Journal of Chromatography, 424 (1988) 325–336
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3962

DETERMINATION OF β-LACTAMS AND THEIR BIOSYNTHETIC INTERMEDIATES IN FERMENTATION MEDIA BY PRE-COLUMN DERIVATISATION FOLLOWED BY FLUORESCENCE DETECTION

A.J. SHAH* and M.W. ADLARD

The Polytechnic of Central London, School of Biotechnology, 115 New Cavendish Street, London W1M 8JS (U.K.)

(First received May 18th, 1987; revised manuscript received September 14th, 1987)

SUMMARY

This paper describes a novel and sensitive pre-column derivatisation method for the detection and quantitation of β-lactams and their biosynthetic precursors at trace levels in fermentation media. Filtered broths from fermentations of strains of *Penicillium chrysogenum* and *Cephalosporium acremonium*, after deproteination and centrifugation, were incubated with 9-fluorenylmethylchloroformate for 5 min at 20°C in 0.2 *M* borate buffer at pH 7.7. Following two-fold pentane extraction of the reagent hydrolysis product, the aqueous layer was injected directly onto a C_{18} reversed-phase column, and products were detected spectrofluorimetrically with excitation and emission wavelengths of 260 and 313 nm, respectively. Detection limits of 0.01 and 0.05 μg ml^{-1} were achieved for both 6-aminopenicillanic acid (6-APA) and isopenicillin N in borate buffer and filtered fermentation broths, respectively, using a 10-μl injection volume. A linear calibration for 6-APA in fermentation broth was obtained for a very wide concentration range (0.05–100 μg ml^{-1}). Detection limits for solutions of cephalosporin C, deacetylcephalosporin C and deacetoxycephalosporin C in broth were all 0.25 μg ml^{-1}. The detection limit for the β-lactam precursor δ-(L-aminoadipyl)-L-α-cysteinyl-D-valine (ACV) dimer in borate buffer was 0.5 μg ml^{-1}. The cephalosporins and ACV dimer gave linear plots in the ranges 3–25 and 1–100 μg ml^{-1}, respectively. Repeated analysis of 6-APA at a concentration of 10 μg ml^{-1} in filtered broth gave a mean peak area of $2.5 \cdot 10^6$ with a standard deviation of $2.6 \cdot 10^5$ using a 10-μl injection volume. Ampicillin spiked into deproteinated blood serum gave a linear calibration in the concentration range 2–100 μg ml^{-1}.

INTRODUCTION

Our research has been directed towards the detection and quantitaton of β-lactams and their biosynthetic precursors as they are produced in liquid media during microbial fermentations (see Figs. 1 and 2). In the last decade high-performance liquid chromatography (HPLC) has become the preferred technique for the quantitative determination of β-lactam antibiotics. It is an efficient, rapid

0378-4347/88/$03.50 © 1988 Elsevier Science Publishers B.V.

