CHROM. 6098

SEPARATION AND DETECTION OF DEGRADATION PRODUCTS OF PENICILLINS AND CEPHALOSPORINS BY MEANS OF THIN-LAYER CHROMATOGRAPHY

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(Received March 8th, 1972)

SUMMARY

Thin-layer chromatography procedures are described which permit the simple and rapid separation and detection of different spontaneous, chemical and enzymatic degradation products of penicillins and of two cephalosporins. The right combination of solvent system and spray reagent allows the identification of these products, even in the presence of the parent antibiotics, in aqueous preparations as well as in biological fluids and microbiological culture broths.

INTRODUCTION

The widespread action of microbial enzymes (β-lactamases, acylases) on penicillins[1-5], together with the instability of these antibiotics in aqueous solutions[6-8] accentuated the need for a convenient and rapid method for the detection of the end products in the presence of the parent antibiotics.

Spontaneous degradation of the penicillins in acid solution consists of a rearrangement into penillic acids. In aqueous alkaline solution, degradation mainly results in the formation of penicilloic acids, which can further decarboxylate into penilloic acids. β-Lactamase activity also results in the formation of penicilloic acids. Both processes cause a loss in the antibacterial activity of the antibiotics[9].

The chemistry of the β-lactam cleavage process in cephalosporins has received some attention[10-15]. However the exact nature of the enzyme-degraded products has not yet been elucidated[15-17].

Penicillin acylases transform the biosynthetic penicillins V and G into the very important 6-aminopenicillanic acid (6-APA), the starting material for the industrial production of semisynthetic penicillins[2,4,5,18-20]. This 6-APA is also susceptible to polymerization processes and to spontaneous and β-lactamase-directed hydrolysis, resulting mainly in the formation of 6-aminopenicilloic acid (penicic acid), which can further decarboxylate into CDAT (D-4-carboxy-5,5-dimethyl-2-aminomethylthiazolidine)[21,22]. 6-APA has only recently been prepared by chemical methods[23,24]. On the other hand, only chemical methods are available at the moment for the transformation of Cephalosporin C into 7-aminocephalospor-

