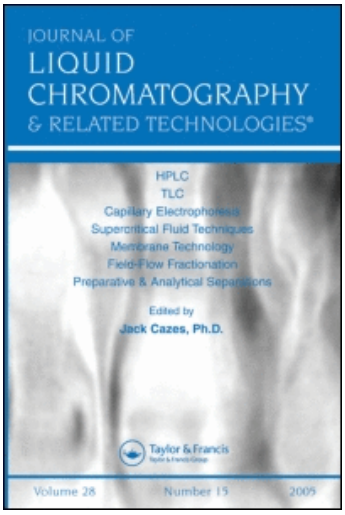

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THE DETERMINATION OF NATURALLY PRODUCED PENICILLINS AND THEIR BIOSYNTHETIC PRECURSORS USING PRE-COLUMN DERIVATISATION WITH DANSYLAZIRIDINE

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ABSTRACT

A novel method of pre-column derivatisation has been developed for the trace-level HPLC determination of penicillins and their biosynthetic intermediates in complex biological samples. The derivatisation is reliant on the reaction between dansylaziridine and a thiolate anion produced by alkaline degradation of the penicillin ring systems. This reaction results in the formation of stable fluorescent products, which may be detected spectrofluorimetrically. Optimisation of both the derivatisation reaction and the chromatographic conditions has been investigated. Using this technique we have been able to simultaneously measure both intra- and extra-cellular levels of penicillins and their biosynthetic intermediates produced in fermentations at concentrations below 1 µg/ml.

INTRODUCTION

The early stages in the biosynthetic pathways leading to penicillin and cephalosporin antibiotics are known to proceed by the formation of isopenicillin N (isopen N) from the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) (1,2). ACV undergoes an oxidative cyclisation catalysed by the enzyme isopen N synthetase to produce isopen N (2,3), which is the first of the bioactive intermediates of the pathway. The biosynthetic pathways diverge at this point dependent on the producing organism. In the fungus Cephalosporium acremonium isopen N is converted in a multi-stage process to cephalosporin C (4) and in Streptomyces species to various other cephalosporins (5). In the fungi Penicillium chrysogenum and Aspergillus nidulans, however, isopen N undergoes either deacylation to produce 6-aminopenicillanic acid (6-APA), or transacylation where the hydrophilic α-aminoadipyl side-chain is exchanged for a hydrophobic chain, resulting in more bioactive compounds like penicillin G (pen G; benzylpenicillin) (6). Structural variations within naturally produced penicillins arise due to differences in the acyl side-chain found at the C-6 position (7, 8).

A knowledge of the intra- and extra-cellular levels of biosynthetic intermediates and products is of importance with regard to understanding the flux through biosynthetic pathways and how it is controlled. Currently there are no established analytical methods available which are capable of the simultaneous determination of both intermediates and products of the penicillin biosynthetic pathway, individually, at trace-levels in complex fermentation broths or intracellular extracts. We have recently described a

HPLC, pre-column derivatisation procedure which allows the quantification of ACV in biological media (9). The derivatisation reaction occurs between dansylaziridine and the free sulphydryl group of the ACV monomer in aqueous solution at pH 8.8 to yield stable fluorescent products. We now describe a modification of this procedure which facilitates determination of hydrophilic and hydrophobic penicillins in addition to ACV in samples from fungal fermentations.

The derivatisation of penicillins by dansylaziridine is shown to occur optimally at pH 12 and relies upon the derivatisation of thiolate anion-containing intermediates produced from the penicillins under these alkaline conditions. Figure 1 shows the molecular species thought to be present when a penicillin is subjected to high pH in aqueous solution (10,11,12). Rupture of the β-lactam amide bond produces the corresponding penicilloic acid. The acid derivative initially retains the 5R,6R stereochemistry of the parent molecule but spontaneously converts to an equilibrium mixture of the 5R,6R and 5S,6R penicilloic acids, through thiazolidine ring opening (11,12).

MATERIALS AND METHODS

Fungal Strains and Growth Media

Penicillium chrysogenum strain number P2 (Pan Laboratories) was grown in Jarvis and Johnson defined medium (13) at 26°C for the required time prior to harvesting. *Aspergillus niger* wild type, strain number IMI 17454 was grown in Aspergillus Complete Medium (14) for five days with both the broth and mycelial pellet used as blanks where appropriate (this species is known to be a penicillin non-producer).