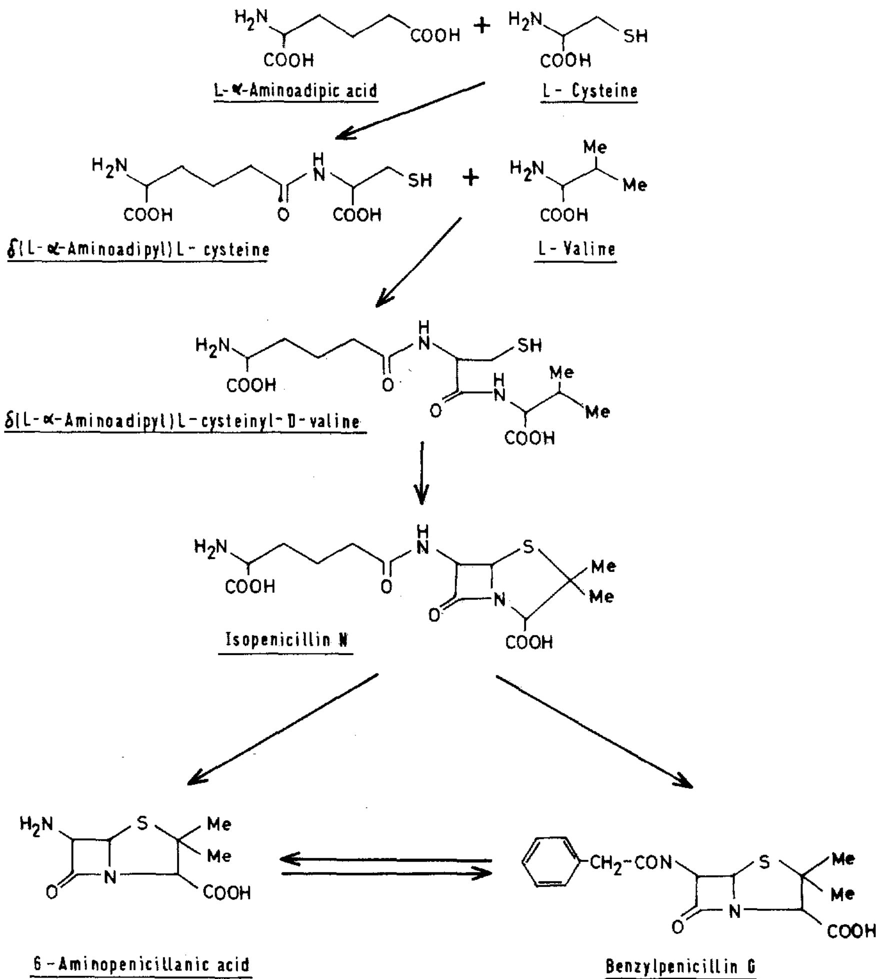


Fig. 1. Biosynthesis of penicillins.

and versatile separation technique, providing an alternative to the classical methods of microbial plate assay, radioimmunoassay and spectrophotometry [1]. UV absorbance and fluorescence detection methods have been used; the latter is favoured due to its inherently greater sensitivity. However, since many of the compounds of interest lack fluorophores, derivatisation is necessary. This is easily accomplished for the compounds which possess primary or secondary amine functions. For present purposes, the ideal derivatising reagent would (i) give highly fluorescent derivatives without itself being fluorescent and (ii) react rapidly with both primary and secondary amine groups to give stable products. The fluorogenic reagents, dansyl chloride [2], *o*-phthaldialdehyde (OPA)-mercaptoethanol [3], phenylisothiocyanate [4] and fluorescamine [5] have all served as relatively sensitive reagents in the HPLC analysis of amino acids. OPA-mercaptoethanol has also been used in the detection and quantitation of β-lactams [6]. The reaction is rapid and the reagent is non-fluorescent;

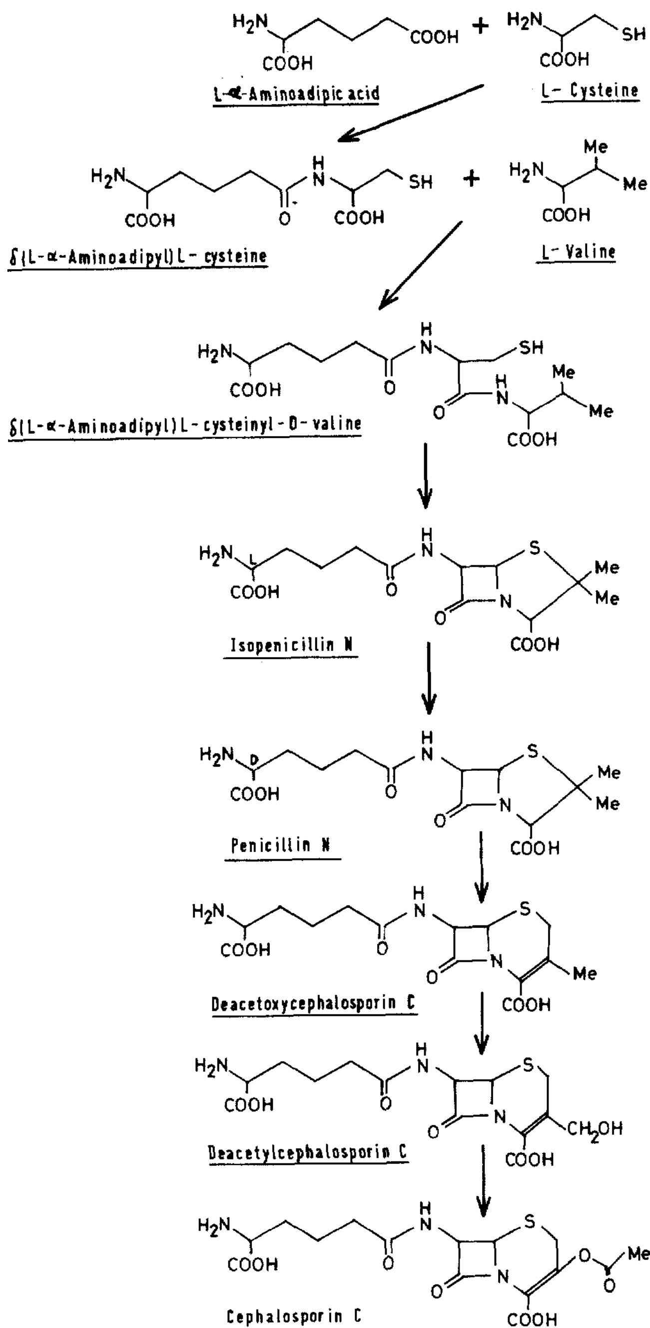


Fig. 2. Biosynthesis of cephalosporins.

however, it reacts only with primary amines. Furthermore, the products are unstable and this limits the method to either post-column or fully automated pre-column derivatisation.

