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# A Method for Testing for Synergy with Any Number of Agents

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The standard checkerboard titration for detecting synergy between antibiotics is practicable for combinations of two antibiotics, laborious for combinations of three, and not feasible for combinations of four or more. Nevertheless, methods for testing of combinations of several antibiotics are urgently needed because some combinations might be superior to those in use and enable the successful treatment of infections resistant to current therapy. A simple method for measurement of synergy (or antagonism) with combinations of any number of agents has been developed which requires less effort than the standard checkerboard titration of two agents. With this method, the concentrations of each of $n$ agents producing some specified effect (such as minimal inhibitory concentration or minimal bactericidal concentration) are determined. A reference combination made up of $1/n$ of each of these concentrations is titrated to find a dilution that produces the specified effect. The degree of dilution required is equal to the sum of the fractional inhibitory concentrations (concentration of each agent in combination/concentration of each agent alone) as conventionally determined by checkerboard titrations; sums of $<1$, 1, and $>1$ indicate synergy, additivity, and antagonism, respectively.

The use of synergistic combinations of antibiotics is often needed in the treatment of serious infections. Testing of combinations for synergy is therefore widely practiced, and there is much literature on the subject. The general procedure is to conduct a so-called checkerboard titration, in which two antibiotics are tested in serial dilutions and in all combinations of these dilutions together to find the concentrations of each antibiotic, both alone and in combination, that produce some specified, easily determined effect.

The nature of the interaction between the two antibiotics is then determined either algebraically or geometrically. In the former method, the concentration of each antibiotic in the combination that produces the specified effect is expressed as a fraction of the concentration that produces the same effect when the antibiotic is used alone, i.e., its fractional inhibitory concentration. When the sum of these fractions is 1, the combination is additive; when the sum is $<1$, the combination is synergistic; and when the sum is $>1$, the combination is antagonistic. In the second method, which is the geometric counterpart of the first, a graph is constructed with the axes representing antibiotic concentrations on linear scales. When the combination is additive, the isobole (i.e., the line joining the points that represent all combinations with the same effect, including the equally effective concentrations of the antibiotics used alone) is straight. Synergistic combinations give concave isoboles, and antagonistic combinations give convex isoboles [1].

However, these procedures are rarely, if ever, carried out with combinations of three antibiotics, and there does not appear to have been any attempt to apply them to combinations of four or more. The reason is obvious. If, for instance, each antibiotic is tested in 10 dilutions (including a control), 100 tests are required for a combination of two antibiotics, 1,000 tests for a combination of three, 10,000 tests for a combination of four, and so on. Testing of combinations of three antibiotics is therefore laborious, and testing of four or more antibiotics appears to be unfeasible. Furthermore, although it is easy to plot isoboles for

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combinations of two antibiotics, plotting them for combinations of three antibiotics entails a three-dimensional construction (see below), and it is physically impossible to plot isoboles for combinations of more than three agents.

However, there is unquestionably a need for methods for testing for synergy with combinations of any number of agents, because such testing might lead to the discovery of antibiotic combinations considerably more potent than those now in use and effective against infections resistant to current therapy. The method described herein allows synergy or antagonism to be measured for combinations of several antibiotics with less effort than is involved in a conventional checkerboard titration of two agents. The principles are not restricted to the use of antibiotics [2].

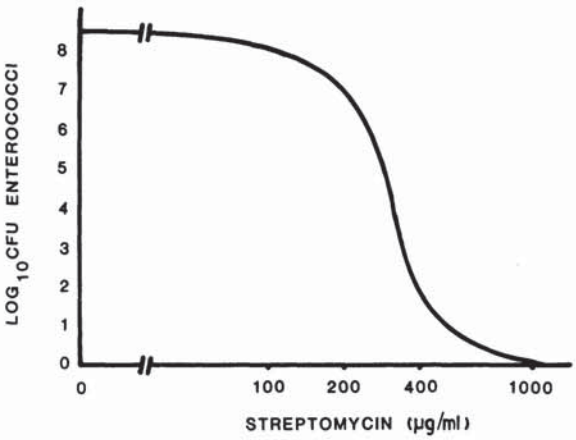


Figure 1. Relation between concentrations of streptomycin and growth of enterococci (data from [3]).

