THE MICROBIOLOGICAL ASSAY OF MIXTURES OF PENICILLIN AND DIHYDROSTREPTOMYCIN

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Considerable interest has recently been aroused by reports of the superiority of preparations of mixed antibiotics over single substances in the treatment of certain infections. Not only do these preparations often yield a wider antibacterial activity but also, with certain combinations, enhanced activity over that of the individual components. Jawetz and Gunnison[1] divided the commoner antibiotics into two groups, those which were mutually synergistic with each other and never antagonistic, and those which were either indifferent or demonstrated simple additive effects. The first group comprised penicillin, streptomycin, bacitracin and neomycin, and the second aureomycin, chloramphenicol and terramycin. The effects of members of one group with those of the other depended largely on the relative susceptibility of the micro-organism. The reliability of *in vitro* assays of these various combinations might also be expected to be conditioned by similar considerations.

Using a serial dilution method of assay with a *Staphylococcus aureus*, Chopra and Gupta[2] found the minimal inhibitory concentration of penicillin reduced to one-tenth by the addition of 0·002 mg. of streptomycin per ml. and to one-thousandth by 0·007 mg. of streptomycin per ml. In these experiments the ratio of penicillin to streptomycin was of the order of 1 : 50, whereas therapeutic mixtures are generally in the ratio of about 1 : 2. These findings, therefore, may not be relevant to the present problem, but they suggest a possible interference in the assay of the penicillin. Using a plate diffusion technique, Uraya[3] successfully assayed penicillin in the presence of streptomycin by using organisms such as a staphylococcus or "dried grass spores". The method using the "dried grass spores" was not only said to be the simpler method to carry out but also to be capable of assaying low concentrations of penicillin in the presence of high concentrations of streptomycin and to be applicable in assaying blood serum.

Peyré and Velu[4], employing both plate and linear diffusion methods, reported values for mixtures of penicillin and streptomycin using a *Staph. aureus*, and of chloramphenicol and neomycin using *Bacterium coli*, which were practically identical with those of the more rapidly diffusing components. These observations pointed to the absence of interference, stimulatory or otherwise, between the pairs of antibiotics. The American Food and Drug Regulations[5] avoid microbiological estimations and rely on physical methods of assay.

Since mixed antibiotics are administered in previously determined proportions, it is essential that the concentrations of both components

should be accurately known; it is not enough to determine the *in vitro* activity of the mixture and from it assume that the correct proportions are present. With the mixture at present under consideration, the two antibiotics concerned exhibit different antibacterial activities, penicillin being selective against Gram-positive and dihydrostreptomycin active against both Gram-positive and Gram-negative organisms. It would seem reasonable,, therefore, to assay the dihydrostreptomycin component by means of a suitably sensitive Gram-negative organism. Alternatively, should the mixture how a synergistic effect, penicillinase could always be used to inactivate the penicillin, after which treatment any streptomycin-sensitive organism should be effective.

The problem with the assay of the penicillin component appeared to be somewhat more difficult at the outset, because both penicillin and dihydrostreptomycin are sensitive to the same groups of organisms. Arquie *et al.*[6] have described a "streptomycinase" derived from several organisms including *Pseudomonas pyocyanea*, and Lightbown[7], whilst not agreeing that it is an enzyme, states that it is equally effective against dihydrostreptomycin; unfortunately it is also inhibitory to bacterial growth. Several other substances, such as hydroxylamine, semicarbazide and cysteine, known to inhibit the activity of streptomycin, are either ineffective against dihydrostreptomycin or are also active to some degree against penicillin. It was eventually established that the success of any microbiological method for the assay of penicillin in the presence of dihydrostreptomycin must depend on the absence of interference, synergistic or otherwise, of the dihydrostreptomycin.

EXPERIMENTAL

Assay of Dihydrostreptomycin. Weighed amounts of dihydrostreptomycin sulphate with a potency of 745μg/mg. and of sodium benzylpenicillin with a potency of 1690 I.U./mg. were mixed intimately and

TABLE I

RESULTS OF ASSAYS OF DIHYDROSTREPTOMYCIN WHEN MIXED WITH PENICILLIN

Test material	Assay values for dihydrostreptomycin using	
	B. subtilis	*Bact. coli*
Dihydrostreptomycin	745 μ/mg.	745 μ/mg.
Dihydrostreptomycin + penicillin ..	734, 776 } = 755 μ/mg.	752, 758 } = 755 μmg.

submitted to assay by the cylinder-plate method. Two test organisms were used, *Bacillus subtilis* (N.C.T.C. 6752) and *Bacterium coli* (N.C.T.C. 86). Since penicillin is known to be more active, weight for weight, than dihydrostreptomycin against *B. subtilis* it was obvious that in carrying out assays with this organism, the penicillin should be first inactivated. Accordingly, a known amount of the mixed solid was dissolved in water and treated with excess penicillinase for 30 minutes. The solution was then diluted further with appropriate amounts of buffer solution and the

assay continued in the usual manner. The results obtained are given in Table I.