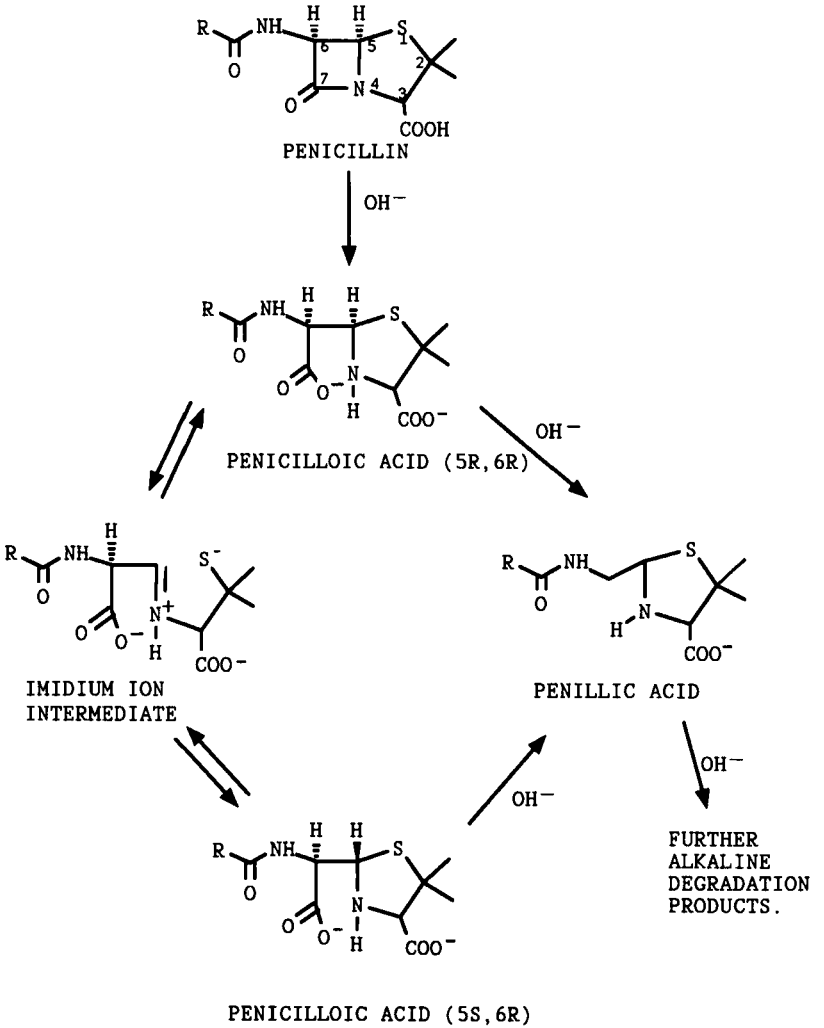

FIGURE 1 Proposed Reaction Mechanism for Alkali-Induced Penam Ring Opening.

Materials and Methods

Pen G, penicillin V (pen V; phenoxymethyl-penicillin), dansylaziridine (5-dimethyl-naphthalene-1-sulphonylaziridine), L-cysteine, dimethylsulphoxide (DMSO) and sodium borohydride were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Ethylenediaminotetracetic acid (EDTA), sodium hydroxide, sodium acetate and glacial acetic acid were purchased from British Drug Houses (BDH) (Dagenham, Essex U.K.) and were of the highest analytical grade available. HPLC grade acetonitrile and methanol were supplied by Rathburn Chemicals (Walkerburn, Peebleshire, U.K.). AnalaR grade dioxan was purchased from Hopkin and Williams Chemicals (Chadwell Heath, Sussex, U.K.). The ACV, isopen N, penicillin K (pen K), penicillin X (pen X)and 6-APA were all kindly donated by Glaxo Group Research (Greenford, Middlesex, U.K.). Benzylpenicilloic acid was a gift from Beecham Pharmaceuticals (Betchworth, Surrey, U.K.). Distilled deionised water was obtained using a Nanopure II system (Fisons, Loughborough, Leicestershire, U.K.).

