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Flow microcalorimetric assay of antibiotics — I. Polymyxin B sulphate and its combinations with neomycin sulphate and zinc bacitracin on interaction with *Bordetella bronchiseptica* (NCTC 8344)

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Abstract: A flow microcalorimetric assay for polymyxin B sulphate has been developed which has a better reproducibility (relative standard deviation <3%) and sensitivity (0.35 $\mu g\ ml^{-1}$) than conventional microbiological assays, and requires an assay time of ca. 4.5 h. The combinations with zinc bacitracin, with neomycin sulphate, and with both zinc bacitracin and neomycin sulphate indicate antagonism between these antibiotics upon interaction with *Bordetella bronchiseptica* (NCTC 8344). The combinations of all three antibiotics assayed were: (1) equimolar proportions; and (2) those proportions present in the commercial preparation Trisep^R (ICI, Macclesfield, UK).

Keywords: *Flow microcalorimetry; polymyxin B sulphate; neomycin sulphate; zinc bacitracin; combined antibiotic therapy; spray-powder preparation; Bordetella bronchiseptica.*

Introduction

Since the early days of the antibiotic era, the quantitative assay techniques of choice have been biological. The methods currently in use for antibiotic assay (British and US Pharmacopoeias) rely upon established procedures, e.g. agar plate diffusion assay/turbidimetric assays, the sensitivity of which is dependent upon the choice of test organism. Such traditional bioassay techniques are "end-point" methods (i.e. they record only a single datum point: inhibition zone diameter, presence or absence of growth after fixed time) and frequently have poor reproducibility [1]. An improvement may accrue from methods which record some parameter which reflects the antibiotic–cell interaction as a function of time.

Such a technique which has received increasing attention is microcalorimetry [2]. Reviews [3, 4] of microcalorimetric investigations of drug–microorganism interactions

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show that no satisfactory antibacterial drug bioassay procedures have been described. This is surprising given that those proposed for antifungal antibiotics [3, 4] have described the advantages of the microcalorimetric technique as being improvements in sensitivity, rapidity, reproducibility and simplicity. There has been only one example described [5] of the microcalorimetric investigation of the consequences of combined antibiotics–cell interaction. This study showed that clotrimazole reduced the effectiveness of amphotericin B against *Saccharomyces cerevisiae*. This antagonism was shown to result from preferential uptake of clotrimazole by the yeast, thus effectively reducing uptake of amphotericin B.

Problems are present in many attempts to assay the bioactivities of the separate components of combined antibiotic therapies. These problems range from attempts to identify synergy, indifference of antagonism amongst the components of the antibiotic mixture to the choice of test organisms (e.g. Gram positive/Gram negative at the first level) which interact with the antibiotic matrix. Methods which may be effective for the individual members of the antibiotic matrix may fail when applied to the mixture.

In an attempt to explore the utility of microcalorimetry in the bioassay of antibacterial antibodies (based on continuous observation of the time course of interaction), and to extend the investigation into the bioassay of antibacterial antibiotics in combination, the work reported in the present and subsequent papers was undertaken.

The system selected for study was the combination of polymyxin B sulphate (PolB), neomycin sulphate (Neo) and zinc bacitracin (ZnB), which constitute a post-surgery spray powder preparation (Trisep^R, ICI Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire, UK). The test organisms for each antibiotic in conventional antibiotic assays are: PolB, *Bordetella bronchiseptica* (NCTC 8344); Neo, *Bacillus pumilus* (NCTC 8241); ZnB, *Micrococcus luteus* (NCTC 7743). The assay method usually employed (ICI, Macclesfield, UK) is the agar diffusion technique. In attempting to assay the antibiotics in combination, some discrimination in these assays is achieved by suitable choice of appropriately sensitive organisms and by pH control of the applied antibiotic solution/agar [6].

The present and subsequent papers describe the flow microcalorimetric bioassay of each antibiotic on interaction with the appropriate test organism, and of the binary and ternary antibiotic combinations upon interaction with that same test organism.

Greater simplicity in bioassay could perhaps be achieved by having only one test organism for all three antibiotics, and the four possible different combinations. Hence a later paper in this series describes attempts to assay, by flow microcalorimetry, each antibiotic and the combinations upon interaction with *Escherichia coli* (NCTC 10418).

