Journal of Chromatography, 504 (1990) 359–367
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 221

Precolumn derivatization technique for high-performance liquid chromatographic determination of penicillins with fluorescence detection

KAZUO IWAKI*, NORIO OKUMURA and MITSURU YAMAZAKI
School of Pharmacy, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa-shi, Ishikawa 920-11 (Japan)
and
NORIYUKI NIMURA and TOSHIO KINOSHITA
School of Pharmaceutical Science, Kitasato University, 9-1 Shirokane-5, Minato-ku, Tokyo 108 (Japan)
(First received March 6th, 1989; revised manuscript received November 27th, 1989)

SUMMARY

A precolumn derivatization method was developed for the high-performance liquid chromatographic (HPLC) determination of penicillins using fluorescence detection. Penicillins were derivatized by a two-step reaction, the β-lactam ring being opened by hydrolysis in aqueous sodium carbonate solution in the first step to give a secondary amine functionality, and the secondary amino group being reacted with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole in the second step to give a fluorescent derivative. The resulting reaction mixture was injected directly onto a reversed-phase column and analysed by HPLC. At a penicillin concentration of 10 μg/ml, the precision (relative standard deviation) ranged from 1.49 to 2.20%. In the concentration range 0.2–100 μg/ml, a linear response was observed. The detection limits of this method were 30–85 ng/ml for five different penicillins at a signal-to-noise ratio of 3:1. The proposed method was applied to the determination of penicillins added to serum following pretreatment by deproteinization and removal of compounds containing amino functionalities with a cation-exchange resin.

INTRODUCTION

Numerous pre- and postcolumn derivatization methods have been developed for the high-performance liquid chromatographic (HPLC) determination of penicillins[1–13]. These methods can be classified into two categories: spectrophotometric methods with the use of mercury(II) chloride combined with alkaline, imidazole or triazole[1–7] and fluorimetric methods with the use of labelling agents for amino compounds such as fluorescamine or *o*-phthalaldehyde (OPA)[8–11]. As the former methods allow the derivatization of various penicillins, this type of derivatization method has recently been applied to the analysis of numerous β-lactam compounds. In

0021-9673/90/$03.50

contrast, the latter methods allow the detection of β-lactams at picomole levels, but they are not applicable to penicillins that have no primary or secondary amino residues for the attachment of the label.

It is well known that the β-lactam ring is easily hydrolysed in weakly alkaline solution to give a corresponding penicilloic acid having a secondary amino functional group[14]. This allows the indirect labelling of β-lactam compounds that have no primary or secondary amino residue with labelling agents for amino compounds, and suggests the possibility of their highly selective determination in biological fluids when penicillins are derivatized after all interfering amino compounds in the biological sample have been removed by suitable pretreatment.

The present paper describes the precolumn fluorimetric derivatization of penicillins by using the above-mentioned reactions. Penicillins hydrolysed by aqueous sodium carbonate were labelled with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) and the derivatives were separated by reversed-phase HPLC and detected fluorimetrically. The method was applied successfully to the determination of penicillins added to serum.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile and water were purchased from Kanto Chemicals (Tokyo, Japan). Sodium cloxacillin (Cl-PC) and sodium dicloxacillin (diCl-PC) were donated by Meiji Seika Kaisha (Tokyo, Japan). Sodium piperacillin (PI-PC) was purchased from Toyama Chemical Industries (Toyama, Japan) and sodium methicillin (Me-PC) from Sigma (St. Louis, MO, U.S.A.). Hyland Q-pack Chemistry Control Serum I was obtained from Cooper Biomedical (Tokyo, Japan). NBD-F was obtained from Dojindo Labs. (Kumamoto, Japan) and potassium benzylpenicillin (PC-G) and other reagents from Wako (Osaka, Japan). A cation-exchange resin column (55 × 5.0 mm I.D.) packed with AG 50W-X8 (H^+) (100–200 mesh) (Bio-Rad Labs., Richmond, CA, U.S.A.) was washed with 10 ml of 50% acetonitrile solution prior to use.