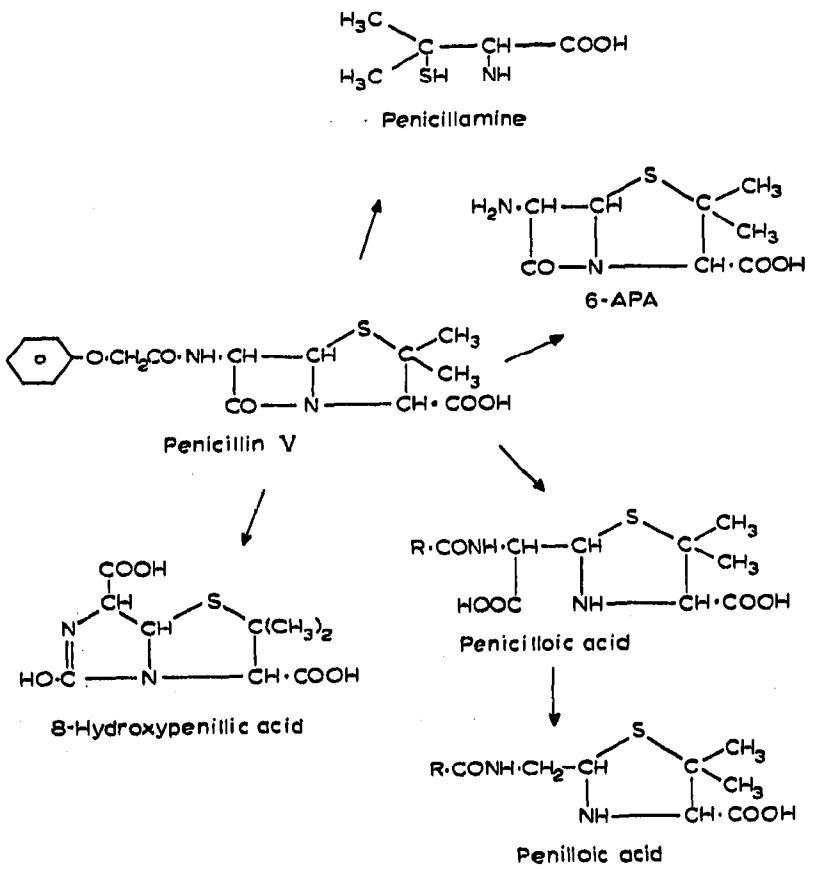
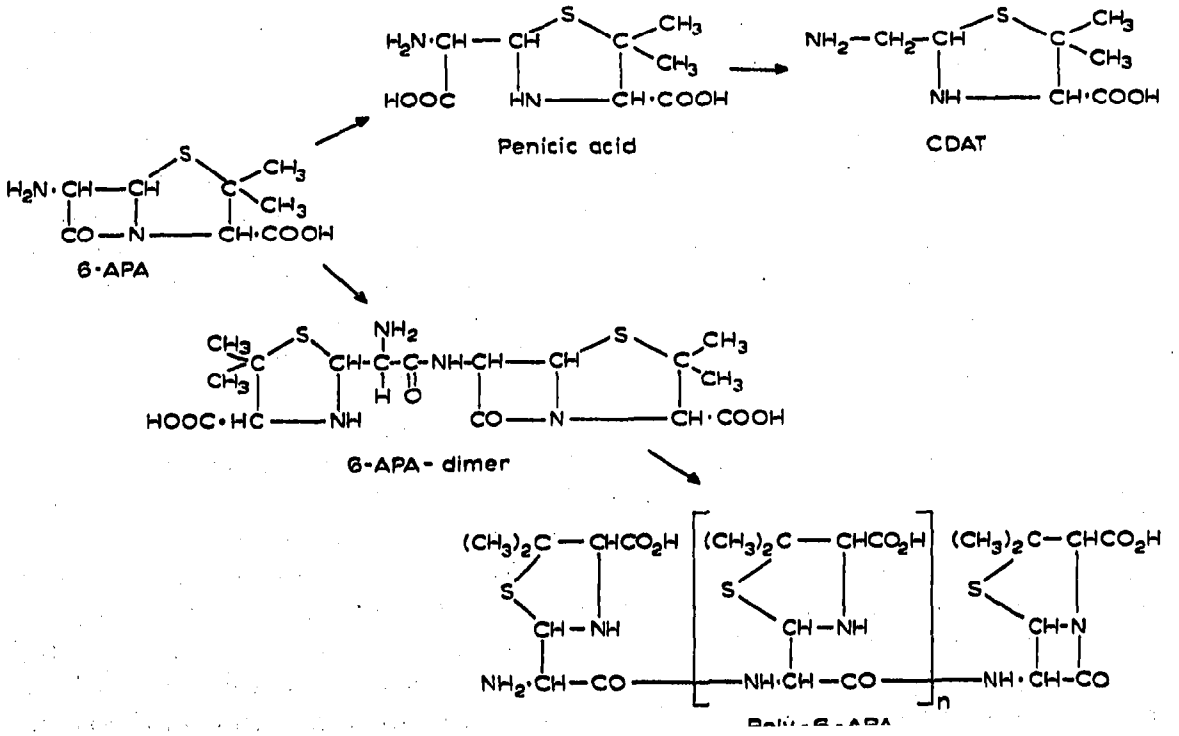


Fig. 1. Degradation patterns of penicillin.



In addition to the action of β-lactamases and acylases, which can be produced by the same microbial strain, penicillins could be transformed by the action of *Enterobacteriaceae* into penicillamine during the spheroplast-induction process[28].

These different degradation patterns are illustrated in Figs. 1, 2 and 3. Although time-consuming, paper chromatographic procedures, sometimes combined with bioautographic methods, are at this moment widely used to detect these degradation products[9,29-31]. However thin-layer chromatography (TLC) has mostly been used to separate and identify the different penicillins or cephalosporins[32-35].

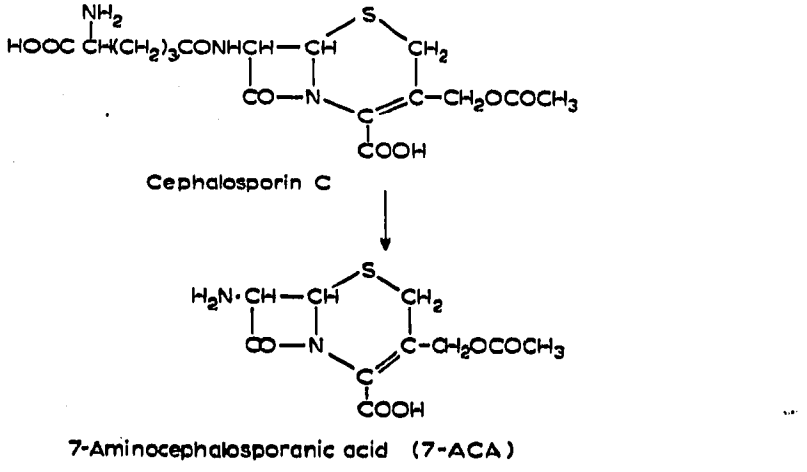


Fig. 3. Transformation of Cephalosphorin C into 7-ACA.

