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Studies on the evaluation of preservative efficacy. IV. The determination of antimicrobial characteristics of some pharmaceutical compounds in aqueous solutions *

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Summary

An investigation of the antimicrobial activity and its concentration dependence of some pharmaceutical compounds other than preservatives (mercuric oxycyanide (MO), ethyl alcohol (EA), propylene glycol (PG) and sodium metabisulphite (SM)) has been performed. For this purpose the kinetic approach to the screening of antimicrobial activity of preservatives was successfully applied. *D*-values and concentration exponents (η) were used as parameters in determining microbiocidal activity. *Aspergillus niger, Candida albicans, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* were employed as challenge organisms. All the substances tested exerted antimicrobial activity within the concentration ranges usually applied in pharmaceutical preparations. EA and PG had about the same η-value (3–6), while SM and MO had values of 1–2 and 0.4–3, respectively.

Introduction

In previous publications (Karabit et al., 1985, 1986, 1988) studies of antimicrobial activity of preservatives were successfully performed by applying a kinetic screening technique. Three types of preservatives were used as model compounds, phenol (acidic), benzylalcohol (neutral) and benzalkonium chloride (quaternary ammonium compound).

Ingredients other than preservatives can have an antimicrobial activity so that the preparation itself has adequate antimicrobial properties, which official preservation directions usually take into consideration. The effects of many pharmaceutical compounds on the efficacy of preservatives have been reported (Barr and Tice, 1957; Prickett et al., 1961; Richards and Reary, 1972; Richards and McBride, 1973; Yousef et al., 1973; Chiori and Ghobashy, 1983). However, the knowledge of the direct antimicrobial activity of these materials alone is by no means complete. Further information is therefore needed.

The present investigation deals with the study of the antimicrobial activity of some pharmaceutical compounds such as active ingredients (mercuric oxycyanide), co-solvents (ethyl alcohol and propylene glycol) and antioxidants (sodium metabisulphite).

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Materials and Methods

Pharmaceutical compounds and reagents

Mercuric oxycyanide (MO) (Pfaltz & Bauer, Inc. U.S.A.). Ethyl alcohol (EA) (Finsprit 95%, Kemetyl, Sweden). Propylene glycol (PG) (1,2-propylenglykol, purum, Kebo lab AB, Sweden). Sodium metabisulphite (SM) (Natriumdisulfit, pro analysi, Merck, F.R.G.). All other chemicals were of reagent grade.

Test organisms

Cultures of the following micro-organisms were used: *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 16404), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 6538), which are the micro-organisms recommended for the challenge test of the USP XXI.

Media

Tryptone soya broth (TSB, Oxoid), tryptone soya agar (TSA, Oxoid), bacteriological peptone (Oxoid), dextrose (Oxoid), sabouraud dextrose agar (SAB, Oxoid).

Preparation of test solutions

Data on the test solutions are given in Table 1. Portions of 9 ml of EA and PG solutions were dispensed in 20 ml vials which, after sealing with rubber stoppers, were sterilized by autoclaving at 121°C for 20 min. MO and SM solutions were dispensed in sterile 20 ml vials by filtration through Millex GS sterile filter (0.22 μm) under aseptic conditions. The vials were then sealed with sterile rubber stoppers. After sterilization the pH values were determined by the use of a Metrohm E632 Digital pH-Meter (Switzerland), equipped with a type EA120 combined electrode.

TABLE 1

Concentrations and pH of test solutions

Compound	Concentration [a] (w/v%)	pH
Mercuric oxycyanide	0.00011; 0.00028; 0.00055; 0.0011; 0.011; 0.033; 0.044; 0.055	7.1 [b]
Ethyl alcohol	5.55; 11.11; 16.67; 22.22; 27.78	7.1 [b]
Propylene glycol	33.33; 44.44; 55.55; 66.66	7.1 [b]
Sodium metabisulphite	0.11; 0.22; 0.33; 0.44; 0.55	5.1 [c]

[a] The test procedure involves a 10% dilution of the solutions.
[b] Phosphate buffer 0.05 M.
[c] Acetate buffer 0.05 M.