Apparatus

The HPLC system consisted of an L-6200 delivery system (Hitachi, Tokyo, Japan), a Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.), an ODS-80T_M pre-packed column (150 × 4.6 mm I.D.) (Tosoh, Tokyo, Japan) and an L-1200 fluorescence spectrophotometer (Hitachi). The detector excitation and emission wavelengths were set at 470 and 530 nm, respectively. All chromatographic studies were performed at room temperature.

Derivatization procedures

The derivatization reagent was prepared by dissolving NBD-F in acetonitrile at a concentration of 80 m*M*. A 1 mg/ml stock solution of each penicillin was prepared with 50 m*M* phosphate buffer (pH 7.4.). Standard solutions of each penicillin were prepared by diluting the stock solution to appropriate concentrations with 50 m*M* phospate buffer (pH 7.4) or control serum.

For standard sample. To 45 μl of the sample solution were added 45 μl of

acetonitrile and 10 μl of 5% sodium carbonate solution. The reaction mixture was allowed to stand for 60 min at 60°C and then cooled in an ice-bath. To the reaction mixture were added 15 μl of 0.2 *M* phosphate buffer (pH 6.0) and 35 μl of the derivatization reagent and then it was allowed to stand for 10 min at 60°C. After being cooled in an ice-bath, the mixture was mixed with 30 μl of 1 *M* hydrochloric acid solution. An aliquot (5–10 μl) of the resulting mixture was injected directly onto the HPLC column.

For serum. An aliquot of serum (600 μl) was pipetted into a tube for centrifugation. After addition of 600 μl of acetonitrile, the tube was vortex mixed for 1 min. Following centrifugation at 2000 *g* for about 5 min, 1 ml of the supernatant was passed through the cation-exchange resin column. The effluent from column was collected, after the first 200 μl had been discarded. To 450 μl of this solution were added 50 μl of 10% sodium carbonate solution and the mixture was allowed to stand for 60 min at 60°C. After cooling in an ice-bath, 100 μl of the resulting hydrolysis mixture were derivatized as described above.

RESULTS AND DISCUSSION

Fig. 1 shows the reaction course for the hydrolysis of the β-lactam ring and the formation of NBD derivatives. The hydrolysis and derivatization reactions should proceed in a weakly alkaline aqueous medium. The five penicillins were hydrolysed in 5% sodium carbonate solution at 60°C to give the corresponding penicilloic acids (II) and were further reacted with NBD-F at 60°C to give fluorescent NBD derivatives (III). The formation of by-products interfering with the detection of the NBD derivatives was not observed in the hydrolysis reaction. An excess of NBD-F gave NBD-OH and a few other products in the derivatization, but they did not interfere with the detection of the NBD derivatives, as they were eluted earlier than any of the NBD derivatives of penicillins. Consequently, the resulting reaction mixture could be injected directly into the chromatograph without further treatment. The NBD derivatives eluting from column were monitored spectrofluorimetrically as described by Watanabe and Imai[15]. Typical chromatograms of the NBD derivatives of five penicillins are shown in Fig. 2.

The hydrolysis reaction also proceeds in β-lactamase solution, but penicillins having β-lactamase resistance cannot be hydrolysed. Indeed, in this study, this enzyme

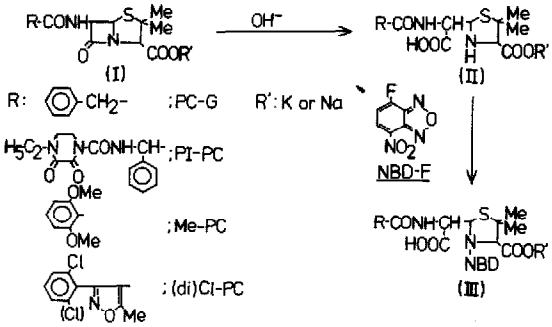


Fig. 1. Reaction scheme for the hydrolysis of β-lactam ring and formation of NBD derivatives.