9-Fluorenylmethylchloroformate (FMOC-Cl) has been widely used as a blocking agent in peptide synthesis [7] and more recently in derivatisation in HPLC analysis of amino acids [8]. In these applications, the reagent reacts with both primary and secondary amino groups and it gives stable products. During derivatisation, excess FMOC-Cl is quantitatively converted to 9-fluorenylmethanol (FMOC-OH) which is also fluorogenic. Consequently the use of the reagent has been limited for the most part to pre-column derivatisation.

We now report the application of FMOC-Cl to the detection and quantitation of natural penicillins, cephalosporins and their precursors at low levels in microbial fermentation broths and of a penicillin in blood serum. Broths from fermentations of mutant strains of *Penicillium chrysogenum* and *Cephalosporium acremonium* were selected due to the availability of these strains through a collaborative projects with industrial sponsors. Determination of a penicillin in blood serum was undertaken to test the applicability of the method using an alternative biological fluid.

EXPERIMENTAL

Materials and reagents

FMOC-Cl and dithiothreitol (DTT) were supplied by Sigma (Poole, U.K.). 6-Aminopenicillanic acid (6-APA) (sodium salt), isopenicillin N (barium salt, δ-(L-aminoadipyl)-L-α-cysteinyl-D-valine (ACV) dimer, cephalosporin C (sodium salt), deacetylcephalosporin C and deacetoxycephalosporin C (potassium salt) were kindly donated by Glaxo (London, U.K.). The semi-synthetic β-lactam ampicillin trihydrate was a gift from Beecham Pharmaceuticals (Brockham Park, U.K.). Acetonitrile (HPLC grade) was purchased from Rathburn (Walkerburn, U.K.). Analytical-reagent-quality (AnalaR) boric acid, sodium hydroxide, formic acid (90%, w/v) and hydrogen peroxide (30%, w/v) were acquired from BDH (Poole, U.K.). Glacial acetic acid, triethylamine and pentane, all of chromatographic grade, were also supplied by BDH.

Biological media

Selected strains of *P. chrysogenum*, strain No. P2 (Pan Labs.) and strain No. SC6140 [9] (Squibb Institute for Medical Research, Princeton, NJ, U.S.A.) were grown in Pan Labs. fermentation medium [10] and liquid fermentation medium described by Allsop [11], respectively. Broth samples were also taken from cultures of a *C. acremonium* N2 mutant strain, supplied by Glaxo. This mutant is a non-producer of isopenicillin synthetase [12], and hence broths were used as blanks in cephalosporin determination because they contain no cephalosporins. The mutant was grown by inoculating 1 l of the appropriate growth medium [12] with conidiation medium (100 ml) [9]. All cultures were incubated at 26°C for five days at 200 rpm in a fermentation incubator–shaker (Model MKX, LH Fermentation, Stoke Poges, U.K.) using four 250-ml shake-flasks. Cultures were filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) under vacuum immediately before deproteination and analysis.

Samples of human blood serum obtained from a healthy volunteer were provided by the Middlesex Hospital (London, U.K.).

Preparation of buffer and derivatising reagent

All aqueous solutions were made up in deionised water obtained using a Nanopure II system (Fisons, Loughborough, U.K.). Borate buffer (0.2 *M*) was prepared from boric acid and adjusted to pH 7.7 with 1.0 *M* sodium hydroxide solution. The derivatising reagent was prepared by dissolving FMOC-Cl (11.6 mg) in dry acetone (3 ml) to give a final concentration of 15 m*M*. The acetate buffer component of the aqueous mobile phase was prepared by dissolving glacial acetic acid (3 ml) and triethylamine (1 ml) in deionised water and diluting to 1 l. The pH was then adjusted to 4.1 with 1.0 *M* aqueous sodium hydroxide. A 50 m*M* acetate buffer of pH 5.0 was used for the analysis of cephalosporins.