Essential components of these studies were held to be: (1) the development (where necessary, and possible) of a defined medium for growth of each organism which satisfied the needs of the microcalorimetric technique (see below); and (2) the employment of liquid nitrogen stored inocula [7] for use in the flow microcalorimetric experiments. The use of liquid nitrogen stored inocula has been shown [1] to greatly improve reproducibility in both traditional and microcalorimetric bioassay procedures. Defined media are considered preferable to undefined media, as their composition is not subject to variation. Moreover, by suitable control of temperature, aeration and other factors, growth rate can be controlled.

This paper describes the use of flow microcalorimetry to examine the effect of PolB on *B. bronchiseptica* and the combined antibiotics upon interaction with this same organism.

PolB, a polypeptide antibiotic, is active almost exclusively against Gram negative bacteria. It has some neurotoxic and nephrotoxic reactions [8–11]. The structure [12–17] and clinical applications have been reviewed [8–11, 18].

Materials and Methods

Organism

Bordetella bronchiseptica (NCTC 8344) was obtained from ICI Pharmaceutical Division (Macclesfield, UK). This strain is the recognized pharmacopoeial test organism for PolB assay.

Medium

Bordetella bronchiseptica was maintained on nutrient agar slopes. A defined medium for growth of *B. bronchiseptica* was developed and used in calorimetric experiments; the medium was based (with modification) on the defined medium developed for *Bordetella pertussis* [19–21] and the known requirement of *B. bronchiseptica* for nicotinic acid [22] and had the following composition (in g l^{-1} except where stated): glutamic acid, 2 (4 for growth experiments); methionine, 0.01; nicotinic acid, 0.001; KH_2PO_4, 8.75; K_2HPO_4 3.75; $(NH_4)_2SO_4$, 2.0) $MgSO_4 \cdot 7H_2O$, 0.125; $CaCO_3$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.0045; $ZnSO_4 \cdot 7H_2O$, 0.0014; $MnSO_4 \cdot 4H_2O$, 0.0011; $CuSO_4 \cdot 5H_2O$, 0.0002; $CoSO_4 \cdot 7H_2O$, 0.0003; H_3BO_3, 6×10^{-5}. After adjustment to pH 7.0 the complete medium was autoclaved (121°, 15 min).

Growth experiments

Growth experiments were carried out in 250 ml baffled conical flasks containing 25 ml medium on a rotary shaker (Gallenkamp, UK; 220 rpm) at 37°. Growth was followed by measurement of absorbance at 540 nm (EEL colorimeter).

Antibiotics

All three antibiotics were stored at −20°. Standard solutions were also stored at −20° without detectable loss of activity over 1 year, as determined by the microcalorimetric bioassays reported in this series of papers. Both PolB and Neo (1.0 g l^{-1}) were stored in solution at pH 7.0 (phosphate buffer 0.1 M). Weighed portions (0.1 g) of ZnB were dissolved in water (5 ml, deionized) acidified (0.5 ml, 3 M HCl) and then made up to 1 l. The certified activities (ICI, Macclesfield, UK) of these solutions were as follows: PolB, 846.4 IU ml^{-1}; Neo, 69.9 IU ml^{-1}; ZnB, 6.327 IU ml^{-1}.

MIC and MBC determinations

Tubes containing a series of two-fold dilutions of PolB in 5 ml of medium were inoculated with 5×10^5 colony-forming units (cfu) and growth, i.e. increase in turbidity at 540 nm (EEL colorimeter) was determined after 24 h incubation at 37°. The lowest concentration of antibiotic preventing growth was deemed to be the minimum inhibitory concentration (MIC). The minimum bactericidal concentration (MBC) was determined by transfer of 0.1 ml of suspension from tubes showing no growth to tryptone soya agar (Oxoid Cat. No. CM 131) plates. Both MIC and MBC were found to be 2 μg ml^{-1}.

Microcalorimetry

The calorimeter (LKB type 10700-1; 0.45 ml flow-through vessel), its operation and

the establishment of the calorimetric incubation have been described previously [23]. The total volume of the incubation medium was 50 ml; the inoculum was 1 ml and incubations were conducted at 37°.

