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Studies on the evaluation of preservative efficacy V. Effect of concentration of micro-organisms on the antimicrobial activity of phenol

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Summary

An investigation of the effect of the concentration of *Staphylococcus aureus* on the antimicrobial activity of phenol has been performed. For this purpose, a kinetic approach to the screening of antimicrobial activity of phenol was successfully applied. The values of the parameters *D*, *E* (the time for elimination of any size population of organism) and *È* (the time for elimination of 10^2 organisms/ml) were calculated. The results show that the preservative efficacy is not affected by the concentration of challenge organisms up to the point where the system becomes overloaded.

Introduction

Studies on the antimicrobial activity of preservatives have been successfully performed by applying a kinetic screening technique (Karabit et al., 1985, 1986, 1988, 1989). These investigations covered the influence of various environmental factors such as pH, temperature of solution, concentration of preservative and the presence of extraneous compounds.

The number of contaminating micro-organisms is among the factors modifying preservative efficacy. A higher degree of microbial contamination, longer exposure time and/or higher concentration of preservative may be needed for elimination of the contaminative organisms (Russell, 1982; Hugo and Russell, 1987). In addition, criticism of the BP and USP challenge tests on the microbial load in challenge inoculi is widespread (Leitz, 1972; Cowen and Steiger, 1976; Moore, 1978). However, the rate of inactivation of challenge organisms is independent of the concentration of organisms in the test sample (Orth, 1979). Therefore, further information about the effect of concentration of micro-organisms on preservative efficacy is desired.

The present investigation examines the effect of micro-organism concentrations on the antimicrobial activity of phenol.

Materials and Methods

Preservative and reagents: Phenol was of pharmacopoeial grade (Ph. Nord. 63), all other

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chemicals being of reagent grade. The test organism was *Staphylococcus aureus* (ATCC No. 6538) as cultures.

Media: Tryptone soya broth (TSB), tryptone soya agar (TSA) and bacteriological peptone were all obtained from Oxoid.

Test solution: A 0.5% phenol solution was used, the pH being adjusted to pH 7.1 using 0.05 M phosphate buffer. After sterilization, pH values were determined by using a Metrohm E632 digital pH-meter, equipped with a EA120 combined electrode (Switzerland).

Preparation of inoculum: The micro-organisms were maintained by subculturing on nutrient agar at monthly intervals. For initial cultivation, the test organism was grown in TSB at 32° C for 24 h.

Organisms were harvested from the liquid media by centrifugation, and washed twice with sterile saline. Stock suspensions were prepared by dilution with sterile saline to give a cell concentration of 10^7–10^9 organisms/ml. The extinction coefficient of the suspension was determined using a spectrophotometer (Spectronic 20, Bausch and Lomb, U.S.A.) at 470 nm with saline as a blank. In the calibration of the instrument, it was found that an extinction coefficient of 0.2, 0.5 and 1.0 corresponded to 10^7, 10^8 and 10^9 cells per ml of *S. aureus*, respectively. Concentrations of 10^5–10^6 organisms/ml were prepared by dilution of 10^7 organisms/ml with sterile saline.

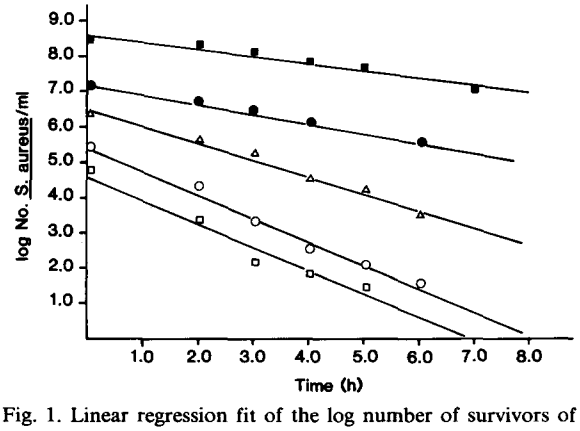

Fig. 1. Linear regression fit of the log number of survivors of different concentrations of *S. aureus* in 0.5% phenol vs time (h). (□) 5.38×10^4/ml, (○) 2.39×10^5/ml, (△) 2.08×10^6/ml, (●) 1.29×10^7/ml, (■) 2.8×10^8/ml.

Test procedure: Portions of 9 ml of the test solution were dispensed into 20 ml vials which, after sealing with rubber stoppers, were sterilized by autoclaving at 121° C for 20 min. A sample (1 ml) of the test organism stock suspension was added to each vial. At specified time intervals, 1 ml of the content was transferred to a membrane filter (Gelman GN-6, 0.45 μm, 47 mm diameter), rinsed thereafter with 300 ml sterile peptone water (0.1%) and incubated at 32° C for 24 h on TSA.

The number of viable micro-organisms present at each time point was determined by the plate-count procedure, beginning with a zero time count, after which the survival curve was plotted. Dilution was necessary with some samples to give a measurable number of colonies per plate (30–300). All dilutions were prepared in sterile saline. In every case, tests were carried out at room temperature (approx. 20° C).

