International Journal of Pharmaceutics, 46 (1988) 141–147
Elsevier

IJP 01558

Studies on the evaluation of preservative efficacy *. III. The determination of antimicrobial characteristics of benzalkonium chloride

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(Received 4 January 1988)
(Accepted 24 February 1988)

Key words: Preservative; Preservative efficacy; Benzalkonium chloride; Antimicrobial characteristic; *D*-value; Activation energy; Temperature coefficient; Concentration exponent

Summary

Aspergillus niger, Candida albicans, Escherichia coli, Pseudomonas aeruginosa and *Staphylococcus aureus* were used as test organisms for the study of preservation efficacy of benzalkonium chloride alone, or in combination with 0.1% EDTA. The influence of different environmental factors, such as the pH of the solution and the temperature and the concentration of the preservative, on the antimicrobial activity of benzalkonium chloride were also investigated. *D*-value, activation energy (E_a), temperature coefficient (Q_{10}) and concentration exponent (η) were used as parameters in determining the preservative effect. The results showed that the kinetic approach to the screening of the antimicrobial activity of benzalkonium chloride – a quaternary ammonium compound – is feasible and appropriate.

Introduction

In two earlier publications (Karabit et al., 1985, 1986) we demonstrated the feasibility of a systematic kinetic approach to the screening of preservative characteristics. Phenol – an acidic type of preservative – and benzylalcohol – a neutral type of preservative – were used as model compounds. The studies covered the influence of various environmental factors as the pH and temperature of the solution and the concentration of preservatives. Death-rate curves, decimal reduction time values (*D*-values), pH-rate profiles, activation energies, temperature coefficients (Q_{10}) and concentration exponents (η) were employed as a measure of the parameters influencing the in-use activity of antimicrobial agents.

The purpose of this investigation was to expand the applicability of the kinetic approach by using benzalkonium chloride, a quaternary ammonium compound, alone and in combination with sodium edetate as model compound.

Materials and Methods

Preservative and reagents

Benzalkonium chloride (Bc) (alkylbenzyldimethyl ammonium chloride. Merck, F.R.G.).

Part I: *Acta Pharm. Suec.*, 22 (1985) 281–290. Part II: *J. Clin. Hosp. Pharm.*, 11 (1986) 281–289.

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Sodium ethylenediaminetetraacetate (EDTA) (Ph. Nord. 63). All other chemicals were of reagent grade.

Test organisms

Cultures of the following micro-organisms were used: *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027) and *Staphylococcus aureus* (ATCC No. 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).

Solution preparation

Data on the test solutions of Bc are given in Table 1. The solutions were hypotonic (100–151 mOsm/litre).

The pH values were determined after sterilization (Metrohm E632 digital pH-meter, Switzerland), equipped with a type EA120 combined electrode.

Preparation of inoculum

The micro-organisms were maintained by subculturing 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, 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) 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

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. 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:

TABLE 1

Test solutions of benzalkonium chloride

%Bc [a]	pH of the solution					
	Acetate buffer 0.05 M			Phosphate buffer 0.05 M		
0.00055	–	–	–	6.1	–	–
0.00083	–	–	–	6.1	–	–
0.0011	3.3	4.0	5.2	6.1	7.1	8.1
0.00167	–	–	–	6.1	–	–
0.0022	–	–	–	6.1	–	–
0.0156	3.3	4.0	5.2	6.1	7.1	8.1
0.0178	–	–	–	6.1	–	–
0.0200	–	–	–	6.1	–	–
0.0011 + 0.11% EDTA	3.3	4.0	5.2	6.1	7.1	8.1

[a] The test procedure involves a 10% dilution of the Bc solutions.

(a) In the case of bacteria or *C. albicans,* to a membrane filter (Gelman GN-6, 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.

(b) In the case of *A. niger,* it was 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.

Test conditions

All experiments were carried out at room temperature (about 20 °C) unless otherwise specified. In the tests carried out at different temperatures, a refrigerator (+3 °C), room temperature and two thermostatically controlled cabinets, with ±0.15 °C in precision (30 ° and 40 °C), were used. The solutions in these tests were kept at the test temperature at least 24 h prior to the experiments.

Calculations

The theoretical and mathematical details of preservation kinetics are described in our previous papers (Karabit et al., 1985, 1986).

