Technical methods

Assay of β -lactam antibiotics when used therapeutically in combination with aminoglycosides

BH POSTMA* *Department of Medical Microbiology, University Hospital and State University, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands*

In 1977, Stevens and Young¹ reported the use of the cation-exchanger cellulose phosphate (CP) as an inactivator of aminoglycosides in serum samples which also contained a β -lactam antibiotic. After inactivation of the aminoglycoside the β -lactam antibiotic was subsequently determined in a microbiological assay. This method has advantages over the use of aminoglycoside-resistant test strains, in which the specificity of the assay may become doubt-

ful.² Our initial experiences with the CP method were disappointing. We attempted to find the cause of this and succeeded in obtaining reliable results when two simple modifications were introduced. This paper discusses the effect of these modifications.

Material and methods

The original method as described by Stevens and Young¹ involved adding 100 mg cellulose phosphate powder to 1 ml of patient's serum. After thorough mixing the sample was centrifuged and the supernatant was taken for microbiological assay. This technique was modified in two respects: (i) serum was diluted 1/3 with 0.1 M phosphate-buffered saline (PBS) pH 7.0 and (ii) 20 mg of CP instead of 100 mg/ml was used.

Discs for obtaining standard curves were prepared using serial twofold dilutions of β -lactam antibiotics in normal human serum-PBS diluent. An aliquot (1 ml) of each dilution was transferred to a tube containing 20 mg CP, shaken for a few seconds on a whirl mixer, left at room temperature for 30 min and then centrifuged at 3000 rpm for 10 min. Discs for standard curves were prepared using this supernatant as well as the original working dilutions.

*Present address: Laboratory for Medical Microbiology, Herman Gorterlaan 2, 5644 SW Eindhoven, The Netherlands.

Accepted for publication 17 February 1982

Table 1 Concentrations of β -lactam antibiotics in the presence of aminoglycoside antibiotics. Results, derived from standard curves, are the mean of six observations. Standard deviations are shown in parentheses

β -lactam antibiotic	Solution concentrations (mg/l)	Gentamicin (10 mg/l)		Tobramycin (10 mg/l)	
		C	T	C	T
Penicillin-G	6	6.3(0.36)	6.4(0.5)	5.5(0.6)	5.4(0.8)
	30	28.5(3.4)	31.5(1.9)	28.1(3.1)	29.5(4.5)
Cloxacillin	5	5.5(0.4)	5.6(0.3)	4.9(0.4)	4.9(0.3)
	20	19.3(1.2)	21.2(2.5)	19.8(1.0)	19.8(1.1)
Cephalothin	5	4.7(0.3)	4.9(0.2)	5.4(0.6)	5.0(0.3)
	20	18.4(0.7)	20.8(1.1)	19.4(1.3)	21.9(1.9)
Carbenicillin	20	18.5(1.3)	19.1(1.5)	18.3(1.9)	19.2(3.1)
	400	389.2(48.8)	435.2(45.6)	384.2(55.6)	380.4(69.7)

β -lactam antibiotic	Solution concentrations (mg/l)	Kanamycin (30 mg/l)		Amikacin (30 mg/l)	
		C	T	C	T
Penicillin-G	6	6.9(1.3)	7.4(1.5)	6.7(0.8)	6.4(0.8)
	30	31.9(7.1)	32.6(6.1)	28.3(5.2)	28.1(3.7)
Cloxacillin	5	5.2(0.2)	5.3(0.3)	5.0(0.3)	4.9(0.3)
	20	19.5(1.3)	18.3(1.6)	19.7(0.6)	19.9(0.5)
Cephalothin	5	5.3(0.2)	5.3(0.2)	4.8(0.2)	4.6(0.1)
	20	20.3(1.4)	21.4(1.8)	19.7(1.2)	19.5(1.7)
Carbenicillin	20	16.5(1.0)	19.3(1.1)	17.2(0.8)	20.5(2.1)
	400	390.5(98.6)	433.8(72.6)	408.1(42.0)	427.3(29.6)

Control (C): discs prepared with serum containing only β -lactam antibiotic; not treated with cellulose phosphate.

