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SEPARATION OF PENICILLINS OF CLOSELY RELATED STRUCTURE BY PARTITION CHROMATOGRAPHY ON PAPER AND CELLULOSE THIN-LAYER PLATES*

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SUMMARY

By applying the technique of real partition chromatography[1] to systems in which aqueous buffer solutions are used as the stationary phase, natural or semi-synthetic penicillins of closely related structure can be satisfactorily separated. Water-saturated alkanols of proper polarity and buffers of proper pH are chosen as the mobile and stationary phases, respectively, according to the polarity of the penicillin to be chromatographed. Examples of penicillins separated are ampicillin *vs.* epicillin, their N-acetyl derivatives, penicillins G *vs.* penicillin V, *m*- *vs.* *p*-hydroxybenzylpenicillins and *m*- *vs.* *p*-hydroxyampicillins.

INTRODUCTION

Many paper and thin-layer chromatographic (TLC) methods have been described for the separation of natural, as well as of semi-synthetic, penicillins[2,3,5–9]. I wish to report that, by a proper modification of the earliest reported method, *i.e.*, that of GOODALL AND LEVI[6], which was thoroughly studied by KARNOVSKY AND JOHNSON[7], penicillins of very closely related structure, *e. g.*, ampicillin *vs.* epicillin and *m*- *vs.* *p*-hydroxyampicillin, are satisfactorily separated on paper as well as on precoated cellulose plates. The modification involves introducing the stationary phase, which is an aqueous buffer solution, as it is, rather than in the form of air-dried buffer salts with subsequent equilibration against water vapor. The stationary phase, therefore, is visibly present during the development of the chromatogram. In other words, the technique of "real partition chromatography"[1] exemplified by the ZAFFARONI type of paper chromatography[11,12] and reversed-phase chromatography has been adopted.

MATERIALS AND PROCEDURES

All the penicillin samples, including epicillin[4], 6-(D-α-amino-1,4-cyclohexadienylacetamido)-penicillanic acid, are from the Squibb collection.

The mobile phases of the solvent systems are water-saturated lower alkanols,

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TABLE I[a]

PAPER CHROMATOGRAPHIC SEPARATION OF PENICILLINS OF CLOSELY RELATED STRUCTURE

Solvent system	*Mobile phase*[b]	*Stationary phase, pH of buffer*	*Spot A*	*Spot B*	*Duration of development (h)*	*Solvent front*	R_F[c]		*Distance moved (cm)*	
							A	*B*	*A*	*B*
1	*n*-Butyl alcohol–*tert.*-amyl alcohol (6:1)	4.1	Ampicillin	Epicillin	16	dripped off	0.22	0.29	9.0	11.3
2	*n*-Amyl alcohol	6.7	N-Acetyl-ampicillin	N-Acetyl-epicillin	16	dripped off	0.22	0.31	10.5	14.0
2	*n*-Amyl alcohol	6.7	Penicillin G	Penicillin V	4.5	at F	0.47	0.63	8.6	11.4
3	*n*-Amyl alcohol–amyl acetate (3:1)	6.7	*p*-Hydroxy-benzylpenicillin	*m*-Hydroxy-benzylpenicillin	30	dripped off	0.08	0.09	10.6	13.2
4	*n*-Butyl alcohol–*tert.*-amyl alcohol (2:1)	6.7	*p*-Hydroxy-ampicillin	*m*-Hydroxy-ampicillin	30	dripped off	0.06	0.07	8.1	11.3

[a] Referring to Fig. 1.
[b] All mobile phases were water-saturated.
[c] R_F values were measured when solvent front was at F in every case.

e.g., *n*-butyl alcohol, *n*-amyl alcohol, etc. The stationary phases consist of half-strength McIlvaine's or 0.1 *M* phosphate buffers. As will be described below, the choice of the pH of the buffer and the polarity of the alkanols depend upon the polarity of the penicillins to be chromatographed. Four solvent systems were developed and they are described in Table I.

Solutions of the penicillins (1.0 mg/ml) are prepared in 0.1 *M* phosphate buffer of a pH the same as that to be used as the stationary phase. Proper dilutions are made with water whenever necessary. For chromatograms where the spots are detected by the starch-iodine reagent[10], a sample spot of 2–4 μg of penicillin is used while a sample spot of approximately 100 ng was shown to be enough for bioautography on a *Staphylococcus aureus* plate.