Derivatisation procedure

Standards. Fresh stock solutions of penicillins and cephalosporins were prepared daily, each at a concentration of 100 μg ml^{-1} in 0.2 *M* borate buffer at pH 7.7, and stored at 0°C until required. Standard solutions were made by appropriate serial dilution of stock solutions, using the same borate buffer as diluent. Each standard (1.0 ml) was treated with borate buffer (0.1 ml), acetone (2.0 ml) and FMOC-Cl (0.4 ml). After incubation for 5 min at room temperature, the FMOC-Cl hydrolysis product (FMOC-OH) was removed by extraction with pentane (3.4 ml). The upper organic layer was discarded. After a second similar extraction, 10 μl of the remaining aqueous layer were injected onto the HPLC column.

In an alternative procedure, the ACV dimer was reduced to the monomeric thiol by treatment of the sample solution (1.0 ml) with a four-fold excess of solid DTT. The solution was then treated with borate buffer, acetone and FMOC-Cl as described previously. In yet another variation, the ACV monomer was oxidised with performic acid to the corresponding sulphonic acid, prior to derivatisation. This was accomplished by treatment of the DTT-reduced sample solution (1.0 ml) successively with 90% (w/v) formic acid (90 μl) and 30% (w/v) hydrogen peroxide solution (400 μl). The solution was then maintained at 4°C for 2 h, prior to adjustment of the pH to 7.7 with concentrated (10 *M*) sodium hydroxide solution. The mixture was then subjected to the derivatisation procedure as described previously.

Biological samples. Filtered fermentation broths containing no residual penicillins were used as blanks in the analytical procedure for penicillins. These were obtained by allowing broths to stand for 24 h at 25°C, previous experiments having established that no detectable analytes other than the ACV dimer remain in the broth after such incubation. Standard solutions of β-lactams in borate buffer (0.1 ml) were spiked into the blank broth samples (1 ml) precooled to 4°C. Acetone (2 ml) was then added to precipitate endogenous proteins, which were then removed by centrifugation at 2600 *g* for 10 min using a refrigerated centrifuge (BR401, Denley Instruments, Billingshurst, U.K.).

Filtered fermentation broths containing naturally produced β-lactams and precursors were similarly deproteinated and analysed in the same way as the spiked

broths, except that the solutions of standard were replaced by 1.0-ml portions of borate buffer.

The blood serum samples (1 ml) were spiked with ampicillin to give concentrations in the range 0–100 μg ml^{-1}, prior to deproteination, centrifugation and derivatisation as described previously for spiked broths.

HPLC instrumentation and procedure

A Perkin-Elmer HPLC system consistsing of a Series 4 pump and programmer, an ISS-100 injector and LCI-100 integrator was used. The detector employed was a Shimadzu Model RF-530 fluorescence spectromonitor fitted with an 18-μl flow-cell. The excitation and emission wavelengths were set at 260 and 313 nm, respectively. The analytical column employed was a 150 mm×4 mm I.D. Hypersil ODS (3 μm) reversed-phase column (Capital HPLC Specialities, Edinburgh, U.K.). This was preceded by a 10 mm×4 mm I.D. guard column (HPLC Technology, Macclesfield, U.K.), containing identical packing material to that of the main column. A solvent-saturating scavenger column, 50 mm×4 mm I.D., Spherisorb ODS (5 μm) (Thames Chromatography, Maidenhead, U.K.), was placed between the sample injection valve and the pumping system. Complex gradient programmes were required for all analyses. Three different programmes were used and they are described in Table I. Each programme was based on a flow-rate of 1 ml min^{-1}, and analysis times varied from 17 to 60 min.

RESULTS AND DISCUSSION

The analytical procedure for β-lactams described was optimised with respect to excitation and detection wavelengths, reaction time with FMOC-Cl, extraction of reagent hydrolysis product and HPLC elution conditions for 6-APA in complex fermentation media.

Excitation and detection wavelengths

Manual operation of the HPLC detector monochromators, under stopped-flow conditions, was used to determine the excitation and emission maxima of FMOC-Cl and of the derivatives of the β-lactams and their precursors. In each case, the excitation and emission maxima were 260 and 313 nm, respectively. This result is to be expected, given the lack of conjugation between the fluorophoric fluorene group and the point of attachment of the chlorine atom in FMOC-Cl or the moiety which replaces it in derivative formation.

Reaction times with derivatising reagent and extraction of reagent hydrolysis product

Variation of incubation times between 30 s and 2 h at 20 °C for 6-APA standard showed that 80% of the reaction was complete after 1 min (see Fig. 3). Although prolonged incubation times resulted in an increase of analyte peak areas, background peaks arising from broth components increased similarly, creating more complex chromatograms and non-linear baselines. The rates of derivatisation of the ACV dimer and of the other penicillins and cephalosporins studied were found to be substantially similar to those of 6-APA. An optimum reaction time of 4 min

TABLE I

ELUTION CONDITIONS OF THE THREE DIFFERENT GRADIENT PROGRAMMES

Each programme was based on a flow-rate of 1.0 ml min^{-1}.