Test (T): discs prepared with serum containing a mixture of a β -lactam antibiotic and an aminoglycoside treated with cellulose phosphate (20 mg/ml).

All discs had a content of 10 μ l, except those for cloxacillin assay, which had 20 μ l, to increase sensitivity. Control and test discs with different mixtures (Table 1) of β -lactam antibiotics and aminoglycosides were prepared in the same way.

Test organisms were *Bacillus subtilis* I³ for the assay of aminoglycosides, penicillin-G, cloxacillin and cephalothin, *Pseudomonas aeruginosa* NCTC 10490 for the assay of carbenicillin⁴ and *Sarcina lutea* ATCC 9341 for the assay of cloxacillin.⁵

Antibiotic medium No 5 (Oxoid) was used for the assay of kanamycin and streptomycin; the same medium supplemented with 5% NaCl⁶ was used for the assay of gentamicin, tobramycin and amikacin. Antibiotic medium No 1 (Oxoid) was used for assay of β -lactam antibiotics. Supplement was used to hasten germination of spores of *B subtilis*.⁶

Results and discussion

It is well known that the activity of β -lactam antibiotic increases at lower pH. Therefore, the effect of different concentrations of CP on the pH of normal human serum was investigated. Addition of 100 mg CP per ml serum resulted in a drop in pH from 7.75 to 5.78 (Table 2). At the lower pH, cloxacillin assay of serum showed an increase in activity by 30–55% of the actual concentration. By testing at lower CP

Table 2 Effect of cellulose phosphate concentrations on the pH of pooled normal human serum

	Concentration of cellulose phosphate (mg/ml)					
	0	10	15	20	25	100
pH	7.75	7.36	7.16	7.02	6.97	5.78

concentrations it was found that a minimum of 10 mgCP/ml was required to inactivate the aminoglycosides (Table 3). However, to ensure that all of the aminoglycoside was inactivated, we used 20 mgCP/ml in subsequent experiments. The change in pH of normal human serum, from 7.75 to 7.02, produced by this lower concentration of CP was still sufficient to affect the activity of the β -lactam anti-

Table 4 Effect of 20 mg cellulose phosphate (CP) per ml normal human serum (NHS) on the activity of some β -lactam antibiotics

Antibiotic concentration (mg/ml)	Diameter of inhibition zone (mm)	
	without CP	with CP (20 mg/ml)
Penicillin-G (60)	27.5	29.8
Ampicillin (40)	21.2	24.5
Cephalothin (100)	32.3	34.8
Carbenicillin (100)	22.0	23.5
Cloxacillin (20)	17.6	18.6

Differences between diameters of inhibition zones with and without CP for each antibiotic are statistically significant (two-sided Student's *t* test; *p* < 0.05).

biotics (Table 4). Serum diluted 1 in 3 with 0.1 M PBS (pH 7.0) prevented a decrease in pH. With this additional modification no increase in the activity of the β -lactam antibiotics studied was found in the test system.

With the two modifications mentioned above, penicillin-G, cloxacillin, cephalothin and carbenicillin can be assayed microbiologically in sera containing therapeutic concentrations of aminoglycosides with an accuracy of $\pm 15\%$ (Table 1).

We are aware that other polyanions like sodium polyanethol have been used for aminoglycoside inactivation in assays of other antibiotics,⁷ but comparative tests were not performed.

I wish to thank Professor RP Mouton, Department of Medical Microbiology, University Hospital, Leiden for his critical support, and Dr H Mattie, Department of Infectious Disease, University Hospital, Leiden, for his comments.

References

- Stevens P, Young L. Simple method for elimination of aminoglycosides from serum to permit bioassay of other antimicrobial agents. *Antimicrob Agents Chemother* 1977;12:286–7.
- Holt HA, Reeves DS. Assays of mixtures of antibiotic. In: Reeves DS, Phillips I, Williams JD, Wise R, eds. *Laboratory methods in antimicrobial chemotherapy*. Edinburgh, London and New York: Churchill-Livingstone, 1978:164–7.

