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Kinetic evaluation of claimed synergistic paraben combinations using a factorial design

D. Gilliland, A. Li Wan Po and E. Scott

The Drug Delivery Research Group, The School of Pharmacy, The Queen's University of Belfast, Northern Ireland

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D. GILLILAND, A. LI WAN PO AND E. SCOTT. 1992. The antimicrobial effects of methyl and propyl parabens are investigated, with *Escherichia coli* as test organism, with a view to determining whether the parabens act synergistically. At appropriate concentrations, the parabens killed *E. coli* cells according to first order kinetics and the bactericidal effects were quantified by the first order kill rate constants. Combinations of methyl or propyl parabens, at concentrations which slow down or inhibit bacterial growth when used singly, produced definite kill. In this sense, the parabens are therefore synergistic since in combination they produce an effect which is not observed when they are used singly. This effect is not true synergism as shown by the results of our experiments with a factorial design. Analysis of variance indicated no significant interaction between the two parabens.

INTRODUCTION

Combinations of antimicrobial agents are widely used both for treating diseases and for preserving pharmaceutical systems. The rationale is that by using combinations, the activity spectrum may be broadened and the agents involved may act additively or synergistically. Sometimes one of the agents in a combination may by itself be inactive. Despite the widespread use of antimicrobial combinations, clinical evidence for synergy is difficult to generate even with *in vitro* systems. The best method for demonstrating synergism is the subject of controversy (Berenbaum 1977). A widely used combination preservative system for which there is much in-use evidence for at least an additive antimicrobial effect is the methyl paraben/propyl paraben mixture. In this report we describe studies designed to evaluate whether the two parabens act additively or synergistically. A kinetic method and a factorial experimental design were used.

MATERIALS AND METHODS

Preparation of media

The chemically defined medium contained (g/l): Na_2HPO_4, 11·45; KH_2PO_4, 1·4025; $(NH_4)_2SO_4$, 1·87; M_5SO_4, 0·187; D-glucose, 0·909; $CaCl_2(2H_2O)$, $1{\cdot}245 \times 10^{-5}$; $FeSO_4$ $(7H_2O)$, 5×10^{-7}. The pH was adjusted to 6·9 with dilute HCl. All chemicals were of analytical reagent quality.

Correspondence to: Prof. A. Li Wan Po, The Drug Delivery Research Group, The School of Pharmacy, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9, Northern Ireland.

Preparation of inoculum

Escherichia coli NCIB 8545 was maintained on Tryptone Soya Agar (Oxoid) slants at 4°C. A loopful of the organism was added to 100 ml of sterile media and grown at 37°C overnight in a shaking waterbath at 100 rev/min. Transfers of organism were made daily for 2 d. On the third transfer the organisms were allowed to grow to an optical density reading of 0·1 at 540 nm (Corning colorimeter 254). This provided cells in the exponential phase of growth. The absorbance value of 0·1 at 540 nm was found to be approximately equal to 1×10^8 cfu/ml. An inoculum of 1×10^9 cfu/ml was prepared by filtering the culture (100 ml), under aseptic conditions, through a 0·45 μm membrane filter and washing with 100 ml of fresh, pre-warmed media. The organisms were then resuspended in 10 ml of media to give the final inoculum.

Preparation of test solutions

The appropriate weights of the methyl and propyl esters of *p*-hydroxybenzoic acid (Sigma) were added to 1 l of medium and placed in a sonic bath for up to 4 h to aid solubilization. The solution was filter-sterilized and 100 ml of test solution dispensed into 250 ml flasks. Medium without any parabens was employed as control. Before inoculation, the test solutions were maintained at 37°C in a shaking waterbath for at least 18 h.

Measurement of microbial numbers

After inoculation of the test solutions viable counts of microbial numbers were made at regular intervals. A 1 ml sample was removed from the test solution and serial 10-fold dilutions were made in 0·1% peptone water. One ml volumes of the dilutions were plated by the pour plate method with Isosensitest Agar (Oxoid). After incubation at 37°C for 20 h, colonies were counted and the number of cfu/ml evaluated.

Statistical analysis

A 2^2 factorial design was used in our study. The low paraben levels corresponded to 0·12% w/v for methyl paraben and 0·012% w/v for propyl paraben. The high concentrations corresponded to 0·14% w/v for methyl paraben and 0·014% w/v for propyl paraben. In total 80 kinetic runs were performed over 16 d and each day involved five kinetic runs. One kinetic run consisted of an enumeration of the number of colony-forming cells at predetermined time intervals. Within-day variability was of the same order as between-day variability. Therefore blocking was not required.

