Procedures for the Assay of Carbenicillin in Body Fluids

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The assay of carbenicillin in clinical specimens is complicated by the fact that carbenicillin also contains a small amount of benzylpenicillin, thereby precluding the use of conventional penicillin assay organisms. This report gives details of a microbiological assay method involving the use of a strain of *Pseudomonas aeruginosa* which is very sensitive to carbenicillin but insensitive to benzylpenicillin. The outline of a microassay method with this organism is presented, and a method for the assay of specimens containing mixtures of carbenicillin and other antibiotics is described.

Carbenicillin (α-carboxybenzylpenicillin; "Pyopen," Beecham Research Laboratories) is a broad-spectrum penicillin which is of interest because of its activity against gram-negative bacteria in general and against *Pseudomonas aeruginosa* in particular (1). The treatment of severe infections generally requires the administration of large doses of carbenicillin (2, 11), and it may be desirable to measure the carbenicillin content of appropriate specimens of body fluids to ensure that the required inhibitory concentrations are being achieved or maintained.

The microbiological assay of carbenicillin is complicated by the fact that the material available at present contains a small proportion of benzylpenicillin (about 1%) which is produced as a result of decarboxylation of carbenicillin during manufacture. Consequently, after the administration of carbenicillin to patients, specimens of body fluids will contain a small quantity of benzylpenicillin as well as carbenicillin; since benzylpenicillin is many times more active than carbenicillin against the assay organisms normally used in the assay of penicillins in body fluids, the presence of even small quantities of benzylpenicillin could have a significant effect on the assay of carbenicillin with these organisms. It is desirable, therefore, that carbenicillin be assayed with an organism which is sensitive to carbenicillin and resistant to benzylpenicillin. In early studies with carbenicillin (1, 7), an assay method involving a carbenicillin-sensitive strain of *P. aeruginosa* NCTC 10490 was described briefly. This report gives further details of assay methods of carbenicillin in body fluids.

MATERIALS AND METHODS

Carbenicillin, the disodium salt of α-carboxybenzylpenicillin, is readily soluble in water (85 g/100 ml at 20°C), giving a clear neutral solution. Its structure is shown in Fig. 1. Specimens of body fluids may be kept in the refrigerator at 5°C for up to 1 week or at −20°C for several weeks without significant loss of carbenicillin activity. Carbenicillin powder should be kept in a cool place because degradation occurs more rapidly at temperatures above 15°C.

**Basic assay method.** Large, rectangular glass plates (30 by 30 cm) were filled to a depth of approximately 4 mm with molten nutrient agar (Oxoid no. 2) seeded with a 0.05% inoculum of an overnight broth culture of *P. aeruginosa* NCTC 10490 (ATCC 23389). When the agar was solid, holes 7 to 8 mm in diameter were punched in the seeded agar with a modified cork borer, and the plugs of agar were removed with a lancet or broad needle. The holes were filled in a random fashion in replicate with suitable dilutions of the test specimens or with standard solutions of carbenicillin. Serum specimens were diluted in pooled human serum or with 3% bovine albumin V in 0.05 M phosphate buffer (pH 7.0). Urine specimens were diluted in 0.05 M phosphate buffer (pH 7.0). Standard solutions of carbenicillin were prepared in serum or buffer as appropriate over a range of 100 to 1.0 µg of carbenicillin per ml. The plates were incubated overnight at 30°C; diameters of the zones of inhibition were measured, and the carbenicillin concentrations of the test specimens were derived from the standard line.

**Microassay method.** Molten nutrient agar was seeded as above with *P. aeruginosa* NCTC 10490. Assay plates were prewarmed and were filled rapidly with a minimal volume of the seeded agar to give an agar layer 1 mm or less, if possible, in depth. The test specimens, serum, lysed blood, heparinized blood, or cerebrospinal fluid were diluted, if considered necessary and 0.02 ml of specimen was pipetted onto filter-

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paper discs (Whatman AA discs) 6 mm in diameter with a standard dropping pipette. Standard solutions of carbenicillin (100 to 1.0 μg of carbenicillin per ml), prepared in the appropriate diluent, were pipetted onto paper discs in a similar fashion. The discs containing the test specimens and standard solutions were pressed on the surface of the seeded agar plate with sterile forceps. The plates were incubated overnight at 30 C, and the zones of inhibition were measured as described above.

