapidity of the bactericidal action due, in part, to zinc and also the physicochemical conditions of the preparation. They were, on the one hand, to replace T0 (sample taken usually immediately after the contamination of the preparation) by a control in which any contact of the micro-organisms with the non-neutralised preparation was prevented. This constitutes the surest method for determining the number of micro-organisms introduced into the sample (the value of which is necessary for calculating the logarithmic regression after different storage times). On the other hand, we suggested that the germ count using a solid medium be complemented with a quantitative enrichment procedure, carried out in a liquid medium. This procedure makes it possible to count those bacteria which were damaged but still viable, to lower the limits of detection and to more reliably assess whether or not the acceptance criteria are satisfied.

The test proposed here is more time consuming and requires more equipment than the conventional method. However, it conforms with the objectives and the test criteria set out in the European Pharmacopoeia. With some adaptation it may be possible to apply this new method to other solid or semi-solid preparations.

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