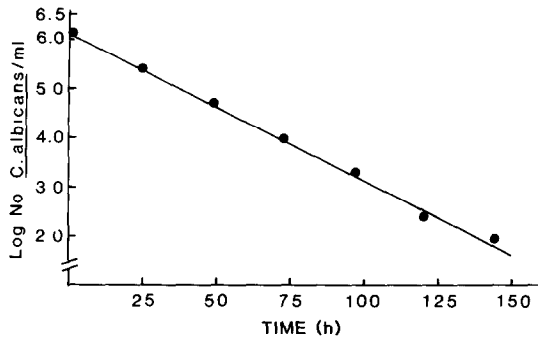

Fig. 1. Linear regression fit of the log number of survivors of *C. albicans* at pH 5.2 and in 0.001% Bc vs time (h). ●, Experimental points; (———) regression fits; $r = -0.999$.

Results and Discussion

The death-rate curves were plotted for each organism in each test sample. A typical curve for *C. albicans* is shown in Fig. 1. From the slopes of the lines, obtained by linear regression, the decimal reduction times (*D*-values) were calculated. Fits of the linear models were good with correlation coefficients, $r = 0.993 \pm 0.0055$ (mean ± S.D., $n = 112$).

Bc had antimicrobial activity against Gram-positive as well as Gram-negative bacteria,

TABLE 2

D-value for the test organisms in benzalkonium chloride solutions with various pH

Micro-organism	*D*-values in hours at pH [a]					
	3.3	4.0	5.2	6.1	7.1	8.1
Aspergillus niger [b]	9.35	5.58	7.46	5.72	40.26	12.67
Candida albicans	26.78	39.23	25.14	9.66	5.43	2.68
Escherichia coli	0.23	0.19	0.12	0.063	0.039	0.035
Pseudomonas aeruginosa	c	0.203	0.24	3.01	1.5	0.47
Pseudomonas aeruginosa [d]	c	e	0.09	0.081	0.13	0.05
Staphylococcus aureus	5.42	5.95	7.09	3.12	0.75	0.13

[a] Mean values of two determinations.
[b] In 0.014% Bc.
[c] Growth was inhibited without preservative.
[d] In 0.001% Bc + 0.1% EDTA.
[e] The elimination of the organism occurred so fast that the *D*-values could not be determined.

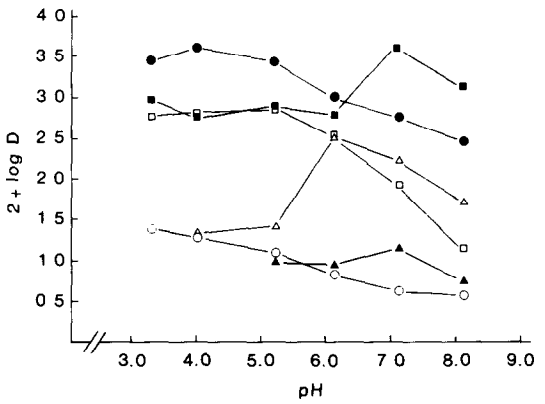

Fig. 2. pH-rate profile for the test organisms in 0.001% Bc solutions (room temperature). ■, *A. niger* (in 0.014% Bc); ●, *C. albicans*; ○, *E. coli*; △, *P. aeruginosa*; □, *S. aureus*; ▲, *P. aeruginosa* (in 0.001% Bc + 0.1% EDTA).

E. coli was significantly most sensitive (Table 2 and Fig. 2). It also possessed antifungal properties, but the fungi were more resistant than the bacteria. At low concentrations Bc was fungistatic rather than fungicidal against *A. niger* (see Table 4).

Effect of pH

D-values for Bc within the pH range of 3–8 at room temperature are reported in Table 2. Plottings of log *D*-values vs pH gave the pH-rate profiles (Fig. 2). Solutions of 0.001% Bc were used for determining the pH-rate profiles of *C. albicans* and bacteria. For *A. niger* a concentration lower than 0.014% Bc only had fungistatic effect. Therefore 0.014% Bc was used for the study of the pH-profile of *A. niger*.

Bc was found to be less active at low pH. The antimicrobial activity was higher in neutral and especially in slightly alkaline (pH 8) solution, indicated by lower *D*-values (Table 2 and Fig. 2). Similar pattern of results has been reported earlier (Monkhouse and Groves, 1967), they assumed that these findings can be explained by a cationic interaction of Bc with the cell wall, which is negatively charged at higher pH.

Higher *D*-values for *P. aeruginosa* at pH 6–7 confirm the resistance of the organism against Bc, which might be due to the biochemical modification of the cell envelope. Thus, the integrity of the cell envelope of *P. aeruginosa* depends substantially upon the outer membrane, which is stabilised by divalent metal ions, particularly Mg^{2+}.

