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Note

A case study of preservation of semi-solid preparations using the European Pharmacopoeia test: comparative efficacy of antimicrobial agents in zinc gelatin

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Abstract

The present study was undertaken with the aim of finding an alternative preservative system to methyl parahydroxybenzoate in zinc gelatin, which was described in the monographs of the Swiss Pharmacopoeia (until Ph. Helv. 8) and in previous editions of the German Pharmacopoeia (until DAB 7). This antimicrobial agent has now been withdrawn in the DAB, because of its potential allergy risks. As for the USP and DAB–DDR zinc gelatin preparations, they have always been devoid of any preservative agent, probably relying on the mild antimicrobial activity of zinc. A literature survey did not reveal if such an aqueous preparation containing the water-insoluble zinc oxide shows efficacious antimicrobial activity by itself. Thus, a comparative evaluation of differently preserved zinc gelatin preparations was performed using a test for the efficacy of antimicrobial preservation that has been modified with regard to the European Pharmacopoeia (EP) test to take into account the solid state of the preparations and the bactericidal effect of the zinc. Three zinc gelatin preparations were checked, either: (i), without any agent; or (ii), with 0.1% methyl parahydroxybenzoate; or (iii), with 0.5% phenoxyethanol, a broad-spectrum antimicrobial agent almost devoid of allergy risks. The three preparations behave quite differently, in particular with respect to fungi. All three preparations passed the modified EP test as far as bacteria are concerned. Even zinc gelatin without preservative is very effective, not only because of the mild antimicrobial activity of zinc (the soluble fraction of zinc oxide in the liquid phase of zinc gelatin was determined to be 13 μg/ml), but most probably because of the low water activity of the preparation (measured as around 0.81), as shown by the absence of growth of a zinc-resistant strain of *Pseudomonas aeruginosa*. Zinc gelatin preserved with methyl parahydroxybenzoate has a weak, although satisfactory, activity against *Staphylococcus aureus*. Regarding fungi, gelatin without an antimicrobial agent and that preserved with methyl parahydroxybenzoate meet the requirements for efficacy against *Candida albicans*, but are only bacteriostatic against *Aspergillus niger*. As for zinc gelatin preserved with phenoxyethanol, it displays the best activity against *C. albicans* and, above all, appears to be the only formulation exhibiting fungicidal activity against *A. niger*. It is therefore recommended to preserve zinc gelatin with this antimicrobial agent, as recently adopted in Supplement 2000 of the Swiss Pharmacopoeia. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Zinc gelatin; Semi-solid preparations; Antimicrobial preservation; Methyl parahydroxybenzoate; Phenoxyethanol

1. Introduction

Different strategies have been adopted by the compendia for the protection of zinc gelatin preparations against microbial activity. Thus, zinc gelatin (*zinci gelatina*) is devoid of any preservative agent in the latest editions of the German Pharmacopoeias (up to DAB 1999 and DAB–DDR 7, respectively), in the USP (monograph existing up to USP XXI), as well as in various formularies (British Pharmaceutical Codex, Neues Rezeptur-Formularium).

In contrast, some other zinc gelatin monographs do mention parabens as preservative agents. For instance, 0.1% methyl parahydroxybenzoate is mentioned in the monographs in earlier editions of the German Pharmacopoeia (up to DAB 7) and in the Swiss Pharmacopoeia (up to Ph. Helv. 8). As for the monographs of the Austrian Pharmacopoeia (e.g. OAB 1990), they mention 0.1% methyl parahydroxybenzoate and 0.05% propyl parahydroxybenzoate.

The deletion of the parabens, as in the German Pharmacopoeia, is justified by the ability of this class of antimicrobial agents to elicit skin sensitization and to induce

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cutaneous allergic responses [1,2]. Note that this preservative belongs to class A3 according to the Blaue Liste [3]. As for the absence of any preservative in zinc gelatin, it probably relies on the mild antimicrobial activity of zinc compounds [4,5].