Equipment

HPLC was performed using a model 2150 HPLC pump connected to a 2152LC controller and a 11300 Ultragrad mixer (LKB, Milton Keynes, Buckinghamshire, U.K.) to produce the gradient. Integration was carried out on a model 4290 integrator (Spectra Physics, St Albans, Herts, U.K) and detection was achieved using a Shimadzu RF-50 spectrofluorimeter.

Chromatographic Parameters

Chromatographic separations were carried out on a 5 μm, C_{18} Spherisorb column (0.46 x 25 cm) purchased from Hichrom Ltd (Reading, Berks, U.K.). A guard column

(0.46 x 5 cm, cartridge system) containing the same stationary phase was used to protect the analytical column and was supplied by Chrompack (Middelburg, The Netherlands). The mobile phases were prepared as acetate buffers (20 mM, containing 0.5 mM EDTA) at the required pH (between 3.6 and 5.0) mixed in a multi-stage gradient over thirty minutes (gradient programme shown in Figures) using a flow rate of 1 ml min^{-1}. A 20 µl injection volume was used in all HPLC analyses.

Sample Preparation

Standards

Standard solutions of the ACV monomer and L-cysteine were prepared as described previously (9). The dansylaziridine was prepared as a 3 mM stock solution in the appropriate solvent. Penicillins were dissolved in deionised water. Both reagents and penicillins were prepared on a daily basis before use and were kept at 4°C.

Biological Samples

The biomass density was determined by removing a 1 ml aliquot from the fermentation flask and drying it on a filter paper (Whatman No.1, 5 cm diameter, Whatman, Maidstone, U.K.) by suction filtration for 20-30 min. The air-dried mycelial mat was then weighed to provide an estimate of biomass.

Fungal fermentation broths were harvested by centrifugation (5000g, 20 min, 4°C). The supernatant fraction (approximately 1 ml) was passed through a 0.45 µm filter (Millipore, Massachusetts, U.S.A.) and the pH adjusted to that required for derivatisation by addition of sodium hydroxide (50 µl, 1 M). The mycelial pellet was thoroughly washed with deionised water and then resuspended in the appropriate volume of water to give

the original biomass concentration in the harvested broth. A sample (1 ml) was taken out and the interstitial water removed by suction filtration. The dry mycelium was transferred into boiling water (1 ml, 100°C) to extract the intracellular contents. After 5 min an aliquot (200 µl) was removed and centrifuged at 2000g for 3 min. The cell debris was discarded and the pH of the sample adjusted to 12.3 by addition of sodium hydroxide (5 µl, 1 M) and used for dansylaziridine derivatisation immediately.

Optimisation of Derivatisation Procedure for Penicillin G

The parameters of pH, time and temperature of the derivatisation procedure were optimised for pen G, employing the same chromatographic and detection conditions previously described for ACV monomer (9). Instead of methanol, however, the dansylaziridine stock solution was prepared in dioxan.

(i) pH used for thiazolidine ring opening.

A series of MOPS solutions (0.1 M) were prepared over the pH range 7.5 to 10.0 with higher pH's obtained by further additions of aqueous sodium hydroxide (1 M). Solutions of penicillin G (2 mM) were prepared as described earlier and aliquots (200 µl) removed and reacted in turn with an equal volume of one of the above buffer solutions. The reactants were mixed and then immediately incubated with the dansylaziridine stock solution (3 mM, 400 µl) at 60°C for 60 min. After rapid cooling to 20° and HPLC analysis, the pH that was found to give the maximum yield of fluorescent derivative was used in the subsequent studies.

(ii) Temperature and time of the derivatisation reaction. The optimimum temperature for the derivatisation reaction was investigated using the

previously optimised pH conditions. Alkali-treated penicillin solutions were incubated with the aziridine as described above for 60 min at the appropriate temperature. The reactants were rapidly cooled to 20°C and then analysed by HPLC. The temperature that gave a maximum peak yield at the highest signal-to-noise ratio was then used to determine the rate of formation of derivative, and hence establish the optimum incubation period at the temperature and pH used.

