Detection and Separation of Penicillins by Thin-Layer Chromatography

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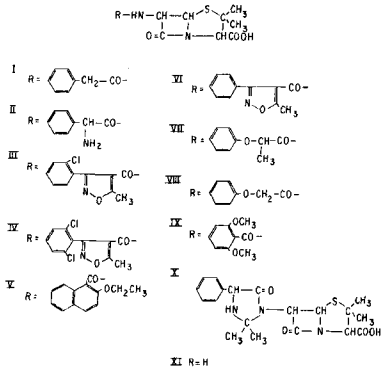
Thin-layer chromatographic systems and spray reagents are described for the rapid differentiation of 10 penicillins. Comparative behavior of 18 other antibiotics under these conditions is presented together with other supporting data.

THE INTRODUCTION of new semisynthetic penicillins, two of which are official in U.S.P. XVII (1) (sodium methicillin and sodium oxacillin), has accentuated the need for convenient methods for microdetection and differentiation of members of the penicillin group. The current U.S.P. (1) and Canadian regulatory tests (2) depend upon solubility characteristics and time-consuming microbiological methods.

Paper and thin-layer chromatography have both been cited in the literature as methods for the separation of "natural" penicillins and other antibiotics (3–8). Detection techniques quoted include treatment with iodine (6, 7), iodine–sodium azide (3) and permanganate (9), and bioautography (4, 5, 8). The penicillins examined in these investigations included only those obtained from direct microbiological fermentation, although paper chromatography has been used in metabolic studies of semisynthetic analogs (10, 11). However, as far as the authors are aware, the differentiation of all the penicillins in current use—many of them manufactured by semisynthetic methods from 6-aminopenicillanic acid (6APA) (XI)—has not yet been achieved.

This paper describes some thin-layer chromatographic procedures which permit the identification of the following 10 penicillins: sodium benzylpenicillin (penicillin G) (I), sodium



ampicillin (II), sodium cloxacillin (III), sodium dicloxacillin (IV), sodium nafcillin (V), sodium oxacillin (VI), potassium phenethicillin (VII), potassium phenoxymethylpenicillin (penicillin V) (VIII), sodium methicillin (IX), and potassium hetacillin (X). Certain of these are well established clinically, while others show promise (12, 13). For purposes of comparison, the same procedures were applied to other readily available antibiotics (Table I).

EXPERIMENTAL

Preparation of Plates.—The chromatoplates (20 × 20 cm.) were coated to a thickness of 250 μ using the standard Desaga spreader. Silica Gel G (Merck) was applied according to Stahl's method (14). The plates were dried at room temperature and activated at 110° for 30 min. before use on the same day. The cellulose plates were coated with a smooth slurry prepared by stirring cellulose MN 300 without binder (Macherey and Nagel) (30 Gm.) mechanically at high speed for 15 min. with distilled water (200 ml.). They were then dried overnight at room temperature and used without preactivation.

The following systems were used. (*A*) Layer: cellulose MN 300, solvent: 0.1 *M* sodium chloride solution; (*B*) layer: cellulose MN 300, solvent: 0.3 *M* citric acid solution saturated with *n*-butanol [0.3 *M* citric acid (100 ml.) and *n*-butanol (20 ml.) were shaken and left to separate]; (*C*) layer: Silica Gel G, solvent: organic phase of isoamyl acetate–methanol–formic acid–water (65:20:5:10) (8); (*D*) layer: Silica Gel G, solvent: acetone–acetic acid (95:5) (9).

Spray Reagents.—(*a*) Ten per cent aqueous ferric chloride (20 ml.) and 5% aqueous potassium ferricyanide (10 ml.) were mixed with 20% sulfuric acid (70 ml.) and used on the day of preparation.

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(*b*) Ninhydrin (0.3 Gm.) in acetone (100 ml.). (*c*) Fifty per cent sulfuric acid.

Preparation of Spotting Solutions.—Samples of each of the 10 penicillin powders (10 mg.) were dissolved in methanol (10 ml.). Similarly, samples (10 mg.) of the 18 antibiotics listed in Table I were shaken with methanol (10 ml.) and the supernatants spotted. Tenfold dilutions of the penicillin solutions with methanol were made when required for determining the sensitivity of the detection method.