The chromatographic jar is made from a diphtheria toxin bottle, having the dimensions 11.5 × 9.0 × 23 cm. The aqueous phase of the solvent system is used to wet the paper linings on two sides of the jar. A Whatman No. 1 paper strip (9 × 23 cm) on which sample spots have been applied at the origin, is drawn through a bath of a mixture of the buffer–acetone (1:3). This mixture is slightly turbid because the phosphate is not soluble in aqueous acetone, and must be used immediately before the turbidity settles. It is not necessary to wet the end of the paper strip that is going to be placed in the trough, but the origin must be wetted. After being hung in air for 3 min to permit evaporation of the acetone, the paper strip, which is still wet, is transferred into the jar, and the upper phase (alkanol phase) of the solvent system is introduced into the trough to develop a descending chromatogram.

Essentially, the same procedure is used for TLC on a cellulose plate. Precoated Analtech Cellulose MN-300 plates (5 × 20 cm, 0.25 mm thick) were used. The plates are immersed quickly in a deep jar filled with the buffer–acetone mixture in such a manner that the end of the plate to which sample spots have been applied becomes immersed last. After the plate has been air-dried for 5 min, an ascending type of chromatogram is developed with the upper phase of the solvent mixture, in the same manner as for paper chromatography.

RESULTS

For the separation of ampicillin and epicillin, the solvent system consists of *n*-butyl alcohol–*tert.*-amyl alcohol–water (6:1:4) and the stationary phase is a buffer of pH 4.1 (System 1). It takes 4.5 h for the solvent front to reach the lower end of the paper strip. The R_F values are ampicillin, 0.22 and epicillin, 0.29. A mixture of the two appears only partially resolved. By developing the papergram for 16 h, complete separation of these two penicillins is achieved as shown in Fig. 1 and Table I (System 1). Since the papergram pictures of all the experiments reported in this communication look alike, Fig. 1 is used to represent all the results. Details of each individual case are described in Table I.

For less polar penicillins, *e.g.*, N-acetylampicillin *vs.* N-acetylepicillin or acid unstable penicillins, *e.g.*, benzylpenicillin *vs.* phenoxymethylpenicillin, use of buffer of pH 6.7 as the stationary phase and water-saturated *n*-amyl alcohol as the mobile phase was shown to be suitable. The results are shown in Fig. 1 and Table I (System 2). It should be mentioned here that if these less polar penicillins are chromatographed in System 1, they all appear near the solvent front and conversely, if an

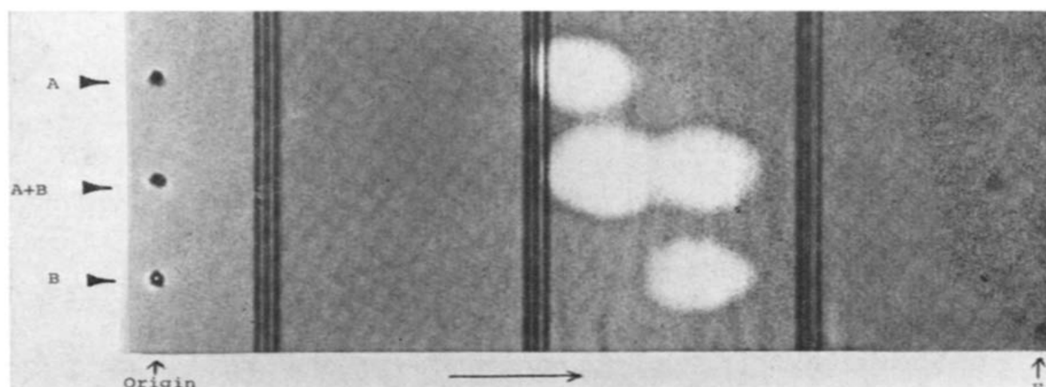

Fig. 1. Paper chromatographic separation of penicillins of closely related structure. Detection of spots: bioautography on *S. aureus* plate.

amphoteric penicillin, *i.e.*, ampicillin, is chromatographed in System 2, it barely moves off the origin.

