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Keyphrases

Dienestrol analysis
Bis-(trimethylsilyl) ether, dienestrol—derivative
GLC—analysis
Bis-(trimethylsilyl) ether, alizarin—internal standard
Analysis—dienestrol–methyltestosterone combination

New Developments in Antibiotic Interference Thresholds of Microbial Assays

By B. ARRET and J. ECKERT

Interference thresholds have been determined for a number of antibiotics in the microbial assays of specific antibiotics. Two aspects were considered in order to facilitate the quantitative analysis of mixtures of antibiotics: the "interference threshold," which is the concentration of an antibiotic causing a significant interference in the presence of another antibiotic in a given assay, and the "sensitivity threshold," which is the concentration of an antibiotic causing a response in the assay for a different antibiotic.

In 1957, Arret *et al.* (1) described the problems encountered in assaying pharmaceutical preparations in which two or more antibiotics are combined, and proposed solutions to these problems. If the test organism used for the assay of one antibiotic (*a*) is not affected by a second antibiotic (*b*), the assay for (*a*) is uncomplicated. But if the test organism is affected by (*b*), erroneously high or low values for (*a*) may be obtained. Consequently, methods for eliminating the effect of (*b*) were developed. In general, one of the following five methods was used: (*1*) inactivating by biological or chemical means; (*2*) using a test organism which is sensitive to (*a*) and relatively resistant to (*b*); (*3*) artificially making the organism resistant to (*b*); (*4*) separating antibiotics by differential solubility techniques; or (*5*) compensating for the presence of (*b*) by adding it to every solution of (*a*) used for the standard curve.

The analyst needs definite quantitative knowledge of the interfering effects of various antibiotics in certain widely used assay procedures. He must be aware of such effects if confidence is to be maintained in the specificity of an assay.

Since the earlier publication (1), new antibiotics have been discovered, and combinations of these antibiotics together with the older ones make it necessary to update this publication. As before, all the pertinent data are in tabular form for easy reference.

The data obtained from this work simplify the problems of assaying antibiotic combinations Although combinations of two antibiotics have specifically been considered here, the approach can be used as a general guide to the assay of combinations of three or more antibiotics.

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EXPERIMENTAL

The effects of 15 antibiotics were determined by assaying them according to the official microbiological assay methods found in the *Code of Federal Regulations* (2). The following antibiotics (assay organisms are given in parentheses) were studied: Bacitracin[1] (*Sarcina subflava*), chloramphenicol (*Escherichia coli*), colistin (*Bordetella bronchiseptica*), erythromycin (*Sarcina lutea*), kanamycin (*Staphylococcus aureus*), neomycin (*S. aureus*, *S. epidermidis*) oleandomycin (*S. epidermidis*), paromomycin (*S. epidermidis*), penicillin (*S. aureus*), polymyxin (*B. bronchiseptica*), streptomycin (*Klebsiella pneumoniae*, *Bacillus subtilis*), tetracycline (*S. aureus*), and viomycin (*K. pneumoniae*).

For each experiment, the antibiotic being assayed was regarded as (*a*). Solutions were prepared containing the reference concentration of (*a*) and various concentrations of the second antibiotic (*b*). The diluent in every case was that ordinarily used for the assay of (*a*). These solutions were assayed against the reference concentration of (*a*) alone as the standard of comparison. Solutions containing various concentrations of (*b*) alone were also prepared.

The lowest concentration of (*b*) which, in com-

[1] Since the previous publication, a new assay for bacitracin was reported (3) in which *Sarcina subflava* is used as the test organism instead of *Micrococcus flavus*. This method is now used routinely in our laboratory.

TABLE I—INTERFERENCE THRESHOLDS: CONCENTRATION OF ANTIBIOTIC *B* INTERFERING IN GIVEN ASSAY METHOD WHEN MIXED WITH THE REFERENCE CONCENTRATION OF ANTIBIOTIC *A*. ANTIBIOTIC *A*: ASSAY ORGANISM USED IN EACH METHOD[a] AND REFERENCE CONCENTRATION OF *A* PRESENT

