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# Factorial designs in the evaluation of preservative efficacy

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**Summary**

An approach to evaluate preservative efficacy using a $2^3$ factorial design is presented. The influence of pH and tonicity of the solution and the presence of EDTA on the antimicrobial activity of phenol was studied. *C. albicans* and *E. coli* were employed as test organisms. Statistically significant effects ($P < 0.005$) of pH and the presence of EDTA on the efficacy of phenol were obtained with both organisms. The tonicity of the solution had a significant effect ($P < 0.005$) only with *E. coli*. Significant interactions ($P < 0.005$) were found between pH and tonicity of the solution with both organisms, and between tonicity of the solution and the presence of EDTA only with *C. albicans*. Furthermore, interactions among all 3 factors were detected with *C. albicans* ($P < 0.005$). The application of a factorial design study can be useful in the evaluation of preservative efficacy at the preformulation stage.

## Introduction

The antimicrobial activity of preservatives is influenced by several factors, such as the pH, tonicity and temperature of the solution, the concentration of preservative, the presence of EDTA, etc. Several studies on the effect of these factors have been reported (Entrekin, 1961; Wickliffe and Entrekin, 1964; Brown and Richards, 1965; Monkhouse and Groves, 1967; Richards and McBride, 1972; Davies et al., 1976; Karabit et al., 1985, 1986, 1988). These studies were based on a "one factor at a time" procedure which is often tedious and does not allow for the possibility of discovering interactions between different variables.

The factorial design provides a means to evaluate simultaneously the influence of individual variables and their interactions at several levels with a minimum of experiments, and subsequently in a shorter time and with less cost. The application of factorial design to stability studies on various pharmaceutical systems has been reported (Bolton, 1983; Bolton et al., 1984; Dévay et al., 1985; Waltersson, 1986; Ahlneck and Waltersson, 1986; Gupta, 1988; Hung et al., 1988).

The aim of this study was to demonstrate the applicability of factorial design analysis by investigating the effect of different factors on the antimicrobial activity of a preservative.

## Materials and Methods

*Preservative and reagents.* Phenol and sodium ethylenediaminetetraacetate (EDTA, Natrii edetas)

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were of pharmacopeial grade (Ph. Nord. 63). All other chemicals were of reagent grade.

*Test organisms.* Cultures of *Candida albicans* (ATCC No. 10231) and *Escherichia coli* (ATCC No. 8739).

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

*Test solution*

0.3% and 0.5% phenol solutions were used, for *E. coli* and *C. albicans*, respectively. The solutions were prepared at pH 5.1 and 8.1 using 0.05 M acetate and phosphate buffer, respectively. Test solutions were made hypotonic (216 mOsm/liter) and hypertonic (400 mOsm/liter) by addition of adequate amounts of sodium chloride. The concentration of EDTA applied was 0.1%.

After sterilization the 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 of the test organisms, *E. coli* was grown in TSB at 32° C for 24 h, and *C. albicans* at 20–25° C for 48 h in a medium consisting of 2% dextrose and 1% bacteriological peptone.

The organisms were harvested from their 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 $10^7$–$10^8$ 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 an absorbance of 0.1 and 0.7 corresponded to $10^7$–$10^8$ cells per ml of *E. coli* and *C. albicans*, respectively.

*Test procedure*

Portions of 9 ml of the test solution were dispensed in 20 ml vials which, after sealing with rubber stoppers, were sterilized by autoclaving at 121° C for 20 min. 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 (Schleicher and Schuell, 0.45 μm, 47 mm diameter), thereafter it was rinsed with 300 ml sterile peptone water (0.1%) and then incubated at 32° C for 24 h on TSA and at 20–25° C for 48 h on SAB for *E. coli* and *C. albicans* growth, respectively.

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.

All tests were carried out at room temperature (approximately 20° C).

*Factorial design*

In this study a $2^3$ factorial design was applied. The effect of 3 factors on the efficacy of phenol was studied at two levels. These factors are pH (A), the tonicity of the solutions (B) and concentration of EDTA (C), and values for the two levels are summarized in Table 1. In Table 2, the calculation matrix for a $2^3$ factorial design is presented (Bolton, 1984). For the 3 factors A, B and C, the 8 combinations are designated; (1), a, b, ab, c, ac, bc, and abc, where (1) refers to all factors at their low level; if factor A is at its high level, and B and C are at their low level, the combination is denoted as a; and so on.