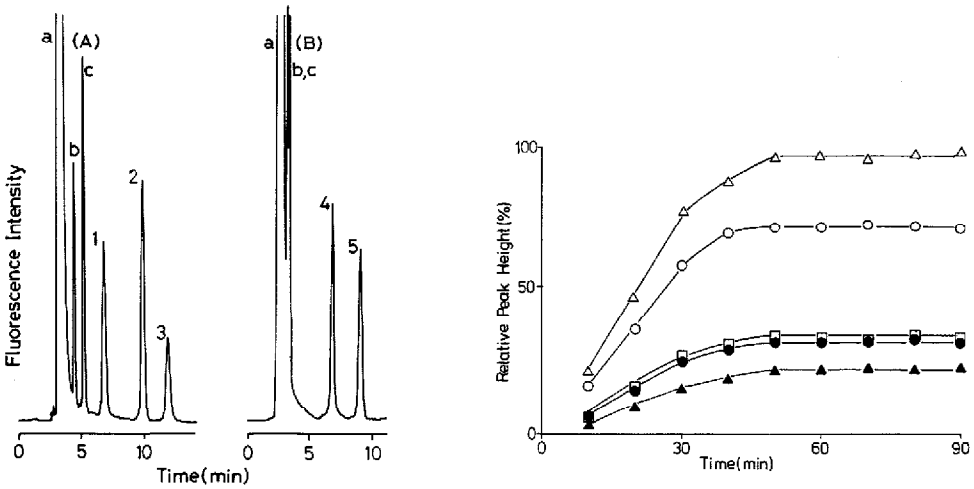


Fig. 2. Chromatographic profiles of the NBD derivatives of five penicillins. 1 = PI-PC; 2 = PC-G; 3 = Me-PC; 4 = Cl-PC; 5 = diCl-PC; a = NBD-OH; b, c = unknowns. Mobile phase, 0.1 *M* phosphate buffer (pH 3.0)–methanol, (A) 60:40 and (B) 45:55; flow-rate, 1.0 ml/min; injection volume, 5 μl; sample concentration, 20 μg/ml for each penicillin; detector sensitivity, 0.5.

Fig. 3. Effect of hydrolysis time on the fluorescence intensity of the NBD derivatives. Reaction temperature, 60°C; sample concentration, 50 μg/ml. △ = PC-G; ○ = PI-PC; □ = Me-PC; ● = Cl-PC; ▲ = diCl-PC.

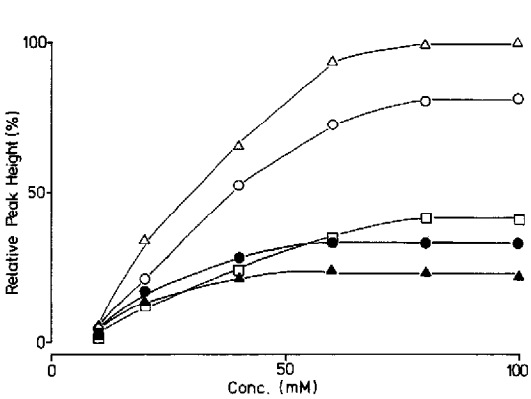
applied to all five compounds. PC-G and Me-PC were easily hydrolysed, but with the other three compounds this reaction hardly proceeded. Therefore, weakly alkaline conditions were adopted in the procedure.

The effects of the precolumn reaction conditions, time of hydrolysis and derivatization reactions, pH of reagent buffer added to the resulting hydrolysis mixture prior to the derivatization and concentration of NBD-F were investigated by HPLC with spectrofluorimetric detection of the reaction products. The results obtained with five penicillins are illustrated in Figs. 3–6.

The time course for hydrolysis was tested at 60°C. In 5% sodium carbonate solution, hydrolysis of all penicillins was completed in 50 min, and longer periods of hydrolysis at this temperature produced no significant improvement in the fluorescence intensity (Fig. 3).