## Methods

*Combinations of one agent.* The simplest approach to this problem is first to define additivity and then to see how synergy and antagonism differ from additivity. Agents that act additively are no more and no less effective in combination than they are separately; in other words, there is no advantage in combining them. There is one sort of combination that must always behave in this way, i.e., the spurious combination of any agent with itself. Such "combinations" are therefore convenient models for additivity, and, by examining their behavior, we can see how additive combinations of different agents should behave. Furthermore, if synergy is broadly defined as a condition in which the effectiveness of agents is increased when they are combined and antagonism as the reverse, this additive model provides a frame of reference by which we can see how synergistic and antagonistic combinations should behave.

For illustration, let us consider the effect of streptomycin on enterococci as illustrated in figure 1 from the data of Moellering et al. [3]. Suppose this antibiotic were placed in two containers, A and B, and we conducted an experiment to find the effects of A and B alone and in combination. To reduce $\log_{10}$ cfu of the enterococci from 8.2 to 2.0, we need a concentration of either 400 $\mu$g of A/ml or 400 $\mu$g of B/ml. This same effect is produced by a combination of 200 $\mu$g of A/ml and 200 $\mu$g of B/ml. Let the concentrations of A and B that each produces the same specified effect (equally effective concentrations) when the antibiotics are used alone be called $A_e$ and $B_e$, and let $A_c$ and $B_c$ be their concentrations when used in combination. Then $A_e = B_e = 400$ $\mu$g/ml and $A_c/A_e + B_c/B_e = 200/400 + 200/400 = 1$.

We also know (because A and B are in fact the same agent) that other combinations having this effect are, for instance, 100 $\mu$g of A/ml + 300 $\mu$g of B/ml or 350 $\mu$g of A/ml + 50 $\mu$g of B/ml: $A_c/A_e + B_c/B_e = 100/400 + 300/400 = 1$ and $350/400 + 50/400 = 1$. Alternatively, if we specify that the effect we wish to produce is a reduction in $\log_{10}$ cfu of the enterococci from 8.2 to 7.0, then $A_e = B_e = 200$ $\mu$g/ml. This effect will be produced by a combination of, for example, 100 $\mu$g of A/ml and 100 $\mu$g of B/ml or 150 $\mu$g of A/ml and 50 $\mu$g of B/ml: $A_c/A_e + B_c/B_e = 100/200 + 100/200 = 1$ and $150/200 + 50/200 = 1$. In other words, with this "pair" of antibiotics, which we know can only behave additively, equation 1 ($A_c/A_e + B_c/B_e = 1$) always holds.

Equation 1 can also be expressed geometrically, because it is an equation of a straight line. Figure 2 is an isobologram, showing the effects of the various combinations mentioned above and illustrating the fact that in this spurious case in which the two constituents of the combination are in fact identical, the points representing all combinations with an equal effect lie on the straight line joining the concentrations of the two constituents that are equally effective when used alone.

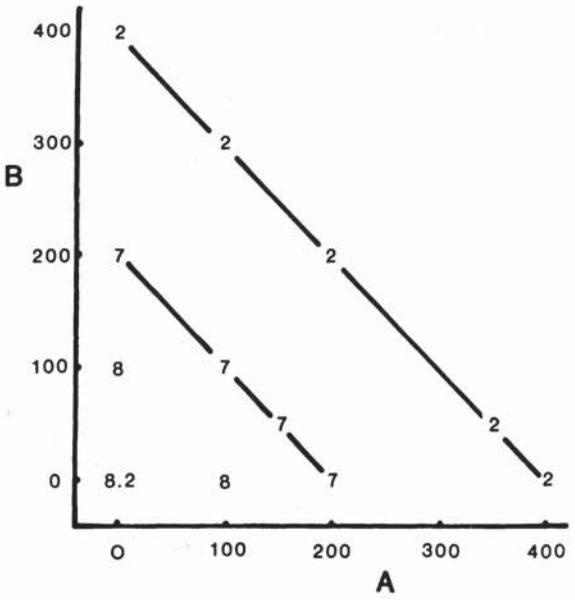


**Figure 2.** Isobologram showing effects of combinations of two identical preparations, A and B, of streptomycin on the growth of enterococci (data from figure 1). Values are $\log_{10}$ cfu of the organisms after culture for 24 hr with the antibiotic preparation. Points representing combinations with equal effects lie on the straight line joining the concentrations of A and B alone that produce the same effect (equally effective concentrations).

