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DERIVATIZATION TECHNIQUES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF β-LACTAMS

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SUMMARY

High-performance liquid chromatographic techniques are described for the determination of β-lactam antibiotics. Reversed-phase chromatography is performed on a chemically bonded silica support, often employing a mobile phase containing an organic modifier and ion-pairing reagent. Novel derivatization procedures are described, including pre-column reaction with an imidazole–metal salt reagent and post-column, fluorescent labelling with *o*-phthaldialdehyde. Each method is investigated and discussed in terms of sensitivity and chromatographic efficiency, with application to natural and semi-synthetic β-lactams.

INTRODUCTION

High-performance liquid chromatographic (HPLC) techniques have developed rapidly over the past decade, allowing determination of an extensive range of compounds, including β-lactam antibiotics[1]. Chromatography of these antibiotics is predominantly influenced by their carboxylic acid function, and has included ion-exchange[2] and reversed-phase ion-pair HPLC[3]. Detection by UV absorption has been commonly employed[4,5], and for those β-lactams which lack a chromophore, greatest sensitivity is normally achieved at wavelengths below 230 nm. However, at these wavelengths selectivity is poor, resulting in high background interference when trace analyses are required for samples such as complex fermentation media.

Recently, derivatization techniques have been described which enhance the detection properties and selectivity of certain analytes, and which may be used with HPLC[6–8]. A specific, pre-column derivatization method has been developed for clavulanic acid[9], whilst post-column derivatization with fluorescamine has been successfully applied to the determination of semi-synthetic cephalosporins[10].

In recent papers we described the pre-column derivatization of penicillins with a combined imidazole–mercury(II) chloride reagent[11] and post-column derivatization of β-lactams with *o*-phthaldialdehyde[12]. Both methods are especially applicable to the analysis of microbial fermentation broths. In this paper, some new aspects of each technique are investigated in an attemt to improve the sensitivity and chromatographic performance.

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EXPERIMENTAL

Materials and reagents

Reagent-grade imidazole and concentrated hydrochloric acid, used in the pre-column derivatization reagent were obtained from Sigma (London, U.K.).

3-Mercaptopropionic acid, *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol, which were employed in studies on post-column derivatization, were also supplied by Sigma. Acetonitrile (HPLC grade) and tetra-*n*-butylammonium hydroxide were obtained from Rathburn Chemicals (U.K.) and Fisons (U.K.), respectively, and all other materials and reagents ('Analar' grade) were acquired from BDH (U.K.).

Penicillins V, G (potassium salts), X (zinc salt), N (barium salt), 6-aminopenicillanic acid (6-APA) (potassium salt), cephamycin C (sodium salt) and cephalosporin C (sodium salt) were kindly donated by Ciba-Geigy (Switzerland) and Glaxo (U.K.). The semi-synthetic β-lactams, ampicillin, cephalexin and cephradine, were obtained from Sigma.

Apparatus and operating conditions

Pre-column derivatization. The reagent was prepared as described in a previous communication[11] and was prepared by dissolving 4.125 g of imidazole in 2.5 ml of distilled water. After addition of 1.0 ml of concentrated HCl, 0.5 ml of the metal salt (0.11 *M*) were added, and finally a further 1.5 ml of concentrated HCL. A volume of 0.1 ml of the reagent was added to each 1-ml sample and, following incubation at 50°C for 50 min, the products of the reaction with β-lactams were analysed by reversed-phase HPLC, with detection at 325 nm.

Chromatography was performed on a 20 cm × 4.6 mm I.D. analytical column, packed with 5-μm Spherisorb C_{18} (Phase Separations, U.K.). Elution at 1.5 ml/min was carried out with a mobile phase consisting of acetonitrile–0.01 *M* sodium phosphate buffer pH 6.5 (20:80, v/v), containing a complexing agent (0.01 *M*). Different metal salts and complexing agents were investigated. Semi-preparative HPLC was performed on a 10 cm × 9.4 mm I.D. 'RAC' column (Whatman, U.S.A.), packed with 5-μm Partisil C_8, by using the mobile phase described above, containing sodium thiosulphate as the complexing agent. Following derivatization of penicillin G (100 μg/ml) with an imidazole–silver nitrate reagent, 100-μl samples were injected and fractions (2 ml) were collected prior to, during, and following peak elution. This was repeated five times and corresponding fractions were pooled. Each combined fraction was analysed for silver content by using a Varian-Techtron AA120 atomic absorption spectrophotometer. The instrument was optimized to achieve maximum sensitivity (hollow cathode lamp current = 4mA; air–acetylene flame; slit width = 100 nm; and detection wavelength = 328.0 nm). Calibrants, prepared by dissolving silver nitrate in the mobile phase, indicated a detection limit below 0.01 ppm.