Preparation of inocula

Baffled flasks (500 ml) containing 100 ml of medium were inoculated with 1 ml of an overnight culture, grown in the same medium, and incubated on a rotary shaker at 37°. At an absorbance (EEL colorimeter, 540 nm) equivalent to 2.5 g dry wt cells l^{-1} (corresponding to the late logarithmic phase of growth) cells were centrifuged (3000***g***, 15 min), washed and resuspended to a cell density of 2.00×10^{10} cfu ml^{-1} in 10% w/v DMSO. The suspension was dispensed in 2-ml aliquots in 12 × 35 mm polypropylene, screw-in cap ampoules (Sterilin, UK), frozen to −90° at a rate of 7° min^{-1} and cooled rapidly to −196° in liquid nitrogen as described previously [7]. Ampoules (stored in liquid nitrogen for up to 1.5 years) were thawed by immersion in water (40°, 4 min). The pre-freeze and post-freeze counts were $2.00 \pm 0.1 \times 10^{10}$ cfu ml^{-1} and $2.01 \pm 0.11 \times 10^{10}$ cfu ml^{-1}, respectively; this represents 100% survival. Post-freeze counts were not affected by length of storage in liquid nitrogen. Viable counts were determined by a surface spread-plate method [24] using quarter-strength Ringer's solution as diluent and plating on tryptone soya agar.

Results and Discussion

A defined medium has been described for *B. pertussis* [8–11] that contains proline, cysteine and glutamic acid as carbon and nitrogen sources, plus salts, Tris buffer, ascorbic acid, nicotinic acid and glutathione. However, only limited growth was obtained in this medium with *B. bronchiseptica*. Modification of proline and glutamic acid concentrations in the presence and absence of glucose resulted in no improvement in growth yield.

Growth in a medium containing glucose (0.2%), methionine (0.01 g l^{-1}) and nicotinic acid (0.001 g l^{-1}) plus salts and trace elements (see Materials and Methods) was poor, but replacement of glucose by glutamic acid or proline (within the range 0.05–0.5% for each) resulted in high yields. No improvement in cell yield was achieved by using proline and glutamic acid together, although doubling times were improved (25 min compared with 40 min in medium containing only glutamic acid). The simplest medium (i.e. without proline) was selected for further study (see Materials and Methods). Following study of lag times and doubling times under the conditions obtaining in microcalorimetric incubations, 0.2% glutamic acid was selected for use in the antibiotic assay. At concentrations of glutamic acid in excess of 0.2% there was a significant change in pH (>0.2 pH unit) during growth, whereas at lower concentrations lag time was increased but culture doubling time was not affected by glutamic acid concentrations in the range 0.1–0.4%.

Following growth, storage in liquid nitrogen and recovery of inocula of *B. bronchiseptica* calorimetric incubations were studied using the 0.2% glutamic acid medium described above. The results (Fig. 1) show that after an exponential rise in power a small peak in the power–time (p–t) curve was observed at 6.17 h, followed by a broad peak between 11–13 h.

The choice of antibiotic assay time and conditions are empirical and are determined *a priori* by convenience and simplicity, since there is, as yet, no theoretical basis for

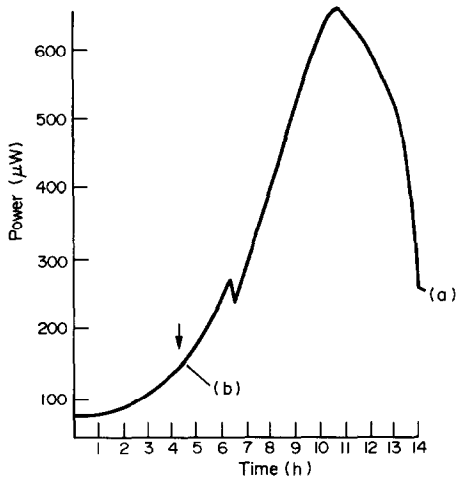

Figure 1
Power–time (p–t) curves for control and treated incubations: (a) control curve, i.e. no antibiotic addition; and (b) addition of 1 μg ml^{-1} of PolB at the time indicated and its effect on the p–t curve.

interpretation of calorimetric results of drug–cell interactions. Inoculation into medium was followed by a three-fold increase in viable counts (from $1.1 \pm 0.06 \times 10^8$ to $3.5 \pm 0.178 \times 10^8$ cfu ml^{-1}) and an exponential increase in power (four-fold increase) to the peak; 61.2 μW (±2.6% over 10 measurements) to the first peak at 6.17 h. This presumably indicates that mean cell size increases during the lag phase (1.3 h) and the early logarithmic phase of growth, as reported for other bacteria [25]. The pH remained constant throughout the period up to the first peak in power. Investigation of pH effects in the pH range 6–7.5 indicated small differences in response to antibiotic addition. The pH optimum (pH 7) for PolB–lipid binding [20] was selected for further study.