RESULTS

Combination of methyl paraben and propyl paraben at 0·12% w/v and 0·014% w/v concentrations, respectively, produced definite kill of the *E. coli* cells (Fig. 1). There was no obvious lag phase in the kill curve and the data closely fitted first order kinetics. Table 1 lists the appropriate statistics for the rate constants at all combinations studied.

In order to investigate whether the two parabens in combination produced a synergistic effect we chose 0·12 and 0·14% w/v for methyl paraben and 0·012 and 0·014% w/v for propyl paraben because pilot studies showed that at lower concentrations the combinations were often only bacteriostatic and consistent kill rate constants could not be calculated. Combinations of higher paraben concentrations on the other hand killed the bacteria too quickly for the required sampling to be carried out satisfactorily. Consequently, the kill rate constants derived from the kill curves were again imprecise.

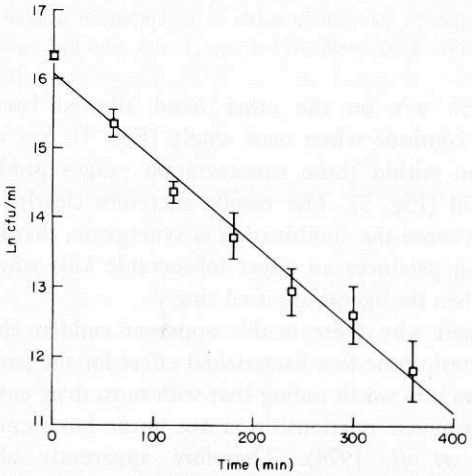

Fig. 1 A semi-logarithmic plot of the effect of 0·12% methyl paraben and 0·14% propyl paraben, in combination, on

Table 1 The effect of concentration of methyl and propyl parabens alone and in combination on *Escherichia coli*

Concentration (% w/v)	First order rate constant (/min)	S.E. $n = 20$
Control	0·0143	0·0003
0·012 propyl paraben	0·0105	0·0003
0·014 propyl paraben	0·0110	0·0002
0·12 methyl paraben		
+0·012 propyl paraben	−0·0091	0·0005
0·12 methyl paraben		
+0·014 propyl paraben	−0·0289	0·0032
0·14 methyl paraben		
+0·012 propyl paraben	−0·0385	0·0034
0·14 methyl paraben		
+0·014 propyl paraben	−0·0649	0·0026

Evaluation of the results showed that the within-day variability was of the same order as the between-day variability. Blocking was therefore not advantageous and each experimental kill curve was considered to be an independent run.

Analysis of variance of the rate constants showed that there was no synergistic effect with the two parabens at the concentrations used (Table 2). In a factorial experiment such as the present one, interaction is shown by a change in slope of the kill rate constant and concentation plot when the concentration of the interactant is changed. Absence of interaction is conversely shown by parallel lines (Cochran & Cox 1957) as was demonstrated in this study when the methyl paraben concentration was increased while maintaining the propyl paraben concentration at either 0·012% w/v (low) or 0·014% w/v (high) (Fig. 2) and, correspondingly, when the propyl paraben concentration was increased while maintaining the methyl paraben concentration constant.

Antimicrobial synergism is an elusive concept (Moellering 1979). Although the most widely accepted definition of this term is 'the joint action of two or more antimicrobial agents to produce an effect which is greater than the sum of the individual effects when the drugs are used alone', the most appropriate methodology used to prove synergy is still controversial. Norden (1982) showed that

	DF	Seq SS	Adj SS	Adj MS	F	*P*
Methyl paraben concentration	1	0·21316	0·021316	0·021316	152·37	<0·001
Propyl paraben concentration	1	0·010512	0·010512	0·010512	75·14	<0·001
Combination	1	0·000914	0·000914	0·000914	1·39	0·243
Error	76	0·010632	0·010632	0·000140		
Total	79	0·042654				

DF, Degrees of freedom; Seq SS, uncorrected sum of squares; Adj SS, adjusted sum of squares; Adj MS, adjusted mean squares; F, F-ratio.