*Combination of two agents.* If the antibiotic in container B is mixed with an equal part of an inert material that has no effect on the antibiotic, the antibiotic's actions, or the bacteria, the equally effective concentrations of A and B that reduce the $\log_{10}$ cfu of enterococci from 8.2 to 7.0 are 200 and 400 $\mu$g/ml, respectively. Thus combinations producing this effect are, for instance, 100 $\mu$g of A/ml + 200 $\mu$g of B/ml or 50 $\mu$g of A/ml + 300 $\mu$g of B/ml: $A_c/A_e + B_c/B_e = 100/200 + 200/400 = 1$ and $50/200 + 300/400 = 1$.

It appears, therefore, that when a combination of two agents is simply additive, equation 1 holds, irrespective of the effect specified or whether the equally effective concentrations of the two agents are the same or different, and that the equation does not depend on any particular relation between concentration and effect. This case may also be expressed geometrically. It is clear from figure 3 that all additive combinations producing the same effect as $A_e$ and $B_e$ lie on the straight line between $A_e$ and $B_e$ whether $A_e$ and $B_e$ are the same or different.

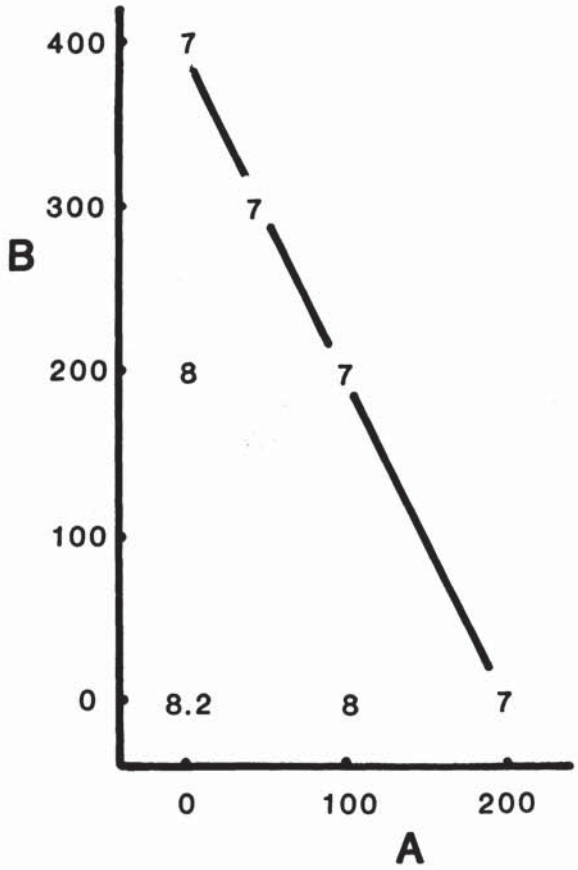


**Figure 3.** Isobologram showing effects of combinations of streptomycin (A) and streptomycin mixed with an equal part of inert material (B). Values are $\log_{10}$ cfu of the organisms after culture for 24 hr with the antibiotic preparation. Points representing combinations with equal effects lie on the straight line joining the concentrations of A and B that are equally effective when used alone.

An additive combination of two agents may therefore be defined as one that satisfies equation 1 and that therefore, in an isobologram, lies on the straight line joining the concentrations of the two agents that, when the agents are used alone, produce the same effect as the combination (the equally effective concentrations).

Unequivocal and precise definitions of synergy and antagonism follow from this definition of additivity. Synergistic agents are more effective in combination than they are separately, i.e., less is required to produce a given effect when the antibiotics are used together than when they are used separately, and the sum of the fractions in equation 1 is therefore $<1$. Conversely, for antagonistic agents, more is required to produce a given effect