Time (min)	Acetonitrile (%)	Acetate buffer (%)	Gradient
Separation of FMOC-derivatised deacetoxycephalosporin C, deacetylcephalosporin C and cephalosporin C			
0	18.0	82.0	Isocratic
20.0	20.0	80.0	Linear
45.0	23.0	77.0	Linear
50.0	100.0	0.0	Linear
Separation of FMOC-derivatised 6-APA, ACV dimer, ACV monomer, ACV sulphonic acid and ampicillin			
0	20.0	80.0	Isocratic
10.0	30.0	70.0	Linear
30.0	30.0	70.0	Isocratic
50.0	35.0	65.0	Linear
55.0	100.0	0.0	Linear
60.0	100.0	0.0	Isocratic
Separation of FMOC-derivatised isopenicillin N			
0	20.0	80.0	Isocratic
7.0	25.0	75.0	Linear
17.0	20.0	80.0	Linear
37.0	30.0	70.0	Linear
40.0	100.0	0.0	Linear

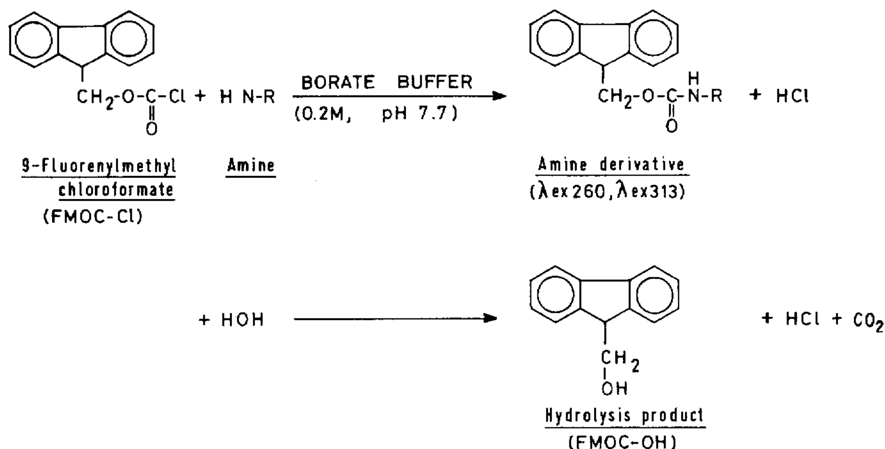
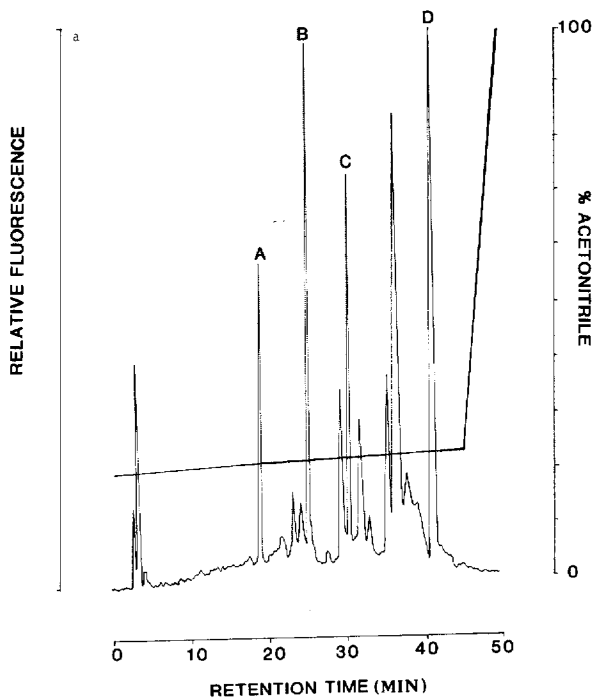
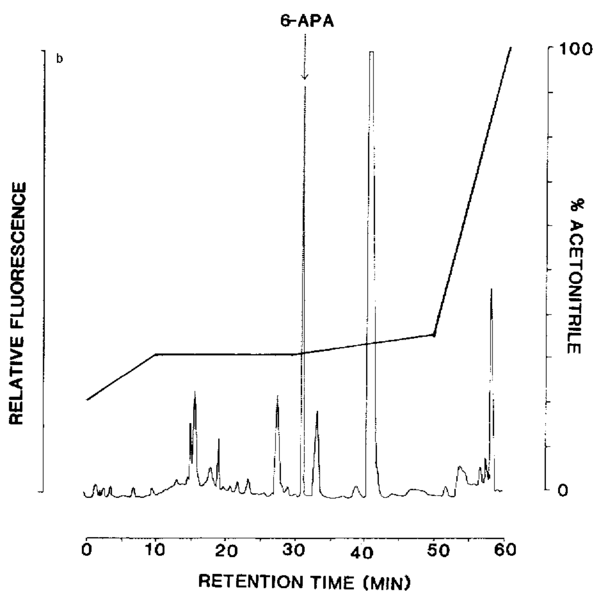
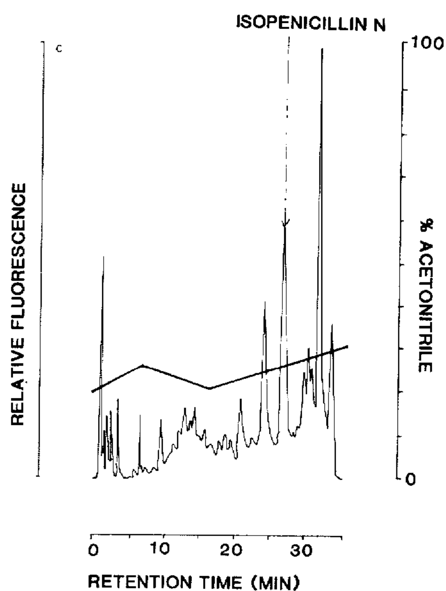