Standard Derivatisation Procedure for ACV and Penicillins

Standard solutions of the ACV monomer and penicillins were treated with an equal volume of sodium hydroxide (100 mM) and an aliquot (100 µl) immediately reacted with the aziridine stock (100 µl). The reactants were incubated at 100°C for 30 min, removed and then stored at -10°C prior to analysis. Fermentation samples were centrifuged and filtered as outlined earlier. The pH of the clarified extracts was raised to 12.3 by addition of sodium hydroxide and a sample removed (50 µl) and immediately reacted with an equal volume of dansylaziridine solution. The reaction mixture was transferred to a boiling water bath and incubated for 30 min. The reacted broth was then rapidly cooled to room temperature (20°C).

Control blanks were prepared by treating the penicillin standard or fermentation samples solutions with an equal volume of sodium hydroxide (100 mM) and standing overnight at room temperature (20°C, 16 h).

Optimisation of Chromatographic Conditions

Using the standard derivatisation procedure, the HPLC running conditions were investigated.

(i) Excitation and emission wavelengths.

An aqueous solution of penicillin G (2 mM) was derivatised and introduced directly into the spectrofluorimeter flow-cell. The wavelengths found to give a maximum signal for the derivative were determined by manual scanning of first the excitation and then the emission monochromaters.

(ii) pH of the aqueous component of the mobile phase for optimum separation of derivatised standard.

A series of acetate buffers (10 mM) were prepared over the pH range 3.6-5.0 (pH's given in Table 1). The HPLC column was equilibrated with each mobile phase (85% buffer, 15% acetonitrile) for thirty minutes and then a mixture of derivatised standards (isopen N, 10 $\mu g\ ml^{-1}$; 6-APA, 15 $\mu g\ ml^{-1}$; ACV, 55 $\mu g\ ml^{-1}$; pen G, 130 $\mu g\ ml^{-1}$) was injected. The capacity factors of each component were then determined. Penicillins K, X and V (each at 130 $\mu g\ ml^{-1}$) were included in the mixture when using ph 4.4 buffer in the mobile phase. A broth sample (1 ml) was spiked with an aliquot of the mixed standards (100 µl) and then derivatised as outlined earlier. This allowed investigation of the resolution of the analytes in complex biological media using mobile phases of various pH's.

Confirmation of Analyte Peak Identity in Chromatographs of Derivatised Fermentation Samples

Analyte concentrations in fermentation samples were determined from calibration graphs. A suitable dilution of the mixture of underivatised standards at concentration described above was added to fermentation samples (100 µl to 1 ml). After derivatisation the calculated increases in analyte concentrations were compared with experimental values determined on the basis of peak areas (peak areas increased by a factor of 2-4 fold).

RESULTS AND DISCUSSION

Optimisation of the Derivatisation Reaction Conditions for Penicillin G

Preliminary experiments with pen G suggested that elevated temperatures were required to produce a significant degree of dansylation compared to the conditions previously described for ACV (9). The use of methanol as the solvent for dansylaziridine proved unsatisfactory due to extensive solvent evaporation during incubation at temperatures greater than 60°C, resulting in a poor analytical reproducibility. The less volatile dioxan was found to be a suitable replacement for methanol.

Optimisation of derivatisation reaction conditions was performed with pen G as this proved to be one of the most difficult penicillins to quantify at low concentrations. When the pH of the aqueous component of the derivatization mixture was increased from 8 to 12.6, the fluorescence intensity of the derivative peak was found to increase up to pH 12.3. This pH was obtained by treating the penicillin standard with an equal volume of 100 mM sodium hydroxide and was subsequently used in all further derivatisations.

Derivatisation temperatures between 20 and 100°C were investigated for pen G with incubation times up to 90 minutes. After 30 minutes incubation at 100°C the peak yield was approximately 93% of that found after 80 minutes, but much much greater than after 90 minutes at 60°C. For convenience, a 30 minute incubation at 100° using penicillin solutions prepared at pH 12.3 was employed as a standard protocol in all subsequent analyses.

TABLE 1

The Influence of pH on Capacity Factors.

The mobile phases were all prepared as acetate buffers (20 mM) containing EDTA (0.5 mM). Elution of the analytes from the column was achieved by application of a gradient of acetonitrile at an initial concentration of 15%. Detection was effected spectrofluorimetrically using a sensitivity setting of 2 and an injection volume of 20 µl. Measurements of pen X, K and V were made at pH 4.4 only.