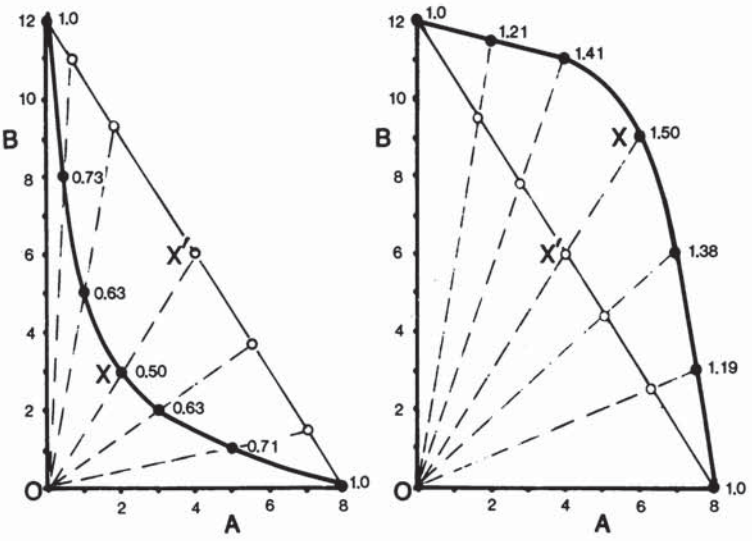


**Figure 4.** Isobolograms showing the relation between sums of fractional inhibitory concentrations and isobolar shapes for combinations of two agents. In this example equal effects are produced by 8 units of agent A and 12 units of agent B. The sum of fractional inhibitory concentrations for each combination is readily calculated by reference to the dose axes of A and B and is shown to the right of the point (●) representing that combination. When the sum of the fractional inhibitory concentrations is <1 (*left*), the point lies below the additive line, the isobole is concave, and the combination is synergistic. When the sum is >1 (*right*), the point lies above the additive line, the isobole is convex, and the combination is antagonistic. Note that, if O is the origin of the isobologram, C the point representing any combination (●), and C′ the intersect (○) of OC or its extrapolate with the additive line, then the sum of fractional inhibitory concentrations for C equals OC/OC′. Synergy and antagonism are usually most marked along OX′ or its extrapolate, where X′ is the midpoint of the additive line.

when the antibiotics are used together than when they are used separately, and the sum of the fractions is >1.

Figure 4 shows the connection between equation 1 and the shape of the isobole for combinations of two agents. When for any given combination the sum of the fractions is <1, the point representing that combination lies below the additive line, and when all combinations with the same effect are synergistic, the isobole for that effect is concave. Conversely, when the sum of the fractions for a combination is >1, the point representing it lies above the additive line, and when all combinations with the same effect are antagonistic, the isobole for that effect is convex.

*Combination of three agents.* Let us now add to our containers of streptomycin (A and B) a third, C, in which the antibiotic is mixed with twice its weight of inert material. The concentrations of A, B, and C required to reduce the $\log_{10}$ cfu of enterococci from 8.2 to 7.0 are now 200, 400, and 600 μg/ml, respectively. Possible combinations of the three that will result in a final concentration of 200 μg of streptomycin/ml are 100 μg of A/ml + 100 μg of B/ml + 150 μg of C/ml and 50 μg of A/ml + 100 μg of B/ml + 300 μg of C/ml: $100/200 + 100/400 + 150/600 = 1$ and $50/200 + 100/400 + 300/600 = 1$. Thus equation 1 can be extended for use with combinations of three agents by adding a term for the third, as long as the sum of the fractions is 1 (equation 2): $A_c/A_e + B_c/B_e + C_c/C_e = 1$.

Equation 2 describes a plane in three dimensions, and figure 5 accordingly shows that, when the sum of the fractions in this equation is 1, the points representing the combinations lie in the flat plane joining $A_e$, $B_e$, and $C_e$.

When three agents are synergistic, the sum of the fractions in equation 2 is <1, and when they are antagonistic, the sum is >1. In the former case, the isobolar surface is concave, and in the latter, convex (figure 6).

*Combinations of any number of agents.* It is evident that the argument used to extend equa-