In carrying out assays with *Bact. coli* it did not seem necessary to inactivate the penicillin, consequently the solutions were made in standard buffer and the plate assay continued in the usual manner. The medium used in this case was a modified McConkey's agar as suggested by Sykes and Lumb[8]. Results of these assays are also given in Table I. Both methods yielded satisfactory values, hence either can be considered suitable for the assay of the dihydrostreptomycin component.

Assay of Penicillin. From previous experience, the zones of inhibition produced in the plate assay are much smaller with dihydrostreptomycin than with the same amount of penicillin. The ratio varies with the *p*H of the test solutions, the culture medium used and the test organism, the value being greater with *Staph. aureus* than with *B. subtilis*. *Staph. aureus* (N.C.T.C. 6571) was therefore chosen as the test organism using a nutrient agar made from the tryptic digestion of ox heart. In the first trials simple assays of the penicillin-dihydrostreptomycin mixtures were made against pure penicillin standard. These consistently revealed fluctuations in the slope of the log dose-response curve which were sufficiently great to render any attempt at assessing potencies valueless (Fig. 1). In an attempt to overcome this, certain substances believed to inhibit, but not to destroy, the dihydrostreptomycin were added to the assay medium. Solutions of penicillin and of the mixture were assayed over the range 20 I.U./ml. to 0·2 I.U./ml. using nutrient agar containing either 0·03 per cent. of sodium thioglycollate or 0·03 per cent. of semicarbazide

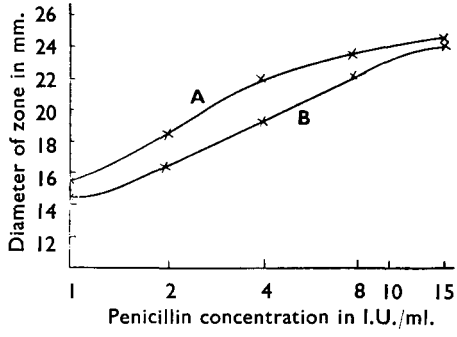

FIG. 1. Cylinder-plate assay responses of penicillin and of penicillin-dihydrostreptomycin mixtures with trypsin broth agar medium.

A. Penicillin with dihydrostreptomycin.
B. Penicillin.

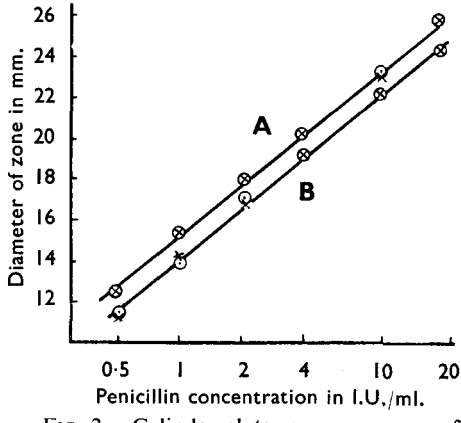

FIG. 2. Cylinder-plate assay responses of penicillin and of penicillin-dihydrostreptomycin mixtures with different agar media.

A. Using B.P. assay medium.
B. Using peptone-yeast extract medium.
⊗ Penicillin.
○ Penicillin dihydrostreptomycin mixture.

hydrochloride. In both cases the relationships between the two slopes were improved, but were not satisfactory. With thioglycollate they still lacked consistent parallelism and with semicarbazide they remained parallel but did not coincide, giving an average of 10 per cent. apparent stimulation of the penicillin activity. The zone edges were also rather diffuse, making the diameters difficult to measure.

A more important factor in countering the stimulatory or other influence of the mixture appeared to be the assay medium. When tryptic digest agar was replaced with that prescribed for the assay of aureomycin in the British Pharmacopœia, 1953, or with a simple peptone-yeast extract medium, (peptone 2 per cent., yeast extract 0·2 per cent., sodium chloride 1 per cent., agar 1·5 per cent., adjusted to *p*H 7·0), the log dose-response slopes for penicillin and for an equi-unit penicillin-dihydrostreptomycin mixture were found to coincide exactly, and the slope over the range 0·5 I.U./ml. to 20 I.U./ml. was almost a straight line (Fig. 2). The aureomycin assay medium, however, yielded zones with large halos which made their measurement difficult and tedious, whereas those from the peptone-yeast extract medium were clearly defined.

Increase in the unit concentration of the dihydrostreptomycin component to twice that of the penicillin or decrease to one half did not affect the results beyond the normal limits of error of the assay.