In this perspective, the need for a convenient TLC method to detect and to identify the different degradation products of the antibiotics in enzymatic as well as in chemical reaction mixtures is urgent. This paper describes TLC procedures which allow the simple and rapid separation and identification of the different (spontaneous, chemical and enzymatic) degradation products of penicillins and cephalosporins. The TLC procedures have proved to be useful when screening for β-lactamase and/or acylase producing microorganisms.

EXPERIMENTAL

Preparation of plates

The chromatoplates (20 × 20 cm) were coated to a thickness of 500 μ using a standard Desaga spreader. Silica Gel G (Merck) was applied according to STAHL's method[36]. The plates were dried at room temperature and activated at 120° for 30 min.

Solvent systems

Four different solvent systems were used: (A) *n*-butanol–water–ethanol–acetic acid (5:2:1.5:1.5); (B) *n*-butanol–water–acetic acid (4:1:1); (C) acetone–acetic acid (95:5); (D) 85 % aqueous acetone.

Solvent A was used by VANDAMME AND VOETS[4,5] in TLC experiments to detect Penicillin G destroying enzymes among different bacterial strains. Solvent

by BIRNER[37] in a TLC procedure to determine phenoxymethylpenicilloic acid in urine in the presence of the parent penicillin, while solvent D has been used in paper chromatographic procedures by DENNEN *et al.*[38] to detect *Cephalosporium* arylamidase activity on cephalosporins.

Reference products

Penicillin V, Penicillin G, Cephalosporin C and 7-ACA were kindly supplied by RIT, Genval (Belgium). 6-APA was a gift from Gist-Brocades, Delft, The Netherlands. Penicic acid was prepared from 6-APA by the procedure described by KULHÁNEK AND TADRA[20]. DL-Penicillamine·HCl and L(+)-cysteine·HCl were from BDH Chemicals Ltd, Poole, Great Britain. Benzylpenicilloic acid, phenoxymethylpenicilloic acid, Cloxacillin, Methicillin and Ampicillin acid were gifts from Beecham Research Lab., Betchworth, Surrey, Great Britain. Oxacillin was a Bayer, Leverkusen, G.F.R., product. Penilloic acids were prepared according to PRUESS AND JOHNSON[18] from the corresponding penicilloic acids.

Spray reagents

Each TLC plate was sprayed consecutively with the following spray reagents: (a) 2 *N* NaOH; (b) the iodine–azide reagent of AWE *et al.*[39]; (c) 1 % starch solution.

The presence of the different products is demonstrated by the appearance of pale spots on a blue-purple background. Some amino acid-related compounds could also be visualized by the ninhydrin reagent, prepared as described by PATTON AND CHISM[40].

Preparation of spotting solutions

Samples of each product were dissolved in a 1/15 *M* phosphate solution of pH 5.6 (2 mg/ml). 6-APA and 7-ACA were dissolved in a 2 % $NaHCO_3$ solution. Ten-fold dilutions were made when required for determining the sensitivity of the detection method. Samples of 2.5 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 each solvent was allowed to rise to a height of 14 cm (approximate times taken: (A) 150 min; (B) 120 min; (C) 20 min; (D) 30 min).

RESULTS

In order to separate and detect the possible degradation products (spontaneous, chemical or enzymatic) of different penicillins and Cephalosporin C, TLC experiments using Silica Gel G were performed.

All the preparations examined produced only one single spot on the TLC plates. The R_F values of the different products in solvent systems A to D are summarized in Table I.

Solvent system A permits the separation of the different spontaneous, chemical or enzymatic degradation products of the penicillins. However, this system needs a development time of about 2 h, although the R_F values are quite stable. 6-APA solutions in this system yield tailing-spots and, originally, this was thought to be a result of dimerization and polymerization processes[22,41]. However, the

TABLE I

R_F VALUES OF PENICILLINS, CEPHALOSPORINS AND DEGRADATION PRODUCTS IN DIFFERENT SOLVENT SYSTEMS

Product	R_F *values* × 100			
	Solvent A	*Solvent B*	*Solvent C*	*Solvent D*
Penicillin V	84	78	66	70
Penillic acid	60	50	15	32
Phenoxymethylpenicilloinate	64	55	10	25
Phenoxymethylpenilloinate	78	70	60	50
6-Aminopenicillanic acid	57	43	50	43
6-Aminopenicilloic acid	43	35	0	10
CDAT	55	40	7	14
Penicillin G	84	78	66	70
Benzylpenicilloinate	64	57	10	25
Benzylpenilloinate	78	70	60	50
Oxacillin	84	78	66	70
Penicilloic acid of Oxacillin	64	57	10	30
Cloxacillin	84	78	66	70
Penicilloic acid of Cloxacillin	64	57	10	30
Methicillin	79	70	60	60
Penicilloic acid of Methicillin	64	57	10	20
Ampicillin	64	50	20	57
Penicilloic acid of Ampicillin	44	28	0	14
DL-Penicillamine	60	43	58	43
L-Cysteine	47	32	23	36
Cephalosporin C	28	14	0	10
Ceporin	10	7	0	7
7-Aminocephalosporanic acid	40	21	43	40

degradation process is initiated during the run in solvent A. All the penicillins and their degradation products, except Methicillin and Ampicillin, behave in the same way as Penicillin V.

