Antibiotic bioassay by flow microcalorimetry

A. E. Beezer, R. J. Miles, Elizabeth J. Shaw and Lynne Vickerstaff

Chemistry Department, Chelsea College, University of London, Manresa Road, London SW3 6LX, and Department of Bacteriology, St Bartholomew's Hospital Medical School, West Smithfield, London EC1A (England), 11 February 1980

Summary. A rapid, sensitive microcalorimetric bioassay of good reproducibility has been developed for the antibiotics penicillin-G, carbenicillin, ampicillin and cefoxitin. The use of growing organisms as responding agents allows discussion of the results for systems which may involve biological fluids.

Calorimetry, and particularly flow microcalorimetry, has been proposed as a useful addition to the armoury of biochemical and biological assay systems available to the analyst[2,3]. Applications involving microbial systems have been; monitoring of microbial growth[4]; the identification and characterisation of microorganisms[5]; and the bioassay of metabolic modifiers[6,7]. This last application has almost exclusively concentrated upon the bioassay of antifungal antibiotics. There has been no publication describing a quantitative assay system based upon microcalorimetry for an antibacterial drug. Moreover, the reports that do exist on the quantitative bioassay of antifungals describe microcalorimetric observations on the effect of the drugs upon non-growing (respiring) yeast cells. There have, in addition, been a number of accounts[8] (for a review see 9) which have only given qualitative descriptions of the calorimetrically observed interactions of antibiotics with bacteria. There is, therefore, nowhere to be found a quantitative microcalorimetric investigation of a bioassay system involving growing bacterial cells as the responding agents. Antibiotic synergy has been the subject of a limited microcalorimetric investigation[10].

More important for clinical situations have been the publications on the calorimetric diagnosis of bacteriuria[11,12] and 2 studies of antibiotic sensitivity testing procedures[13,14]. The study of Beezer et al.[13] showed that rapid, potentially automatable antibiotic sensitivity testing was possible by flow microcalorimetry. This study indicated, too, that the microcalorimetric response was dependent upon the concentration of the antibiotic applied, although no quantitative results were reported.

Conventionally antibiotic sensitivity testing and bioassay are performed by the agar plate diffusion technique which requires overnight incubation (16–18 h)[15]. This paper reports the extension of previous work[6,7] to the quantitative bioassay of 3 penicillins and 1 cephalosporin.

Materials and methods. The minimum inhibitory concentrations (MIC) were determined by the broth dilution technique[16].

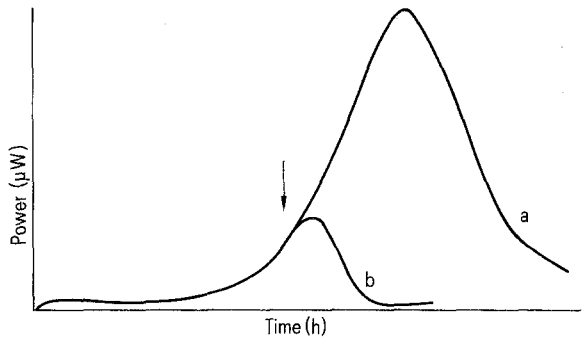
Calorimetry: The design[17] of the calorimeter was similar to that originally reported except that the thermostatted air bath supplied was exchange for a thermostatted water bath maintained, in this work, at 303 °K. The calorimetric unit was placed in a submarine contained in the water bath (Grant Instruments, Cambridge). The microcalorimeter was operated as described previously[18]. Briefly, the method consists of pumping (48 cm^3/h) medium from a magnetically stirred incubator contained within a thermostatted (303 °K) water jacket through the microcalorimeter (LKB Produkter AB, Bromma, Sweden, type 10700-1) and returning the outflow to the fermenter. An instrumental baseline was created before inoculation to a level of 5×10^3 organisms/cm^3 from an overnight culture. After about 1 h a deflection on the recorder equivalent to 10 μV was achieved (f.s.d. for 105 μW) at which point either 1 cm^3 of water or 1 cm^3 of an aqueous solution of the selected antibiotic at an appropriate concentration was added. In the absence of added antibiotic the power output continued to rise exponentially as has been observed previously[4,5].