To the authors, it is not known whether this aqueous preparation, which contains the water-insoluble zinc oxide but no preservative agent, would pass a test for efficacy of antimicrobial activity. It was thus decided to compare the antimicrobial activity of three zinc gelatin preparations: (i), without any preservative agent (as in DAB 10); (ii), with 0.1% methyl parahydroxybenzoate (as previously in Ph. Helv. 8); and (iii), with 0.5% phenoxyethanol. The selection of the latter agent was based on a literature survey and expert opinions. Phenoxyethanol is well accepted for topical use because it is nearly devoid of any safety concern and belongs to class A1 of the Blaue Liste [3]. However, minimal inhibitory concentrations of phenoxyethanol are generally higher than those of methyl parahydroxybenzoate [1,6].

Any preservative system of a preparation needs to be validated by a test for efficacy of antimicrobial activity, consisting of artificially contaminating the preparation by the selected micro-organisms, and monitoring their reduction with time. This implies that the preparation be contaminated homogeneously, and aliquots are sampled for counting the viable micro-organisms after the homogenization. The standard methods, e.g. those of the European Pharmacopoeia (EP), are essentially aimed at liquids, and thus cannot be applied to solid or semi-solid preparations, such as zinc gelatin which needs to be to liquefied before use. A modified method was thus proposed for non-fluid preparations [7]. Three modifications were introduced compared with the standard method: (i), the splitting of the preparation into aliquots following its warming; (ii), the introduction of a control sample in which the preservative is neutralized at the inoculation; and (iii), a quantitative enrichment procedure with micro-organisms as a complement to the conventional counts. The modified test is thus used here to compare the efficacy of antimicrobial preservation of the three zinc gelatin preparations.

Some additional experiments are also carried out in order to substantiate any chemical interaction between methyl parahydroxybenzoate and zinc that might explain the surprising antimicrobial activity of both compounds in their mutual presence.

2. Materials and methods

2.1. Materials

Zinc oxide, gelatin, glycerol 85%, methyl parahydroxybenzoate and phenoxyethanol were purchased from Fluka (Buchs, Switzerland). The basic composition of the three zinc gelatin preparations was that of the Swiss Pharmacopoeia 8, which is equivalent to those of the DAB and USP.

The micro-organisms used were of the American Type Culture Collection (ATCC) — *Staphylococcus aureus* (ATCC 6538 P), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404) — were those recommended by the EP. A strain of *P. aeruginosa* resistant to zinc, isolated in our laboratory, was also used in order to assess the specific influence of this metal.

As growth media for the test, Tryptone soja agar (TSA, Oxoid) was used for the bacteria, Sabouraud–dextrose (SDA, Oxoid) for the yeast and malt (2%) agar for the fungus. Growth media for enrichment were Tryptone soja broth (TSB, Oxoid) for the bacteria, Potato dextrose broth (PDB, Difco) for the yeast and liquid malt (2%) for the fungus. All growth media contained the same concentrations of inactivators as in the neutralizing fluid.

The neutralizing fluid used to eliminate the residual antimicrobial activity of the preparations was an aqueous solution containing lecithin (0.07%), polysorbate (0.5%) and L-histidine (0.05%).

2.2. Methods

2.2.1. Procedures for the test for efficacy of antimicrobial preservation

In order to take into account the solid state of the preparations and the bactericidal effect of the zinc, a modified test for efficacy of the antimicrobial preservation with regard of the EP test was used. Details can be found in a previous paper [7]. In particular, a neutralized control (a preparation which is first neutralized then contaminated), rather than the sample at 'time zero' (T0, a preparation which is contaminated and then neutralized), was used to estimate the initial micro-organism population. The germ count using a solid medium was also systematically complemented with a quantitative enrichment procedure, carried out in a liquid medium.