Second Antibiotic Present (*B*)	Bacitracin 1 unit/ml. *S. subflava*	Chloramphenicol 2.5 mcg./ml. *E. coli*	Colistin 1 mcg./ml. *B. bronchiseptica*	Erythromycin 1 mcg./ml. *S. lutea*	Kanamycin 5 mcg./ml. *S. aureus*	Neomycin 10 mcg./ml. *S. aureus*	Neomycin 1 mcg./ml. *S. epidermidis*	Oleandomycin 5 mcg./ml. *S. epidermidis*	Paromomycin 1 mcg./ml. *S. epidermidis*	Penicillin 1 unit/ml. *S. aureus*	Polymyxin 10 units/ml. *B. bronchiseptica*	Streptomycin 30 mcg./ml. *K. pneumoniae*	Streptomycin 1 mcg./ml. *B. subtilis*	Tetracycline 0.24 mcg./ml. *S. aureus*	Viomycin 100 mcg./ml. *K. pneumoniae*
Bacitracin, units/ml.	...	...	100	...	4	...	...	0.3	70	...	...	...	...	...	75
Chloramphenicol, mcg./ml.	...	...	9	...	3[b]	...	...	5	20	...	...	...	...	...	0.2
Colistin, mcg./ml.	390	0.1	...	>500	450	150	7	290	90	>500	0.07	0.7	51	15	0.3
Cycloserine, mcg./ml.	97	130	0.5	...	90	210	250[b]	>500	>500	90	0.3	140	45[b]	20	100
Erythromycin, mcg./ml.	...	...	40	...	0.2[b]	...	...	0.4	0.8	...	...	...	...	...	0.5
Kanamycin, mcg./ml.	8	3	>500	400	...	2	0.3	3	0.3	>500	0.1[b]	3	0.2	3	1
Neomycin, mcg./ml.	...	...	>500	...	0.5	...	...	4	0.4	...	...	...	...	...	0.6
Novobiocin, mcg./ml.	0.3	6	20	10	4	2	2	0.7	0.7	4	200	0.9	9	0.2	2
Oleandomycin, mcg./ml.	3	3	270	0.4	0.3[b]	3	1	...	2	72	190	4	0.3[b]	0.1	2
Paromomycin, mcg./ml.	250	3	40[b]	140	0.5	0.9	0.2	2	...	>500	0.5[b]	3	0.2	2	0.9
Penicillin, units/ml.	...	...	0.2[b]	...	0.4	...	...	90	85	...	...	...	...	...	95
Polymyxin, units/ml.	...	...	8	...	>500	...	...	>500	>500	...	...	...	...	...	3
Streptomycin, mcg./ml.	...	...	60[b]	...	2	...	...	290	110	...	...	...	...	...	3
Tetracycline, mcg./ml.	...	...	3	...	1	...	...	125	50	...	...	...	...	...	0.07
Viomycin, mcg./ml.	36	5	0.1[b]	>500	20	60	3	25	10	>500	0.5[b]	20	0.8[b]	60	...

[a] All assays are plate methods except chloramphenicol, streptomycin *K. pneumoniae*, tetracycline, and viomycin. Streptomycin and dihydrostreptomycin may be considered as acting identically. To find how bacitracin interferes with the *S. aureus* assay for kanamycin, find "bacitracin" in the left-hand column headed "Second Antibiotic Present" and follow that row horizontally across to the column headed "Kanamycin, 5 mcg./ml., *S. aureus*." The figure 4.0 units/ml. indicates that samples containing bacitracin–kanamycin ratios of less than 4:5 (units:mcg.) could be assayed accurately for kanamycin by this method. [b] Indicates a depression rather than an enhancement of activity.

TABLE II—SENSITIVITY THRESHOLDS: CONCENTRATION OF ANTIBIOTIC ALONE[a] PRODUCING MINIMUM MEASURABLE GROWTH INHIBITION IN GIVEN ASSAY METHOD

Antibiotic Tested	Bacitracin *S. subflava*	Chloramphenicol *E. coli*	Colistin *B. bronchiseptica*	Erythromycin *S. lutea*	Kanamycin *S. aureus*	Neomycin *S. aureus*	Neomycin *S. epidermidis*	Oleandomycin *S. epidermidis*	Paromomycin *S. epidermidis*	Penicillin *S. aureus*	Polymyxin *B. bronchiseptica*	Streptomycin *K. pneumoniae*	Streptomycin *B. subtilis*	Tetracycline *S. aureus*	Viomycin *K. pneumoniae*
Bacitracin, units/ml.	...	...	15	...	7	...	...	20	9	...	...	...	...	...	110
Chloramphenicol, mcg./ml.	...	...	10	...	>500	...	...	6	7	...	...	...	...	...	0.3
Colistin, mcg./ml.	91	0.2	...	90	110	130	2	2	5	>500	0.08	1	7	2	0.5
Cycloserine, mcg./ml.	51	65	0.3	200	100	80	240	220	210	32	0.3	70	400	25	75
Erythromycin mcg./ml.	...	...	80	...	0.3	...	...	0.05	0.3	...	...	...	...	...	1
Kanamycin, mcg./ml.	3	2	120	25	...	2	0.4	0.5	0.3	1	120	5	0.2	2	2
Neomycin, mcg./ml.	...	...	70	...	0.7	...	...	0.09	0.07	...	...	...	...	...	2
Novobiocin, mcg./ml.	0.2	3	20	3	0.6	1	0.3	0.5	0.4	0.2	20	0.8	20	0.2	0.7
Oleandomycin, mcg./ml.	3	4	>500	0.2	0.6	0.5	0.6	...	2	7	150	4	1	0.06	3
Paromomycin, mcg./ml.	6	2	400	8	0.6	0.9	0.06	0.4	...	18	400	6	0.2	0.7	2
Penicillin, units/ml.	...	...	>500	...	0.2	...	...	>500	180	...	...	...	...	...	75
Polymyxin, units/ml.	...	...	1	...	400	...	...	25	35	...	...	...	...	...	6
Streptomycin, mcg./ml.	...	...	>500	...	2	...	...	480	>500	...	...	...	...	...	4
Tetracycline, mcg./ml.	...	...	1	...	0.3	...	...	>500	410	...	...	...	...	...	0.04
Viomycin, mcg./ml.	191	4	>500	170	40	30	2	9	5	200	>500	30	3	30	...

