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Note

Paper chromatographic determination of benzylpenicillin in carbenicillin preparations

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Preparations of carbenicillin are known to contain trace amounts of benzylpenicillin, the concentration of which increases during storage of the preparations owing to decarboxylation of the carbenicillin into benzylpenicillin. Because of the different ranges of antibacterial activity of the two penicillins, this transformation may lead to incorrect results when the susceptibility of pathogenic gram-negative bacteria to carbenicillin is tested *in vitro*, *e.g.*, using the disc plate method.

The paper chromatographic separation of benzylpenicillin from carbenicillin is described in this paper. A modification of this method can be used for the quantitative determination of benzylpenicillin in samples of carbenicillin.

EXPERIMENTAL

Carbenicillin samples

The following preparations of carbenicillin were analysed: Carbenicillin sodium (Beechams, Brentford, Great Britain, lot No. F 436, keg No. 1); Carbenicillin sodium (Beechams, laboratory reference standard No. 7, 790 μg/mg); Carbenicillin sodium (Beechams, laboratory reference standard No. 8, 795 μg/mg); and Carbenicillin natrium (Bayer, Leverkusen, G.F.R., 828 μg/mg). Amounts of 2 μg in aqueous solutions (10 μl) were applied on to the chromatograms.

Carbenicillin discs (Lachema, Brno, Czechoslovakia), each containing 100 μg of carbenicillin, were extracted with 70% aqueous *n*-propanol (100 μl per disc) at room temperature and volumes of 10 μl of the extract were applied on to the chromatograms.

Benzylpenicillin

A standard of sodium benzylpenicillin (Upjohn, Kalamazoo, Mich., U.S.A., lot No. 2218 F) was used in all experiments. In qualitative analyses, amounts of 0.5 μg in aqueous solutions (10 μl) were applied on to separate chromatograms. In quanti-

tative evaluations, volumes of 10 μl containing 0.125, 0.25, 0.50 and 1.0 μg were applied on to separate chromatograms.

Solvent systems

The following systems were used to separate benzylpenicillin and carbenicillin: A, 70% aqueous *n*-propanol[1]; B, 60% aqueous *n*-propanol[1]; C, methanol–*n*-hexane (6:4, bottom layer)[2]; D, *n*-butanol saturated with water; E, methanol–*n*-propanol–0.013 *M* aqueous sodium citrate (6:2:1).

Form, development and detection of chromatograms

Strips of Whatman No. 1 paper (35 × 1 cm) were used. The chromatograms were developed at room temperature without equilibration by the ascending technique in narrow glass cylinders containing 25 ml of the solvent system. When the front of the system had reached a distance of 15 cm from the origin (about 3.5 h with systems A and D), the development was terminated. Bioautographic detection of the air-dried chromatograms with *Bacillus subtilis* was carried out on large agar plates (36 × 25 cm) as described earlier[3,4]. The zones of inhibition were made visible by spraying the growing culture on the plates with 0.5% aqueous 2,6-dichlorophenolindophenol, which resulted in dark blue spots on the decolorized background[5].

Quantitative determination of benzylpenicillin in carbenicillin samples

Separate chromatograms were run in triplicate for each sample and each standard concentration using solvent system A. The developed and air-dried chromatographic strips were cut into squares of side 1 cm, which were then placed on a large agar plate (36 × 25 cm) seeded with *Bacillus subtilis*. The plate was incubated overnight at 37° and the diameters of the circular inhibition zones around the squares corresponding to the positions of benzylpenicillin were measured. The total area (A mm^2) of the zones from each chromatogram was calculated according to the equation

$$A = \pi (r_1^2 + r_2^2 + \cdots r_n^2)$$

and the diameter (d mm) of the total area was calculated as

$$d = 2\sqrt{\frac{A}{\pi}}$$

The mean values of the diameters (d) of the total areas (A), as obtained after detection on triplicate chromatograms, were plotted semi-logarithmically against the corresponding doses of standard benzylpenicillin. The resulting standard line and the mean values of d from the samples were used to calculate the amounts of benzylpenicillin in the samples of carbenicillin.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of benzylpenicillin from carbenicillin in two samples using solvent systems A and D. Similar results were obtained with the other two sam-

