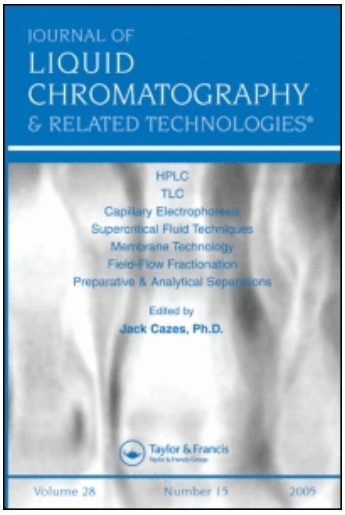

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PENICILLINS FOLLOWING DERIVATIZATION TO MERCURY-STABILIZED PENICILLENIC ACIDS

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ABSTRACT

A high-performance liquid chromatographic, pre-column derivatization procedure is described, allowing the determination of natural and semi-synthetic penicillins by absorption at 325nm. Derivatization with an imidazole/mercuric chloride reagent is followed by reverse-phase chromatography of the resulting mercury-stabilized penicillenic acids on an octadecylsilane, chemically-bonded stationary phase. Gradient elution is employed with a mobile phase containing organic modifier, buffer and ethylenediaminetetraacetic acid. Pre-column reaction conditions including time and temperature are examined. Accuracy, reproducibility and detection limits of the method are discussed. The technique is applied to the analysis of fermentation media following sample preparation by centrifugation and deproteinization.

INTRODUCTION

The efficacy of penicillin and its derivatives has resulted in their widespread use for many years. With all such antibiotics, assay systems are required which enable accurate and precise quantitation.

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Several physico-chemical techniques for the determination of penicillins have been developed (1). These include the iodometric titration (2), and colorimetric assays such as the hydroxylamine (3) and molybdoarsenic acid-mercuric chloride methods (4). The isomerism of penicillins to their corresponding penicillenic acids which absorb at 325nm, has also been used in the determination of β-lactams (5). However, many of these assay systems are severely limited in their application. The methods often lack intrinsic sensitivity or fail to distinguish individual penicillin levels, when two or more β-lactams are present. Also, the occurrence of interfering components in biological media such as serum or microbial fermentation broths often produce misleading results due to lack of reagent specificity and/or the presence of high background readings.

The development of high-performance liquid chromatography (HPLC), has enabled the rapid, sensitive and quantitative determination of many antibiotics, including penicillins (6).

An extensive range of stationary and mobile phases has been reported for the analysis of β-lactams (7-9). Optimum chromatographic conditions appear to have been achieved using reverse-phase systems, often employing ion-pairing reagents to increase the capacity factor of highly polar compounds (10). Many HPLC detection methods are now available (11), although the most widely used is U.V. absorption. Detection of penicillins by this technique is usually performed at 254nm or 220nm. However, our interest lies in the microbial biosynthesis of β-lactams (12-14), and we have found the presence of interfering components in complex fermentation media to be problematic when using these wavelengths.

A derivatization procedure was therefore considered, to enable detection of penicillins at a longer wavelength, there-

by reducing interference from other media constituents. Such problems have been overcome to some extent, by use of post-column derivatizing agents, including fluorescamine (15) and o-phthaldialdehyde (16). However, both reagents are limited to the detection of β-lactams possessing a primary amino function.

Treatment of penicillins with an imidazole/mercuric chloride reagent was investigated as a possible pre-column derivatization method. The slow reaction rate prevented the use of post-column derivatization.

The reaction is highly specific, and the resulting penicillenic acids, after stabilization as mercury (II) complexes, can be detected at 325nm (17) following HPLC.

EXPERIMENTAL

Materials and Reagents

Acetonitrile was HPLC S grade and obtained from Rathburn Chemicals (U.K.). Other mobile phase constituents, including ethylenediaminetetraacetic acid (disodium salt), and sodium dihydrogen orthophosphate were of "analar" grade and supplied by B.D.H.(U.K.).

Reagent grade imidazole was supplied by Sigma, London, (U.K.), whilst the mercuric chloride and hydrochloric acid (concentrated) used in the pre-column derivatization reagent were of "analar" grade and obtained from B.D.H.(U.K.).