The reaction conditions of the derivatization were also tested at a 60°C. The maximum fluorescence intensity for PC-G, PI-PC and Me-PC was obtained at NBD-F concentrations of 80 m*M* or above, and that for Cl-PC and diCl-PC at concentrations of 60 m*M* or above (Fig. 4). The buffer solutions were investigated in the pH range 5.0–7.0. In this range, the fluorescence intensity for Cl-PC and diCl-PC was almost constant. The fluorescence intensity for PC-G, PI-PC and Me-PC was also constant in the pH range 5.0–6.0, but higher pH decreased the fluorescence intensity (Fig. 5). Fig. 6 shows the effect of time on the yield of the fluorescent derivative. The maximum fluorescence intensity for PC-G and PI-PC was obtained after 10 min and that for Me-PC, Cl-PC and diCl-PC after 5 min. In both instances, longer periods of reaction gave no significant improvement in the peak height.

In order to determine the reproducibility of the present method, several analyses



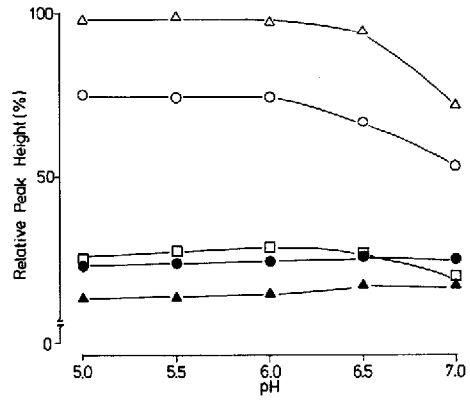


Fig. 4. Effect of NBD-F concentration on the fluorescence intensity of the NBD derivatives. Conditions and symbols as in Fig. 3.

Fig. 5. Effect of pH of reagent buffer on the fluorescence intensity of the NBD derivatives. Conditions and symbols as in Fig. 3.

were performed. Table I lists the relative standard deviations of the peak height for five penicillins at a concentration of 10 μg/ml. Detection limits (signal-to-noise ratio = 3) for all the NBD derivatives are also listed in Table I. A linear response was observed in the range 0.2–100 μg/ml. The regression equations and the correlation coefficients (r^2) of calibration graphs for the penicillins are given in Table II. Highly sensitive detection for all five penicillins was achieved.

Our findings demonstrate that penicillins that have no primary or secondary amino function can be derivatized with NBD-F as a fluorimetric labelling agent after hydrolysis. This suggests that the method may be useful for the highly selective determination of penicillins that have no amino function in a biological fluid or fermentation medium. Thus penicillins can be easily derivatized after all amino

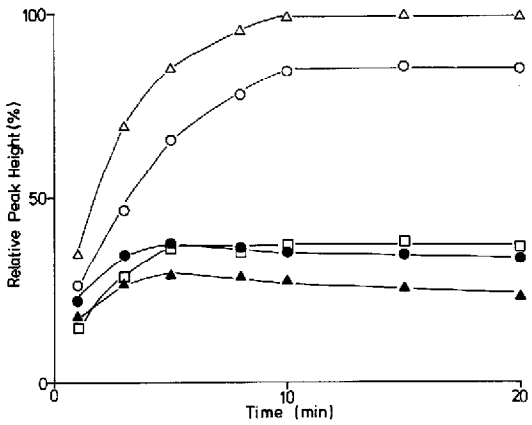


Fig. 6. Effect of reaction time of the derivatization on the fluorescence intensity of the NBD derivatives. Conditions and symbols as in Fig. 3.

TABLE I

REPRODUCIBILITIES OF PEAK HEIGHT AND DETECTION LIMITS FOR PENICILLINS

Sample	*Relative standard deviation (%)*[a]		*Detection limit (signal-to-noise ratio 3) (ng/ml)*
	Intra-assay	*Inter-assay*	
PI-PC	0.99	1.49	50
PC-G	1.32	1.82	30
Me-PC	1.70	2.20	85
Cl-PC	1.01	1.51	30
diCl-PC	1.23	1.73	45

[a] 10 μg/ml, $n = 12$.