In addition to the test for efficacy of antimicrobial preservation, an in-use test was performed to simulate the effect of using the preparation in normal conditions on the micro-organism viability. Samples of zinc gelatin of 56 g were prepared and contaminated with a suspension of *A. niger*, as described before. One gram of preparation was withdrawn in order to count the viable micro-organisms (T0), the rest of the sample being kept at 4°C. After 24 h, the samples were tempered (30 min at room temperature) and then immersed in a water bath until fluidification (about 10 min). One gram was withdrawn for counting, the sample was then cooled again. The same procedure was repeated for 3 days.

2.2.2. Tests for assessing chemical interaction between zinc and methyl parahydroxybenzoate

Due to the particular antimicrobial activity found for zinc gelatin containing methyl parahydroxybenzoate, it was decided to investigate a possible interaction between the

Table 1
Log reduction in the number of viable micro-organisms in the three zinc gelatin preparations: *Staphylococcus aureus*

Time	EP criteria[a]	Without preservative	Methyl parahydroxybenzoate	Phenoxyethanol
Day 2	2	>5	3.2	> 5
Day 7	3	>5	>5.8	>5
Day 14	–	–	–	–
Day 28	NI	>5	>5.8	>5

[a] NI, no increase.

metal and the preservative. In this respect, it is worth recalling that the phenolate compound zinc bis(methyl parahydroxybenzoate) is known to exist [8].

As an interaction is assumed to occur between soluble entities, it was decided first to determine the soluble fraction of zinc oxide in the liquid phase of zinc gelatin. Accordingly, zinc oxide was dispersed in a mixture of glycerol and water (respecting the proportions of the preparation). The dispersion was filtered and the filtrate was assayed for zinc using atomic absorption spectrophotometry (Pye Unicam SP9, Philips, Gland, Switzerland). The concentration of soluble zinc was estimated to be 13 μg/ml.

The interaction between methyl parahydroxybenzoate and zinc was examined using four different techniques.

First, UV spectrometry was used anticipating some shift of the absorption peak that could result from some complexation. The spectra of a zinc chloride solution, of a methyl parahydroxybenzoate solution and of a solution of both solutes in the glycerol–water mixture were recorded using a HP 8452A diode-array spectrophotometer (Hewlett–Packard, Meyrin, Switzerland). No wavelength shift could be observed, but of course, this does not exclude any complex formation.

HPLC and capillary electrophoresis were also used, assuming that some change in retention time of methyl parahydroxybenzoate would indicate some complexation.

HPLC analyses were performed using a Merck–Hitachi Lachrom system (Darmstadt, Germany), consisting of a L-7400 UV-visible detector set at 263 nm, a L-7300 oven fixed at 30°C, a L-7200 automatic sample injection system and a L-7100 pump. Instrument control and data acquisition were carried out by a Compaq Deskpro 5150 microcomputer equipped with a Merck–Hitachi L-7000 interface. The mobile phase was acetonitrile–phosphate buffer (0.05 M (pH 7); 35:65 v/v) delivered at a flow-rate of 1 ml/min. A Nucleosil 100 C_{18} column (125 × 4 mm inner diameter; Macherey–Nagel, Düren, Germany) was used and volumes of 20 μl were injected.

A ^{3D}CE automatic apparatus (Hewlett–Packard, Waldbronn, Germany) equipped with a diode-array detector set at 195 nm was used for capillary electrophoresis experiments. Runs were carried out in an untreated fused silica capillary 64.5 cm (effective length, 56 cm) × 75 μm inner diameter, 375 μm outer diameter, Bubble Factor 3 (Hewlett–Packard, Waldbronn, Germany). The applied voltage was 30 kV (constant) and the capillary temperature was 25°C. Sample injection was done, at the anodic end of the capillary, by pressure as follows: (i), injection of the sample (50 mbar, 5 s); (ii), injection of the running buffer (50 mbar, 5 s). One series was carried out with 50 mM of a phosphate buffer (pH 2.5), and an another series was analyzed using 50 mM of a phosphate buffer (pH 6.0). No significant change in retention time was noticed when zinc chloride was added to methyl parahydroxybenzoate.