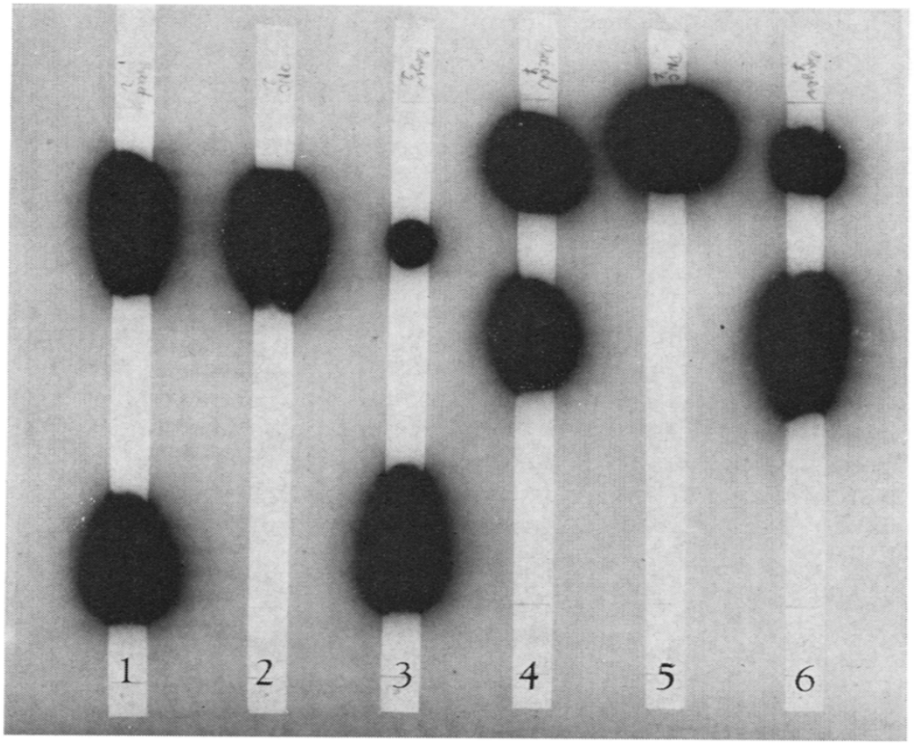

Fig. 1. Paper chromatographic separation of benzylpenicillin in two carbenicillin preparations. Development: ascending in 70% aqueous *n*-propanol (strips 1–3) and in *n*-butanol saturated with water (strips 4–6). Detection: *Bacillus subtilis*, zones made visible with 2,6-dichlorophenolidophenol. 1 and 4, Carbenicillin sodium (Beechams, lot No. F 436, keg No. 1); 2 and 5, benzylpenicillin; 3 and 6, Carbenicillin natrium (Bayer).

ples of carbenicillin. Similarly to the laboratory standard from Bayer, the laboratory standards from Beechams contained less benzylpenicillin when compared with the Beecham sample shown in Fig. 1. $R_{\text{benzylpenicillin}}$ values of the two penicillins in five systems are given in Table I.

Analyses of extracts from carbenicillin sensitivity discs in solvent system A resulted, after bioautography of the chromatograms, in two distinctive zones with the R_F values of 0.41 and 0.88, corresponding to carbenicillin and benzylpenicillin, respectively.

Fig. 2 shows the principle of the quantitative determination of benzylpenicillin in the chromatographed carbenicillin preparations. Usually, benzylpenicillin was present in three or four squares from one chromatogram. After measuring the diameters of the zones of inhibition and calculating the mean values of the diameters (d) of the total areas (A), calculations were made as described above. In a typical analysis, 0.24 μg (*i.e.*, about 11%) of benzylpenicillin was found in 2.0 μg of Carbenicillin sodium lot No. F 436, keg No. 1 (Beechams).

The results indicate the necessity for checking carbenicillin preparations and sensitivity discs for their contents of benzylpenicillin, and the technique described might be of value in such analyses.

TABLE I

PAPER CHROMATOGRAPHIC $R_{benzylpenicillin}$ VALUES OF CARBENICILLIN AND BENZYLPENICILLIN FROM A CARBENICILLIN PREPARATION*

Solvent systems: A = 70% aqueous *n*-propanol; B = 60% aqueous *n*-propanol; C = methanol–*n*-hexane (6:4, bottom layer); D = *n*-butanol saturated with water; E = methanol–*n*-propanol–0.013 *M* aqueous sodium citrate (6:2:1).

Solvent system	$R_{benzylpenicillin}$	
	Carbenicillin	*Benzylpenicillin*
A	0.41	1.01
B	0.68	0.93
C	0.12	1.02
D	0.10	0.96
E	0.38	0.97

* Carbenicillin sodium (Beechams, lot No. F 436, keg No. 1).

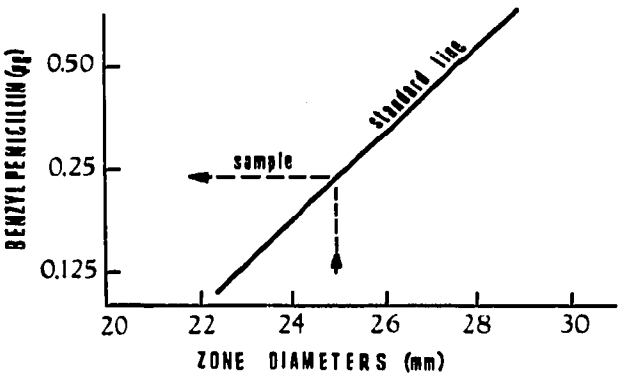

Fig. 2. Quantitative determination of benzylpenicillin in samples of carbenicillin after chromatographic separation in 70% aqueous *n*-propanol and detection with *Bacillus subtilis* (schematic; see text for details).

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