TABLE II

REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS OF CALIBRATION GRAPHS FOR PENICILLINS

Concentration range of 0.2–100 μg/ml.

Sample	*Regression equation*[a]		*Correlation coefficient* (r^2)
	Slope	*Intercept*	
PI-PC	8.37	2.63	0.998
PC-G	11.60	4.21	0.998
Me-PC	3.90	2.29	0.996
Cl-PC	5.26	1.82	0.998
diCl-PC	4.34	0.54	1.000

[a] Peak height = intercept + slope × concentration (μg/ml).

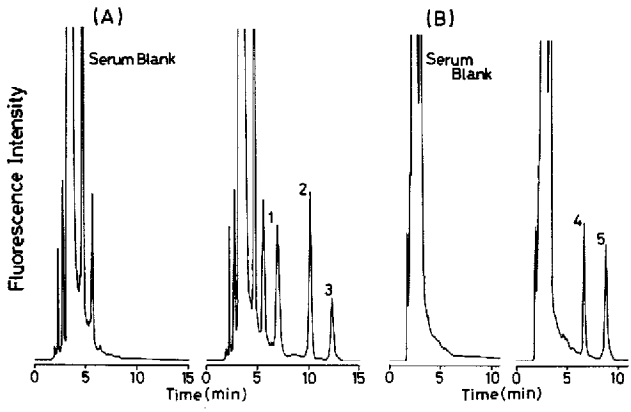


Fig. 7. Chromatographic profiles of serum blank and five penicillins added to serum. Mobile phase, 0.1 *M* phosphate buffer (pH 3.0)–methanol, (A) 60:40 and (B) 45:55. Peak assignments and other conditions as in Fig. 2.

TABLE III

RECOVERY OF PENICILLINS FROM SERUM

Penicillin	*Recovery (%, n = 6)*		
	5 μg/ml	*20 μg/ml*	*50 μg/ml*
PI-PC	95.0	93.7	92.5
PC-G	93.8	91.0	93.9
Me-PC	90.4	91.2	92.8
Cl-PC	95.1	97.0	98.7
diCl-PC	95.8	96.2	100.7

TABLE IV

REPRODUCIBILITIES OF DETERMINATION OF PENICILLINS SPIKED IN SERUM

Penicillin	*Relative standard deviation (%, n = 6)*		
	5 μg/ml	*20 μg/ml*	*50 μg/ml*
PI-PC	1.97	1.90	1.92
PC-G	2.00	1.95	1.80
Me-PC	2.36	2.35	2.01
Cl-PC	3.04	2.40	2.25
diCl-PC	2.85	2.80	2.11

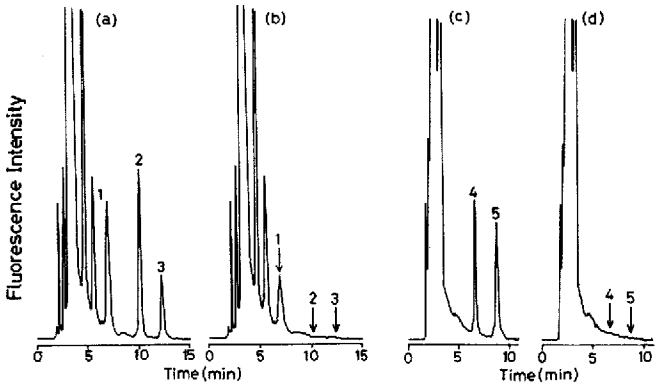


Fig. 8. Chromatographic profiles of five penicilloic acids added to supernatant of deproteinized serum, derivatized (b and d) after pretreatment by the cation-exchange resin and (a and c) without the pretreatment. Sample concentration, 20 μg/ml as corresponding penicillins. Peak assignments and other conditions as in Fig. 7.