Solvent system B behaves very similarly to system A, although the separation of 6-APA from penicilloic acids is much better than in system A.

System C was especially selected for the separation of penicillins, penicilloic acids, 6-APA and penicic acid from their mixtures and for the detection of 7-ACA in the presence of the parent cephalosporins. As in system A, all penicillins and degradation products behave in the same way as did Penicillin V, except Methicillin and Ampicillin. System C also allows the detection of penicillamine and cysteine among the other degradation products. This system permits the rapid detection of acylase activity in the presence of β-lactamase and *vice versa*. The running time of the system is 20 min, so the risk of the eventual hydrolysis of the products taking place during the chromatography procedure is very small. However, volatilization of the solvent is a drawback of this system. Due to this, the R_F values are not at all constant, and vary markedly with changes in temperature, although in all cases the different products are well separated.

System D was also found useful for the separation of Cephalosporin C and 7-aminocephalosporanic acid.

The iodine–azide detection system has proved adequate for routine use in the

mixtures containing a penicillin or Cephalosporin C and their degradation products. Furthermore, it was found that amino acids (except L-cysteine and analogues) and the normal constituents of microbiological growth media or fermentation media do not interfere with the detection of these products in culture broths.

After spraying with the iodine–azide–starch reagent, the blue background of the TLC chromatograms fades as a function of time and also depends on the solvent system used.

The ninhydrin detection system has proved adequate for the detection of the following compounds: L-cysteine, 6-APA, 7-ACA, penicic acid, Cephalosporin C, penicillamine, Ampicillin and the penicilloic acid of Ampicillin, with this restriction — that the mixtures may not contain other ninhydrin-positive interfering products. 6-APA and 7-ACA yield brown and yellow spots, respectively, while the other products mentioned above yield red-purple spots. Other intact penicillins and other degradation products were scarcely visible.

The ninhydrin reagent can be used for the detection of the products mentioned above in aqueous solutions and pharmaceutical preparations, but not in culture broths.

The sensitivity of the method was determined by spotting dilutions of the standard solutions in amounts down to 0.1 μg. The limit of detection was found to be in the range of 0.5–1 μg of product. However, the sensitivity of the spray reagents towards the penicillins is considerably less than that obtained by bioautographic or microbiological procedures. Nevertheless, some degradation products of 6-APA and 7-ACA could be demonstrated in amounts not detectable by microbiological methods[4,42].

DISCUSSION

Thin-layer chromatography of the degradation products of one kind of penicillin or cephalosporin has only recently been developed, although TLC procedures to separate different penicillins or cephalosporins are widely used.

FOOKS AND MATTOK[35] described a TLC system which allows the separation and identification of Procaine–Penicillin G and degradation products, and BONDAREVA *et al.*[43] used TLC to detect 6-APA in penicillin–acylase reaction mixtures. Separation of Penicillin V from its corresponding penicilloic acid by TLC was first described by BIRNER[37].

The systems described here allow a rapid separation and identification of a wide range of degradation products (including spontaneous, chemical and enzymatic) of the penicillins and allow the detection of 7-ACA in the presence of cephalosporins. However, none of the systems is able to separate mixtures of penicillins.

Although TLC is a useful technique for examining the degradation of penicillins and cephalosporins, the possibility of generation of artefacts with this method —just as with paper chromatography—must always be considered.

With the unstable nature of these antibiotics in mind, decomposition of the drugs or polymerization processes may occur during the run (although in TLC to a lesser degree than in paper chromatographic and bioautographic methods) and shadow spots and tailing may result from interactions between the sample and the

the corresponding esters); however, these disadvantages are inherent in any chromatography system.

The R_F values, given in Table I, are also susceptible to variations due to uncontrollable circumstances such as relative humidity, the method of manufacture of silica gel, the degree of tank-saturation and temperature variations. Using the technique described by GALANOS AND KAPOULAS[44] and by DHONT AND MULDERS-DIJKMAN[45] to standardize R_F values, these disadvantages can be eliminated.

With the restrictions mentioned above, the solvents and the two spray reagents proposed here allow a rapid and convenient separation and detection of various important degradation products of a number of penicillins and cephalosporins.

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