Preparation of inoculum

The micro-organisms were maintained by sub-culturing on nutrient agar at monthly intervals. For the initial cultivation of the test organisms, the bacteria were grown in TSB at 32°C for 24 h, *C. albicans* at 20–25°C for 48 h in a medium consisting of 2% dextrose and 1% bacteriological peptone and *A. niger* at 20–25°C for one week on SAB plates.

Bacteria and *C. albicans* were harvested from their liquid media by centrifugation, washed twice with sterile saline. Stock suspensions were prepared by dilution with sterile saline to give a cell concentration of 10^7–10^8 organisms/ml. The absorbance of the suspension was measured in a spectrophotometer (Spectronic 20, Bausch and Lomb, U.S.A.) at 550 nm with saline as a blank. In the standardization of the instrument it was found that absorbance of 0.1 and 0.7 corresponded to 10^7–10^8 cells per ml of bacteria and *C. albicans* respectively.

A. niger was harvested by washing the cells from the plates with 10 ml of sterile saline containing 0.05% polysorbate 80, which was found to give a stock suspension containing 10^7–10^8 cells/ml.

Test procedure

One ml of the test organism stock suspension was added to each vial. At specified time intervals, 1 ml of the content was transferred as follows.

In the case of bacteria or *C. albicans*, to a membrane filter (Schleicher and Schuell, 0.45 μm, 47 mm diameter), after which it was rinsed with 300 ml sterile peptone water (0.1%) and then incubated either at 32°C for 24 h on TSA or at 20–25°C for 48 h on SAB for bacterial and *C. albicans* growth, respectively.

In the case of *A. niger*, into Petri plates and embedded in SAB and then incubated at 20–25°C for 5 days.

The number of viable micro-organisms present at each time interval was determined by the plate-count procedure, beginning with a zero time count, after which the survivor curve was plotted.

Dilution was necessary with some samples to give a countable number of colonies per plate (30–300). All dilutions were prepared in sterile saline.

The entire test procedure was carried out at room temperature (about 20 °C).

Calculations

The theoretical and mathematical details of preservation kinetics are described in earlier publications (Karabit et al., 1985, 1986). The decimal reduction time (*D*-value) is used for expressing the resistance of a micro-organism to a preservative. The *D*-values were calculated from the negative reciprocal of the slope of the survivor curves obtained by linear regression of the plot of log number of surviving organisms vs exposure time. The concentration dependence was evaluated by determining the concentration exponent (η) (Bean, 1967, 1972). The slope of the straight line obtained by plotting the logarithm of the *D*-values vs the logarithm of the preservative concentrations represents the concentration exponent.

Results and Discussion

All the pharmaceutical compounds tested exerted antimicrobial activity. As expected the degree of activity was related to the concentration tested. Death rate curves were obtained for each combination of test organism and pharmaceutical compound. An example is shown in Fig. 1 for *A. niger* in 50% PG solution. The decimal reduction times (*D*-values) were calculated by linear regression from the slopes of these curves (Tables 2–5). Fits of the linear models were acceptable with correlation coefficients, $r = 0.981 \pm 0.0183$ (mean $\pm$ S.D., $n = 120$).

The relationship between concentration and antimicrobial activity was studied at 3 concentration levels and expressed by the concentration exponent (dilution coefficient or η-value) (Tables 2–5). As the activity is found to be exponentially related

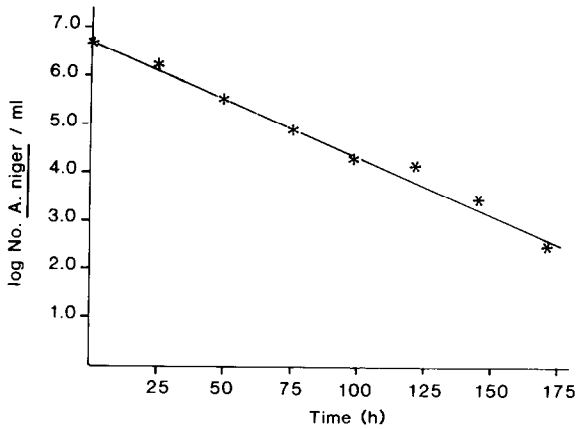

Fig. 1. Linear regression fit of log number of survivors of *A. niger* in 50% PG vs time (h) ($r = -0.994$).

to the concentration, the η-values were calculated from the slopes of the linear regression lines for the plots of log *D*-values vs log concentrations, with acceptable correlation coefficients, $r = 0.974 \pm 0.0295$ (mean $\pm$ S.D., $n = 20$). In Fig. 2 such a plot for PG with *E. coli* as test organism is presented.

