Journal of Chromatography, 161 (1978) 417–420

CHROM. 11,311

Note

Thin-layer chromatographic method for the identification of the polymyxins

A. H. THOMAS and I. HOLLOWAY

Division of Antibiotics, National Institute for Biological Standards and Control, London NW3 6RB (Great Britain)

(Received May 26th, 1978)

The polymyxins are a group of closely related decapeptide antibiotics produced by species of *Bacillus*. They differ from each other in amino acid composition and on the basis of the fatty acid attached to the peptide, some may be sub-divided into two or more active components. The composition and structure of the polymyxins have been reviewed by Vogler and Studer[1] and by Storm *et al.*[2]; recently, polymyxins F, S and T have been isolated and described[3–5].

The antibiotics used therapeutically, polymyxin B and colistin (polymyxin E), both consist essentially of two active components. A spectrophotometric test based on the absorbance of phenylalanine can be used to differentiate polymyxin B and colistin. Qualitative amino acid analysis of the hydrolysed polymyxin is used to distinguish polymyxin B from other polymyxins[6,7]. The components of polymyxin B and colistin can be separated by the paper chromatographic method used by Suzuki *et al.*[8]. Recently, the main components in polymyxin B and colistin have been separated by high-pressure liquid chromatography[9]. This paper describes a simple thin-layer chromatographic method which will differentiates the polymyxins and their semi-synthetic derivatives sulphomyxin and colistin sulphomethate.

EXPERIMENTAL

Materials

Polymyxin B and colistin were international standards, sulphomyxin and colistin sulphomethate were pharmaceutical materials. Fractions of polymyxin B_1, B_2 and polymyxin E_1, E_2 isolated by counter-current separation were kindly supplied by Miss P. Newland, Division of Antibiotics, National Institute of Biological Standards and Control, London, Great Britain. The following were kindly provided: polymyxins A, D and M by Dr Knud Andersen, Dumex A/S, Copenhagen, Denmark; polymyxin F by Dr W. Parker, The Squibb Institute for Medical Research, Princeton, N.J., U.S.A., and polymyxins S and T by Dr J. Shoji, Shionogi and Co. Ltd., Osaka, Japan.

Commercially available precoated silica gel silanised plates (Merck, Darmstadt, G.F.R.; 20 × 20 cm, 0.25 mm thickness) were used. Plates were also prepared with silica gel 60 H silanised (Merck) according to the manufacturer's instructions, but with the addition of 13% (w/w) of calcium sulphate dihydrate. The solvent system

consisted of acetone–0.1 *N* hydrochloric acid (25:75) containing 1% (w/v) sodium chloride. The spray reagent was composed of ninhydrin (300 mg) dissolved in 2,4,6-trimethylpyridine (5 ml) and ethanol (95 ml). Antibacterial activity was detected using *Bordetella bronchiseptica* (NCTC 8344) inoculated in Medium B, pH 7.3[10]. In order to ensure adequate buffering of the medium, because of the acidic nature of the chromatographic plate, after development, potassium dihydrogen phosphate (0.1778 g) and disodium hydrogen orthophosphate dihydrate (9.55 g) were added to a litre of the medium. The phosphates precipitated above 60° but redissolved when the medium cooled to 45° prior to inoculation with the bacterial suspension. The inoculated medium contained approximately $5 \cdot 10^5$ colony-forming units per millilitre.

Method

The plates were heated at 105° for 60 min before use. The samples were dissolved in distilled water (the polymyxins 10 mg/ml, sulphomyxin and colistin sulphomethate 15 mg/ml) and volume of 2 μl were applied to the plate. A plate was placed in a filter-paper-lined chromatographic tank which had been saturated with solvent vapour. The samples were chromatographed over a 15-cm path, and the plate then removed and allowed to dry at room temperature. The plate was sprayed with the ninhydrin reagent and heated for 10 min at 100° to reveal purple spots on a white background.

