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STUDIES ON RESIDUAL ANTIBACTERIALS IN FOODS

III*. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PENICILLIN G IN ANIMAL TISSUES USING AN ON-LINE PRE-COLUMN CONCENTRATION AND PURIFICATION SYSTEM

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SUMMARY

A simple and reproducible method for the determination of residual penicillin G in edible animal tissues by high-performance liquid chromatography (HPLC) is described. The method consists in an off-line clean-up step using a basic aluminium oxide column and a Sep-Pak C_{18} cartridge and an on-line pre-column concentration and purification system. The procedure shows good sensitivity and precision. The recoveries from cattle liver, kidney and muscle fortified with 1 μg/g of sodium penicillin G were 75.0–92.6% and the relative standard deviations were 2.35–4.06%. The detection limit corresponded to 0.05 μg/g of sodium penicillin G in animal tissues.

INTRODUCTION

Penicillin G is the most widely used antibiotic and continues to play an important role in human and veterinary medicine. Because of the deposition of residual penicillin G in edible animal tissues, a simple, sensitive and selective method for its determination has been particularly required.

For measuring residues of penicillin G in foods, microbiological assays are most often used as they are relatively sensitive, but these methods are difficult to quantify accurately, are time consuming and lack specificity.

A great variety of chemically based techniques for the analysis of penicillin G have been reported, including iodine titration[1], hydroxylamine colorimetry[2], gas chromatography[3,4], thin-layer chromatography[5,6] and high-performance liquid chromatography (HPLC)[7–12]. However, most of these methods were developed for clinical applications and are inadequate for determining trace levels of penicillin G in milk and animal tissues.

Recently, Moats[13] described an HPLC determination of penicillin G, penicillin

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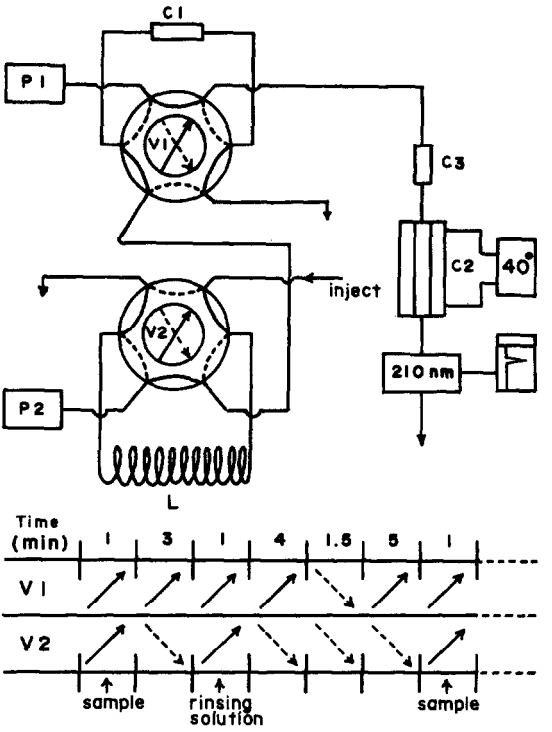


Fig. 1. Diagram of the chromatographic system and column switching programme. C1 (pre-column): LiChrosorb RP-18 (5 μm), 1 cm × 4.0 mm I.D. C2 (analytical column): LiChrosorb RP-18 (5 μm), 15 cm × 4.0 mm I.D. C3 (guard column): Permaphase ETH, 5 cm × 2.1 mm I.D. P1: pump 1. P2: pump 2. L: sample loop, 4 m × 0.8 mm I.D. Mobile phase 1: methanol–water–0.2 *M* phosphate buffer pH 5.0 (7:12:1). Mobile phase 2: methanol–water–0.2 *M* phosphate buffer pH 5.0 (1:18:1). Flow-rate 1:1.0 ml/min. Flow-rate 2: 1.5 ml/min. Rinsing solution: methanol–water–20% sodium chloride solution (9:27:4).

V and cloxacillin in milk. Although this method is sensitive, it is inapplicable to animal tissues because tissues such as liver, kidney and muscle are subject to higher background readings than milk.

The purpose of this study was to establish a rapid and selective method for the determination of penicillin G in animal tissues by combining a simple pre-treatment with the use of a Sep-Pak C_{18} cartridge and on-line pre-concentration and purification.