Post-column derivatization. The OPA reagent was prepared with borate buffer (0.4 *M*), containing 2-mercaptoethanol (0.28 *M*) and *o*-phthaldialdehyde (3.5 *M*), at pH 12. Details of the method have been previous described[12]. The reagent is introduced into the column eluent via a T-piece and reaction takes place in a coiled-tube reactor, normally maintained at 90°C. Chromatographic conditions are given in Fig. 2.

The thiol reagents, 3-mercaptopropionic acid and dithioerythritol (DTE) were

investigated in the post-column derivatization system. For these thiols the fluorescence intensities of 6-APA, penicillin N and cephamycin C derivatives were studied as a function of pH (between 9 and 12) and reactor coil temperature (between 50°C and 90°C).

A cation-exchange column (15 cm × 4.6 mm I.D.) was tap-packed with Amberlite IRA-120 (BDH), and was fitted in-line, between the T-piece for reagent addition and the reactor coil. During these studies, a reagent pH of 12 was used, and the cation-exchange column was thermostated at 50°C, in common with the reactor coil.

RESULTS AND DISCUSSION

Pre-column derivatization

The influence of metal ion and complexing agent on HPLC efficiency and sensitivity is detailed in Table I. Although only data for penicillin X are given, results from penicillins G and V showed similar trends.

When reagents were prepared with nickel, zinc or cadmium salts in place of mercury(II) chloride, no product was detected, and cuvette studies showed the absence of any significant absorption at 325 nm (or any other wavelength). The inability of these metals to produce stable penicillenic acid derivatives may reflect their relative 'hardness'[14], and consequent poorer tendency to complex with 'soft' ligands, such as penicillenic acids which are thought to coordinate metal ions through the sulphur atom[15].

An intense absorption was recorded at 325 nm when silver, gold or mercury

TABLE I

THE EFFECTS OF METAL ION AND COMPLEXING AGENT ON THE HPLC OF PENICILLIN X, FOLLOWING PRE-COLUMN DERIVATIZATION

Chromatographic efficiency calculated by $N = 2\pi (h' \cdot t_R/A)^2$, where A = peak area; h' = peak height; t_R = retention time[14]. Peak area from a 3-μl injection of derivatized penicillin X (100 μg/ml). – = no derivatized product detected by HPLC, or cuvette studies. ND = no derivatized product detected by HPLC, although intense absorption, at 325 nm, recorded by cuvette studies.

Complexing agent in mobile phase	*Metal ion used in the derivatization reagent*					
	Ni^{2+}	Zn^{2+}	Ag^{+}	Cd^{2+}	Au^{+}	Hg^{2+}
EDTA						
Chromatographic efficiency	–	–	ND	–	ND	554
Peak area ($\times 10^{-3}$)						957
$Na_2S_2O_3$						
Chromatographic efficiency	–	–	1527	–	ND	1912
Peak area ($\times 10^{-3}$)			2577			2346
NaCN						
Chromatographic efficiency	–	–	1621	–	358	2104
Peak area ($\times 10^{-3}$)			2530		1912	2614

Fig. 1. HPLC of penicillin X (30 μg/ml) (A), penicillin G (30 μg/ml) (B) and penicillin V (40 μg/ml) (C), following pre-column derivatization with an imidazole–mercury(II) chloride reagent. Flow-rate = 1.5 ml/min; injection volume = 20 μl; detector sensitivity = 0.2 a.u.f.s.; detection wavelength = 325 nm. Mobile and stationary phases are given in the Experimental section.

salts were used. When the derivatization reagent was prepared with silver nitrate, detection of the product following HPLC, was only possible when the mobile phase contained sodium thiosulphate or sodium cyanide, whereas only the latter solvent system revealed the product when gold chloride was used. In contrast, detection at 325 nm was achieved with all complexing agents employed, when mercury(II) chloride was present in the reagent. Irrespective of the metal ion, both chromatographic efficiency and sensitivity increased with the sequence of complexing agents, ethylenediaminetetraacetic acid (EDTA), sodium thiosulphate and sodium cyanide. Optimum conditions were thus established by using mercury(II) chloride in the reagent, and sodium cyanide in the solvent. although due to the extreme toxicity of sodium cyanide, it was considered that for most purposes, sodium thiosulphate was satisfactory. Fig. 1 illustrates the isocratic elution of penicillins X, V and G following pre-column derivatization with an imidazole–mercury(II) chloride reagent, when sodium thiosulphate was used in the mobile phase.