β-lactamase Type I, used in the preparation of fermentation broth "controls", was obtained from Sigma, London, (U.K.).

Penicillins K (sodium salt), N (barium salt), V, G (potassium salts), X (zinc salt) and methicillin (sodium salt) were kindly donated by Ciba-Geigy (Switzerland) and Glaxo (U.K.).

Apparatus and Operating Conditions

The solvent delivery system used consisted of two, double reciprocating pumps (model No. 750/04; Applied Chromatography

Systems, U.K.) controlled by a high pressure mixing unit (model No. 750/36; Applied Chromatography Systems, U.K.), enabling gradient formation. Injections were made using a Rheodyne valve (model No. 7125; Anachen, U.K.) fitted with a 20µl loop. Chromatography was performed on a 20 x 4.6 mm ID analytical column slurry-packed (18) with 5µm Spherisorb C18 (Cat. No. S5ODS2; Phase Separations, U.K.). A 4 x 10 mm ID guard-column was fitted prior to and in series with the analytical column and was tap-packed (18) with 5µm Spherisorb C18.

U.V. detection was carried out at 325nm, using a variable wavelength detector (Spectromonitor III; Laboratory Data Control, U.S.A.) fitted with a 12µl flow cell. Integration of peak areas and data handling was performed by a Hewlett-Packard reporting integrator (model No. 3390A; Hewlett-Packard, U.S.A.).

The mobile phase was prepared by mixing various proportions of CH_3CN with an aqueous solution containing 0.01M NaH_2PO_4 and 0.01M EDTA. The solvent was then adjusted to pH 6.5 with 2.0M NaOH. Degassing was performed by the continual passage of helium through the solution.

The imidazole/$HgCl_2$ reagent was prepared in plastic-capped, glass vessels by dissolving 4.125g of imidazole (recrystalized twice form propan-2-ol) in 2.5ml of distilled water. After addition of 1.0ml of HCl, 0.5ml of $HgCl_2$ (0.11M) were added, and finally a futher 1.5ml of HCl.

Pre-Column Reaction System

To each 1ml sample aliquot, 0.1ml of the imidazole /$HgCl_2$ reagent (prepared as previously described) was added. The reaction mix was then placed, for the period of time described, in a water bath thermostatically controlled to the required temperature. Samples were then rapidly cooled to room temperature by immersion in an ice bath and injected onto the column.

Sample Preparation

Preparation of fermentation broths involved centrifugation, (10mins. at 8,000xg) following adjustment of the pH to 7. The supernatant was then removed, and subjected to deproteinization by the method described by Rudrick and Bawdon (19), involving protein precipitation by acetonitrile, subsequent to re-extraction with methylene chloride. After final centrifugation (15mins. at 8,000xg) and removal of the aqueous layer, the sample was then treated with the derivatization reagent, as described above.

Using an analytical microsyringe, fermentation broths were spiked, prior to deproteinization, with standard solutions of the penicillins. These were prepared daily by dilution with distilled water to the required concentration from a stock of 1mg/ml.

"Control" samples were prepared by addition of 50µl of β-lactamase (final concentration 4 units/ml) to 1ml of the fermentation broth. Following incubation at 25°C for 2 hours, samples were then treated by deproteinization and pre-column derivatization as previously described.

RESULTS AND DISCUSSION

Detection Wavelength

Detection of the penicillenic acids which were produced by reaction with imidazole and stabilized by complexation with mercury (II), was carried out at 325nm. Cuvette studies had previously shown this to be the absorbance maximum of these derivatives (20). This was confirmed by using a "stop-flow" HPLC technique which produced an absorption band centred at 325nm.