The combination of Bc and 0.1% EDTA decreased the resistance to Bc (Table 2 and Fig. 2). Similar results have been reported earlier in several studies (Brown and Richards, 1965; Monkhouse and Groves, 1967; Adair et al., 1971; Russell, 1971; Richards and MacBride, 1972). This potentiating effect of EDTA might be attributed to the release of lipopolysaccharide and loosely bound lipid from the cell wall by EDTA (Gray and Wilkinson, 1965; Cox and Eagon, 1968; Russell, 1971; Leive, 1974), and by the chelation of calcium or magnesium ions in the outer cell membrane (Brown and Richards 1965; Leive 1974; Boggis et al. 1979; Kenward et al. 1979). Thus, the cell wall seems unable to maintain cell integrity. Furthermore, Fig. 2 shows, as expected, that antimicrobial activity is higher at higher pH levels, findings supported by observations of Monkhouse and Groves (1967).

The growth of *P. aeruginosa* was inhibited without preservative at pH 3.3 (Karabit et al., 1985, 1986). At pH 4 the high activities of the combination of 0.001% Bc and 0.1% EDTA resulted in the elimination of the same organism in a much shorter time that the death-rate constant could not be determined.

Effect of temperature

The effect of temperature on the antimicrobial activity of Bc was studied at pH 6.1 within the temperature range of 3°–40°C (Table 3). The concentration of Bc used was 0.001% except for *A. niger* where a concentration of 0.014% was required for fungicidal effect. However, in spite of the increased concentration, Bc had only a fungistatic effect on *A. niger* at 3°C. At 40°C *E. coli* was eliminated so fast that the death-rate constant could not be determined.

The death-rate constants obtained at various temperatures are also treated according to the Arrhenius equation (Pflug, 1972). The plots of log death-rate constant vs the reciprocal of absolute temperature gave straight lines (Fig. 3). From the slopes of these lines, obtained by linear regression, the activation energies (E_a) and temperature coef-

TABLE 3

D-, E_a- and Q_{10}-values for benzalkonium chloride at pH 6.1

Test organism	*D*-values (h) [a]				E_a (kJ/mol)	Q_{10}
	3 °C	20 °C	30 °C	40 °C		
Aspergillus niger [b]	[c]	5.72	1.39	0.16	133.9	6.1–5.5
Candida albicans	64.78	9.66	5.92	0.81	76.7	3.2–2.7
Escherichia coli	2.66	0.063	0.036	[d]	85.9	3.7–3.2
Pseudomonas aeruginosa	19.92	3.01	0.64	0.44	76.3	3.2–2.7
Staphylococcus aureus	22.71	3.12	0.44	0.25	92.3	4.1–3.2

[a] Mean values of two determinations.
[b] In 0.014% Bc.
[c] Benzalkonium chloride had fungistatic effect only.
[d] The elimination of the organism occurred so fast that *D*-value could not be determined.

ficients (Q_{10}) were calculated (Table 3). The temperature coefficients lies in the range of 3–6 (Table 3). From the Arrhenius plots (Fig. 3), it could be estimated that the death-rates decreased with a factor of 11–20 for the test organisms with a temperature change from 25 °C to 3 °C (refrigerator temperature). Similar findings are reported by others (Hugo and Foster, 1964). This indicates that preserved multidose unit containers may be stored at room temperature between withdrawals, if it is appropriate from the drug stability point of view (Karabit et al. 1985, 1986). This recommendation is, however, not supported by Davies et al. (1976).

The activation energy values (E_a) for Bc at pH 6.1 (Table 3) were found to be on the same level (80–90 kJ/mol) as those for phenol and for benzylalcohol (Karabit et al. 1985, 1986).

Effect of preservative concentration

The concentration dependence was evaluated by determining the concentration exponents (dilu-

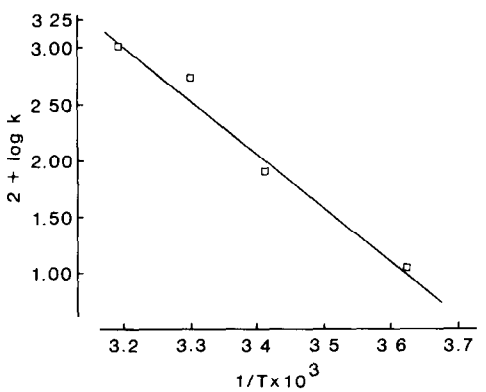

Fig. 3. The Arrhenius plot for *S. aureus* at pH 6.1; $r = -0.988$.