[a] All assays are plate methods, except chloramphenicol, streptomycin-*K. pneumoniae*, tetracycline, and viomycin. Streptomycin and dihydrostreptomycin may be considered as acting identically. To find the amount of bacitracin alone which produces an inhibitory zone on *S. aureus* kanamycin plates, find "bacitracin" in the left-hand column headed "Antibiotic Tested" and follow that row horizontally across to the column headed "Kanamycin, *S. aureus*." The fig. "7" indicates that bacitracin alone (7 units/ml.) produces a minimum measurable inhibitory zone.

bination with the reference concentration of (*a*), gave a relative potency [as compared to the reference concentration of (*a*)] of greater than 110% or less than 90% has been termed the "interference threshold." These limits were selected because the 95% confidence range of an average microbiological assay is ±10%. Any assay within this range of theoretical is considered normal variation and any assay beyond this range is considered significant.

The lowest concentration of (*b*) alone which caused a measurable response in the assay procedure of (*a*) is called the "sensitivity threshold."

The interference thresholds and the sensitivity thresholds for the antibiotics and methods tested are given in Tables I and II, respectively.

DISCUSSION

The information given in Table I makes it possible to predict whether the assay of an antibiotic will be complicated by interference from a second antibiotic included in the formulation, which then must be eliminated.

Table II describes the effects of single antibiotics on various assays. The information given in this table can be applied in numerous ways, such as: (*i*) a guide to determine if interference can be expected from different antibiotics in a given assay procedure; (*ii*) a guide to the specificity of the given assay procedure; (*iii*) a means of qualitative identification of unknowns; and (*iv*) selection of an alternative organism for various antibiotics and combinations of them. In dealing with the latter, caution must be exercised, because in many cases the zones are not as clear and well defined as those usually obtained with the original method.

It was noted in many cases that (*b*) alone produced inhibition in the assay for (*a*) at a much lower concentration than that which caused interference when combined with the reference concentration of (*a*). For example (see Table II), 200 mcg./ml. of viomycin will cause inhibition in the penicillin assay without penicillin, but (Table I) more than 500 mcg./ml. of viomycin is necessary to cause interference in the assay with penicillin present. This is due to the fact that assay conditions may be more favorable to (*a*) so that they mask the effect of (*b*). Factors such as pH and ionic strength of the solvent, composition of the nutrient medium, and incubation temperature are significant. Therefore, the data should not be construed to indicate any synergistic or antagonistic relationships.

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Keyphrases

Antibiotic combinations—microbial analysis

Interference threshold—antibiotics

Sensitivity threshold—antibiotics

Analysis specificity—interference elimination

Conversion of Griseophenone A to (±)-Dehydrogriseofulvin in the Presence of Horseradish Peroxidase and Hydrogen Peroxide

By ALVIN SEGAL and ELMORE H. TAYLOR

The horseradish peroxidase catalyzed conversion of griseophenone A (I) to (±)-dehydrogriseofulvin (V) has been demonstrated. The results support a one-electron oxidative coupling mechanism previously proposed.

Barton and Cohen (1) first suggested that in the biosynthesis of the antibiotic (+)-griseofulvin (VII) (Scheme I), the chlorobenzophenone (I) could conceivably be converted to the spiran, (−)-dehydrogriseofulvin (V), *via* a 1-electron oxidative coupling mechanism proceeding through the formation of the intermediate diradical (III). Chemical synthesis of (±)-V using 1-electron oxidizing agents supports this hypothesis (2–4). The final step was the stereospecific enzymatic reduction of (−)-V to form (+)-VII (5–7).

(+)-Griseofulvin (VII) was first isolated from the mycelium of *Penicillium griseofulvum* (8). It was subsequently shown to be a metabolic product of many species of *Penicillia* (9).

The enzyme peroxidase has been demonstrated to be present in species of *Penicillia* (10), and has been implicated in the biosynthesis of fungal metabolites such as the ergot alkaloids (11). The peroxidase catalyzed O—C oxidative coupling of phenols has been reported (12). In addition, 1-electron transfer mechanisms have been proposed for coupled oxidations involving peroxidase (13). On the basis of this information the authors decided to investigate the possibility of converting I to V *via* peroxidase catalysis.

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