

A rapid assay method for cephalosporins

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SYNOPSIS A simple, rapid assay for cephalosporins is described. The method is based on the inhibition by cephalosporins of the fermentation of glucose or inositol by a strain of *Providencia* resistant to aminoglycoside antibiotics.

The method gives answers which are as accurate as those obtained by standard agar diffusion techniques within four hours, and utilizes skills and resources readily available in most routine bacteriology departments. Results are not affected by gentamicin or kanamycin concurrently administered to the patient.

This method will be of value in helping to monitor cephalosporin therapy in patients with serious sepsis, especially those with impaired renal function, and may help in elucidating and preventing the problem of nephrotoxicity associated with cephalosporin administration.

In recent years it has become generally recognized that the use of potentially toxic antimicrobial agents in the treatment of serious sepsis needs to be monitored by the estimation of serum concentrations both to avoid toxicity and to ensure adequate dosage. With antibiotics such as aminoglycosides and cephalosporins which are excreted via the kidneys, the need for monitoring is increased in patients with impaired renal function or those undergoing dialysis. Moreover, if meaningful monitoring is to be achieved, it has become essential to employ rapid assay techniques.

Several rapid assay methods, designed principally for aminoglycosides, have been described, based on a four to six hour agar diffusion (Sabath, Casey, Ruch, Stumpf, and Finland, 1971; Reeves, 1972); on the inhibition of pH change by bacteria metabolizing lactose or urea (Faine and Knight, 1968; Noone, Pattison, and Samson, 1971; Noone, Pattison, and Slack, 1972); and on the adenylation of aminoglycosides with radioactive ATP by an R-factor-mediated enzyme (Smith, Van Otto, and Smith, 1972).

Rapid assay techniques for cephalosporins have not yet received as much attention. This situation may change as cephalosporins become increasingly used, usually in combination with gentamicin, as first-line drugs in the treatment of serious sepsis, such as Gram-negative septicaemia (Martin, Cuomo, Geraghty, Zager, and Mandes, 1969).

Standard doses of cephalosporins in such cases are in the range 6 g per 24 hours and more. Peak serum concentrations of 50 to 100 $\mu\text{g/ml}$ and more are probably achieved. Nephrotoxicity attributable to cephalosporins, especially cephaloridine but also cephalothin, has been described (Kabins and Cohen, 1965; Benner, 1969; Foord, 1969; Pickering, Spooner, de Quesada, and Cade, 1970; Opitz, Herrmann, Herrath, and Schaefer, 1971) the incidence being increased by concurrently administered frusemide, the most widely used diuretic (Dodds and Foord, 1970). It has been suggested recently that nephrotoxicity is possibly associated with serum concentrations of cephaloridine in excess of 80 $\mu\text{g/ml}$ (Winchester and Kennedy, 1972). This assumption has been questioned (McAllister, 1972) but much more will be learned about such matters when cephalosporin therapy is more intensely monitored.

Wardell and his coworkers have described the basis of a pH method of antibiotic assay based on glucose fermentation by the Oxford H strain of *Staph. aureus* (Wardell, Yule, Ballard, and Forsyth, 1971). As described, this method has limitations both in terms of its sensitivity to concomitantly administered antibiotics, including aminoglycosides, and its failure to consider the problem of variations in the buffering capacity of individual human sera (Faine and Knight, 1968; Noone *et al.*, 1971).

A method giving results in three to four hours for the assay of cephalosporins has been developed, based on the inhibition of the fermentation of either glucose or inositol by a strain of *Providencia* highly

resistant to the aminoglycosides, which overcomes the individual differences of particular patients' sera.