The results in Table I parallel the association constants of the various complexing agents with metal ions used in the reagent preparation[16].

It was shown by atomic absorption analysis that fractions corresponding to peaks detected at 325 nm did not contain silver. The evidence suggest that incorporation of a complexing agent in the mobile phase results in complete or partial removal of metal ion from the penicillenic acid–metal complex.

In the absence of complexing agent, it may be concluded that during chromatography various interactions occur between the stationary phase and metal ion–penicillenic acid complex in solution, causing band-broadening and peak skew.

Poor chromatographic efficiency and low sensitivity also result when the association constant between complexing agent and metal ion is relatively low (*e.g.*, gold with EDTA) and in extreme circumstances, no product is detected, presumably due to excessive peak broadening.

Accuracy, reproducibility, linearity and sensitivity limits were investigated, employing HPLC conditions as described under Fig. 1 with the exception that EDTA replaced sodium thiosulphate in the mobile phase. Reproducibility was studied by (a) repeated injection ($n = 10$) of penicillin X (25 μg/ml) which gave a 2% error from the mean, and (b) repeated calibration on a day-to-day basis constructed from peak area measurements following injections of penicillins X and G, both in the range 0–50 μg/ml. The calibration lines gave a correlation coefficient of 0.997. Accuracy was assessed by addition of penicillin G (to give a final, known concentration between 4.5 and 50 μg/ml) to fermentation broth samples. Penicillin X (10 μg/ml) was also incorporated into each sample. The peak area ratio, determined from the calibration lines of penicillin X and G, was used to calculate experimental penicillin G concentrations. The mean difference between experimental and known concentrations of penicillin G was 4.9%. Using EDTA in the mobile phase, mercury(II) chloride reagent, and 20-μl injection volumes, the detection limits for penicillins X and G were 1 μg/ml. The alternative use of sodium thiosulphate resulted in even lower limits of 0.5 μg/ml.

Post-column derivatization

In order to increase sensitivity and reduce the severity of the reaction conditions described for post-column derivatization of β-lactams with OPA[12] two approaches have been made.

The first involved use of two alternative thiol reagents, 3-mercaptopropionic acid, and DTE. The fluorescent derivatives of cephamycin C, penicillin N and 6-APA with both reagents all produced approximately 30% lower emission intensities than the corresponding 2-mercaptoethanol products irrespective of reaction temperature and pH. Also the reagent prepared with 3-mercaptopropionic acid, itself appeared less stable and precipitation was noted after 2–3 h at room temperature, especially above pH 11.

The second approach has been to hydrolyse the β-lactams after elution from the analytical column but prior to reaction with OPA, by introducing an in-line column, containing a strong cation-exchange resin. It was thought that the resin surface may provide a localized nucleophilic environment, thus promoting hydroxyl and/or metal ion catalysed hydrolysis of the antibiotics. Fig. 2 illustrates the effects of such a column. An increase in sensitivity was realised for all β-lactams studied with the exception of penicillin N, which showed a slight decrease in peak area. A maximum increase in sensitivity was achieved for 6-APA (approximately 1.5-fold). The difference in behaviour shown by penicillin N may be attributed to its relatively high instability towards hydrolysis, resulting in extensive hydrolysis before entry into the cation-exchange column. When the additional column was employed some line-broadening was observed for all the peaks in each recorded chromatogram (for 6-APA, reduction in system efficiency was equivalent to 15% of the theoretical plates). This was principally due to the increased dead-volume resulting from the additional column. A potentially more effective hydrolytic system, at present under consider-