Capacity Factors

pH	Isopen N	6-APA	ACV	Pen G	Pen X	Pen K	Pen V
5.0	2.37	4.14	7.40	8.10	--	--	--
4.8	2.01	3.49	6.36	7.33	--	--	--
4.4	2.03	3.95	5.60	8.30	6.63	6.88	8.61
4.0	2.10	4.60	4.90	9.18	--	--	--
3.6	2.40	5.90	3.60	10.30	--	--	--

Optimisation of Chromatographic Conditions

The maxima for absorption and emission were found to occur at 324 and 520 nm respectively. Due to increased baseline noise levels at these wave lengths, slightly off-maximum settings of 339 nm for excitation and 540 nm for emission were adopted.

Optimal chromatographic separation conditions for ACV, 6-APA, isopen N and pen G were investigated by measurement of the capacity factors for each derivatised standard as a function of the pH of the aqueous buffer used in the mobile phase. A standard solvent gradient elution programme using acetonitrile as the organic modifier was adopted for each chromatographic run. These data are presented in Table 1.

It can be seen that at pH 4.4, the peaks corresponding to all analytes are suitably separated, including those penicillins formed in fermentations from endogenous side-chain precursors. A chromatogram illustrating the separation of a mixture of the first four analytes after pre-column derivatisation with dansylaziridine using a mobile phase at pH 4.4, is shown in Fig. 2. When analysing spent fermentation broths or extracted intracellular contents, chromatograms obtained after derivatisation were found to be considerably more complex than those found for the simple mixture of standards. For biological samples, the use of an acetate buffer of pH 4.4 in the elution programme enabled all four compounds to be optimally resolved from close-lying background peaks.

Linearity of Response and Detection Limits

The optimised reaction and chromatographic conditions were used to produce calibration graphs for pen G, 6-APA, isopen N and ACV. The plots were fitted by regression analysis and found to be linear, passing through the origin. Detection limits and the regression equations are given in Table 2.

Reproducibility of the Analytical Procedure

The stabilities of the derivatised products of the four previously mentioned analytes were investigated by repeat injection of the samples. The reproducibility of each injection was similarly determined using samples that were stored at - 10°C between analyses. Results are given in Table 3 and were determined using replicate injections. Since coefficient of variance values are similar in magnitude and lower than ten percent, the procedure is proved suitable for all four analytes.