Materials and Methods

A strain of *Providencia* (group A) designated BS 750, isolated from the leg ulcer of a geriatric patient, was chosen for the assay: (a) because of its resistance to the aminoglycosides including gentamicin; and (b) because of its ability to ferment glucose (and inositol) rapidly, compared with other coliforms tried, causing a fall in pH of from 1.0 to 1.5 units over a three-hour period. This fall in pH was inhibited by rising concentrations of cephalosporin, the rate of fall being greatest over the therapeutic range of cephalosporins (fig 1). The organism was identified

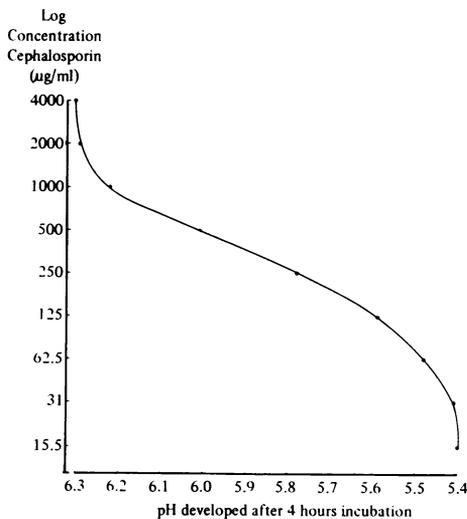


Fig 1 Inhibition of pH change by cephalosporin: 5 ml of 4% glucose broth plus 0.4 ml cephalosporin standards in pooled human serum inoculated with *Providencia* (BS 750) and incubated at 37°C.

as *Providencia* spp according to the criteria of Cowan and Steel (1966) and was found by disc testing on lysed blood agar to be totally resistant (growth up to the margins of the disc) to gentamicin (10 µg), kanamycin (5 µg), streptomycin (10 µg), and tetracycline (10 µg). It is sensitive to ampicillin (10 µg), carbenicillin (100 µg), and cephaloridine (5 µg). The minimum inhibitory concentrations of cephaloridine and cephalothin were 0.8 µg/ml and 1.6 µg/ml respectively, using as an inoculum 0.03 ml of a 1/200 dilution of an overnight Todd Hewitt broth culture of BS 750.

A standard inoculum of from 0.03 to 0.3 ml of an overnight Todd Hewitt broth culture of BS 750 exhibited maximum fermentation of 4% glucose and 3% inositol broth solutions, 5% and greater concentrations of glucose and 4% and greater concentrations of inositol, increasingly inhibited fermentation for a given inoculum. Concentrations below 4 and 3% respectively failed to stimulate maximum fermentation.

MEDIUM

Either 4% glucose broth (Oxoid CM67 with sterile 4% glucose added after autoclaving) or 3% inositol broth (made similarly) was used containing 0.4 mg of Andrade's indicator per 100 ml. Both kinds of solution were buffered to pH 7.1 and stored in 100 ml aliquots at room temperature.

STANDARDS

Initially these were made fresh every day from cephaloridine (Glaxo) or cephalothin (Eli Lilly) in sterile phosphate buffer at pH6. In later assays by the 'x/x/2 method' (see below), solutions of cephalosporins of 10 mg/ml or of 100 mg/ml were kept for periods of up to one week at -20°C. For the assay, standards were made from 1000 µg/ml to 62.5 µg/ml.

METHOD

Either 4% glucose or 3% inositol broth is dispensed aseptically in 5 ml aliquots into each of 10 8 ml plastic, disposable, screw-top tubes (Sterilin Ltd) All tubes in any given test are supplied with broth from the same bottle. The tubes are labelled '1000x', '500x', '250x', '125x', and '62.5x' in one series and '100x/2', '500 x/2', etc, in the second series.

Of freshly prepared cephalosporin standards, 0.4 ml is added to the appropriate tubes—1000 µg/ml to the '1000x' and '1000x/2' tubes and so on, then 0.2 ml of antibiotic-free pooled human serum is added to each of the x/2 tubes. Of patients' serum, 0.4 ml is added to each of the x tubes and 0.2 ml of patients' serum to each of the x/2 tubes, ie, 3.0 ml of patients' serum in all.