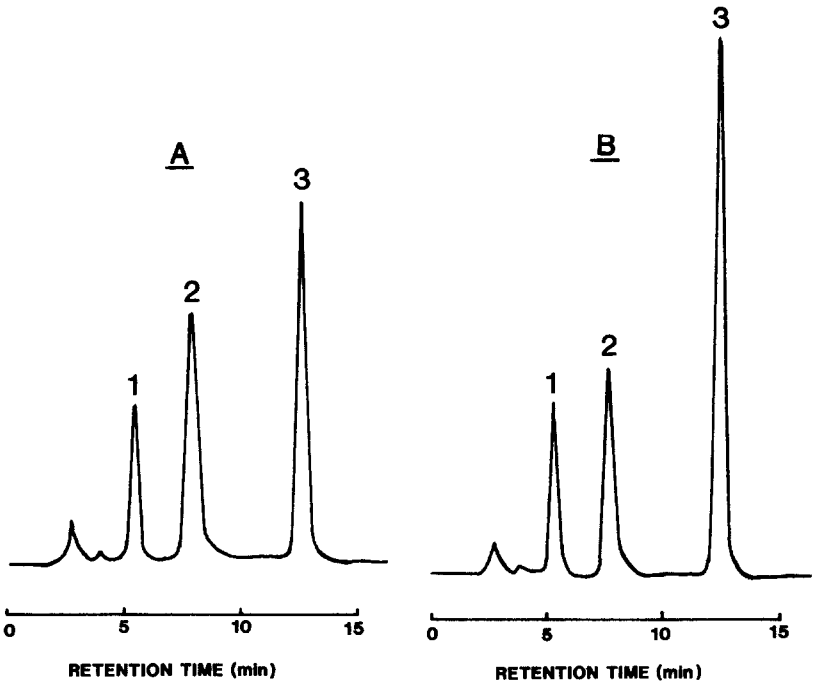

Fig. 2. HPLC of cephamycin C (333 μg/ml) (1), penicillin N (111 μg/ml) (2) and 6-APA (333 μg/ml) (3), (A) using post-column derivatization with fluorescence detection; (B) the effects of an in-line column, containing a cation-exchange resin. Stationary phase: 5 μm Spherisorb C_8 in a 20 cm × 4.6 mm I.D. column. Mobile phase: acetonitrile–phosphate buffer (0.01 *M*), pH 5.5 containing tetra-*n*-butylammonium hydroxide (0.01 *M*) and 0.2% acetic acid (5:95, v/v). Flow-rate = 1.5 ml/min; injection volume = 10 μl; reactor coil length = 20 m (0.3 mm I.D.); detector sensitivity = 512 λ_{ex} = 350 nm; λ_{em} = 450 nm.

ation by the authors, may be in the use of a post-column enzyme reactor, prepared by immobilization of β-lactamase.

Linearity, accuracy, reproducibility and detection limits of the post-column method were determined under the conditions described under Fig. 2. Calibration lines, constructed from peak areas obtained when known concentrations (between 0–100 μg/ml) of antibiotic were injected, gave correlation coefficients greater than 0.999 for both penicillin N and cephamycin C. Reproducibility of a repeated 20-μl injection (n = 20) of cephamycin C (100 μg/ml) gave a 2% error from the mean. The absolute accuracy of the method was investigated by using an identical procedure to that previously described for pre-column derivatization, based on the use of an internal standard. Fermentation broths were spiked with known concentrations of cephamycin C final concentration (10–130 μg/ml). Using penicillin N (50 μg/ml) as an internal standard, the ratio of peak areas was used to calculate experimental cephamycin C concentrations. The mean error between experimental and known concentrations was 2%. Detection limits of between 0.5 and 1.0 μg/ml were obtained for cephamycin C and penicillin N when 20-μl injections were used. Sensitivity limits for the semi-synthetic β-lactams, ampicillin, cephalexin and cephradine were 26 μg/ml, 14 μg/ml and 8 μg/ml respectively.

CONCLUSIONS

The derivatization methods described allow rapid, accurate, reproducible and quantitative determination of β-lactams in complex aqueous media.

Pre-column derivatization with an imidazole–mercury(II)chloride reagent is applicable to penicillins with a side chain at C-6 (*e.g.*, penicillins X, G, V and K). Improvements in terms of sensitivity and chromatographic efficiency have been made by replacing the complexing agent, EDTA, in the mobile phase, with sodium thiosulphate or sodium cyanide. Evidence suggests that the product detected by UV absorption in the HPLC procedure does not contain a metal.

Post-column derivatization with OPA may be applied to β-lactams containing a primary amino function (*e.g.*, 6-APA) isopenicillin N and cephamycin C. Attempts to increase sensitivity by use of various thiol reagents including 3-mercaptopropionic acid were not successful. However, increased sensitivity was achieved for certain β-lactams by using a cation-exchange resin, incorporated within the HPLC system.

With few exceptions, the majority of β-lactams are potentially capable of determination by one or the other method, although simultaneous use of the derivatization techniques has proven unsuccessful (data not given).

Due to enhancement of sensitivity and selectivity by these methods, detection of novel antibiotics may be possible.

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