Removal of benzylpenicillin from carbenicillin. Benzylpenicillin was eliminated from laboratory standard carbenicillin by treatment with a penicillin acylase enzyme preparation, which hydrolyzed benzylpenicillin to the relatively inactive 6-aminopenicillanic acid but had no effect on carbenicillin. Penicillin acylase was prepared as described by Cole and Sutherland (4). A conical flask (500 ml) containing 50 ml of a nitrogen-enriched (Yeatec) medium plus 0.15% phenylacetic acid was inoculated with 0.5 ml of an overnight nutrient broth culture of Escherichia coli NCIB 8473 and incubated overnight at 30 C on a rotary shaker. After incubation for 18 hr, 0.5 ml of n-butyl acetate was added to the culture, and shaking was continued for a further hour by which time the culture was nonviable. A 2.0-ml volume of the suspension of killed cells was added to 20 mg of carbenicillin in 18.0 ml 0.05 M phosphate buffer (pH 8.0). The mixture of cells and carbenicillin was incubated at 37 C for 4 hr and filtered through a Seitz filter to remove the E. coli cells and to eliminate penicillin acylase activity. The treated carbenicillin and standard solutions of untreated carbenicillin and benzylpenicillin were spotted (6 μl) on filter-paper strips (Whatman no. 1, 1 cm wide) and chromatographed with a butanol-ethyl alcohol solvent system (n-butanol-ethanol-water, 4:1:5, v/v, top phase). Chromatography was carried out overnight at 5 C and the chromatograms were dried at 40 C. Carbenicillin and benzylpenicillin were located by placing the dried chromatograms on nutrient agar seeded with a spore suspension of Bacillus subtilis ATCC 6633, which was incubated overnight at 37 C. The enzyme-treated carbenicillin and standard solutions of benzylpenicillin and carbenicillin were also assayed for activity against Sarcina lutea NCTC 8340 and P. aeruginosa NCTC 10490.

Inactivation of carbenicillin with penicillinase. Inactivation of carbenicillin in specimens containing carbenicillin and other antibiotics was achieved by using a commercial preparation of B. licheniformis penicillinase ("Wellcome" penicillinase, Burroughs Wellcome & Co., London). The preparation, 1 ml of which was stated to inactivate 1,000,000 units of penicillin, was diluted to give a 2% solution when added to the specimen, and the mixture of penicillinase and antibiotic was incubated for 2 hr at 37 C prior to assay.

RESULTS AND DISCUSSION

Activity of carbenicillin after removal of benzylpenicillin. The removal of benzylpenicillin from carbenicillin, after treatment with E. coli penicillin acylase, is illustrated in Fig. 2. The bioautogram shows the presence of benzylpenicillin in untreated standard material and its absence after treatment with penicillin acylase.

The results of microbiological assays with untreated and enzyme-treated samples of carbenicillin, using S. lutea NCTC 8340 and P. aeruginosa NCTC 10490 as assay organisms, are illustrated in Fig. 3. When assayed against S. lutea, the zone of inhibition produced by 50 μg of standard untreated carbenicillin per ml was equivalent to that obtained with 1.0 μg of benzylpenicillin per ml. After treatment with penicillin acylase, carbenicillin was distinctly less active against sarcina, and the solution containing 50 μg of carbenicillin per ml only equalled a solution of 0.1 μg of benzylpenicillin per ml. These results suggest that, against S. lutea NCTC 8340, the activity shown
TABLE 1. Antibiotic sensitivity of P. aeruginosa NCTC 10490

| Antibiotic          | Minimal inhibitory concn* (µg/ml) | Minimal concn detectable in assay with P. aeruginosa NCTC 10490 (µg/ml) |
|---------------------|-----------------------------------|------------------------------------------------------------------------|
| Carbenicillin       | 1.0                               | 1.0                                                                    |
| Ampicillin          | >500                              | >500                                                                   |
| Benzylpenicillin    | 500                               | >500                                                                   |
| Cephaloridine       | >500                              | >500                                                                   |
| Cephalothin         | >500                              | >500                                                                   |
| Chloramphenicol     | 250                               | >500                                                                   |
| Colistin methane sulphonate | 25                        | 50                                                                    |
| Gentamicin          | 1.25                              | 12.5                                                                   |
| Kanamycin           | 500                               | 500                                                                    |
| Polymyxin B sulphate | 12.5                             | 12.5                                                                   |
| Streptomycin        | 50                                | 25                                                                     |
| Tetracycline        | 125                               | 200                                                                    |

* Serial dilution in nutrient agar; inoculum, one drop of an overnight broth culture of the assay organism. Values were read after 24 hr at 37°C.

