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Review Article

Synergy in preservative combinations

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Introduction

The complex nature of many pharmaceutical and cosmetic formulations renders the problem of their preservation an exacting challenge. Often a single antimicrobial agent (at a permissible concentration) is ineffective for the complete protection of such products againts microbial contamination. As a result, resort has been made to the development of preservative combinations designed to give a more adequate protection. Occasionally these antimicrobial combinations show synergy, that is their combined effects are greater than would be expected from simple addition. Such a situation is obviously advantageous, providing enhanced activity at lower individual preservative concentrations and its occurrence will form the basis of this review. This phenomenon has also been recognized and exploited in therapeutics where synergistic combinations of antibiotics and other anti-infective agents have been successfully used (Greenwood, 1983). This review will not cover biocide combinations aimed at increasing the spectrum of activity.

Measurement of combined antimicrobial action and the determination of synergy

Several effects are possible when two antimicrobial agents act simultaneously on a uniform microbial population: (a) the combined effect produced by the two agents may be greater than the sum of the individual effects of either compound alone, this is known as synergy; (b) the overall effect may be reduced, in which case the

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combination is antagonistic; or (c) the combined action is no greater than that predicted from the activities of the individual compounds, this response is variously described as additive or indifferent.

Any method used to assess synergy must also be capable of demonstrating these alternative effects. Many of the techniques described below were first applied in the field of antibiotics (Beale and Sutherland, 1983) but are equally applicable to the investigation of preservative combinations. Correlation between methods is not always obtained since they often measure different parameters.

Diffusion tests

In this, the simplest method, paper discs or strips impregnated with the agents under test are placed on an agar plate previously seeded with the test organism. The interaction of the zones of inhibition observed after incubation enable an assessment of the type of interaction, if any, between the two components under study to be made. Any enlarged zones of inhibition may indicate synergy. Hugo and Foster (1963) demonstrated the interactions of 28 combinations of antibacterial agents against six species of organisms by this method.

The major limitations to the diffusion tests are that they rely on the activity of the antimicrobial agents being unaffected by dilution in the solid media and that the results are only qualitative.

Spiral plating method

Uniform deposition of organisms onto a rotating agar plate (spiral plating) followed by the application of a continuously varying dilution of antimicrobial agent over the same track can, after incubation of the plate, be used to determine precisely inhibitory concentrations. This method can be extended to study mixtures of antimicrobial agents where the plated combination is compared against the individual agents thus providing a quantitative assessment of the interaction (Schalkowsky and Schalkowsky, 1981).

Turbidometric methods

This technique involves the continuous monitoring by turbidometric methods of the log phase growth curves of bacteria exposed to individual antimicrobial compounds and their combinations. The success of the combination is determined from the observed deviation in the expected growth curve (Brown and Richards, 1965; Brown, 1966; Richards and Hardie, 1972); results can also be expressed in terms of doubling times (Richards and McBride, 1971a). This test has the added advantage of identifying rapid lytic activity in a preservative system.

Serial dilution technique

This method, sometimes called the chessboard or checkerboard method because of the traditional arrangement of samples and the pattern of resultant growth, involves exposing the test organism to serial dilutions of two antimicrobial agents in a nutrient medium such that, within the range of dilutions used, all possible combinations of concentrations are tested. The range of dilutions used normally