Antibiotic addition at the point in microcalorimetrically monitored growth corresponding to a doubling in cell numbers (corresponding to an increase in power of 27.5 μW) proved convenient and ensured addition of antibiotic to organisms in the exponential phase of growth. The rate of change in decline of the p–t curves was apparently related to antibiotic concentration (Fig. 1). Measurement of the rate of decline in the p–t curve immediately following addition of PolB was found to be linearly related to the logarithm of the concentration of PolB present in the incubation (Fig. 1). With respect to the MIC the upper limit in concentration (above which the decline in p–t curve became invariant with concentration) was 3.5 × MIC (7 μg ml^{-1}). At small concentrations above 0.035 × MIC the discrimination between rates of decline in p–t curve was low.

The useful analytical range over which a linear relationship was found for log dose versus response (slope of p–t curve following antibiotic addition) was 0.35 μg ml (equivalent to 0.175 MIC)–2 μg ml^{-1} (equivalent to the MIC). The relative standard deviation (RSD) was 2.9% (n = 10).

No investigation of the complex sequence of events in metabolism which are responsible for the appearance of the small peak at 6.17 h following exponential increase in power, and the subsequent slower increase to the broad peak, was undertaken. Nor indeed was any investigation of the processes leading to the very marked kinetic changes upon interaction of antibiotic with *B. bronchiseptica*. However, the response of the organism toward antibiotic was observed within 5 min of antibiotic addition (this corresponds approximately to the pumping time from external incubator vessel to the

calorimetric chamber). The sensitivity of the calorimetric technique may be seen by noting that the minimum detectable concentration of PolB was 0.035 × MIC. This sensitive and rapid observation of the interference in metabolism by PolB, a membrane active antibiotic [8–11], can be contrasted with the lack of observable differences between fresh, frozen and antibiotic-treated samples of *B. bronchiseptica* when subjected to electron microscopic investigation [18, 26, 27].

The antibiotics comprising the Trisep^R preparation PolB, Neo and ZnB were investigated in combinations upon interaction with *B. bronchiseptica*. Often some multiples of MIC values are used in making up drugs in combinations. In the work reported here the drugs have been combined in the proportions present in Trisep^R and in equimolar proportions. PolB at 0.5 × MIC (1 μg ml^{-1}) was taken as the controlling concentration. Thus for Trisep^R related concentrations, addition of PolB plus Neo (at 4.03 μg ml^{-1}) and of PolB plus ZnB (at 3.57 μg ml^{-1}) resulted in slower rates of decline in the derived p–t curves (Fig. 1). These effects can be related to the PolB concentration which is equivalent to these observed responses, i.e. 0.45 and 0.39 μg ml^{-1}, respectively. These results indicate antagonism betwen PolB and the other two antibiotics. As the microcalorimeter records the effects of these antibiotics upon overall metabolism, it may be that both Neo and ZnB compete with PolB for available binding sites on the cell surface/membrane. At proportions used in Trisep^R and at equimolar proportions of the three antibiotics added together, this again resulted in lower p–t curve rates of decline (equivalent to 0.47 and 0.5 μg ml^{-1} PolB, respectively). This also indicates some low-level antagonistic interaction between these antibiotics. It would appear, therefore, that equimolar concentrations of the three antibiotics are more effective against *B. bronchiseptica* than the combination used in Trisep^R. No detailed investigation of the relationship between concentration and response for the three antibiotics in combinations was undertaken, hence no conclusion can be drawn about an optimal concentration for each antibiotic in the combination upon interaction with *B. bronchiseptica* in this *in vitro* test. It should be stressed that these conclusions refer only to challenge toward *B. bronchiseptica* and that optimization really extends over challenge of any particular combination towards the three different organisms used in routine bioassay, as far as *in vitro* tests are concerned. Optimal proportions may be different on *in vivo* challenge to a pathogenic bacterium.

In conclusion, therefore, a microcalorimetric assay for PolB has been developed which exhibits improvements in time (4.5 h), reproducibility (RSD 2.9%) and sensitivity (0.035 × MIC 0.07 μg ml^{-1}) compared with conventional microbiological assays. This conclusion is parallel to that reached for the assay of nystatin reported by Cosgrove *et al.* [1] who compared traditional and microcalorimetric assays.

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