EXPERIMENTAL

Apparatus and reagents

The chromatographic system consists of two pumps, pump 1 (Uniflow 211, Jasco, Tokyo, Japan) and pump 2 (NSP-800-18, Nihon Seimitsu Kagaku, Tokyo, Japan), two six-port valves, valve 1 (Jasco VL611) equipped with a pre-column, 1 cm × 4.0 mm I.D., (Umetani, Osaka, Japan) packed by the balanced slurry technique with LiChrosorb RP-18 (5 μm) (E. Merck, Darmstadt, F.R.G.) and valve 2 (Kyowa Seimitsu, Tokyo, Japan) equipped with a 2-ml sample loop (4 m × 0.8 mm I.D.

stainless-steel tube), a UV detector (Jasco Uvidec 100 II) and a recorder (U-125M, Nippon Denshi, Kogaku, Kyoto, Japan).

Chromatography was performed on a 15 cm × 4.0 mm I.D. stainless-steel column (Umetani) packed by the balanced slurry technique with LiChrosorb RP-18 (5 μm). A 5 cm × 2.1 mm I.D. guard column was fitted in front of the analytical column and was tap-packed with Permaphase ETH (DuPont, Wilmington, DE, U.S.A.). The mobile phase for the pre-column methanol–water–0.2 *M* phosphate buffer, pH 5.0 (1:18:1) and that for the analytical column was methanol–water–0.2 *M* phosphate buffer, pH 5.0 (7:12:1). The rinsing solution for the pre-column was methanol–water–20% sodium chloride solution (9:27:4). The analytical column was water-jacketed for temperature control (40°C). The apparatus is shown in Fig. 1.

Sodium penicillin G (1650 U/mg) was obtained from Sigma (St. Louis, MO, U.S.A.), sodium tungstate from Katayama Chemical (Osaka, Japan) and methanol (HPLC grade) from Wako (Osaka, Japan).

The phosphate buffer was prepared from 0.2 *M* potassium dihydrogen phosphate by titration to pH 5.0 with 0.2 *M* sodium monohydrogen phosphate.

The basic aluminium oxide column was prepared by packing 18 g of basic aluminium oxide for column chromatography (Woelm Parma, Eschwege, F.R.G.) into a 30 cm × 15 mm I.D. glass column with water and washing with water until the eluate was clear.

A Sep-Pak C_{18} cartridge was purchased from Waters Assoc. (Milford, MA, U.S.A.).

All the water used was purified by the Mili-Q-Reagent-Grade water system (Millipore, Bedford, MA, U.S.A.).

Sample preparation

To an accurately weighed *ca.* 10-g amount of homogenized sample in a 100-ml glass-stoppered graduated cylinder were added 40 ml of water, 20 ml of 5% sodium tungstate solution and 20 ml of 0.33 *N* sulphuric acid. After vigorous mixing, the volume was adjusted to 100 ml with water. The mixture was transferred into a 100-ml centrifuge tube and centrifuged at 600 *g* for 10 min. The supernatant solution (50 ml) was poured into a basic aluminium oxide column and drained by gravity, then 20 ml of water were passed through the column. The eluates were combined and 20 ml of 20% sodium chloride solution was added to the solution. The mixture was poured into a Sep-Pak C_{18} cartridge at a rate of 2 ml/min, then the cartridge was washed with 10 ml of 2% sodium chloride solution and 10 ml of methanol–water–20% sodium chloride solution (3:15:2). The Sep-Pak C_{18} cartridge was attached to a 20-ml glass syringe and pre-conditioned with 20 ml of methanol, 20 ml of water and 2 ml of 2% sodium chloride solution prior to use. The cartridge was then attached to another glass syringe, penicillin G was eluted into a 20-ml volumetric flask with 19 ml of water and the volume was adjusted with 20% sodium chloride solution. Aliquots of the solution were subjected to HPLC.

Chromatographic procedure

Chromatography was carried out using the chromatographic system and the column switching programme shown in Fig. 1. Valves 1 and 2 were manually switched according to the column switching programme.

RESULTS AND DISCUSSION

Penicillin G was not retained to any extent from water on the LiChrosorb RP-18 reversed-phase column. However, the retention time was greatly increased when salts (phosphate buffer, sodium chloride, potasium chloride, etc.) were present in the mobile phase.

Fig. 2 shows the effect of the concentration of the phosphate buffer (pH 5.0) in the mobile phase on the capacity factor (k') of penicillin G. Sodium chloride and potassium chloride exerted similar effects in the elution of penicillin G (Fig. 3).