*Statistical procedures*

The main effect and the interactions can be calculated (Yates, 1937) from the totals of the

TABLE 1

*Factorial design parameters and experimental conditions*

| Factors | Factor levels | |
|---|---|---|
| | Low (−) | High (+) |
| (A) pH of the solution | 5.1 | 8.1 |
| (B) Tonicity of the solution | 216 mOsm/l | 400 mOsm/l |
| (C) EDTA concentration | 0% | 0.1% |

TABLE 2

*Calculation matrix for a $2^3$ factorial design*

| Factor combination | Level of factor in experiment [a] | | | Level of the interactions [b] | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | AB | AC | BC | ABC |
| (1) | − | − | − | + | + | + | − |
| a | + | − | − | − | − | + | + |
| b | − | + | − | − | + | − | + |
| ab | + | + | − | + | − | − | − |
| c | − | − | + | + | − | − | + |
| ac | + | − | + | − | + | − | − |
| bc | − | + | + | − | − | + | − |
| abc | + | + | + | + | + | + | + |

[a] −, factor at low level; +, factor at high level.

[b] Multiply signs of factors to obtain signs for interaction terms in combination (e.g. AB at (1) = (−) × (−) = (+)).

individual treatment combinations by means of the table of signs (Table 2). The calculation of these values was performed by a program for 3-way analysis of variance on a desk top computer Hewlett-Packard 9825T. The resulting sum of squares and subsequent significance test was also obtained from this program.

## Results and Discussion

Survivor curves were obtained for each combination of test organism and test solution from the plots of the log number of surviving organisms versus exposure time by linear regression (Fig. 1). D-Values were calculated from the slopes of these curves. Fits of the linear models were acceptable with correlation coefficients, $r = 0.984 \pm 0.0204$ (mean ± S.D., $n = 48$). The D-values were then used for expressing the resistance of the microorganisms to phenol under different test conditions.

At a concentration of 0.3%, phenol had only fungistatic activity against *C. albicans*, therefore, 0.5% phenol was used.

Some of the factors included in the experiments have been studied earlier in "one factor at a time" trials. Therefore, the information obtained from factorial analysis should be compared with those results.

If the effects of some or all factors vary with changes in the other factors, the effect is denoted

TABLE 3

*Results and analysis of variance for the $2^3$ factorial experiment (run in triplicate). The effect of pH and tonicity of the solution and presence of EDTA on the D-values for E. coli*

| Factor | D-value (h) | | | d.f. | Mean square | $F$ [a] |
|---|---|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 | | | |
| (1) | 7.9 | 7.1 | 6.7 | | | |
| a | 4.57 | 5.0 | 4.63 | 1 | 85.5 | 209.7 *** |
| b | 10.3 | 9.9 | 12.2 | 1 | 19.40 | 47.67 *** |
| ab | 4.99 | 5.0 | 4.55 | 1 | 21.43 | 52.27 *** |
| c | 4.67 | 5.8 | 6.1 | 1 | 7.752 | 19.32 *** |
| ac | 5.1 | 4.07 | 3.59 | 1 | 1.224 | 2.936 |
| bc | 9.2 | 9.5 | 9.3 | 1 | 0.0104 | 0.029 |
| abc | 3.85 | 4.26 | 3.76 | 1 | 0.160 | 0.455 |
| Experimental error | | | | 16 | 0.408 | |

[a] Significance level based on $F_{0.995} = 10.58$; *** $P < 0.005$.

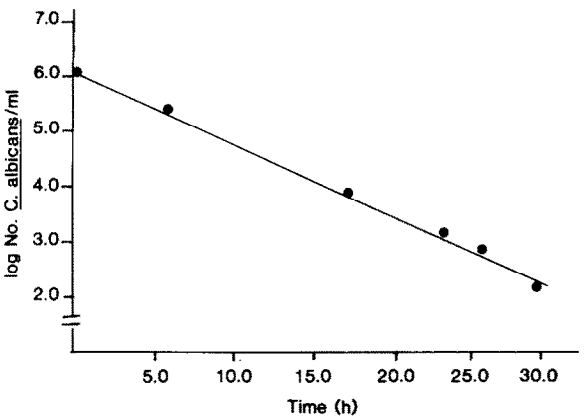


Fig. 1. Linear regression fit of the log number of survivors of *C. albicans* in hypertonic, 0.5% phenol solution at pH 5.1 versus time (h). ●, experimental points; ———, regression fits; $r = -0.998$.