To detect antibacterial activity, a plate, after removal from the chromatographic tank, was left overnight at room temperature to dry and to allow the solvent vapour to evaporate. A 4–5 mm thick inoculated layer of solidified medium was placed in contact with the surface of the chromatographic plate. The plate with its covering layer of medium was incubated at 35° for 24–28 h. Antibacterial activity was revealed as a clear zone of inhibition of growth in the medium.

RESULTS AND DISCUSSION

The thin-layer chromatograms (Figs. 1 and 2) show that most of the polymyxins and the semi-synthetic derivatives may be differentiated from each other. Two or more components were apparent in many of the polymyxins examined. The ninhydrin reacting components, except those at the origin, were biologically active. The polymyxins which could be identified were B_1, B_2, E_1 and E_2 (R_F values: 0.17, 0.25, 0.21 and 0.32, respectively). These polymyxins were obtained by counter-current separation using a coil-planet centrifuge. The fatty acids were identified by gas–liquid chromatography, methyloctanoic acid in polymyxins B_1 and E_1, and methylheptanoic acid in polymyxins B_2 and E_2. The minor components detected in polymyxins B and E by thin-layer chromatography probably correspond to the octanoic acid and heptanoic acid containing components.

Polymyxins A and M could not be differentiated from each other by this chromatographic procedure, but it has been suspected that these two antibiotics are identical[2,11]. When laboratory-prepared plates were used, two components were detected in both these polymyxins, but there was no difference between them. Except for polymyxins A and M the separations obtained on either the precoated or the laboratory-prepared plates were similar.

Polymyxins B and E can be identified by this chromatographic method, using

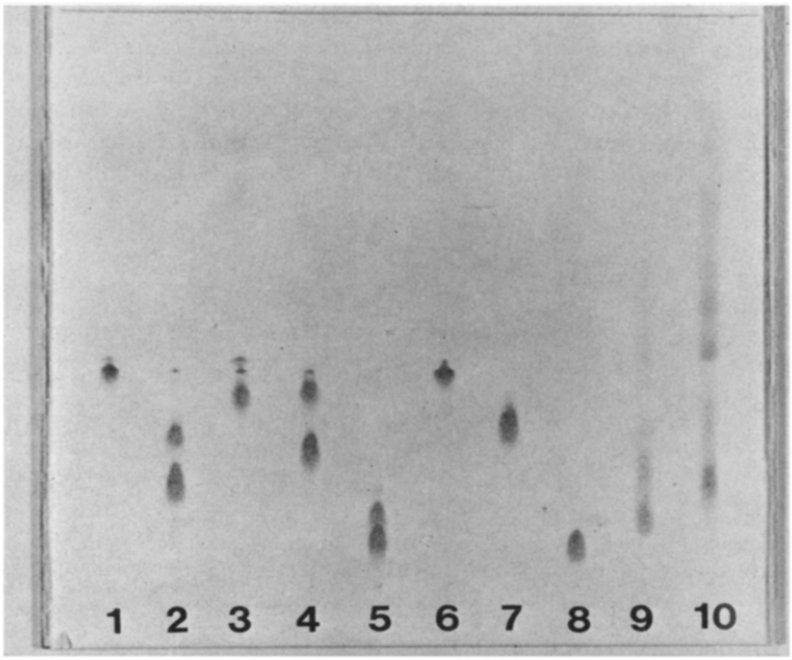

Fig. 1. Chromatogram of the polymyxins detected with ninhydrin. Polymyxins (20 μg applied): 1, A; 2, B; 3, D; 4, E; 5, F; 6, M; 7, S; 8, T; 9, sulphomyxin (30 μg applied); 10, colistin sulphomethane (30 μg applied).