Using sodium tungstate and sulphuric acid as the precipitant for proteins, good deproteinization efficiency could be achieved. The recovery of penicillin G in the clear supernatant was essentially quantitative.

A basic aluminium oxide column eliminated successfully the yellow pigment in the sample extract, which otherwise might cause high background readings on the chromatogram. However, acidic and neutral aluminium oxide could not be used because penicillin G was adsorbed irreversibly to the column and could not be recovered completely.

The use of a Sep-Pak C_{18} cartridge was found to be the most appropriate approach for separating penicillin G before assay. The packing material of the cartridge is similar to that present in the analytical column. Accordingly, penicillin G was not retained on the cartridge from water but adsorbed strongly from aqueous solutions containing salts such as sodium chloride.

Figs. 4 and 5 show the effects of the sodium chloride and methanol concentration, respectively, in the rinsing solution for a Sep-Pak C_{18} cartridge on the peak height of penicillin G. They indicate that the sodium chloride concentration should be more than 0.5% and that of methanol should be less than 15%. Concequently, methanol–water–20% sodium chloride solution (3:15:2) was used to rinse the cartridge.

A linear relationship existed between the sample volume injected into a Sep-

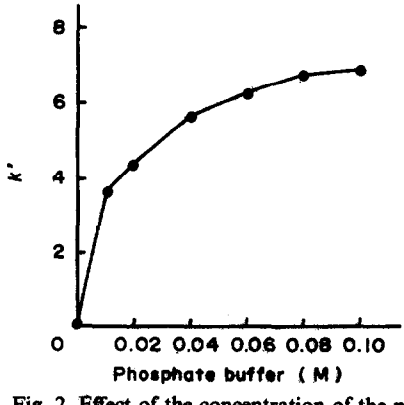


Fig. 2. Effect of the concentration of the phosphate buffer (pH 5.0) in the mobile phase on the capacity factor (k') of penicillin G.

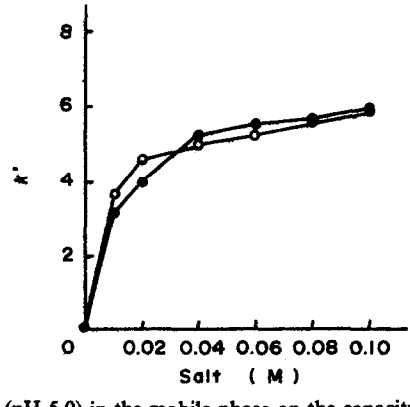
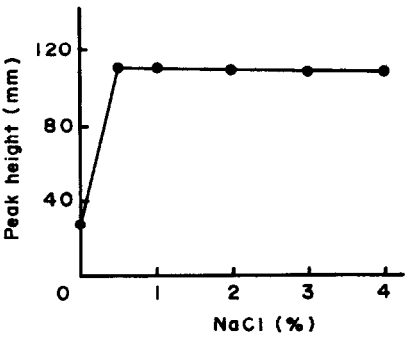


Fig. 3. Effect of the concentration of salts in the mobile phase on the capacity factor (k') of penicillin G. ●, Sodium chloride; ○, potassium chloride.



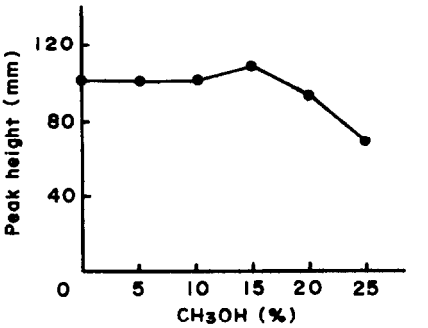


Fig. 4. Effect of sodium chloride concentration in the rinsing solution (15% methanol solution) for Sep-Pak C_{18} cartridge on the peak height of penicillin G.

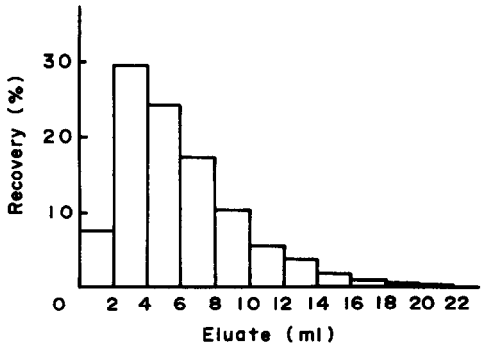
Fig. 5. Effect of methanol concentration in the rinsing solution (2% NaCl solution) for Sep-Pak C_{18} cartridge on the peak height of penicillin G.