Finally 0.2 ml of a Todd-Hewitt broth culture of BS 750, incubated from two to 24 hours at 37°C, is added to each tube. An 18-hour culture is to be preferred and contains 5×10^8 organisms/ml by the Miles and Misra surface counting method (1938). After the addition of the inoculum, each tube is inverted and shaken to mix thoroughly. The tubes are incubated in a water bath at 37°C for three to four hours until a red coloration develops strongly in the tubes with the least cephalosporin and before those with the highest concentration have followed suit.

The reaction is not halted, and the pH of each tube

is read using a Pye Unicam 290 pH meter, measuring accurately to 0.01 of a unit, and in the sequence '1000x', '1000x/2', '500x', '500x/2', etc.

Curves are plotted on semi-log paper of measured pH (horizontal axis) against the log of the nominal concentration of cephalosporin, ie, value of the standard. Two curves are obtained and the difference between them at any given pH is equal to half the cephalosporin concentration in the test serum (Noone *et al*, 1971). The cephalosporin concentration can be worked out as an average value from several readings (fig 2).

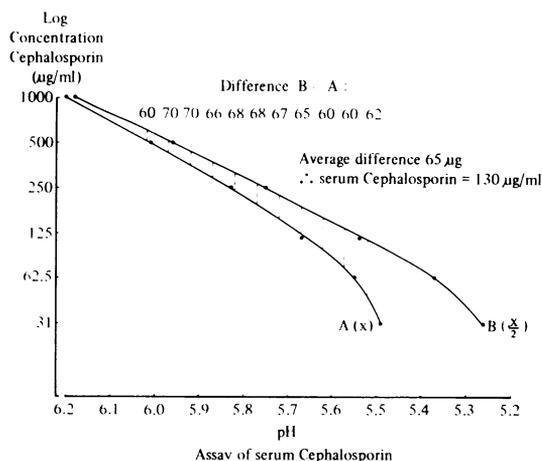


Fig 2 Curves plotted on semi-log paper of measured pH against the log of the nominal concentration of cephalosporin (ie, value of the standard). The difference between the curves at any given pH is equal to half the cephalosporin concentration in the test serum.

Initially 'straight' assays were performed, where the inhibition of pH change by 0.5 ml of cephalosporin standards in pooled human serum, of 0.2 ml of an overnight broth culture of BS 750 in 5 ml of 4% glucose broth, was compared with pH inhibition produced by 0.5 ml of test serum.

PLATE ASSAY

Conventional agar diffusion assays were performed using sensitivity test agar (Difco Bacto antibiotic medium 0270-01) seeded with the Oxford strain of *Staph. aureus* (NCTC 6571). Wells were cut and filled with cephalosporin standards in pooled human serum and dilutions of the test serum. Tests were performed in duplicate or triplicate. After 18 to 24 hours' incubation at 37°C, diameters of zones of inhibition of growth of Oxford *Staph. aureus* were measured using calipers and plotted against the log

concentration of standards. The serum concentrations could be read from the straight line obtained.

Results

The results are set out in the table where comparisons of plate assay and pH assay are made (1) on known amounts of cephalosporin added to antibiotic-free pooled human serum (unknown at time of test to experimenter) and (2) on specimens from patients. It will be seen that the 'straight' pH assay method is unreliable, giving accurate results in some instances but often yielding grossly wrong answers. However the 'x, x/2' results are extremely good and consistent. This is exactly similar to the experience obtained with the urease assay method for aminoglycosides (Noone *et al*, 1971, 1972, and 1973a).

The addition of up to 200 µg/ml of gentamicin to the incubation mixtures failed to affect the fermentation of glucose by BS 750.