Chromatographic Procedure.—Samples of the methanolic solution of each penicillin salt representing 1 mcg. (1 μl.) were applied to a plate by means of a Hamilton micro-syringe. The plates were inserted in a previously equilibrated filter paper-lined tank and the appropriate solvent allowed to rise to a height of 15 cm. (approximate times taken: system, *A*, 70 min.; *B*, 120 min.; *C*, 50 min.; and *D*, 20 min.). The plates were dried thoroughly in a stream of warm air except when system *C* was used, in which case drying was accomplished by heating at 120° for 20 min. and cooling before spraying. The spots were observed as blue areas on a yellow-green background after spraying lightly with reagent (*a*). Full color development took up to 5 min., the speed depending on the nature of the penicillins.

The sensitivity of this spray reagent was determined by spotting dilutions of the 10 penicillin solutions in amounts down to 0.04 mcg. with a Burroughs Wellcome Agla syringe on plates treated in the usual way in the four systems.

A plate loaded with the 10 penicillins was chromatographed as described above with system *A*, sprayed with reagent (*b*) after drying, and heated at 100° for 5 min. Only one violet spot corresponding to sodium ampicillin (II) was observed. Similarly, a plate sprayed with reagent (*c*) revealed only two yellow spots corresponding to sodium nafcillin (V) and sodium methicillin (IX).

The behavior of the 18 antibiotics listed in Table I in systems *A* to *D* was investigated. The methanol supernatants of these substances were applied to plates and chromatographed in the usual way. The plates were sprayed with reagent (*a*) after drying.

RESULTS AND DISCUSSION

Previous chromatographic investigations of the penicillins were not concerned with distinguishing between the different acyl derivatives of 6-aminopenicillanic acid (XI), although various methods have been described for paper chromatography of penicillins G and V (3, 7, 8). Some of the solvents cited in this early work were used in trials with different thin-layer chromatographic adsorbents during the present screening program. Thus, in this study, butyl acetate–*n*-butanol–acetic acid–water (80:15:40:24) used in paper chromatography by

TABLE I.—APPROXIMATE R_f VALUES[a] AND BEHAVIOR OF OTHER ANTIBIOTICS IN SYSTEMS *A* TO *D*

Antibiotic	System *A*	*B*	*C*	*D*
Tetracycline HCl	0.60[b]	0.76	0	0
Chlortetracycline HCl	0.54[b]	0.69	0	0
Oxytetracycline HCl	0.66[b]	0.75	0	0
Demethylchlortetracycline HCl	0.52[b]	0.67	0	0
Pyrrolidinomethyltetracycline	0.59[b]	0.74	0	0
Polymyxin B sulfate	N.S.[c]	N.S.	N.S.	N.S.
Vancomycin HCl	0.91[b]	0.95	0	0
Neomycin sulfate[e]	N.S.	N.S.	N.S.	N.S.
Tyrocidine HCl[f]	0	0	0.01(0.06)	0
Tyrothricin[f]	0	0	0.23	0
Gramicidin[f]	0	0	0.41	0.74[b]
Bacitracin[f]	N.S.	N.S.	N.S.	N.S.
Novobiocin	0	0	0.59	0.89
Amphotericin B	0	0.20	0	0
Cephaloridine	0.97[d]	0.90[b]	...[d]	0.79[b]
Griseofulvin	0.23	0.60	0.59	0.83
Ristocetin	0.74	0.97	0	0
Lincomycin HCl	0.96	0.95	0	0.13(0.03)

[a] R_f Values of secondary spots are shown in parentheses. [b] Streak. [c] No visible spot. [d] Long streak. [e] Purchased from K and K Laboratories, Plainview, N. Y. [f] Purchased from General Biochemicals Ltd., Chagrin Falls, Ohio.

TABLE II.—R_f VALUES[a] OF 10 PENICILLINS IN SYSTEMS *A* TO *D*

Substance	System *A*	*B*	*C*	*D*[b]
I, Benzylpenicillin Na	0.90	0.90	0.61(0.28)	0.58
II, Ampicillin Na	0.97	0.98(0.91)	0.12	0.15
III, Cloxacillin Na	0.65	0.38	0.64	0.77
IV, Dicloxacillin Na	0.47	0.22	0.65	0.77
V, Nafcillin Na	0.47(0.37)	0.22[c]	0.64	0.77
VI, Oxacillin Na	0.74	0.49	0.65(0.37)	0.63
VII, Phenethicillin K	0.84	0.73(0.55)	0.66	0.77
VIII, Phenoxymethylpenicillin K	0.82	0.76(0.56)	0.66	0.75
IX, Methicillin Na	0.93	0.93	0.52(0.18)	0.59
X, Hetacillin K	0.96	0.98	0.30(0.12)	0.64(0.15)

[a] Average of 10 plates. R_f values of secondary spots are shown in parentheses. [b] Results in this system are very susceptible to temperature variations. [c] With streaking.