Pak C_{18} cartridge and the peak height of penicillin G on the chromatogram, when the volume was less than 80 ml, as used in the present method.

Penicillin G could be easily eluted by a solution that was free from salts. Fig. 6 shows the elution pattern of penicilin G from a Sep-Pak C_{18} cartridge. Most of the penicilin G was eluted with 19 ml of water. The use of water as the eluting solution had several advantages over other solvents such as methanol. It eluted the interfering components with difficulty, and penicillin G was more stable in water than in methanol because penicillin is easily degraded by methanol and the corresponding alkyl-α-D-penicilloic acid is produced[14].

Because of the low absorbance in the UV region, the sensitivity was insufficient for determining trace levels of penicillin G when the effluent from the Sep-Pak C_{18} cartridge was subjected to HPLC with no further treatment. Although the sensitivity was closely related to the volume of sample injected on the analytical column, a large volume injection (over 200 μl) caused overloading of the column and provided high background readings on the chromatogram.

On-line pre-column concentration has become widely used in HPLC trace



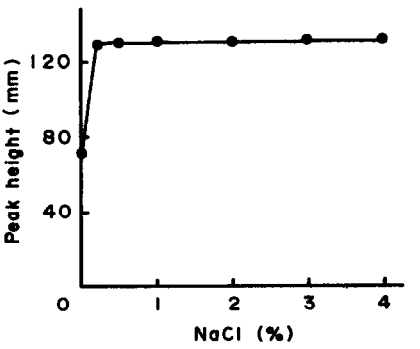


Fig. 6. Elution pattern of penicillin G from Sep-Pak C_{18} cartridge.

Fig. 7. Effect of the sodium chloride concentration in the sample solution on the peak height of penicillin G.

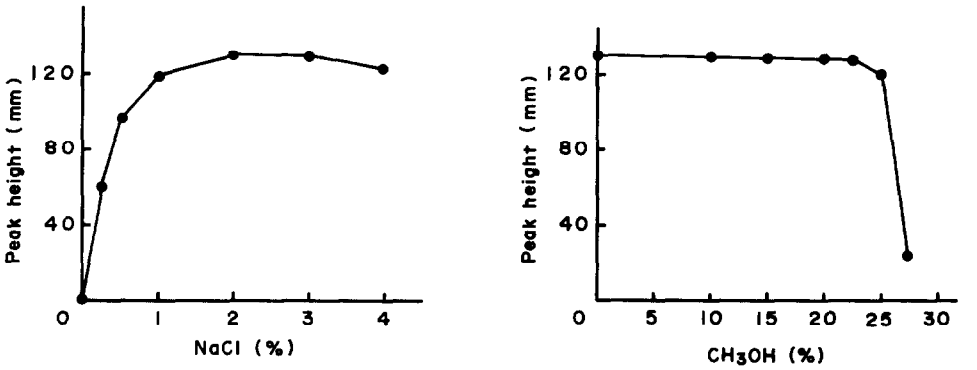


Fig. 8. Effect of the sodium chloride concentration in the rinsing solution (22.5% methanol solution) for the pre-column on the peak height of penicillin G.

Fig. 9. Effect of the methanol concentration in the rinsing solution (2% NaCl solution) for the pre-column on the peak height of penicillin G.

analysis[15–20]. In this technique, the compounds to be analysed have to be strongly adsorbed in the pre-column and require rapid desorption when the column is switched to transfer the pre-concentrated analyte on to the analytical column.

Optimum operating conditions for the pre-column concentration and purification system in HPLC could be chosen by reference to the investigation of Sep-Pak C_{18} cartridge clean-up step, because the pre-column used in the present method,