Finally, possible zinc complexation was studied by measuring its transport through a supported liquid membrane (SLM), which consisted of a microporous support impregnated with a hydrophobic organic solvent containing a cation carrier. This technique has been extensively applied to metal speciation. Here, the equipment and conditions used were described earlier [9]. Again, no difference in zinc flux was noticed when methyl parahydroxybenzoate was present.

3. Results and discussion

As shown in Tables 1 and 2, bacteria do not survive for long in zinc gelatin, even in the absence of preservative. The low water activity of the preparation — a value of 0.81,

Table 2
Log reduction in the number of viable micro-organisms in the three zinc gelatin preparations: *Pseudomonas aeruginosa*

Time	EP criteria[a]	Without preservative[a]	Methyl parahydroxybenzoate	Phenoxyethanol[b]
Day 2	2	>5	>5	>5
Day 7	3	>5	>5	>5
Day 14	–	–	–	–
Day 28	NI	>5	>5	>5

[a] NI, no increase.
[b] Observed only with the enrichment procedure.

Table 3
Log reduction in the number of viable micro-organisms in the three zinc gelatin preparations: *Candida albicans*

Time	EP criteria[a]	Without preservative	Methyl parahydroxybenzoate	Phenoxyethanol
Day 2	–	–	–	–
Day 7	–	–	–	–
Day 14	2	1.6	2.8	>4.9
Day 28	NI	2.8	4.7	>4.9

[a] NI, no increase.

which was determined using a TH-2 Thermoconstanter hygrostat (Novasina, Pfäffikon, Switzerland) — certainly prevents micro-organism growth. Furthermore, reduction is more drastic for the aerobic *P. aeruginosa* than for *S. aureus* because of the low oxygen content of the dense material. Of course, the antimicrobial activity of zinc (at least when soluble) could be invoked. However, when comparing the effect of the preparation on the official *P. aeruginosa* strain and on the zinc-resistant strain, no great differences could be observed (log reduction >5 already after 2 days). This suggests that the preparation is simply not favorable to bacterial growth and that its antimicrobial activity is limited.

Reductions in the number of viable bacteria in zinc gelatin preparations with preservative agents were similar — with one prominent exception — to numbers obtained with the unpreserved preparation. The exception concerns the delayed action of the preparation containing methyl parahydroxybenzoate against *S. aureus*.

If adsorption of this agent on zinc oxide is the reason for the decrease in antimicrobial activity, this would also operate on the other micro-organisms. In fact, absence of adsorption of methyl parahydroxybenzoate on zinc oxide has been reported [10]. The same reasoning also holds for a possible complex formation between methyl parahydroxybenzoate and the zinc fraction soluble in the liquid phase of zinc gelatin (estimated to be 13 μg/ml). Here, no evidence of complex formation was found using the four techniques described before.

Of course, although apparently not reported in the literature, some interaction between methyl parahydroxybenzoate and gelatin can be anticipated as with other hydrocolloids (see the review by Myburgh and McCarthy [11], on the subject), but again, this would also adversely affect the activity against the other micro-organisms. Finally, the diminished effectiveness of methyl parahydroxybenzoate could be explained by the high pH of the liquid phase of the preparation due to zinc oxide (a pH value of 7.0 has been reported for an aqueous suspension [10,11]). In this case, the low activity could be ascribed either to partial hydrolysis of methyl parahydroxybenzoate or to its questionable activity at such a pH value. However, there is again no reason that this would not operate for the other micro-organisms.

The present work shows that no preservative inactivation of methyl parahydroxybenzoate is evident in the presence of zinc oxide with the test organism *P. aeruginosa*. The same has been previously observed when using *Escherichia coli* [10]. It therefore appears to be a specificity for *S. aureus* at the biological level (growth promoting action of methyl parahydroxybenzoate or mutual inhibition of the antimicrobial activity of methyl parahydroxybenzoate and zinc). However, if no satisfactory explanation can be put forward, it should be emphasized that methyl parahydroxybenzoate gives acceptable efficacy of antimicrobial preservation according to EP criteria.