In the present study, *m*- *vs.* *p*-hydroxybenzylpenicillins and *m*- *vs.* *p*-hydroxyampicillins were shown to be two pairs of penicillins most difficultly resolved. An effective technique consists of choosing a solvent system so that these penicillins move with an R_F value of 0.1 or smaller. By developing the chromatogram for an exceedingly long period of time, they eventually become resolved. In the case of *m*- *vs.* *p*-hydroxybenzylpenicillins, water-saturated *n*-amyl alcohol–amyl acetate (3:1) *vs.* half-strength McIlvaine's buffer of pH 6.7 proved effective while in the case of *m*- *vs.* *p*-hydroxyampicillins, water-saturated *n*-butyl alcohol– *tert.*-amyl alcohol (2:1) *vs.* half-strength McIlvaine's buffer of pH 6.7 worked satisfactorily. The data are described in Fig. 1 and Table I (Systems 3 and 4).

These partition systems were shown to be adaptable to precoated cellulose thin-layer plates. For the separation of ampicillin and epicillin, Solvent System 1 was shown to be applicable to Analtech precoated Cellulose MN-300F plates. Fig. 2 shows such a chromatogram. The spots were detected with the starch-iodine spray reagent[10].

Adaptation of System 1 for the separation of ampicillin and epicillin on a silica gel plate failed. Not only were these two penicillins not separated, but another slower-moving starch-iodine positive spot was observed with either ampicillin or epicillin.

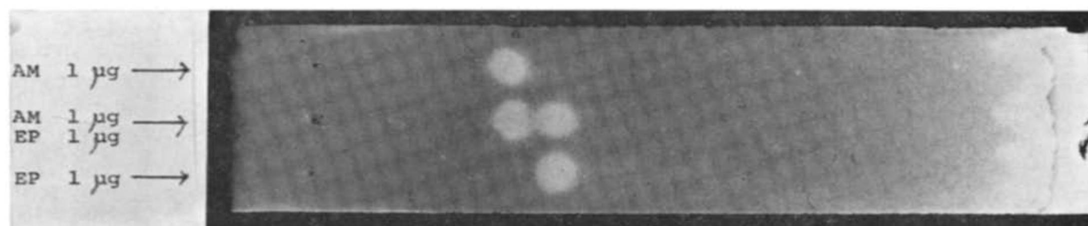

Fig. 2. Thin-layer chromatogram of ampicillin and epicillin on cellulose plate. AM = Ampicillin; EP = epicillin. Solvent system: System 1 (see Table I). Development time: 6 h. Detection of spots: starch-iodine spray reagent.

The spot size is estimated to be approximately 1/10 of that of the main spot and is bio-inactive. It is believed that silica gel causes some degradation of the penicillins.

CONCLUSION AND DISCUSSION

It should be pointed out that ampicillin and epicillin can be separated as satisfactorily as shown in Fig. 1 by an alternative procedure that resembles the original method of GOODALL AND LEVI[6] more closely than the procedure described above. After being drawn through the bath of the buffer–acetone mixture, the paper strip is allowed to air-dry completely. It is then equilibrated overnight against the aqueous phase of the solvent mixture in a jar provided with paper linings as described above and developed with the alkanol phase of System 1. However, repeated tests showed that another spot, which is starch-iodine positive, but bio-inactive, was invariably noted with either ampicillin or epicillin. This degradation product moved with approximately half the R_F value of the main spot. It is known that the ampicillin type of compound is quite acid-stable. This observation is explained on the basis that exposing an air-dried spot on paper to water-vapor-saturated air at pH 4.1 for an extended period of time is quite different from keeping a dilute solution in a test tube at the same pH and temperature. The recommended procedure, therefore, not only simplifies the procedure for the introduction of the stationary phase, but also eliminates the formation of the degradation product during chromatography.

From the examples given in this report, it appears obvious that the behavior of penicillins on these partition chromatographic systems is very similar to that of steroids on ZAFFARONI-type chromatographic systems: *i.e.*, (1) the shape of spots is essentially round with no tailing; (2) compounds with small difference in polarity are resolved as discrete spots; and (3) compounds with large difference in polarity, *e.g.*, ampicillin *vs.* N-acetylampicillin, require two mobile phases of different polarity for them to move with readily measurable R_F values, *e.g.*, between 0.1 and 0.8. These systems are, therefore, useful not only for the separation of compounds of closely related structure but also for judging their polarity and thus their possible structure.

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