Bacterium: A strain of *Escherichia coli,* which had been isolated from a urine specimen, was used as a test organism. The semi-defined medium employed had the following constitution (g/l): glucose, 1; K_2HPO_4, 4.2; NaH_2PO_4, 3.9; peptone, 1; yeast extract, 0.2; $MgCl_2$, 0.13; $CaCO_3$, 0.003; $FeSO_4 \cdot 7\,H_2O$, 0.007; $MnCl_2 \cdot 4\,H_2O$, 0.001.

Antibiotics: Standard solutions of ampicillin, carbenicillin, penicillin and cefoxitin were prepared in distilled water and used within the day of preparation. The MIC was determined for each antibiotic and the results are shown in table 1.

Results. A range of concentrations (from 1 × MIC to 5 × MIC) for each antibiotic were added to exponentially growing cells. The power-time curves (previously termed thermograms[18]) were used to construct dose-response curves. Response was taken[7] to be; 1. the time taken for the power-time curve to return to the baseline level or, 2. the size of the peak observed in antibiotic treated growths compared with the size of the peak in the power-time curve observed in the absence of antibacterial agents (figure). An alternative response measure adopted was that of recording the time taken from addition of the appropriate antibiotic to achieve the maximum in the peak produced. These results are shown in table 2. These arbitrary choices of response were selected largely for convenience and also because the nature and kinetics of the interaction of these antibiotics with growing cells are not well understood. This means that, although the microcalorimeter faithfully records the interaction as a function of time, the process is, as yet, too complex to permit interpretation and hence provide theoretical justification for any selected response parameter.

Discussion. In previous studies[6,7] the response selected was that of time-to-return-to-baseline. The studies reported



Typical power-time curves showing (a) control and (b) antibiotic treated observations.

Table 1. MIC as determined by broth dilution

Antibiotic	MIC (μg/cm^3)	Concentration (M × 10^6)
Ampicillin	2.0	5.72
Carbenicillin	8.0	18.94
Penicillin G.	32.0	89.78
Cefoxitin	2.0	4.68

Table 2. Concentrations of drugs applied and the corresponding response parameters

Antibiotic	Concentration n × MIC	μM	Response* Time to return to baseline (min)	Relative peak size (%)	Time to peak (min)
Penicillin	1	89.78	90	37	32
(MW, 356.4)	2	179.57	60	24.5	18.5
	4	359.15	50	–	–
	5	448.93	45	9	10
Carbenicillin	1	18.94	90	44	32
(MW, 422.4)	2	37.88	75	29	20
	4	75.76	65	–	–
	5	94.70	60	12	10
Ampicillin	1	5.72	95	49	25
(MW, 349.4)	2	11.45	70	28	15
	4	22.90	45	–	–
	5	28.62	40	6.5	5
Cefoxitin	1	4.68	–	–	–
(MW, 427.4)	2	9.36	117	41	35
	4	18.72	90	20	15
	5	23.40	60	16	14

* Mean of at least 3 results. No 2 results for the same system differed by more than 5% (see text for discussion).

here, however, involved growing and not respiring cells. In spite of this substantial variation in experimental design, use of this parameter leads, too, to linear log dose-response curves[15] for all antibiotics except for penicillin-G. Use of the parameters, time-to-peak-maximum and relative size of peak [(treated/untreated) × 100], both lead to linear log dose-response plots except for cefoxitin.

As yet we have no satisfactory explanation for these observations except to note that against the strain of *E. coli* used in this work the MIC of penicillin-G is substantially higher than the MICs of the other antibiotics. Moreover, cefoxitin belongs to a different group of antibiotics than the other 3 penicillins. It was one object of this limited study to attempt to differentiate between closely related antibiotics and related clinically indicated drugs. These results indicate that there appear to be 'mode of action' differences that would merit further microcalorimetric investigation.

As far as bioassay is concerned, however, it should be noted that the longest time-to-peak maximum recorded was 35 min. Thus, adopting this parameter, the microcalorimetric technique is, typically, much more rapid than the classical technique of agar plate diffusion for the bioassay of these antibacterial agents.