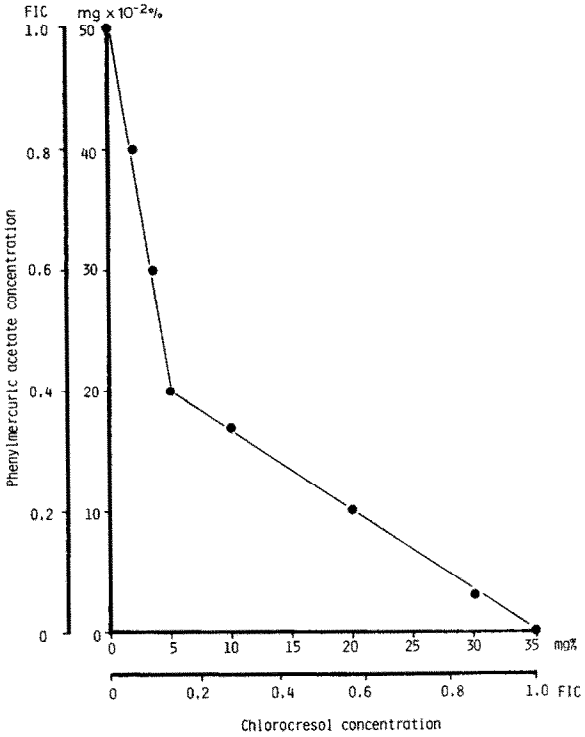


Fig. 1. Isobologram drawn from minimum growth inhibitory concentrations of chlorocresol and phenylmercuric acetate used alone and in combination against *Staphylococcus aureus*.

extends from just above the expected minimum growth inhibitory concentration (MIC) for each compound to zero. The combination first showing no growth after overnight incubation is taken as the endpoint. The method of applying this technique can be seen in the publication of Quesnel et al. (1978)

Results from serial dilution methods can be presented graphically by constructing an isobologram from the MIC values of each agent in a growth inhibitory combination and alone (Fig. 1). The isobologram obtained can be concave indicating synergy, convex demonstrating antagonism, or a straight line which joins the MIC values of the individual drugs alone, which represents an indifferent or additive combination.

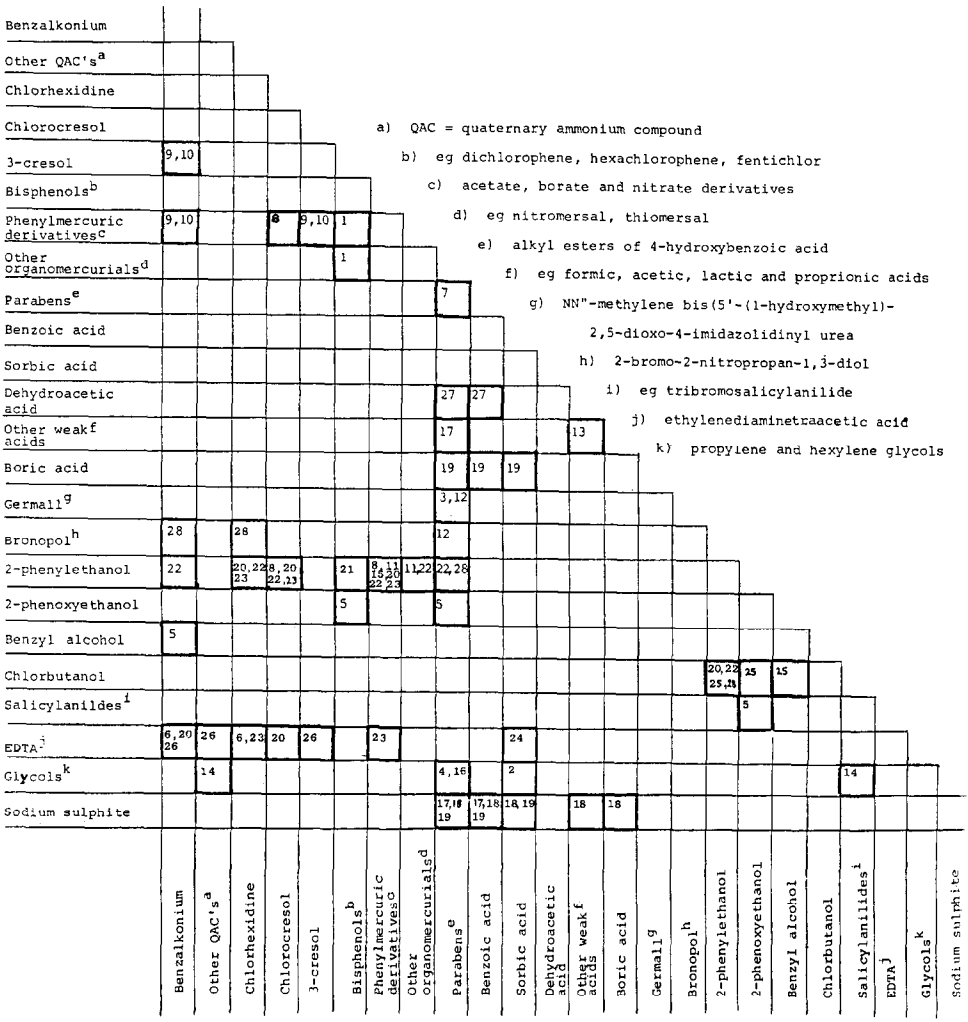
Inhibitory concentrations are sometimes expressed as a ratio of the concentration of an agent in a combination to its individual MIC (termed the fractional inhibitory concentration, FIC). The simultaneous activity of two substances in a combination can then be obtained from the sum of the FICs of each component (ΣFIC). Clearly ΣFIC can be used as an index of synergy since a value of 1 would indicate additivity while values above and below 1 would suggest antagonism and synergy, respectively. FICs can also be used to plot an isobologram (Fig. 1).