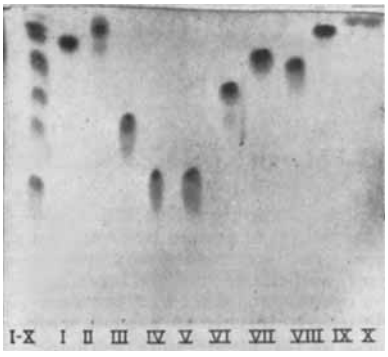

Fig. 1.—Chromatoplate of penicillins (I–X) in system *A*.

Fischer and Lautner (3), and butyl acetate–*n*-butanol–acetic acid–phosphate buffer pH 5.8–methanol (80:15:40:24:5) used by Nussbaumer in quantitative thin-layer chromatography of penicillins G and V (6) failed to separate the 10 analogs completely. Betina (8) in an extensive survey of antibiotic chromatography utilized various solvent systems for penicillins G and V and quoted 3% ammonium chloride and isoamyl acetate–methanol–formic acid–water (system *C*) as giving the best paper chromatographic separations. The authors found that 0.1 *M* sodium chloride was superior to the ammonium chloride system and system *C* was of limited value for separating the different derivatives by thin-layer chromatography.

The R_f values of the 10 penicillin salts with the four systems used are recorded in Table II. The limit of detection with spray (*a*) was found to be in the range of 0.1 to less than 0.04 mcg. System *A* was the system of choice for the separation of the analogs but failed to separate benzylpenicillin (I) from methicillin (IX); ampicillin (II) from hetacillin (X); dicloxacillin (IV) from nafcillin (V), and phenethicillin (VII) from phenoxymethylpenicillin (VIII) (Fig. 1). System *C* was found useful for the separation of I, II, IX, and X, but of all the solvent/adsorbent modifications tried, none separated penicillin IV from V or VII from VIII. However, nafcillin (V) was identified by spraying the chromatographed plate with spray reagent (*c*) when it appeared as an intense yellow spot. Methicillin was the only other penicillin which gave this color but at a very different R_f value. The use of (*a*) after this treatment, revealed the other analogs as previously described. As expected, ampicillin (II) with a primary amino group was the only penicillin to give the ninhydrin reaction which may, therefore, be applied as a confirmatory test for this substance. System *B* was a useful alternative solvent, although some of the spots exhibited variable R_f values due to streaking effects.

Ampicillin (II) and its derivative hetacillin (X) gave similar R_f values in the above systems. Hardcastle *et al.* (9) reported R_f values of 0.15 and 0.65, respectively, for these compounds as free acids in solvent system *D* and noted that dimethylformamide (DMF) should be used as the spotting solvent since hetacillin decomposes in methanol. The authors compared DMF and methanol as spotting solvents for all 10 penicillin salts in the four systems and found that the R_f values for all compounds except hetacillin were not appreciably altered by the different solvents when both freshly prepared and solutions "aged" for 1 week were used. For potassium hetacillin, there was no change in R_f when systems *A* and *B* were used. However, with systems *C* and *D* two spots were evident for the salt with both freshly prepared methanol and DMF spotting solutions. The major, faster running, spot (R_f values 0.30 and 0.64 in systems *C* and *D*, respectively) was found to be diminished in intensity while the slower running spot (R_f values 0.12 and 0.15) was found to be increased in "aged" solutions of both methanol and DMF. Thus, it would appear that hetacillin or its salts decomposed to yield ampicillin in both methanol and DMF (in which the salt is only slowly soluble) on storage and that differentiation of hetacillin from ampicillin is best accomplished using fresh solutions in system *D*. The R_f values in this system, as noted in Table II, vary markedly with changes in temperature, hence, system *D* is quoted only for the differentiation of ampicillin (II) and hetacillin (X).

A number of other solvent systems and adsorbents were tried during the course of this work. Acidic and alkaline solvent mixtures were generally found to give double spot formation and tailing (as with system *B*) probably attributable to hydrolysis. The use of ion-exchange layers such as diethylaminoethylcellulose and buffered layers proved disappointing.

The approximate R_f values of 18 antibiotics in these systems quoted in Table I indicate that these drugs do not interfere with this method of penicillin differentiation. Many of the antibiotics were only slightly soluble in methanol and with such substances the supernatant was used for spotting. The spray reagent (*a*) was much less sensitive in all cases.

These rapid diagnostic procedures will ensure that the more common penicillins are not readily substituted for the more expensive and recently introduced analogs and should also prove useful when applied to the detection of cross-contamination of pharmaceutical formulations by penicillins (15). The application of these procedures to the latter problem is currently under study in these laboratories.

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