Calculations: The decimal reduction time (D value) is used for expressing the effectiveness of a preservative. The D value is calculated from the negative reciprocal of the slope of the survival curve obtained by linear regression of the plot of log number of surviving organisms vs exposure time (Karabit et al., 1985, 1986). The time predicted for complete elimination of any size population of a particular organism (E value) is evaluated from the product of the D value times the log number of organisms/ml (Orth, 1979). The value of $\dot{E}$ (the time required for complete elimination of 10^2 organisms/ml) is calculated from the equation of the linear regression curve obtained via plotting log initial concentrations of organism vs log E value. The linear regression model is given by:

$$Y = A + BX$$

where Y denotes log initial concentration of organism, A is the intercept, B represents the slope of the line, and X is the log E value.

Results and Discussion

The effect of different concentrations of *S. aureus* in 0.5% phenol on the rate of die-off

(death-rate curves) is shown in Fig. 1. From the slopes of the lines obtained by linear regression, the decimal reduction times (D values) were calculated. The mean D values and the predicted times for complete elimination of the organism (E values) are listed in Table 1 for each concentration of *S. aureus*.

The mean of the D values for *S. aureus* was independent of the initial concentrations of organism up to 10^6 organisms/ml (Table 1). This is in accordance with results reported by Orth (1979). Moreover, this finding shows that an inoculum size up to 10^6 organisms/ml (as required by the BP 1988 and USP XXI challenge tests) will not affect preservative efficacy in pharmaceutical or cosmetic products. However, this high value for inoculum size has been used to allow accurate determination of the end-point in terms of the required log reduction (Bloomfield, 1986).

A significant ($p < 0.05$) increase in D value was obtained with 10^8 organisms/ml (Table 1). This increase in D value might be attributed to the large inoculum size which quenches the activity of the preservatives as a result of overloading of the system with extraneous organic matter (Moore, 1978). In addition, no significant changes in D values were observed between 10^6 and 10^7 and between 10^7 and 10^8 organisms/ml.

The time required for complete elimination of micro-organisms (E value) could be calculated from the D value and initial concentration of micro-organisms. For example, the mean D value for 5.38×10^4 *S. aureus*/ml in 0.5% phenol solution was 1.48 h. The time for total elimination of this amount of organism is given by the product of log concentration of organism times the D value, or $4.73 \times 1.48 = 7.00$ h. The E values for the challenge organisms increased with increasing values of the initial concentration of organisms (Table 1). The linear relation between the initial concentration of micro-organisms and E values is illustrated in Fig. 2. When log initial concentrations of *S. aureus* were plotted vs log E value, a linear regression correlation coefficient of 0.988 was obtained. From the linear regression equation, a predicted time for elimination of 10^2 organisms/ml ($\grave{E}$ value) was calculated. The value of 10^2 organisms/ml is the bioburden suggested by Kallings, et al. (1966) as the upper limit for an acceptable total organism count for non-sterile medicine. The $\grave{E}$ value for *S. aureus* is 1.2 h. The calculation of the $\grave{E}$ value in this study was based on five experimental points, and thus the result should be more reliable.

TABLE 1

D and E values for different concentrations of S. aureus in 0.5% phenol solution

Concentration (No. of organisms/ml)	D [a] (h)	E (h)
5.38×10^4	1.48 (±0.34)	7.0
2.39×10^5	1.48 (±0.34)	7.9
2.08×10^6	2.26 (±0.78)	14.3
1.29×10^7	4.15 (±1.24)	29.5
2.8×10^8	6.39 (±2.35)	53.9

[a] Mean values of five determinations. The 95% confidence intervals for the mean are given in parentheses.

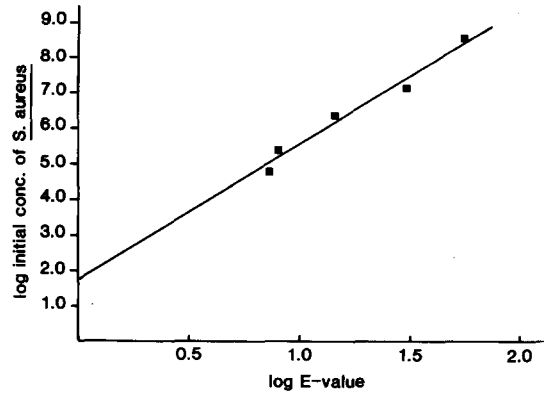

Fig. 2. Relation between the E value and the initial concentrations of *S. aureus* in 0.5% phenol solution.

Conclusions

(1) The preservative efficacy is unaffected by the concentration of challenge organisms up to the point at which the system becomes overloaded.
(2) The study of preservative efficacy by application of the kinetic approach is appropriate and offers rapid and quantitative information on the antimicrobial properties of preservatives.

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