Fig. 3. Reaction scheme for the derivatisation of primary or secondary amines with FMOC-Cl in the presence of borate buffer (0.2 *M*, pH 7.7) and for the formation of reagent hydrolysis product.


A-Deacetylcephalosporin C
B-Deacetoxycephalosporin C
C-Cephalosporin C
D-Ampicillin
a
A
B
C
D
RELATIVE FLUORESCENCE
% ACETONITRILE
100
0
0
10
20
30
40
50
RETENTION TIME (MIN)


b
6-APA
RELATIVE FLUORESCENCE
% ACETONITRILE
100
0
0
10
20
30
40
50
60
RETENTION TIME (MIN)


c
ISOPENICILLIN N
RELATIVE FLUORESCENCE
% ACETONITRILE
100
0
0
10
20
30
RETENTION TIME (MIN)

at 20 °C was adopted for all analytes, representing a compromise between absolute detectability, signal-to-noise ratio and total time for analysis.

The efficiency of pentane extraction of FMOC-OH from reagent-treated broths was studied. HPLC analyses of the aqueous layers after a single extraction gave FMOC-OH peak areas which were seven-fold greater than those following two extractions. This indicates that approximately 86% of FMOC-OH was removed per cycle, leaving only 2% in the aqueous phase after two extractions. The residual 2% quantities presented no chromatographic problems, and the two-fold extraction procedure was adopted. Extractions with hexane gave almost identical results.

Gradient elution programmes

Elution profiles for the various β-lactams in fermentation media are shown in Figs. 4 and 5. Post-run integrator sensitivity changes were used to enhance analyte peaks in the chromatograms shown in Fig. 4 only. Peaks of ACV derivatives in borate buffer are shown in Fig. 6. The profiles indicate that chromatographic windows were successfully obtained for all analytes, hence allowing their unequivocal identification and, except in the case of isopenicillin N, their quantitation.

Calibrations and detection limits

The cephalosporins and ampicillin (the latter having been used as an internal standard) were well separated in single chromatographic runs both in borate buffer (not shown) and in the *C. acremonium* fermentation broth (Fig. 4a). Each cephalosporin, in blank medium, gave a linear plot in the 3–25 $\mu g\ ml^{-1}$ concentration range. The regression equation for seven points in the 0–25 $\mu g\ ml^{-1}$ range was: peak area (arbitary units) = 1.3 × concentration ($\mu g\ ml^{-1}$) − 1.5 (standard deviation = 1.4). Although points corresponding to concentrations less than 3 $\mu g\ ml^{-1}$ did not lie on the line, a detection limit of 0.25 $\mu g\ ml^{-1}$ was established for this β-lactam. Results for the other two cephalosporins in broth were closely similar. Throughout these calibrations, the variation in peak area of the internal standard (ampicillin, 100 $\mu g\ ml^{-1}$) was only 4%.