	Plate Assay (µg/ml)	pH Method (µg/ml)	Known Concentrations (µg/ml)
Straight Assay Cephalothin	72	100	—
	36	39	—
	25	49	—
	52	72	—
Cephaloridine	100	25	—
	70	75	—
	100	128	—
	41	55	—
	70	120	—
x-x/2 Method Cephalothin	60	67	—
	100	96	—
	108	100	—
	600 ¹	160	—
	—	—	—
Cephaloridine	—	210	200
	—	106	100
	210	226	250
	100 ²	124	125
	180	158	180
	96 ²	84	90
	380	388	360
	110	78	100
	270	300	300
	360	324	350
185	180	187	
160	168	175	

Table Straight assay and x-x/2 method compared

¹Patient also receiving benzylpenicillin

²Serum also contained 10 µg/ml of gentamicin

Discussion

The main advantages of this pH assay method are its rapidity, simplicity, and reliance on materials and resources readily available in most routine bacteriology departments. In common with the urease pH

assay method in our hands it is more reliable and at least as accurate as routine plate assay techniques. It is unaffected by concurrently administered aminoglycosides. The method employs 3 ml of patients' serum—about 6 ml of blood—not a large volume from an adult but probably too much if repeated assay is required from neonates. Reducing serum volumes leads to a loss of sensitivity of the method. The 'straight' assay matching 0.5 ml of patients' serum with cephalosporin standards in pooled human serum leads relatively often to strikingly wrong results, attributable to variations in buffering capacity of individual specimens of serum. Work is in progress to overcome this problem.

Nevertheless the $x, x/2$ method not only provides one method of solving this problem, it also has another clear advantage. By measuring a difference in a doubly diluted series of standards with an added aliquot of unknown serum, the $x, x/2$ method does not need strictly accurate standards while still giving accurate answers (although standards must be prepared with as much care and accuracy as possible). Plate assay methods require very accurate standards or else an erroneous result is produced. With aminoglycosides, which are physicochemically stable, the maintenance of accurate standards is possible. Cephalosporins, however, deteriorate fairly rapidly in solution. This necessitates making up fresh standards each day from powdered antibiotic for assay purposes, which is time-consuming and leaves room for introducing error during the making up of standards. When employing the ' $x, x/2$ ' method one is able to keep aliquots of cephalosporin at concentrations of 10 mg/ml or 100 mg/ml at -20°C made up in phosphate buffer at pH 6 and use a separate aliquot each day. In this way one vial of powdered cephalosporin can provide solutions lasting a week, saving time each day, and money too, as cephalosporins are expensive.

In this way the ' $x, x/2$ ' method overcomes the problem of the lability of cephalosporin standards, the individual buffering capacities of particular serum, and, by being based on a method involving the use of five pairs of incubation mixtures each containing test serum, furnishes its own intrinsic multiple replication of the test, so obviating the need for performing the test more than once.

The cephalosporin assay is not as rapid as the urease method (Noone *et al*, 1973b). The splitting of urea by *Proteus* spp occurs more rapidly than the fermentation of glucose and other sugars by coliforms. In fact the urease activity of *Proteus* spp is so rapid that it is unaffected by the presence of cephalosporin, penicillin, and other antibiotics even when the organism is 'sensitive' to their action (Noone, 1972). The aminoglycosides and other antibiotics interfering

with protein synthesis such as chloramphenicol and tetracycline exert their antibacterial effect much more rapidly and can thus inhibit urease production in sensitive organisms (Noone *et al*, 1973a; Rubenis, Kozij, and Jackson, 1973).

Because it depends on the antimicrobial activity of the antibiotic concerned, the pH assay method can be no more rapid than the antimicrobial effect allows. The cephalosporin assay takes up to four hours; the aminoglycoside assay by the urease method takes about one hour. The inhibitory effect of aminoglycoside is thus exerted within one hour illustrating the more rapidly effective antibacterial action of these antibiotics compared with cephalosporins. This is certainly matched by the good clinical results obtained with the proper use of gentamicin (Martin *et al*, 1969). Whatever cephalosporins are developed in the next few years, aminoglycosides will probably remain the drugs of choice for treating serious Gram-negative sepsis. Nevertheless cephalosporins have a useful antimicrobial role and this new assay method should help promote their more effective, safer usage.

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