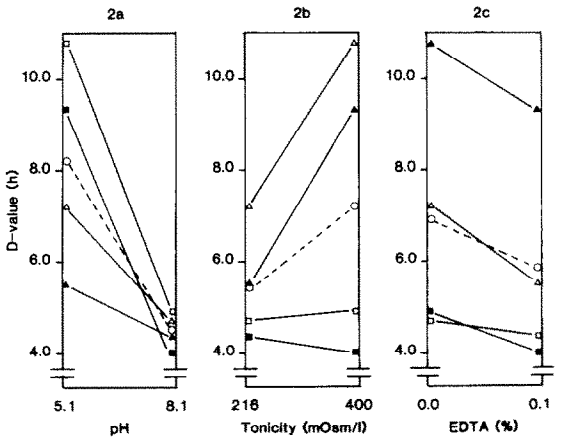


Fig. 2. The effects of pH (2a), tonicity (2b), and concentration of EDTA (2c) on the D-values for *E. coli* in 0.3% phenol solution. (a) △, B−C−; ▲, B−C+; □, B+C−; ■, B+C+; ○, main effect. (b) △, A−C−; ▲, A−C+; □, A+C−; ■, A+C+; ○, main effect. (c) △, A−B−; ▲, A−B+; □, A+B−; ■, A+B+; ○, main effect.

"interaction". According to the matrix in Table 2, the value for the effect of each factor or interaction can be obtained.

The effects of the pH and tonicity of the solution and the presence of EDTA on the antimicrobial activity of phenol against *E. coli* and *C. albicans* are presented in Tables 3 and 4 (triplicate results), and visualized in Figs. 2 and 3, respectively (Bolton et al., 1984). The slope of the lines in the figures is an indicator of the magnitude of the effect of the factor marked on the *x*-axes, thus horizontal lines indicate that no effect was obtained. The dotted lines marked "main effect" are obtained from the average of all data at the low and high levels of the factors in each diagram.

TABLE 4

*Results and analysis of variance for the $2^3$ factorial experiment (run in triplicate). The effect of pH and tonicity of the solution and presence of EDTA on the D-values for C. albicans*

| Factor | D-value (h) | | | d.f. | Mean square | $F$ [a] |
|---|---|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 | | | |
| (1) | 7.9 | 7.8 | 7.9 | 1 | | |
| a | 26.3 | 27.0 | 29.4 | 1 | 1051 | 611.1 *** |
| b | 6.1 | 7.3 | 5.8 | 1 | 15.06 | 8.756 |
| ab | 15.2 | 12.7 | 12.6 | 1 | 43.71 | 25.42 *** |
| c | 7.2 | 8.0 | 10.9 | 1 | 63.34 | 36.83 *** |
| ac | 20.4 | 21.9 | 20.3 | 1 | 0.165 | 0.096 |
| bc | 12.9 | 11.8 | 12.3 | 1 | 229.3 | 133.4 *** |
| abc | 27.8 | 27.0 | 24.3 | 1 | 77.79 | 45.24 *** |
| Experimental error | | | | 16 | 1.719 | |

[a] Significance level based on $F_{0.995} = 10.58$; *** $P < 0.005$.

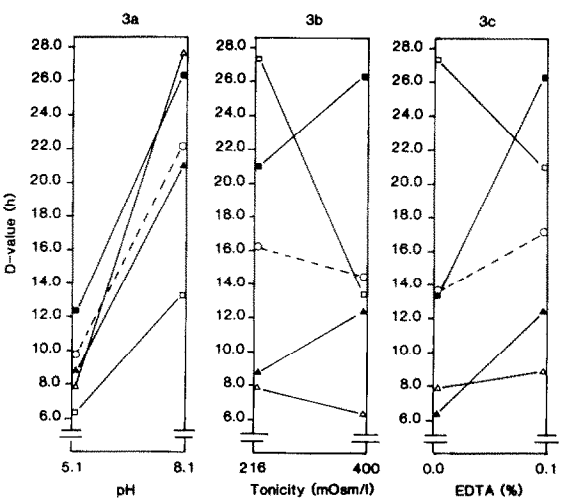


Fig. 3. The effects of pH (3a), tonicity (3b), and concentration of EDTA (3c) on the D-values for *C. albicans* in 0.5% phenol solution. (a) △, B−C−; ▲, B−C+; □, B+C−; ■, B+C+; ○, main effect. (b) △, A−C−; ▲, A−C+; □, A+C−; ■, A+C+; ○, main effect. (c) △, A−B−; ▲, A−B+; □, A+B−; ■, A+B+; ○, main effect.