An approximately linear calibration for derivatised 6-APA in borate buffer was obtained for concentrations in the range 0.01–100 $\mu g\ ml^{-1}$ (i.e. four orders of magnitude). In spiked fermentation broths the results were almost as good. The analyte peaks were well resolved from the background (Fig. 4b). Seven concentrations in the range 0–20 $\mu g\ ml^{-1}$ gave the following regression equation: peak area (arbitary units) = 5.3 × concentration ($\mu g\ ml^{-1}$) + 0.8 (standard deviation = 6.3).

Fig. 4. Reversed-phase HPLC of FMOC derivatives of (a) cephalosporins (5 $\mu g\ ml^{-1}$), (b) 6-APA (50 $ng\ ml^{-1}$) and (c) isopenicillin N (50 $ng\ ml^{-1}$), all in filtered fermentation broths. Chromatographic conditions as described in the Experimental section; injection volume, 10 μl; detector sensitivity, 16; λ_{ex}, 260 nm; λ_{em}, 311 nm. Chromatograms were obtained by post-run reintegration using attenuation factor changes of 1024 to 16, values being preset to change before and after analyte peak(s) elution.

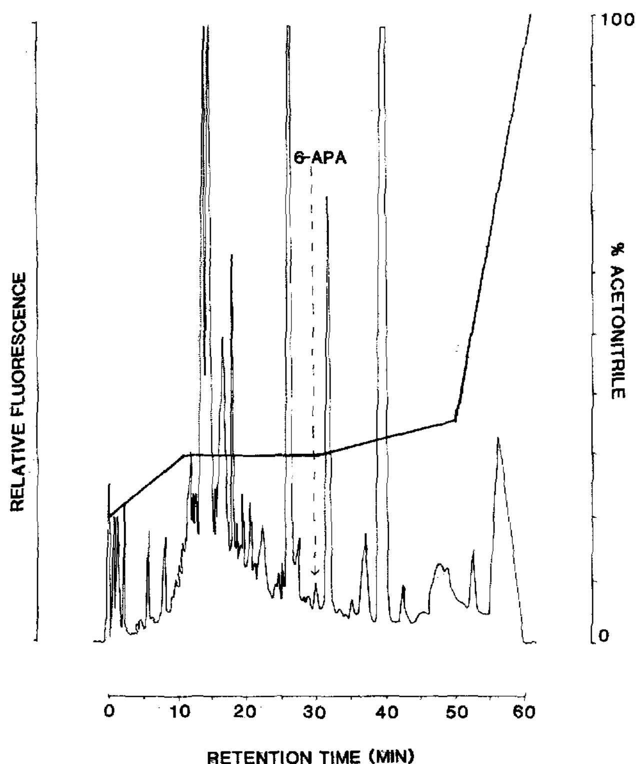


Fig. 5. Reversed-phase HPLC of the FMOC derivative of 6-APA (approximately 1 μg ml^{-1}) produced in broth after growing *P. chrysogenum* for six days at 26°C in complex liquid medium. Operating conditions as described in the Experimental section; injection volume, 10 μl; detector sensitivity, 16.

The chromatogram for the analysis of isopenicillin N is shown in Fig. 4c. Quantitation of this analyte was not attempted due to shortage of material. The applicability of the technique to the analysis of 6-APA produced naturally in fermentation medium is illustrated in Fig. 5. Here no peak enhancement technique has been used, and the peak area corresponds to an analyte level of about 1 μg ml^{-1}. Chromatograms of FMOC-Cl-derivatised ACV dimer, monomer and oxidised monomer, all in borate buffer, are shown in Fig. 6. The retention times of the monomeric thiol and the sulphonic acid derivatives are longer than that of the FMOC dimer, as expected on the basis of relative polarities. The sulphonic acid derivative shows a substantially greater peak area than that of the dimer or monomer. Increased sensitivity of detection of oxidised cysteine derivatives, relative to the thiol form, has been noted by other workers, who attribute it to reduced self-quenching of fluorescence in solutions lacking free thiol groups. In the present work, the DTT and performic acid treatment, prior to derivatisation, allowed quantitation of ACV dimer in borate buffer at concentrations down to 1 μg ml^{-1}. The presence of ACV dimer in each of the fermentation broths used was established by disappearance of the dimer peaks and appearance of monomer peaks following DTT and performic acid treatments. However, quantitative estimation of the dimer in broths was not attempted because, in contrast to the