The serial dilution technique is very flexible and can be used to measure

TABLE 1
SUMMARY OF REPORTED SYNERGISTIC COMBINATIONS OF ANTIMICROBIAL PRESERVATIVE AGENTS

Bold squares indicate synergy, numbers within squares refer to references listed briefly below and in full at end of article.

(1) Barr et al., 1970; (2) Barr and Tice, 1957; (3) Berke and Rosen, 1970; (4) Blanchard, 1980; (5) Boehm, 1968; (6) Brown and Richards, 1965; (7) Gerrard et al., 1962; (8) Harding, 1984; (9) Hugbo, 1976; (10) Hugbo, 1977; (11) Hugo and Foster, 1963; (12) Jacobs et al., 1975; (13) Moon, 1983; (14) Neipp, 1957; (15) Palanichamy et al., 1982; (16) Prickett et al., 1961; (17) Rehm and Stahl, 1960; (20) Richards, 1971; (21) Richards and Hardie, 1972; (22) Richards and McBride, 1971a; (23) Richards and McBride, 1972; (24) Robach and Stateler, 1980; (25) Stock, 1962; (26) Wilkinson, 1975; (27) Winkler, 1955; (28) Woźniak-Parnowska and Krówczyński, 1981.



bactericidal activity, in which case the results are expressed in terms of minimum bactericidal concentrations (Quesnel et al., 1978).

Viable counts and sterilizing times

Richards et al. (1969), Richards and McBride (1971b, 1972), Richards and Hardie (1972), Woźniak-Parnowska and Krówczyński (1981), Palanichamy et al. (1982) and Akers et al. (1984) have used sterilization and killing times as a parameter for monitoring interactions of preservatives. These workers examined the activity of antimicrobial agents against several species of bacteria in ophthalmic and parenteral preparations. Synergy is defined, in this case, as a significant decrease in sterilization time for the combination compared with the individual components, as well as an increased protection of the product when rechallenged with a high inoculum of organisms. This approach has the advantage of working with the pharmaceutical products and approximates very well to an in vivo situation.

Examples of synergistic combinations

An early recognized example of synergy is the enhancement of sporicidal activity by combining acid or alkali with alcohol (Coulthard and Sykes, 1936). A widespread application of the enhancement of activities in combination is the physical/chemical 'synergy' of heating with a bactericide in the sterilization of some pharmaceutical preparations (British Pharmacopoeia, 1980). Synergy within three component preservative systems has been observed (Rehm et al., 1964; Wells and Lubowe, 1964; Boehm and Maddox, 1971) and the use of combinations of up to six agents has been reported, although it is unlikely that such a complex system has any sound theoretical basis (Parker, 1973, 1982).