Table 3 Effect of concentrations of cellulose phosphate (CP) on the activity of some aminoglycosides (discload 10 μ l)

Concentration of aminoglycoside (mg/ml)	Diameter of inhibition zone (mm) at different concentrations of cellulose phosphate						
	Cellulose phosphate (mg/ml)						
	0	1	5	10	15	20	25
Gentamicin (10)	17.0	13.5	12.4	0	0	0	0
Tobramycin (10)	17.3	16.1	7.7	0	0	0	0
Kanamycin (30)	17.1	15.7	12.2	0	0	0	0
Amikacin (30)	17.1	14.9	7.9	0	0	0	0
Streptomycin (30)	14.4	12.8	10.0	0	0	0	0

- ³ Levy D, Russell WE, Middlebrook G. Dosages of isoniazid and streptomycin for routine chemotherapy of tuberculosis. *Tubercle* 1960;**41**:23–31.
- ⁴ Palmer WR, Höffler D. *Carbenicillin. Basisdaten zur Therapie mit einem Antibioticum*. München, Wien, Baltimore: Urban und Schwarzenberg, 1977.
- ⁵ Sutherland R, Rolinson GN. Penicillins. In: Reeves DS, Phillips I, Williams JD, Wire R, eds. *Laboratory methods in antimicrobial chemotherapy*. Edinburgh, London and New York: Churchill-Livingstone, 1978:171–8.
- ⁶ Fabius GThJ, Mouton RP. Rapid microbiological blood level determination of aminoglycoside antibiotics. In: Mouton RP, ed. *Aminoglycoside assays, methods and clinical relevance*. Amsterdam, Oxford: Excerpta Medica, 1979:12–22.
- ⁷ Edberg SC, Bottonbley CJ and Gam K. Use of sodium polyanethol sulfonate to selectively inhibit aminoglycoside and polymyxin antibiotics in a rapid blood level antibiotic assay. *Antimicrob Agents Chemother* 1976;**9**:3:414–7.

Requests for reprints to: Dr BH Postma, Laboratory for Medical Microbiology, Herman Gorterlaan 2, 5644 SW Eindhoven, The Netherlands.

Congo blue: a rapid stain for elastic fibres

JD DAVIES,* EW YOUNG† *University Department of Pathology, Bristol Royal Infirmary, Bristol BS2 8HW*

There are numerous empirical methods for staining elastic fibres.¹ Most require relatively lengthy procedures which involve overnight incubation, and are therefore not adapted to simultaneous evaluation with the corresponding haematoxylin and eosin-stained sections.

Congo red, a dye which was initially used in commercial colouring of cotton fabrics, is often employed for the diagnosis of amyloid in human tissue.^{2,3} Most batches of Congo red, even when used in the standardised saturated salt conditions described by Puchtler *et al*⁴ also stain elastic fibres. Such elastic staining can, of course, be a disadvantage when identification of amyloid is desired. Although the anisotropic and dichroic characteristics of amyloid usually distinguish it from elastic fibres, the inexperienced histopathologist may find the distinction difficult. This problem may be accentuated in the dermis, where the recent demonstration of the P component of amyloid in elastic fibres⁵ could well add to the confusion.

The Congo staining method which we describe is a modification of that of Highman.³ It demonstrates elastic fibres clearly and distinguishes them sharply from amyloid.

Material and methods

The tissues were fixed in unbuffered fresh formal saline. They were processed via xylene for paraffin embedding in the routine manner. Paraffin sections (5 µm) were taken to water.

The sections were stained with 0.5% (wt/vol) Congo red in 50% alcohol according to the method of Highman³ for 10 min. Thereafter the sections were rinsed in distilled water.

Instead of the exposure to alkaline ethanol, appropriate for Highman's Congo red procedure, the sections are then rinsed for about 10 s in 1% hydrochloric acid in 70% ethanol. With this treatment the sections become blue to the naked eye.

*Address for correspondence: Dr JD Davies, University Department of Pathology, Level 9 Phase I, Bristol Royal Infirmary, Bristol BS2 8HW.

†Present address: Department of Neuropathology, Frenchay Hospital, Bristol BS16 1LE.