Fig. 3. Effect of removal of benzylpenicillin from carbenicillin by treatment with penicillin acylase on the activity of carbenicillin against Sarcina lutea NCTC 8340 and P. aeruginosa NCTC 10490.

by this particular sample of carbenicillin was largely or entirely due to that of the benzylpenicillin formed during manufacture. Treatment with penicillin acylase had no effect on the activity of carbenicillin against P. aeruginosa NCTC 10490.

Assay of carbenicillin with P. aeruginosa NCTC 10490. The main reason for employing P. aeruginosa NCTC 10490 as an assay organism for carbenicillin is that the organism is relatively sensitive to carbenicillin and relatively insensitive to benzylpenicillin (Table 1), which is invariably present in small quantities in the material available at present. The results shown here demon-

strate that benzylpenicillin is much more potent against S. lutea NCTC 8340, a standard penicillin assay organism, than is carbenicillin; accordingly, the presence of even small amounts of benzylpenicillin in test specimens of body fluids could significantly influence the results of assays of carbenicillin with S. lutea (or B. subtilis or Staphylococcus aureus).

A disadvantage of using this strain of pseudomonas as an assay organism for carbenicillin is that this organism is relatively insensitive compared with the organisms conventionally employed for penicillin assays. Consequently, it is not possible to assay concentrations lower than 1 to 2 µg of carbenicillin per ml. On the other hand, in most cases of severe infections carbenicillin will be administered at a dosage which will normally produce concentrations in the body readily detectable with P. aeruginosa NCTC 10490.

Microassay of carbenicillin. The punch-hole assay method described above requires a volume of 1.0 to 2.0 ml of specimen, and this is not always readily available, particularly when neonates or infants are involved. The use of paper discs for microassay methods is not novel (5, 6), and the micromethod described here is based on well-tried principles. The assay of small volumes of specimens by this method is reliable, and the sensitivity of the assay is not markedly reduced provided a thin agar layer is used in the plates.

Assay of specimens containing carbenicillin and other antibiotics. The treatment of severe or un-
diagnosed infections may involve therapy with carbenicillin and one or more antibiotics. This is particularly the case with pseudomonas infections for which carbenicillin may well be used in conjunction with gentamicin or one of the polymyxins (2, 3, 8, 11). The general principle involved in the assay of such mixtures is the elimination of carbenicillin with penicillinase and the measuring of nonpenicillin antibiotic with a suitable assay organism, an established procedure for the assay of mixtures containing penicillin and nonpenicillin antibiotics (6, 9). Carbenicillin activity is measured by using an organism insensitive to the nonpenicillin antibiotic.

The use of commercial penicillinase prepared from B. subtilis or B. licheniformis is not advisable for the assay of mixtures of carbenicillin and penicillinase-stable penicillins such as methicillin or cloxacillin, as these latter penicillins are relatively unstable to the penicillinases produced by these organisms. A suitable method of assay of such mixtures involves the use of a penicillinase-producing strain of S. aureus sensitive to methicillin and cloxacillin but resistant to carbenicillin, as has been described for the assay of cloxacillin in specimens containing ampicillin and cloxacillin (9).

The carbenicillin content of antibiotic mixtures may normally be determined by assay with P. aeruginosa NCTC 10490, since most antibiotics are relatively insensitive to this organism compared with carbenicillin (Table 1). This means that antibiotic activity not due to carbenicillin may be eliminated by suitable dilution of the specimen, unless the antibiotic was present at a relatively high concentration and one which was considerably greater than that of the carbenicillin concentration in the mixture. To ensure that the activity attributed to carbenicillin after dilution of the specimen was in fact due to carbenicillin, a confirmatory test (9) could be carried out by treating the diluted specimen with penicillinase and repeating the assay on P. aeruginosa NCTC 10490.

As an example, specimens of serum and urine, containing carbenicillin and gentamicin, have been assayed (i) by diluting the specimen with appropriate diluent to an estimated concentration of 10 μg of gentamicin per ml or less (Table 1) and assaying the carbenicillin content with P. aeruginosa NCTC 10490, and (ii) by inactivating carbenicillin with a commercial preparation of penicillinase, as described, and measuring the gentamicin content with Corynebacterium xerosis NCTC 9455 as assay organism. With this method, it has been possible to assay specimens of serum or urine containing antibiotic concentrations as low as 1.0 μg of carbenicillin and 0.1 μg of gentamicin per ml.

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