*Escherichia coli*

D-Value data and factorial analyses are represented in Table 3 and illustrated in Fig. 2a–c. pH is the main factor which significantly ($P < 0.005$) affects the antimicrobial activity of phenol against *E. coli*. Lower D-values (increased antimicrobial activity) were obtained by changing the pH of the solution from 5.1 to 8.1. Similar results were obtained in an earlier study where a 0.5% phenol solution was tested, using a "one factor at a time" study (Karabit et al., 1985).

In Fig. 2 the effects of pH and tonicity of the solution and concentration of EDTA on the D-values are illustrated. For example, in hypertonic solution (B + ) and in the presence of 0.1% EDTA (C + ), an increase in pH of the solution from 5.1 to 8.1 results in a lowering of the D-value from 9.3 to 3.95 h (mean values of combinations bc and abc) (Fig. 2a). The diagrams in Fig. 2b,c may be interpreted in a similar manner when investigating the main effects.

The effect of tonicity of the solution is statistically significant ($P < 0.005$) (Table 3). The antimicrobial activity of phenol is decreased as the tonicity of the solution changes from hypotonic to hypertonic. This is indicated by an increase in D-values (Fig. 2b). A similar effect has been reported by Monkhouse and Groves (1967) for benzalkonium chloride.

The significant effect ($P < 0.005$) of EDTA on the efficacy of phenol (Table 3) is illustrated by the slope of the lines in Fig. 2c. In other studies (Monkhouse and Groves, 1967; Smith, 1970; Richards and McBride, 1972; Russell and Furr, 1977; Karabit et al., 1988) it has been found that the antimicrobial activities of several preservatives are strongly potentiated by EDTA.

A significant interaction ($P < 0.005$) between pH and tonicity of the solutions was obtained (Table 3) and is illustrated in Fig. 2a by the different slopes of the lines labelled (B + C − ) and (B − C − ), as well as (B + C + ) and (B − C + ). Thus, the activity of phenol is strongly potentiated by an increase in pH in a hypertonic solution compared to that in a hypotonic solution.

The addition of EDTA gave no significant interaction effect in this study with regard to both pH and tonicity of the solution. In Fig. 2b the BC interaction may be assessed visually by comparing the slopes of the lines labelled (A − C − ) and (A − C + ), and also those of labelled (A + C − ) and (A + C + ). These lines are approximately parallel and indicate that the effect of the tonicity is independent of the presence of EDTA. The lack of interaction between the pH of the solution and the presence of EDTA (AC) could be interpreted in the same manner (Fig. 2a).

*Candida albicans*

The pH of the solutions had a significant effect ($P < 0.005$) on the antimicrobial activity of phenol (Table 4), also indicated by the slope of the line labelled "main effect" (Fig. 3a). The D-values increase on changing the pH of the solutions from 5.1 to 8.1, i.e. the antimicrobial activity of phenol is decreased. This is in accordance with results from "one factor at a time" trials reported earlier (Karabit et al., 1985). The main effect curve of the EDTA factor (C) (Fig. 3c) indicates the significant effect ($P < 0.005$) of this factor (Table 4). No plausible explanation was found or discussed for the decrease in efficacy of phenol. On the other hand, the tonicity factor curve (B) (Fig. 3b) indicates no significant effect (Table 4).

Significant interactions ($P < 0.005$) between the pH and the tonicity of the solution (AB), tonicity of the solution and the presence of EDTA (BC), and among 3 factors A, B and C were detected (Table 4).

The BC interaction is illustrated in Fig. 3b, by the increase in D-value in the presence of EDTA in hypertonic solution. The lack of significant interactions between factors, e.g. AC interaction (Table 4), could not be assessed visually by comparing the slopes of the lines (B + C + ) and (B + C − ), or (B − C − ) and (B − C + ) (Fig. 3a). This might be attributed to the interaction among all 3 factors (Table 4).

## Conclusions

Factorial design could be a useful screening tool for the selection of preservatives for pharmaceutical preparations. At the preformulation stage the effects of various formulation factors

and the interactions between them can be studied simultaneously with less experimental effort and cost. Although the mechanisms behind these interactions are not fully understood, the additional information provided by the factorial experimental design could be used for further investigations.

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