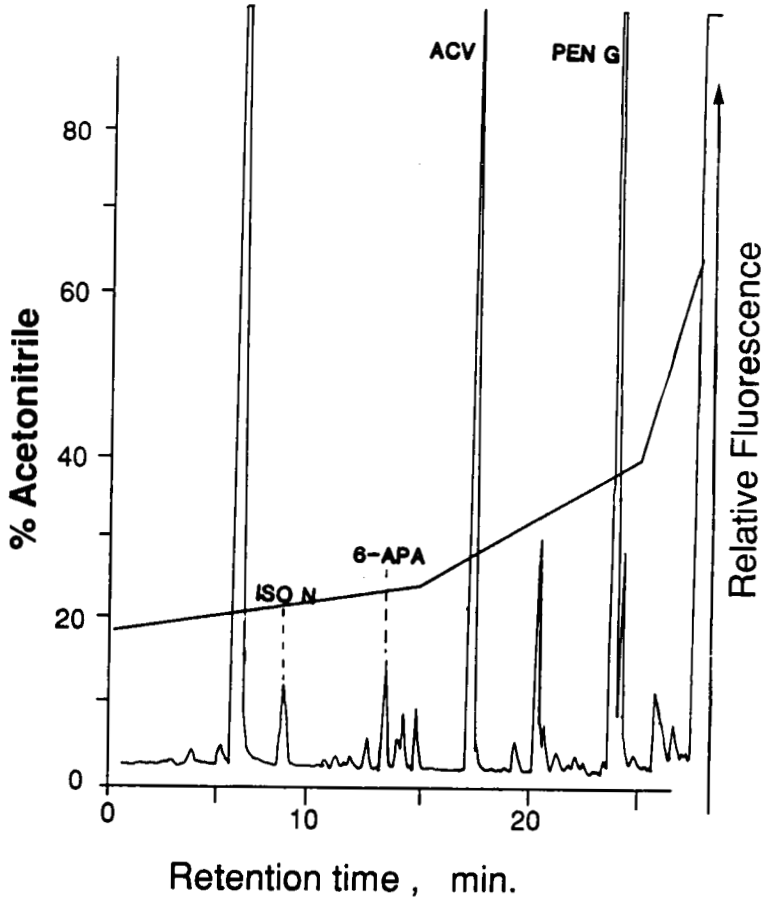

FIGURE 2 HPLC chromatogram of a derivatised mixture of isopen N (10 µg/ml), 6-APA (15 µg/ml), ACV (55 µg/ml) and pen G (130 µg/ml). Derivatisation and HPLC operating conditions given in the Materials and Methods section.

TABLE 2

Detection Limits and Regression Equations.

Detection limits were determined with a signal-to-noise ratio of 3:1. Mobile phase comprised acetate buffer (pH4.4, 20 mM, 0.50 mM EDTA) and acetonitrile. Injection volume = 20 µl; detector sensitivity = 1.

Analyte	Regression Equation *	Detection Limit *
Isopen N	Conc = (area x 10^{-4})- 0.55/0.23	0.045
Pen G	Conc = (area x 10^{-4})+ 0.67/1.05	0.050
ACV	Conc = (area x 10^{-4})+ 0.53/0.71	0.100
6-APA	Conc = (area x 10^{-4})+ 0.66/0.36	0.150

* Values for the concentration in the equation and for the detection limits are quoted in µg ml^{-1}. The area is defined in arbitrary units.

TABLE 3

Reproducibility and Derivative Stability

The derivatisation reactions and chromatographic separations were carried out under the optimal conditions described in the text. Reproducibility of the analytical procedure was determined by consecutive injections of a mixed sample of derivatised standards (20 µl, n=8). A measure of stability of derivatised analytes was obtained using day-to-day injections (20 µl,n=5) of mixed standards stored at -10°C over several days and recording the time taken for a 10% reduction in peak area.

Analyte	Reproducibility (coefficient of variance)	Derivative Stability (Hour)
Isopen N	4.0%	96
Pen G	6.9%	84-96
6-APA	5.0%	84
ACV	3.0%	>120

Once derivatised, samples of analytes may be stored at -10°C for up to 4 days prior to HPLC analyses.

Quantification of Penicillins and ACV in Complex Biological Media

Using the same gradient elution programme illustrated in Fig. 2 and a mobile phase having an aqueous component of pH 4.4, the analytical procedure was used to quantify the levels of pen G, 6-APA and isopen N in the extra-cellular broth and in an intra-cellular extract from a 3-day old culture of P. chrysogenum. It was possible to quantify ACV in the intra-cellular extract from the same analytical run as the three penicillins.

To confirm the identity of the penicillin peaks in chromatograms obtained by the analysis of these P. chrysogenum samples, a series of experiments was conducted. Firstly, a standard addition of a mixture of standards (isopen N, 6-APA, pen G and ACV) was made to samples prior to derivatisation. The observed increase in peak area for each analyte was found to agree with the increase calculated from the analyte concentration derived from the regression curve for each peak. Secondly, a broth sample from the penicillin non-producer Aspergillus niger was subjected to the analytical procedure. No peaks with retention times corresponding to any of the analytes were observed. Thirdly, the above procedures were repeated but the pH of the aqueous component of the mobile phase was varied between pH 3.6 - 5.0. The resultant changes in capacity factors for each of the analytes were compared with the values obtained previously for the standards (Table 1) and found to be identical. Finally, fermentation broths and intracellular extracts were treated with sodium hydroxide (1M) and left at room temperature overnight.