The reproducibility of the microcalorimetric techniques is, in this exercise, set at the limit of reproducibility of the inoculum from overnight cultures (approximately 3–5%). It has been shown[19], however, that by use of liquid nitrogen stored inocula the reproducibility can be improved to approximately 1%. This, together with the possibility of automation of a flowing system, where the response parameter is time, makes the microcalorimetric technique a reasonable alternative to classical bioassay methods.

The wider significance of these results is, however, the demonstration that, in contrast to the previously reported qualitative accounts of bacterium/antibiotic interaction, it is possible to develop satisfactory microcalorimetric bioassay procedures involving actively growing cells. Thus, this demonstration of a bioassay procedure for relatively 'easy' drugs suggests an extension of the method to the more difficult areas of bioassay. The microcalorimetric technique is well suited to this since a general reaction property (enthalpy change) is used to record events occurring in complex reaction systems (cell/drug). Moreover the shape of the p-t curve contains within it detailed kinetic data[6,7] which may shed light upon mode of action theories. In addition, since the calorimeter makes no demand upon the system except that there be a reaction with a measurable enthalpy change (opacity, suspended material, colour of solution, etc. do not present problems) then extension of the technique to the measurement of drug concentrations in serum, blood and tissue becomes possible. Such studies are in progress.

1 Acknowledgment. We thank the Department of Health and Social Security for a grant to support this work.
2 P. Monk, Process Biochem. *13*, 4 (1978).
3 C. Spink and I. Wädso, in: Methods of Biochemical Analysis, p. 1. Ed. D. Glick. Wiley, New York 1976.
4 A.E. Beezer, R.D. Newell and H.J.V. Tyrrell, Microbios *22*, 73 (1979).
5 A.E. Beezer, R.D. Newell and H.J.V. Tyrrell, Antonie van Leeuwenhoek *45*, 55 (1979).
6 A.E. Beezer, R.D. Newell and H.J.V. Tyrrell, Analyt. Chem. *49*, 34 (1977).
7 A.E. Beezer, B.Z. Chowdhry, R.D. Newell and H.J.V. Tyrrell, Analyt. Chem. *49*, 1781 (1977).
8 E. Semenitz and F. Tieffenbrunner, in: Application of Calorimetry in the Life Sciences, p. //// Ed. I. Lamprecht and B. Schaarschmidt, de Gruyter, Berlin 1977.
9 A.E. Beezer and B.Z. Chowdhry, in: Biological Microcalorimetry, p. 195. Ed. A.E. Beezer, Academic Press, London 1980.
10 R.F. Cosgrove, A.E. Beezer and R.J. Miles, J. infect. Dis. *138*, 681 (1978).
11 A.E. Beezer, K.A. Bettelheim, R.D. Newell and J. Stevens, Sci. Tools *21*, 13 (1974).
12 A.E. Beezer, K.A. Bettelheim, S. al. Salihi, and E.J. Shaw, Sci. Tools *25*, 6 (1978).
13 A.E. Beezer, R.J. Miles, E.J. Shaw and P. Willis, Experientia *35*, 795 (1979).
14 J.S. Binford, L.F. Binford and P. Adler, Am. J. clin. Path., *59*, 86 (1973).
15 F. Kavanagh, Ed. Analytical Microbiology. Academic Press, New York 1963.
16 P.M. Waterworth, in: Antibiotics and Chemotherapy, 4th edn, p. 490. Ed. L.P. Garrod, H.P. Lambert and F. O'Grady. Churchill Livingstone, London 1973.
17 P. Monk and I. Wädso, Acta chem. scand. *22*, 842 (1968).
18 A.E. Beezer, R.D. Newell and H.J.V. Tyrrell, J. appl. Bact. *41*, 34 (1976).
19 Preliminary report on recommendations for publication standards produced by the Inter Union Commission on Biothermodynamics.
20 R.F. Cosgrove, A.E. Beezer and R.J. Miles, J. Pharm. Pharmac. *31*, 171 (1979).