Since fungi can accommodate a lower water activity than bacteria, they are less sensitive, in the absence of a preservative agent, to the special conditions of zinc gelatin. There is a slow reduction in viable *C. albicans* in the preparation without preservative (Table 3). In the case of *A. niger*, the effect is only fungistatic, as there is no decrease in the number of spores in 28 days (Table 4). Phenoxyethanol appears to be much more effective against *C. albicans* than methyl parahydroxybenzoate and it is the only condition where a fungicidal activity against *A. niger* has been observed.

Examination of the EP criteria reveals that the log reduction in the number of viable micro-organisms is acceptable for both bacteria, zinc gelatin being preserved or not. The log reductions with gelatin added with methyl parahydroxybenzoate or phenoxyethanol are also acceptable for *C. albi-*

Table 4
Log reduction in the number of viable micro-organisms in the three zinc gelatin preparations: *Aspergillus niger*

Time	EP criteria[a]	Without preservative	Methyl parahydroxybenzoate	Phenoxyethanol
Day 2	–	–	–	–
Day 7	–	–	–	–
Day 14	2	0	0	0
Day 28	NI	0	0	1.4

[a] NI, no increase.

Table 5
Number of viable *Aspergillus niger* in the zinc gelatin preparations when used as prescribed[a]

Time	Without preservative	Methyl parahydroxybenzoate	Phenoxyethanol
T0	5.7	45.5	4.7
1st use	<1	<1	<1
2nd use	<1	<1	<1
3rd use	<1	<1	<1

[a] CFU/ml in log.

cans zinc. In contrast, the criteria for efficacy are not satisfied for all three preparations, as far as *A. niger* is concerned. Although not complying with the EP test carried out with *A. niger*, the preparation with phenoxyethanol displays the best efficacy against this fungus. In this respect, it is recalled that the official loading test does not take into account the conditions of use of the preparation. In fact, when subjecting the three zinc gelatin preparations contaminated with *A. niger*, spores are already killed when placed for the first time in a water bath, by a heat effect (80°C can be lethal) or by activation of the antimicrobial agent, when present (Table 5).

In conclusion, the three zinc gelatin preparations display quite different efficacies of antimicrobial preservation, in particular with fungi. Zinc gelatin without a preservative is effective against bacteria, not so much because of the antimicrobial activity of the zinc fraction of the liquid phase of the preparation, but because the low water activity of the preparation inhibits organism growth. Zinc gelatin without preservative has some efficacy against *C. albicans*, but is again only bacteriostatic against *A. niger*.

Adding methyl parahydroxybenzoate, as in former editions of the DAB or in the Ph. Helv., leads, without any convincing reason, to poor preservation against *S. aureus*, even though the EP acceptance criteria are met. Preservation against *C. albicans* is also satisfactory, in contrast to *A. niger*, where only a fungistatic activity is observed.

The preservative efficacy of zinc gelatin containing phenoxyethanol against bacteria is also optimal, and that against *C. albicans* is the best of all three preparations. In addition, it is the only preparation exhibiting fungicidal activity against *A. niger*.

Thus, preservation with methyl parahydroxybenzoate can be discarded not only because of allergy risks, but also because of its poor antimicrobial activity due to the special characteristics of gelatin zinc. Preservation with phenoxyethanol can be recommended over the absence of an antimicrobial agent, owing to superior activity against fungi. Phenoxyethanol is now present in the monograph of the Supplement 2000 of the Swiss Pharmacopoeia.

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Glossary

DAB	Deutsches Arzneibuch
DAB-DDR	Deutsches Arzneibuch der Deutschen Demokratischen Republik
USP	The United States Pharmacopeia
Ph. Helv.	Pharmacopoea Helvetica

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