Chromatographic Conditions

Fig. 1 indicates a typical chromatogram obtained following pre-column derivatization of four β-lactams with the

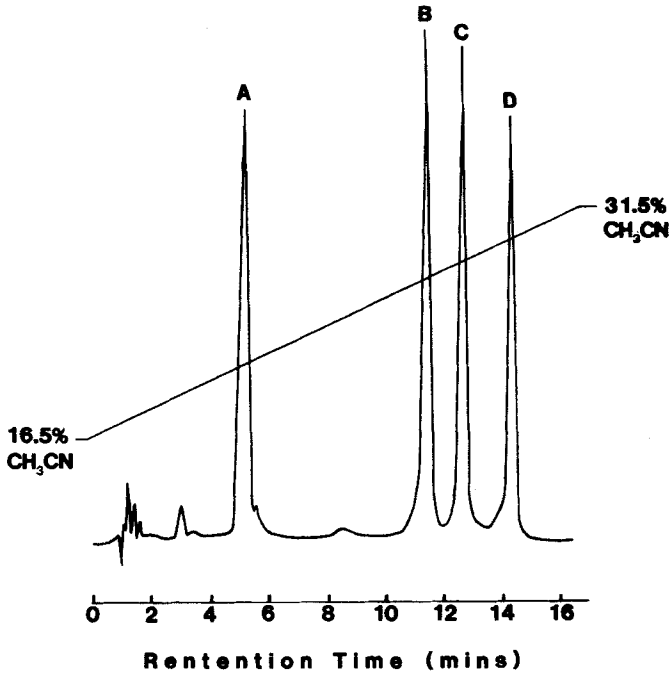

FIGURE 1

HPLC of penicillin X (50µg/ml) (A), methicillin (75µg/ml) (B), penicillin G (50µg/ml) (C) and penicillin V (60µg/ml) (D), following pre-column derivatization with the imidazole/$HgCl_2$ reagent, at 50°C for 50 minutes. Chromatographic conditions were as described in the text, using a gradient of acetonitrile as illustrated. Flow rate was 2.0ml/min. with a detection wavelength of 325nm, and an attenuation of 0.05 AUFS. Injection volume: 20 µl.

imidazole reagent. Chromatography of the products was optimized at a pH of 6.5 and by the inclusion of EDTA in the mobile phase. Although the role of the polyaminocarboxylic acid is unclear, its exclusion resulted in very broad, unsymmetrical peaks. It seems likely that the high complexing ability of EDTA is of importance as its substitution with succinic or citric acid produced poor chromatographic results. Replacement with trans-1,2-diaminocyclohexanetetraacetic acid however,

gave similar results to those obtained with EDTA. The complexing agent may act by removing excess mercury (II) ions or by forming a more stable penicillenic acid-mercury-EDTA complex.

Pre-Column Reaction Conditions

The effects of pre-column reaction time and temperature were investigated by HPLC analysis of the reaction products. Fig. 2 illustrates the results obtained with four, naturally-occurring β-lactams.

At 50°C, the rate of formation of the mercury-stabilised penicillenic acids was slower than at higher temperatures. After 50 mins. the maximum sensitivity for all four penicillins was obtained and longer periods of incubation at this temperature produced no significant increase of decrease in the peak areas.

Raising the incubation temperature to 70°C or 80°C resulted in not only an increase in the rate of formation of the penicillin derivatives but also their decay rate. At 70°C, slightly higher sensitivities than at 50°C were achieved for all penicillins studied. However, the optimum incubation time varied slightly with different penicillins (25-35mins.) and was more critical than at 50°C. For temperatures in excess of 70°C, the maximum sensitivity achievable, irrespective of incubation time, was always lower than that for derivatization at 50°C or 70°C. In further studies, the pre-column reaction conditions of sample incubation for 50 minutes at 50°C, were therefore chosen to provide maximum sensitivity and product stability.

Reaction of the imidazole/$HgCl_2$ reagent with penicillin K resulted in a derivative with relatively low intrinsic sensitivity, whilst with penicillin N the reaction proved completely unsuccessful. As both these β-lactams possess an aliphatic side-chain, it may be that the rate of product