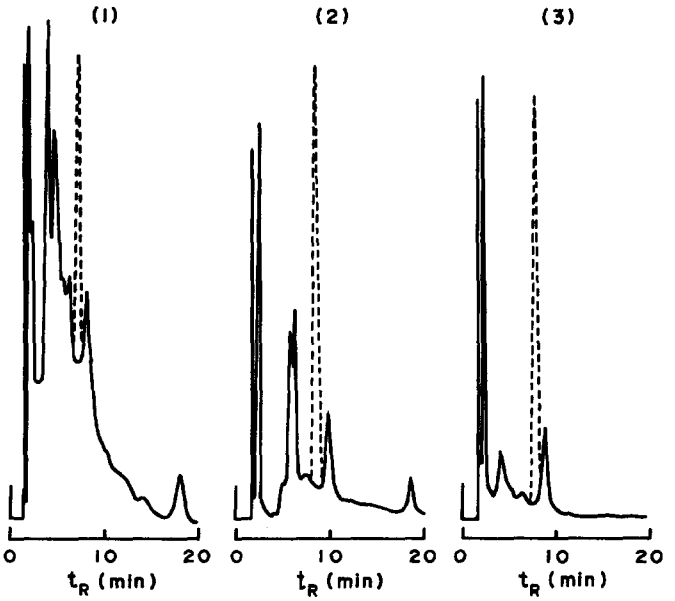


Fig. 10. Typical chromatograms obtained from (1) cattle liver, (2) kidney and (3) muscle by the overall procedure. Broken line: sample spiked with 1.0 μg/g of sodium penicillin G. Operating conditions as in Fig. 1.

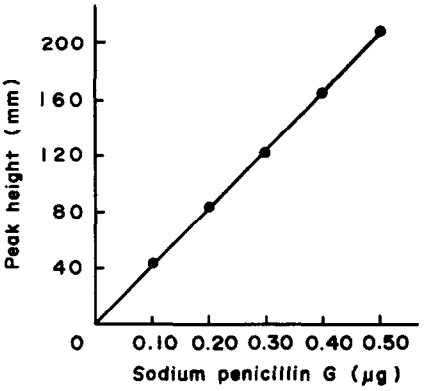


Fig. 11. Calibration graph for penicillin G.

which was packed with LiChrosorb RP-18 as in the analytical column, had similar properties to the Sep-Pak C_{18} cartridge.

Fig. 7 shows the effect of the sodium chloride concentration in the sample solution on the peak height of penicillin G and indicates that this concentration should be greater than 0.2% in order to ensure a constant peak height. Consequently, the effluent from the Sep-Pak C_{18} cartridge (*ca.* 19 ml) was adjusted to 20.0 ml with 20% sodium chloride solution.

Fig. 8 shows the effect of the sodium chloride concentration in the rinsing solution for the pre-column on the peak height of penicillin G. In the range 2.0–3.0%, the peak height was constant and maximum. Fig. 9 shows a similar effect of the methanol concentration. The background readings on the chromatogram decreased with increasing methanol content in the rinsing solution but above 22.5% the peak height decreased sharply because penicillin G leaked from the pre-column. On the basis of these results, methanol–water-20% sodium chloride solution (9:27:4) was used to rinse the pre-column.

The backflush mode was not used to elute the adsorbed material from the pre-column. The problem of band broading was solved by the use of a short (10 mm) and highly efficient column that was packed in a similar manner to the analytical column. The peak broadening is consequently of the same order of magnitude as that obtained with conventional 100-μl injections.

Fig. 10 shows typical chromatograms obtained from cattle liver, kidney and muscle fortified with sodium penicillin G.

TABLE I

RECOVERIES PENICILLIN G FROM CATTLE TISSUES

Sample	*Amount added (μg per 10 g)*	*Recovery* (%)*	*Coefficient of variation (%)*
Liver	10	75.0	2.35
Kidney	10	92.6	4.06
Muscle	10	91.0	3.09

* Average of five determinations.

Sample solutions containing sodium penicillin G were measured according to the procedure described and a linear relative response graph passing through the origin was obtained with sodium penicillin G at concentrations in the range 0.10–0.50 μg in 1.0 ml of solution (Fig. 11).

In recovery tests, the proposed method was applied to samples of cattle liver, kidney and muscle spiked with sodium penicillin G at a level of 1 μg/g. The recoveries and reproducibilities were determined by carring out five identical analyses and the results are given in Table I. The detection limit was 0.05 μg/g when 10 g of tissue sample were used and the detector sensitivity was 0.08 a.u.f.s.

CONCLUSIONS

The off-line pre-treatment with the use of a Sep-Pak C_{18} cartridge and the on-line concentration and purification procedure described here are a practical alternative to conventional preparation and concentration procedures based on extraction and evaporation. These techniques have several advantages over the conventional pre-treatment procijedures. They provide chromatographically cleaner extracts because of the partial separation, they are applicable to unstable substances that are liable to degrade on extraction and evaporation and they effect a significant time saving per pre-treatment because tedious preparations are unnecessary. The technique further permits the sensitive detection of analytes that have low UV absorption.

For these reasons, the present method is especially well suitable for determining trace levels of penicillin G residues in complex samples such as animal tissues.

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