TABLE 4

The concentration dependence of benzalkonium chloride in aqueous solution at pH 6.1

Test organism	*D*-values (h) [a]								Conc. exponent (η)
	0.0005%	0.00075%	0.001%	0.0015%	0.002%	0.014%	0.016%	0.018%	
A. niger	b	b	b	b	b	5.72	2.19	0.59	9.0
C. albicans	71.42	–	9.66	–	5.5	–	–	–	1.8
E. coli	0.75	0.15	0.06	c	c	–	–	–	3.6
P. aeruginosa	b	b	3.01	0.092	0.054	–	–	–	6.0
S. aureus	13.09	–	3.12	–	0.67	–	–	–	2.2

[a] Mean values of two determinations.
[b] Benzalkonium chloride was only fungistatic or bacteriostatic.
[c] The elimination of the organism occurred so fast that *D*-values could not be determined.

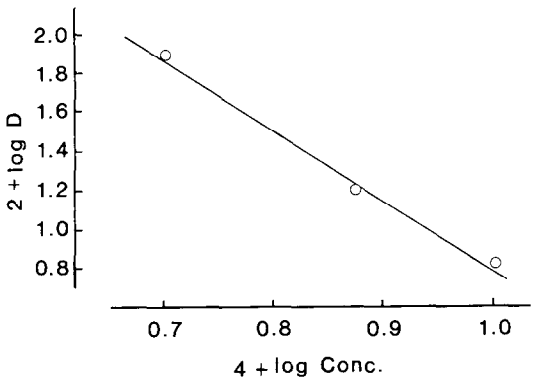

Fig. 4. The relationship between concentration and antimicrobial activity of benzalkonium chloride in aqueous solution at pH 6.1. Test organism *E. coli*.

tion coefficients or η-values) (Bean, 1967, 1972). The relationship between the concentration of Bc and its intrinsic activity was investigated at 3 concentration levels (Table 4).

According to the exponential relationship between concentration and intrinsic activity, the logarithms of the *D*-values were plotted vs the logarithms of Bc concentrations. The slope of the straight lines, calculated by linear regression, gave the concentration exponent. Fig. 4 shows such a plot for *E. coli* at pH 6.1.

For *E. coli* the killing rate was so fast at concentration $\geqslant 0.0015\%$ Bc that the *D*-values could not be determined (Table 4). On the other hand, concentrations $\leqslant 0.00075\%$ Bc had only bacteriostatic effect on *P. aeruginosa* (Table 4).

η-values of Bc with bacteria and *C. albicans* were found to be about 2-6 and about 9 with *A. niger* (Table 4).

Conclusions

Three general principles apply to a microbiological method for testing antimicrobial agents (Croshaw, 1981). If useful information is to be obtained, it should (1) give information that can be interpreted in terms of practical use, (2) give repeatable and reproducible results and (3) be adequately controlled.

The antimicrobial efficiency of a preservative is usually examined at two stages of testing. The first stage concerns laboratory tests in which it is verified whether a chemical compound possesses antimicrobial activity, screening tests. In this paper as well as in two earlier publications (Karabit et al. 1985, 1986) we have studied the applicability of a systematic kinetic approach to the screening of preservation properties using preservatives of different chemical structure as model substances. We have found that this approach for testing preservatives well fulfils the requirements stated above in the 3 principles for a microbiological method in this connection. It can give complete and useful information about the influence of the external physical environment, temperature and pH, as well as of the modification of the concentration of the antimicrobial agent. The antimicrobial activity is determined by death-rate curves, which give high experimental precision and repeatable results. The method is also well controlled. The pH-rate profile presents a clear and illustrative view of the pH-dependence. From the quantitative relation between temperature and death-rate, according to Arrhenius, data can be obtained, which give a complete picture of the influence of temperature on the preservation efficiency. Some of the data obtained may be of theoretical value only. Taken in toto, however, they may lead to a better understanding of the reasons for the sensitivity or resistance of micro-organisms to antimicrobial agents, as well as to possible means of improving the activity of such agents.

Acknowledgements

The authors are grateful to Mr. Ola Flink, ACO Läkemedel AB, for supplying the micro-organisms. We also wish to thank Professor Christer Nyström for fruitful discussions, and Mrs. Eva Nises Ahlgren for preparing the manuscript.

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