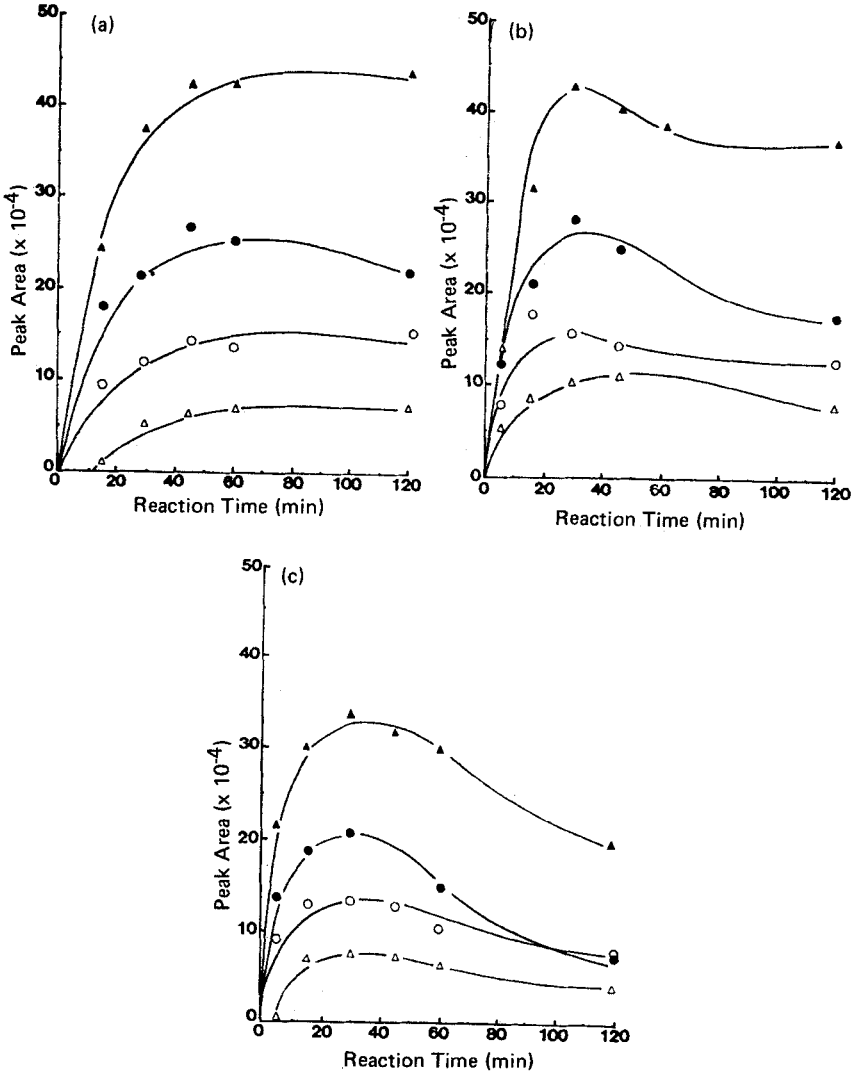

FIGURE 2

The effects of reaction time on the pre-column derivatization of penicillin X (o), penicillin V (●), penicillin K (Δ) and penicillin G (▲), at 50°C (A), 70°C (B) and 80°C (C). Chromatographic conditions were as described under Fig.1.

decay is reduced by aromatic stabilization of the oxazolone ring in derivatives of penicillin G, V, X and methicillin.

Substitution of mercury in the reagent with iron (II), zinc (II) or nickel (II) failed to produce an absorption at 325nm, following penicillin derivatization. However, with copper the reaction yielded two major products, detectable at this wavelength, and resolved by HPLC. Although the use of other metals has yet to be investigated, it is clear that at the present time, mercury, producing one, stable reaction product, is most suited to the assay system.

Accuracy and Reproduceability

During these studies chromatographic and pre-column reaction conditions were maintained as described under Fig. 1.

Linearity of the method with respect to concentration of penicillins X and G was checked. Serial dilutions of each β-lactam between 0 and 50µg/ml were prepared in triplicate, and, following pre-column derivatization, 20µl of each solution injected onto the column. Peak areas were used to construct calibration lines, giving a correlation coefficient of 0.997 for penicillin X and G. Gradients of 14959 area units/µg/ml for penicillin X and 15443 area units/µg/ml of penicillin G were obtained, by regression analysis. Good day-to-day reproducibility was obtained by repeating the calibration lines on several different occasions. Reproducibility of a 20µl repeated injection (n=10) of penicillin X (25µg/ml) gave a 2% error from the mean.