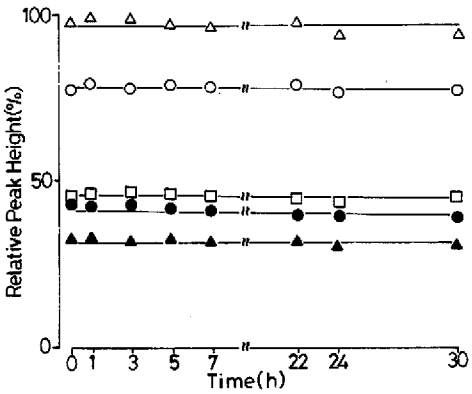


Fig. 9. Stability of the NBD derivatives on the resulting reaction mixture at ambient temperature. Symbols as in Fig. 3.

compounds in the biological sample have been removed by pretreatment with a cation-exchange resin.

Fig. 7 shows chromatograms of serum blank and the five penicillins added to serum, derivatized by the present method following pretreatment by deproteinization and passage through a cation-exchange column. Interference-free chromatograms, sufficient recovery and reproducibilities were obtained (Tables III and IV). In these pretreatment methods, as the pH of the effluent from the cation-exchange column was very low compared with the standard solution, the hydrolysis and derivatization reactions did not proceed. This problem was solved by pH adjustment using 10% instead of 5% sodium carbonate solution.

Penicilloic acid is a major metabolite of penicillins. If it is not completely removed by the pretreatment, it will result in a positive systematic error during the determination of unchanged drug. Fig. 8 shows the chromatograms of the five penicilloic acids (penicillins hydrolysed with 5% sodium carbonate solution) added to the supernatant of deproteinized serum. Apart from that arising from PI-PC, they were completely removed by the present pretreatment. The penicilloic acid from PI-PC was only 60% removed, so the method was unsuitable in this instance. In order to resolve this problem, other pretreatment methods are being studied.

The NBD derivatives of the five penicillins were stable for at least 24 h in the resulting reaction mixture at room temperature (Fig. 9). This stability is sufficient to allow the use of an autosampler, which suggests that the present method may be applied to routine analysis. The results also suggest the possibility of determination by the use of one of the penicillins as an internal standard for other penicillins, except for PI-PC.

REFERENCES

1 M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, *J. Liq. Chromatogr.*, 6 (1983) 2019.
2 M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, *J. Chromatogr.*, 297 (1984) 385.
3 D. Westerlund, J. Carlqvist and A. Theodorson, *Acta Pharm. Suec.*, 16 (1979) 187.
4 J. Carlqvist and D. Westerlund, *J. Chromatogr.*, 164 (1979) 373.

5 J. Haginaka and J. Wakai, *Anal. Chem.*, 57 (1985) 1568.
6 J. Haginaka and J. Wakai, *J. Pharm. Pharmacol.*, 39 (1987) 5.
7 A. J. Shah, M. W. Adlard and G. Holt, *Analyst*, 113 (1988) 1197.
8 S. Lam and E. Grushka, *J. Liq. Chromatogr.*, 1 (1978) 33.
9 T. L. Lee, L. D'arconte and M. A. Brooks, *J. Pharm. Sci.*, 68 (1979) 454.
10 K. Miyazaki, K. Ohtani, K. Sunada and T. Arita, *J. Chromatogr.*, 276 (1983) 478.
11 M. E. Rogers, M. W. Adlard, G. Saunders and G. Holt, *J. Chromatogr.*, 257 (1983) 91.
12 J. Haginaka and J. Wakai, *Anal. Chem.*, 58 (1986) 1896.
13 J. Haginaka, J. Wakai, H. Yasuda, T. Uno, K. Takahashi and T. Katagi, *J. Chromatogr.*, 400 (1987) 101.
14 J. P. Hou and J. W. Poole, *J. Pharm. Sci.*, 60 (1971) 503.
15 Y. Watanabe and K. Imai, *J. Chromatogr.*, 239 (1982) 723.