A selection of antimicrobial agents whose interactions have been shown to be synergistic to varying degrees is illustrated in Table 1. Some reportedly synergistic combinations have been exploited commercially, e.g. Phenonip (2-phenoxyethanol and parabens: Parker et al., 1968; Wallhäusser, 1984) and Lauribic (glyceryl monolaurate and sorbic acid: Kabara, 1980, 1984a).

Mechanisms of synergy

Antimicrobial preservatives that belong to the same chemical groups are believed to produce merely additive effects when used in combination. In contrast, synergism or antagonism has been reported for combinations of preservatives that separately have different mechanisms or sites of action on the microbial cell (Hugbo, 1977).

Several mechanisms have been proposed to account for synergy between antibiotics (Greenwood, 1983) but only one of these, permeabilization synergy, has been widely applied to preservative combinations. In this type of synergy one agent assists the passage of the other through the cell wall or membrane, an example being the combination of an organomercurial with a phenolic agent. Here the phenol is

believed to disrupt the cytoplasmic membrane thus permitting easier access of the mercurial to the cell interior where thiol-containing enzyme systems, a possible target of organomercurials, are located (Hugbo, 1977). With this type of synergy it is not necessary for both components of a combination to possess marked antimicrobial activity. For example, ethylenediaminetetraacetic acid (EDTA), a chelating agent, has been shown to potentiate the activity of many antimicrobial compounds whilst frequently exerting only weak antimicrobial action itself (Sheikh and Parker, 1972; Hart, 1984). In particular, EDTA has been used to decrease the resistance of *Ps. aeruginosa* to various antibacterial agents (Wilkinson, 1975). This action of EDTA is believed to be the result of the compound 'stripping' away a portion of the outer membrane and cell wall of the Gram-negative organism (by chelating Mg^{2+} and Ca^{2+} ions normally responsible for outer membrane stability), thus exposing the underlying cytoplasmic membrane and cell interior to attack by the other agent in the combination (Haque and Russell, 1974).

Conclusions

In the field of preservation one agent is often inadequate to provide protection against contamination especially by pseudomonads. This is particularly true of complex pharmaceutical and cosmetic preparations, where no single agent is entirely satisfactory in reducing sterilization time and protecting against multiple challenges of bacteria at high inoculum levels.

When considering combinations of antimicrobial agents, it should always be remembered that in vitro observations do not always reproduce the same results in vivo and particular attention should be paid to eliminating combinations or concentration ratios which may prove antagonistic. The activity of the system must ultimately be tested in the product to be preserved since many factors, including ingredients and formulation design, could influence the potential for synergy. While these factors often subscribe to produce a reduced preservative capability overall (Parker, 1982; McCarthy, 1984) the enhancement of activity by ingredients has been reported (Kabara, 1981, 1984b). Equally important is the species specificity of some antimicrobial combinations, e.g. boric acid enhances the fungistatic activity of benzoic acid or sorbic acid, but these combinations are antagonistic against *E. coli* (Rehm and Stahl, 1959, 1960; for further examples see Kull et al., 1961; Richards and Hardie, 1972; Robaach and Stateler, 1980).

In the field of preservation few reports have sought to explain the basis of synergy and in those that have permeabilization effects have been most commonly invoked. It is interesting to speculate on other possible mechanisms of synergy and recently effects on microbial energetics and chemiosmotic coupling have been reported (Corner, 1981; Moon, 1983); this action may be of particular importance if repair and recovery processes within the cell are to continue uninterrupted. It is important therefore that in our search for synergy we should exploit our understanding of the mode of action of individual agents as a predictor for possible synergistic combinations.

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