Mercuric oxycyanide (MO)

The antimicrobial activity of MO on test organisms was studied at pH 7.1 and Table 2 shows *D*-values and η-values for MO. At the concentration of 10^{-4}% MO had only bacterio-

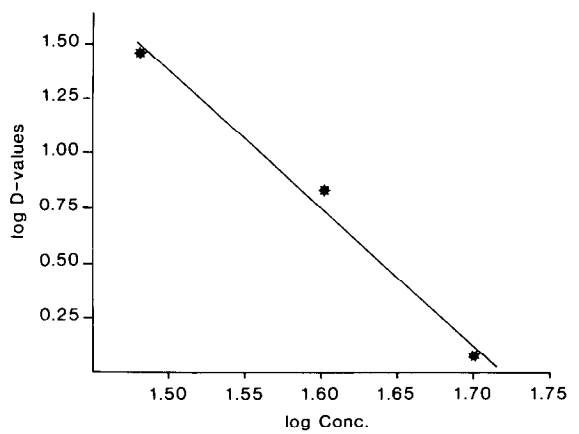

Fig. 2. The relationship between concentration and antimicrobial activity of propylene glycol in aqueous solution at pH 7.1. Test organism *E. coli*.

TABLE 2

D-values and the concentration dependence of mercuric oxycyanide in aqueous solution at pH 7.1

Test organism	*D*-values (h)								η
	0.0001%	0.00025%	0.0005%	0.001%	0.01%	0.03%	0.04%	0.05%	
A. niger	a	a	a	a	a	59	25.6	11.0	3.3
C. albicans	a	a	107	11.1	0.88	–	–	–	1.5
A. coli	0.445	0.201	0.079	b	b	–	–	–	1.1
P. aeruginosa	a	0.87	0.424	0.199	b	–	–	–	1.1
S. aureus	11.7	–	–	5.4	1.95	–	–	–	0.4

D-values are means of two determinations.

–, No experiment was performed.

[a] Mercuric oxycyanide had only fungistatic or bacteriostatic effect.

[b] The elimination of the organism occurred so fast that *D*-values could not be determined.

static effect on *P. aeruginosa,* while at concentrations $\geq 0.01\%$ the death rate for this organism was so high that *D*-values could not be determined. The elimination of *E. coli* occurred too fast, already at the concentrations of $\geq 0.001\%$. The fungi were found to be more resistant against MO than the bacteria. Thus, the concentrations of $> 0.01\%$ and $\geq 5 \times 10^{-4}\%$ were required for elimination of *A. niger* and *C. albicans* respectively (Table 2).

MO is used as topical antiseptic in the concentration range of 0.01–0.025%. As seen in Table 2 MO had a reliable bactericidal effect in this range, while the fungicidal effect is not guaranteed, as MO was only fungistatic at the concentration of 0.01%. However, it may be concluded that aqueous solutions of MO with a concentration in this range does not need an addition of preservative.

The antimicrobial activity of MO might be due to the mercurial attack on the cell wall causing lysis of the cell. In addition it will inhibit enzymes in the membrane and cytoplasm, which contains thiol, –SH, groups (Hugo, 1983).

The concentration exponent found were in the range of 0.4–3 (Table 2). The lower η-value was obtained with *S. aureus,* while a higher one was found for *A. niger*. An η-value of 1.0 has been reported for mercuric chloride (Berry and Michaels, 1950).

Ethyl alcohol (EA)

Table 3 shows the *D*-values and concentration exponents for EA. Ethanol exhibits marked anti-

TABLE 3

D-values and concentration dependence of ethyl alcohol in aqueous solution at pH 7.1

Test organism	*D*-values (h)					η
	5%	10%	15%	20%	25%	
A. niger	–	a	95	58	22.6	2.9
C. albicans	–	73	11.9	1.31	–	5.7
E. coli	–	2.77	0.66	0.0326	–	6.2
P. aeruginosa	34.6	5.7	0.66	b	–	3.5
S. aureus	–	14.1	4.83	1.05	–	3.7

D-values are mean values of two determinations.