Table 2 Analysis of variance for the rate constants from first order kill curves of methyl and propyl parabens against *Escherichia coli*

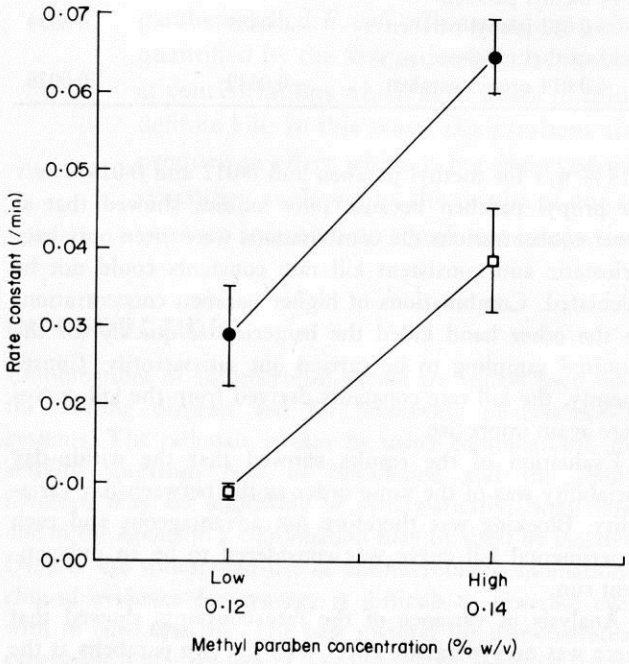

Fig. 2 Results of factorial design experiment to evaluate the effect of methyl and propyl paraben combinations on the kill rate constant of *Escherichia coli*. (Error bars are S.E., $n = 20$.) □, Low propyl paraben; ●, high propyl paraben

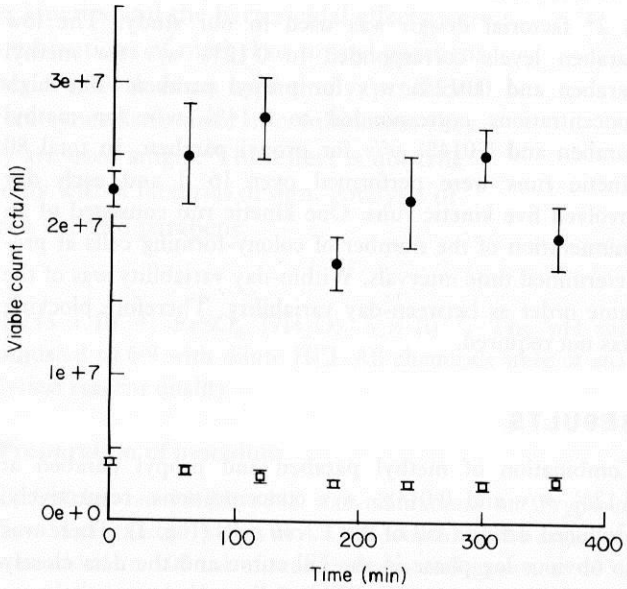

Fig. 3 The survival of exponential phase *Escherichia coli* cells in chemically defined media in the presence of 0·12% w/v and 0·14% w/v methyl paraben. (Error bars are S.E., $n = 5$.) □, 0·12% methyl paraben; ●, 0·14% methyl paraben

checker board method are compared with those from kill curves that used two time points. This is not surprising since the checker board method uses the minimum inhibitory concentration while the kill curve method uses rate of kill as end-points. In our present method we have used the rate constants as end-points since this summary statistic enables us to avoid multiple comparisons of serially correlated data (Matthews *et al.* 1990). With this approach we were not able to show any synergism in the concentration ranges 0·12% w/v and 0·14% w/v for methyl paraben and 0·012% w/v and 0·014% w/v for propyl paraben.

It is interesting to note however that 0·12% w/v and 0·14% w/v methyl paraben when used alone produced a bacteriostatic effect (Fig. 3). Propyl paraben at 0·012% w/v and 0·014% w/v on the other hand allowed bacterial growth to continue when used singly (Fig. 4). Yet every combination within those concentration ranges produced bacterial kill (Fig. 5). The results therefore clearly show that in this sense the combination is synergistic, that is the combination produces an effect (observable kill) which is not seen when the agents are used singly.

To explain why there is this apparent sudden change from a bacteriostatic to a bactericidal effect for the paraben combination it is worth noting that with most drug entities, the dose–response relationship is not linear but sigmoidal (Goldstein *et al.* 1974). Therefore apparently abrupt changes in potency are often observed. The danger of mistaking this change for synergism has been highlighted by Berenbaum (1977).

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