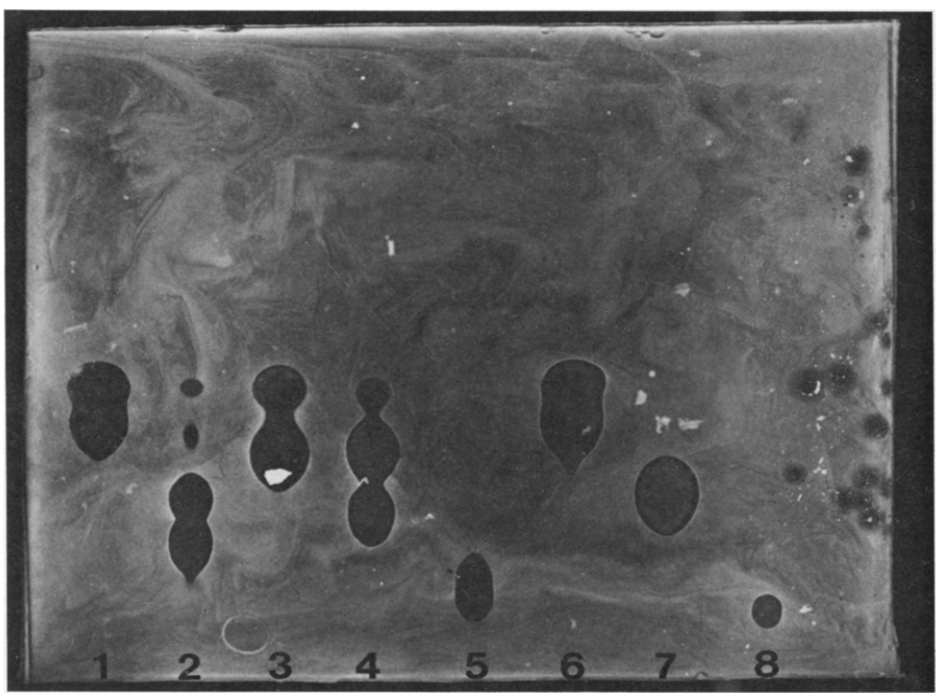

Fig. 2. Chromatogram of the polyxymins detected with *Bordetella bronchiseptica* (NCTC 8344). Polymyxins (20 μg applied): 1, A; 2, B; 3, D; 4, E; 5, F; 6, M; 7, S; 8, T.

ninhydrin; other ninhydrin-reacting antibiotics, *e.g.* aminoglycosides, bacitracin, capreomycin and viomycin, have different R_F values and would not interfere. An advantage of this method as an identification test is that it does not require hydrolysis of the sample prior to chromatography and is therefore quicker and simpler than the current pharmacopoeial test. The possibility of developing the method as a semiquantitative assay for the major components of polymyxin B and E is being investigated.

ACKNOWLEDGEMENT

We are grateful to Mrs C. Smith, Chemistry Section, National Institute for Biological Standards and Control, London, for performing the gas–liquid chromatographic analysis.

REFERENCES

1 K. Vogler and R. O. Studer, *Experientia*, 22 (1966) 345.
2 D. R. Storm, K. S. Rosenthal and P. E. Swanson, *Annu. Rev. Biochem.*, 46 (1977) 723.
3 W. L. Parkes, M. L. Rathnum, L. D. Dean, M. W. Nimeck, W. E. Brown and E. Meyers, *J. Antibiot.*, 30 (1977) 767.
4 J. Shoji, T. Kato and H. Hinoo, *J. Antibiot.*, 30 (1977) 1035.
5 J. Shoji, T. Kato and H. Hinoo, *J. Antibiot.*, 30 (1977) 1042.
6 M. R. Howlett and G. B. Selzer, *J. Chromatogr.*, 30 (1967) 630.
7 *European Pharmacopoeia*, Vol. II, Maisonneuve, Sainte-Ruffine, 1971, p. 344.
8 T. Suzuki, H. Inouye, K. Fujikawa and Y. Suketa, *J. Biochem.*, 54 (1963) 25.
9 K. Tsuji and J. H. Robertson, *J. Chromatogr.*, 112 (1975) 663.
10 *British Pharmacopoeia*, HMSO, London, 1973, p. A102.
11 J. Shoji, H. Hino, Y. Wakisaka, K. Koizumi, M. Mayama and S. Matsuura, *J. Antibiot.*, 30 (1977) 1029.