–, No experiment was performed.

[a] Ethyl alcohol had only fungistatic effect.

[b] The elimination of the organism occurred so fast that *D*-value could not be determined.

TABLE 4

D-values and the concentration dependence of propylene glycol in aqueous solution at pH 7.1

Test organism	*D*-values (h)				η
	30%	40%	50%	60%	
A. niger	a	145	43.5	19.1	4.9
C. albicans	42.3	19.1	1.69	–	6.3
E. coli	28.6	6.8	1.18	–	6.3
P. aeruginosa	5.3	1.45	0.63	–	4.3
S. aureus	12.1	3.88	2.57	–	3.1

D-values are means of two determinations.
–, No experiment was performed.
[a] Propylene glycol had only fungistatic effect.

microbial activity against Gram-positive as well as Gram-negative bacteria over the concentration range 10–20%. This is in accordance with Bruch (1971).

The elimination of *P. aeruginosa* in 20% ethanol solution was so fast that the *D*-value could not be determined, while 10% solution was only fungistatic against *A. niger*. η-Values found were in the range of 3–6. Concentration exponents of about 11 for EA (Tilley, 1939), and about 2–10 for benzylalcohol (Karabit et al., 1986) have been reported.

The germicidal properties of EA might be attributed to alteration in the cell membrane permeability causing the release of intracellular constituents (Hugo, 1982) and/or inhibition of the production of amino acids essential for organism growth (Dagley et al., 1950).

Propylene glycol (PG)

D-values and concentration exponents for PG are reported in Table 4. All concentrations of PG tested exerted a reliable bactericidal action. PG also had a fungicidal effect on *C. albicans*, while *A. niger* was less sensitive, as 30% PG showed only fungistatic activity against this organism. This is in accordance with the findings reported by Barr and Tice (1957). PG acts in a way similar to EA (Rosen and Berke, 1973), and the concentration exponents were also found to be in the same range i.e. about 3–6 (Tables 3 and 4). This is in accordance with the proposition that "preservatives belonging to a particular chemical group have approximately the same concentration exponent" (Bean, 1972).

Both EA and PG are used as co-solvents in pharmaceutical formulation. The concentration of PG in injections usually is $\geq 40\%$. Such solutions have themselves adequate antimicrobial properties and need no further preservation.

Sodium metabisulphite (SM)

The antimicrobial activity of SM was found to be strongly dependent of pH. At pH 7.1 SM had only bacteriostatic/fungistatic effect on the test organisms, while at a pH of 5.1 the organisms were considerably more sensitive. This result is in agreement with findings by Richards and Reary, (1972).

D-values and η-values are reported in Table 5. SM had bactericidal activity in the concentration range tested, while the fungi were less sensitive. At

TABLE 5

D-values and concentration dependence of sodium metabisulphite in aqueous solution at pH 5.1

Test organism	*D*-values (h)					η
	0.1%	0.2%	0.3%	0.4%	0.5%	
A. niger	a	21.3	14.2	6.1	–	1.8
C. albicans	a	a	42.4	26.7	19.6	1.6
E. coli	6.8	5.0	1.89	–	–	1.1
P. aeruginosa	8.3	3.22	1.24	–	–	1.6
S. aureus	8.1	6.4	2.29	–	–	1.1

D-values are mean values of two determinations.
–, No experiment was performed.
[a] Sodium metabisulphite had only fungistatic effect.

the concentration of 0.1% and 0.2% SM had only fungistatic effect on *A. niger* and *C. albicans* respectively. The η-values were about 1–2 (Table 5). The microbiocidal effects of SM are due to its reaction with microbial DNA (Richards and Reary, 1972).

SM has been used as an effective antioxidant for aqueous systems in the past and is still used to a considerable extent. The concentration of SM applied for this purpose is usually below 0.1% in weakly acidic solutions. Therefore, the germicidal activity of SM usually is insufficient for an adequate preservation of such solutions.