Suggested Methods of Assay

From the foregoing experimental observations the following methods for the assay of the components of a penicillin-dihydrostreptomycin mixture are suggested.

Dihydrostreptomycin

(*a*) *Using Bact. coli.* Dissolve a weighed quantity of the mixture in 0·05 M phosphate buffer, *p*H 7·8, and from this prepare dilutions containing approximately 10 and 100μg. per ml. Prepare similar dilutions of the Standard Preparation of Dihydrostreptomycin. Carry out a normal cup-plate assay with a 24 hour broth culture of *Bact. coli* (N.C.T.C. 86) and using a medium containing peptone 2 g., bile salts 0·5 g., neutral red (1 per cent. solution) 0·5 ml., agar 1·5 g., made up to 100 ml. with water and adjusted to *p*H 7·8 to 8·0.

(*b*) *Using B. subtilis.* Prepare a solution of the mixture in 0·05 M phosphate buffer, *p*H 7·8, add sufficient penicillinase to inactivate the penicillin, allow to stand for 30 minutes and then prepare further dilutions to contain 1 and 10μg. of dihydrostreptomycin per ml. Carry out a normal cup-plate assay with a spore-suspension of *B. subtilis* (N.C.T.C. 6752) using a medium containing peptone 0·5 g., meat extract 0·3 g., agar 1·5 g., made up to 100 ml. with water and adjusted to *p*H 7·8 to 8·0.

Penicillin

Proceed with the assay of penicillin as described in the British Pharmacopœia, 1953, p. 796, but use the Oxford strain of *Staph. aureus* (N.C.T.C. 6571) with a medium containing peptone 2 g., yeast extract 0·2 g.,

sodium chloride 1 g., agar 1·5 g., made up to 100 ml. with water and adjusted to *p*H 7·0.

If the preparation is in an oily base, first suspend 1 ml. of the preparation in 10 ml. of chloroform and extract with 3 successive quantities of 20 ml. of phosphate buffer. Bulk the extracts, dilute further as required with the appropriate phosphate buffer and complete the assays described above.

To illustrate the reliability of these methods, a suspension of sodium benzylpenicillin and dihydrostreptomycin sulphate each at a nominal concentration of 100,000 units per ml. in a liquid paraffin base containing 0·75 per cent. aluminium stearate was assayed against the appropriate standards with the following results:—

Dihydrostreptomycin

Nominal value 100,000 U./ml.
Values found with *Bact. coli* (*a*) 96,000 U./ml.
(*b*) 103,000 U./ml.
„ „ „ *B. subtilis* 95,500 U./ml.

Penicillin

Nominal value 100,000 I.U/ml.
Value found with B.P. aureomycin assay medium 96,000 I.U./ml.
„ „ „ peptone-yeast extract medium 93,000 I.U./ml.

SUMMARY

1. Methods have been described for the microbiological assay of dihydrostreptomycin and of penicillin in mixed preparations.

2. The assay of dihydrostreptomycin was carried out with *B. subtilis* or *Bact. coli* and presented no difficulty.

3. The assay of penicillin was carried out with *Staph. aureus* and its reliability was found to be dependent on the assay medium used, satisfactory results being obtained with a peptone-yeast extract medium.

We wish to thank Mrs. H. M. Payne, B.Sc., for her assistance in carrying out many of these assays.

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DISCUSSION

The paper was presented by MR. D. G. LEWIS.

MR. J. W. LIGHTBOWN (Mill Hill) said that one of the most important principles in biological assay was that both standard and the test should contain the same biologically active substances. That was not always

possible, and then any biological changes would affect the test and the standard to a different extent. In the assay under discussion the results depended upon the medium. They would also be dependent on the *p*H, which was controlled by many other factors. Therefore, although it was quite likely that the authors could carry out an assay in their own laboratory and obtain good results, it was probable that if others tried to use it in other laboratories they would run into difficulty. It was difficult to obtain standardised media, and, using the same ingredients, even different batches in the same laboratory varied significantly. Had the authors tried the assay with a number of batches, and had they always obtained satisfactory results? He felt that in the assay of penicillin and dihydrostreptomycin mixtures it would be more satisfactory to isolate penicillin chemically or physically and assay it separately.

MR. H. P. LEGGETT (Liverpool) said that a more satisfactory process in his view would be to remove dihydrostreptomycin by precipitating with silicotungstic acid. With a mixture containing procaine penicillin as well as a soluble salt, precipitation would take out the procaine penicillin as well, enabling one to work through the components individually.

MR. D. G. LEWIS, in reply, said that various manufacturers' peptones had been tried. They all gave satisfactory assays and no difference was found in the laboratory between the batches. The suggestion for removing the penicillin and other ingredients was a good one.