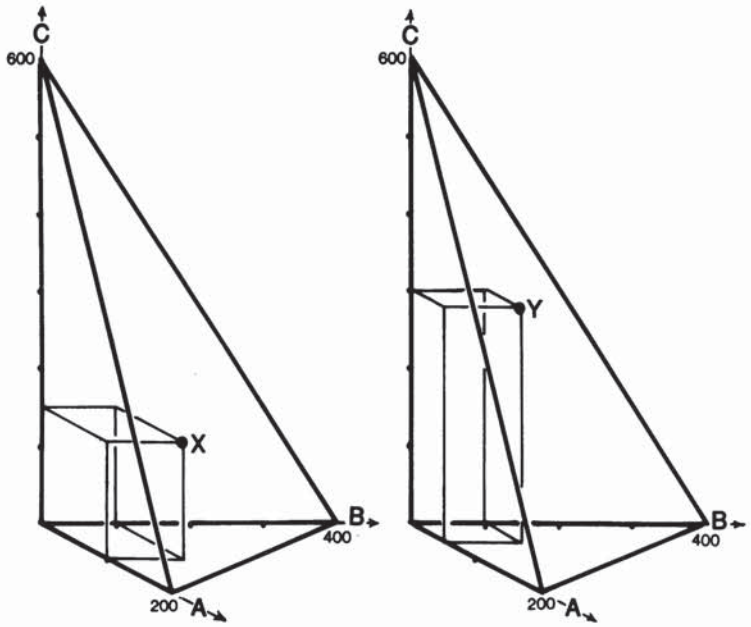


**Figure 5.** Three-dimensional isobolograms showing that the points representing additive combinations of antibiotics lie in the flat plane that joins the concentrations of the three agents that are equally effective when used alone, and that the sum of fractional inhibitory concentrations for such combinations is 1. In this example, equally effective concentrations of A, B, and C are 200, 400, and 600 μg/ml, respectively. Combination X consists of 100 μg of A/ml, 100 μg of B/ml, and 150 μg of C/ml. Combination Y consists of 50 μg of A/ml, 100 μg of B/ml, and 300 μg of C/ml.

tion 1 to equation 2 can be used to extend it for use with any number of agents. For instance, if we had six different mixtures of an antibiotic with an inert material (A–F) such that the concentrations of each required to produce a given specified effect were 200, 400, 800, 900, 1,000, and 1,200 μg/ml, respectively, or six different antibiotics (A–F) with these equally effective concentrations, we could form a combination of all six that would itself have this specified effect if we ensured that $A_c/200 + B_c/400 + C_c/800 + D_c/900 + E_c/1{,}000 + F_c/1{,}200 = 1$.

Therefore, we can write a general equation (3) for use with any number of antibiotics: $A_c/A_e + B_c/B_e + C_c/C_e + \ldots + X_c/X_e = <1$ for synergy, 1 for additivity, or >1 for antagonism.

*Experimental design.* Equation 3 indicates how to measure the degree of synergy or antagonism for any combination of any number of agents, but not which of the infinite number of

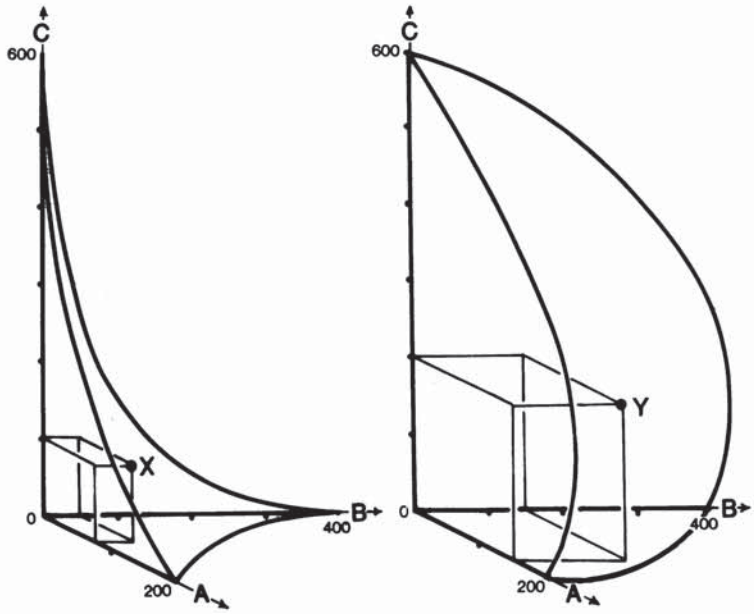


**Figure. 6.** Three-dimensional isobolograms showing that points representing synergistic combinations of three agents lie on concave surfaces, and antagonistic combinations on convex surfaces, that join the concentrations of the three agents that are equally effective when used alone. In this example, equally effective concentrations of A, B, and C are as in figure 5. Combination X consists of 80 μg of A/ml, 50 μg of B/ml, and 100 μg of C/ml, and the sum of the fractional inhibitory concentrations is 0.69. Combination Y consists of 150 μg of A/ml, 150 μg of B/ml, and 200 μg of C/ml and the sum is 1.46.

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