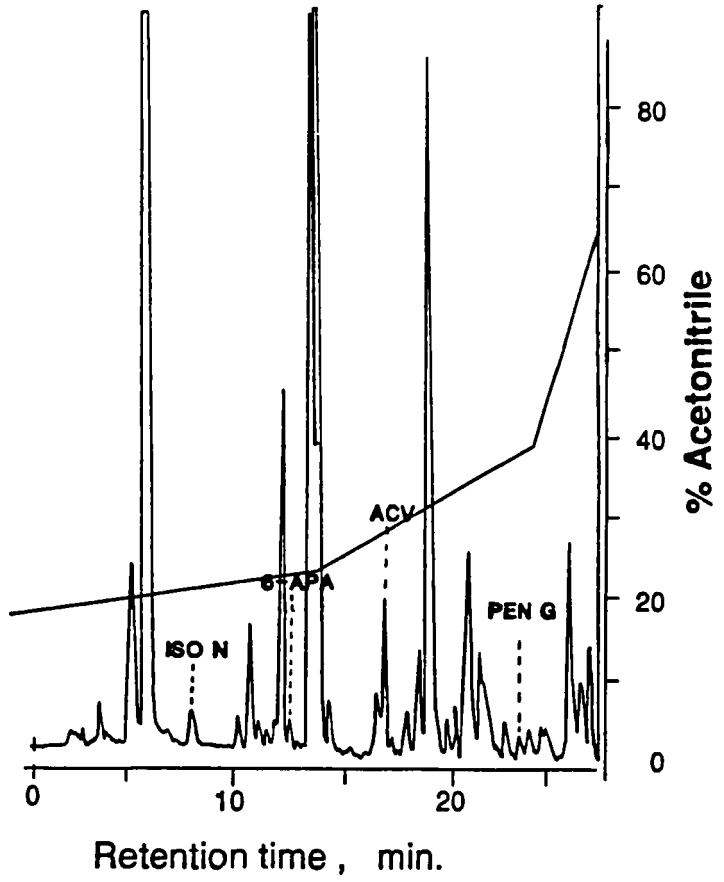

FIGURE 3 HPLC chromatograms of a derivatised sample of an intracellular extract obtained from mycelium of P.chrysogenum P2.

These extreme conditions lead to complete hydrolysis of the β-lactam ring and the subsequent decarboxylation to penillic acid and further alkaline degradation products. When the samples were derivatised and subjected to analysis as usual, no peaks were seen with the retention times of the analytes of interest.

TABLE 4

Concentrations of pen G and biosynthetic intermediates in culture samples of P. chrysogenum P2

Derivatisation, HPLC and fermentation conditions are as described in the Experimental section.

Analyte	Extracellular Level ($\mu g\ ml^{-1}$)	Ratio +	Intracellular Level ($\mu g\ ml^{-1}$) *	Ratio +
Isopen N	2.3	1.0	3.2	1.0
Pen G	14.5	6.3	2.9	0.9
6-APA	8.0	3.5	2.4	0.75
ACV	-	-	4.8	1.5

* Indirect measurement, made from a sample of washed mycelium obtained from 1 ml of culture.

+ Ratios of analyte concentrations relative to isopen N

Fig. 3 shows the chromatogram obtained from the intracellular extract of the P. chrysogenum culture. Table 4 shows the analyte concentrations found in extra- and intracellular samples and the ratios of the concentrations based on isopen N as 1.0.

The chromatogram shown in Fig. 3 demonstrates the simultaneous determination of all four analytes; these are the product and all biosynthetic intermediates of the β-lactam biosynthetic pathway of this industrially important organism. The method is more suited to the study of intracellular preparations, as the chromatograms of the extracellular broth samples were more complex. There is little published information on the relative levels of these metabolites in P. chrysogenum. The presented analytical procedure is suitable for the study of metabolic control of this pathway. In addition it can be applied to the analysis of ACV and isopen N levels in intracellular extracts of

any penicillin or cephalosporin producing organism, since the early part of the various biosynthetic pathways share the steps leading to isopen N.

In the pathways of cephalosporin producing organisms studied to date, pen N is directly formed from isopen N. Since the mechanism of the derivatisation reaction is likely to produce diastereoisomers, this method would be expected to at least partially resolve these epimers. The lack of a pure penicillin N standard has precluded experimental confirmation of this.

In conclusion, we believe that the reported method may be used to shed more light on factors which influence metabolic control of β-lactam biosynthesis, particularly in relation to experiments involving genetic transformation of and gene-cloning in relevant microorganisms.

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