Conclusions

(1) The kinetic approach to the screening of preservative efficacy can be successfully applied to the characterization of the antimicrobial activity of pharmaceutical compounds other than preservatives.

(2) Certain pharmaceutical compounds exert antimicrobial activity to a varying degree depending on their concentration. Therefore, presence of such ingredients in pharmaceuticals should be considered in connection with the design of preservation systems, and furthermore, in the interpretation of the results from preservative efficacy tests.

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References

Barr, M. and Tice, L.F., A study of the inhibitory concentrations of glycerin-sorbitol and propylene glycol-sorbitol combination on the growth of microorganisms. *J. Am. Pharm. Assoc. Sci. Edn.*, 46 (1957) 217–218.

Bean, H.S., Types and characteristics of disinfectants. *J. Appl. Bact.*, 30 (1967) 6–16.

Bean, H.S., Preservatives for pharmaceuticals. *J. Soc. Cosmet. Chem.*, 23 (1972) 703–720.

Berry, H. and Michaels, I., The evaluation of the bactericidal activity of ethylene glycol and some of its monoalkyl ethers against *Bacterium coli*. *J. Pharm. Pharmacol.*, 2 (1950) 105–114.

Bruch, C.W., Cosmetics: sterility vs. microbial control. *Am. Perfum. Cosmet.*, 86 (1971) 45–50.

Chiori, C.O. and Ghobashy, A.A., A potentiating effect of EDTA on the bactericidal activity of lower concentrations of ethanol. *Int. J. Pharm.*, 17 (1983) 121–128.

Dagley, S., Dawes, E.A. and Morrison, G.A., Inhibition of growth of aerobacter aerogenes: the mode of action of phenols, alcohols, acetone, and ethyl acetate. *J. Bact.*, 60 (1950) 369–379.

Hugo, W.B., Disinfection mechanisms. In Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. (Eds.), *Principles and Practice of Disinfection, Preservation and Sterilisation*, Blackwell, London, 1982, pp. 158–185.

Hugo, W.B., The mode of action of non-antibiotic antibacterial agents. In Hugo, W.B. and Russell, A.D., (Eds.). *Pharmaceutical Microbiology*. Blackwell, London, 1983, pp. 258–264.

Karabit, M.S., Juneskans, O.T. and Lundgren, P., Studies on the evaluation of preservative efficacy. I. The determination of antimicrobial characteristics of phenol. *Acta Pharm. Suec.*, 22 (1985) 281–290.

Karabit, M.S., Juneskans, O.T. and Lundgren, P., Studies on the evaluation of preservative efficacy. II. The determination of antimicrobial characteristics of benzylalcohol. *J. Clin. Hosp. Pharm.*, 11 (1986) 281–289.

Karabit, M.S., Juneskans, O.T. and Lundgren, P., Studies on the evaluation of preservative efficacy. III. The determination of antimicrobial characteristics of benzalkonium chloride. *Int. J. Pharm.*, 46 (1988) 141–147.

Prickett, P.S., Murray, H.L. and Mercer, N.H., Potentiation of preservatives (Parabens) in pharmaceutical formulations by low concentrations of propylene glycol. *J. Pharm. Sci.*, 50 (1961) 316–320.

Richards, R.M.E. and Reary, J.M.E., Changes in antibacterial activity of thiomersal and PMN on autoclaving with certain adjuvants. *J. Pharm. Pharmacol.*, Suppl., 24 (1972) 84P-89P.

Richards, R.M.E. and McBride, R.J., Preservation of sulphacetamide eye-drops BPC. *Pharm. J.*, 210 (1973) 118–120.

Rosen, W.E. and Berke, P.A., Modern concepts of cosmetic preservation. *J. Soc. Cosmet. Chem.*, 24 (1973) 663–675.

Tilley, F.W., An experimental study of the relation between concentration of disinfectants and time required for disinfection. *J. Bact.*, 38 (1939) 499–510.

Yousef, R.T., El-Nakeeb, M.A. and Salama, S., Effects of some pharmaceutical materials on the bactericidal activity of preservatives. *Can. J. Pharm. Sci.*, 8 (1973) 54–56.