Accuracy was investigated by spiking fermentation media with known concentrations of penicillin G. Using penicillin X as an internal standard at 10µg/ml, and following sample treatment as described under apparatus and operating conditions, the ratio of peak areas was used to calculate a theoretical penicillin G concentration. The results are listed in Table 1.

TABLE 1

DETERMINATION OF PENICILLIN G IN FERMENTATION MEDIA

SAMPLE No.	PENICILLIN G CONCENTRATION (µg/ml) THEORETICAL	EXPERIMENTAL	ERROR (%)
1	17.0	18.0	5.5
2	4.5	4.3	4.4
3	24.0	22.5	6.25
4	37.5	35.2	6.1
5	50.0	48.9	2.2

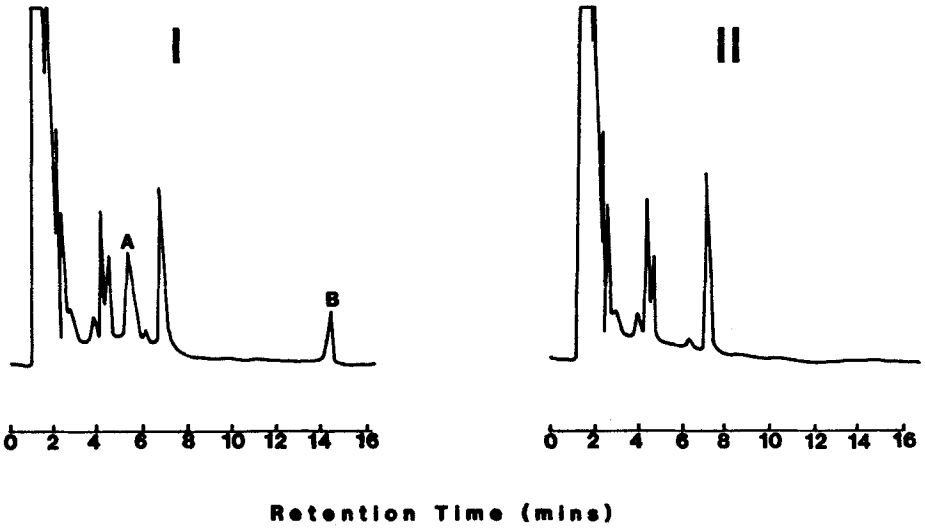

FIGURE 3

(I) HPLC analysis of a typical fermentation broth containing penicillins X (5µg/ml) (A) and G (5µg/ml) (B), following deproteinization and pre-column derivatization with imidazole/$HgCl_2$.

(II) Chromatography of a fermentation broth "control", resulting from β-lactamase treatment of the sample shown in Fig. 3 (I).

Derivatization and chromatographic conditions were as described under Fig. 1.

Sensitivity and Detection Limits

Using 20µl injections, detection limits of 1µg/ml were achieved for penicillin X and penicillin G, in samples of fermentation media. Although higher sensitivities may be obtained by use of larger injection volumes, 20µl was used to prevent possible overloading of the column. Fig. 3A illustrates a typical fermentation broth, containing penicillins X and G (both 5µg/ml), whilst Fig. 3B indicates the use of β-lactamase treatment in preparation of a "control" sample.

Sample preparation by deproteinization was necessary to ensure linearity and high levels of detection. However, this treatment was found to be unnecessary in batch reactions where absorbance at 325nm was measured in a cuvette. A possible explanation to these somewhat contradictory results may lie in reaction of the highly reactive N-penicilloylimidazole intermediate of the imidazole catalysed isomerism, with free amino and thiol groups of proteins (21). The product, whilst still containing the molecular structure necessary for U.V. absorption at 325nm, will undoubtedly have different chromatographic characteristics to the "free" penicillenic acid.

CONCLUSIONS

The pre-column derivatization procedure described allows the rapid and selective determination of semi-synthetic and naturally produced penicillins in a variety of biological media, including microbial fermentation broths. Although the procedure appears inapplicable to β-lactams such as penicillin N, the possibility exists for detection of 6-amino penicillenic acid by its prior acylation.

The method may be used for the determination of known penicillins and also has potential value for the detection of novel penicillins, due to the specificity of the reagent.

ACKNOWLEDGEMENTS

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