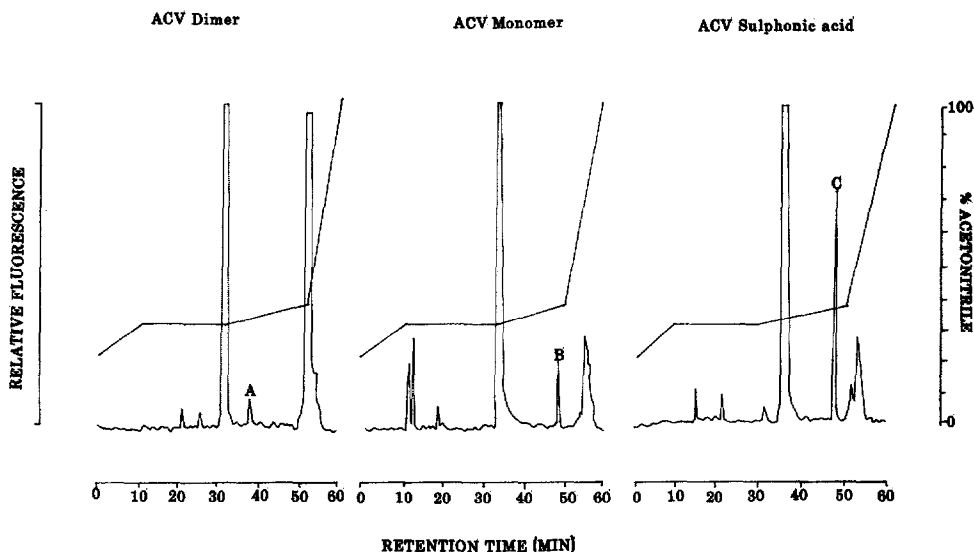


Fig. 6. Reversed-phase HPLC of FMOC derivatives of (A) ACV dimer (100 μg ml^{-1}), (B) ACV monomer (100 μg ml^{-1}) and (C) ACV sulphonic acid (100 μg ml^{-1}), all in borate buffer. Operating conditions as described in the Experimental section; injection volume, 10 μl; detector sensitivity, 16.

penicillins, its removal from blank solutions by incubation at 25 °C (or even higher temperatures) was not achieved.

In order to ascertain the applicability of the method to synthetic β-lactams in a complex biological medium other than fermentation broths, the analysis of ampicillin in human blood serum was studied.

Under elution conditions identical to those used for 6-APA, the peaks corresponding to ampicillin were identified in chromatograms of spiked samples. The peaks were well resolved, and four concentrations in the range 2–100 μg ml^{-1} gave an approximately linear plot, the lowest concentration corresponding to the detection limit. The regression equation was peak area (arbitrary units) = 8.2 × concentration (μg mol^{-1}) − 23 (standard deviation = 28).

CONCLUSIONS

The application of a modified precolumn FMOC-Cl derivatisation procedure for the reversed-phase HPLC analysis of β-lactams including natural penicillins and cephalosporins and precursors in complex fermentation media has been successfully achieved. The procedure requires a solvent extraction step to remove hydrolysis product and complex gradient elution programmes to separate the analyte peaks form interfering peaks resulting from the presence of amino acids and peptides in the medium.

The detection limits are an order of magnitude lower than those previously reported for the antibiotics studied, and the linearity of detection of 6-APA and isopenicillin N covers extremely wide concentration ranges. The principle drawback in the analytical method is the need (and time taken) for extraction of

reagent hydrolysis product. Successful application of the method to analysis of ampicillin in blood serum suggests its feasibility in other biological media.

REFERENCES

1 V. Betina, The Biology of Antibiotics, Elsevier, Amsterdam, 1983, Ch. 3.
2 W.R. Gray and B.S. Hartley, J. Biochem., 69 (1983) 59.
3 M. Roth, Anal. Chem., 43 (1971) 880.
4 G. Frank and W. Stubert, Chromatographia, 6 (1973) 522.
5 K. Samejima, W. Dairman and S. Undenfriend, Anal. Biochem., 42 (1971) 222.
6 M.E. Rogers, M.W. Adlard, G. Saunders and G. Holt, J. Chromatogr., 257 (1983) 91.
7 L.A. Carpino and G.Y. Han, J. Org. Chem., 37 (1972) 3404.
8 S.J. Einarsson, B. Josefsson and S. Lagerkvist, J. Chromatogr., 282 (1983) 609.
9 P.A. Fawcett, J.J. Usher and E.P. Abraham, Biochem. J., 151 (1975) 741.
10 J. Lein, in Z. Vaněk and Z. Hošťálek (Editors), The Overproduction of Microbial Metabolite: Strain Improvement and Process Control Strategies, Butterworths, London, 1986, Ch. 6.
11 A.E. Allsop, Thesis, the Polytechnic of Central London, London, 1982.
12 H. Shirafuji, Y. Fujisawa, M. Kida, T. Kanzaki and M. Yoneda, Agric. Biol. Chem., 43 (1979) 155.