Journal of Chromatography, 348 (1985) 379–387
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 095

STUDIES ON RESIDUAL ANTIBACTERIALS IN FOODS

IV*. SIMULTANEOUS DETERMINATION OF PENICILLIN G, PENICILLIN V AND AMPICILLIN IN MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received June 4th, 1985; revised manuscript received August 5th, 1985)

SUMMARY

A rapid and simple method for the simultaneous determination of penicillin G (PCG), penicillin V (PCV) and ampicillin (ABPC) in milk is described. The retention behaviour of these β-lactam antibiotics in reversed-phase liquid chromatography with mobile phases containing sodium alkylsulphonate was studied. Good separations were obtained with methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 11 m*M* sodium 1-heptanesulphonate and a LiChrosorb RP-18 column. The sample was pre-treated with a Sep-Pak C_{18} cartridge. The peaks corresponding to each β-lactam antibiotics can be confirmed with the treatment using penicillinase.

The recoveries from milk fortified with sodium PCG, potassium PCV and ABCP at levels of 0.5 and 0.1 μg/g each were generally better than 87% and the relative standard deviations were 1.17–4.98%. The detection limits corresponded to 0.03 μg/g of these β-lactam antibiotics in milk.

INTRODUCTION

β-Lactam antibiotics continue to be used in both human and veterinary medicine. In modern agricultural practice, however, frequent utilization of these antibiotics has led to problems with the spread of resistance factors and environmental pollution, and a simple, sensitive and selective method for the determination of residual β-lactam antibiotics in livestock products is therefore required.

Microbiological assays are mainly used for the determination of residual β-lactam antibiotics in foods as they are very sensitive. However, these methods require a long period of incubation, lack specificity and are difficult to quantify accurately.

Although numerous chemical methods[1–6] are available for the determination of β-lactam antibiotics, most of them are inadequate for determining trace levels in

* For Part III, see ref. 17.

0021-9673/85/$03.30

livestock products because they were developed for clinical applications. The detection of β-lactam antibiotics at residue levels requires much higher sensitivity and selectivity.

Recently, high-performance liquid chromatographic (HPLC) techniques for the analysis of β-lactam antibiotics have been developed[7–14] and applied also to the determination of residual antibiotics in foods[15–17]. In previous work, we established an HPLC method[17] for the determination of residual penicillin G (PCG) in animal tissues using an on-line concentration and purification system and successfully applied it to analyses of cattle liver, kidney and muscle. This method, however, is inapplicable to the simultaneous determination of other kinds of β-lactam antibiotics such as ampicillin (ABPC).

The purpose of this work was to investigate the retention behaviour of penicillin V (PCV), PCG and ABPC in a reversed-phase ion-pair HPLC system and to establish a rapid, sensitive and selective method for the simultaneous determination of these β-lactam antibiotics in milk.

EXPERIMENTAL

Apparatus

The HPLC equipment consisted of a Jasco (Tokyo, Japan) Uniflow 211 pump, a VL 611 variable-loop injector with a 100-μl sample loop, a Uvidec 100 II UV detector operating at 210 nm and a Nippon Denshi Kogaku (Kyoto, Japan) U-125M recorder.

Separations were carried out by using a stainless-steel column (15 cm × 4.3 m I.D.) (Umetani, Osaka, Japan) packed by the balanced slurry technique with Li-Chrosorb RP-18 (5 μm) (E. Merck, Darmstadt, F.R.G.). The column was encased in an acrylic jacket connected to a Yamato (Tokyo, Japan) BT-35 circulating water-bath to maintain the temperature at 45°C.

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.).

Reagents

Sodium PCG (1650 U/mg), potassium PCV (1560 U/mg) and ABPC were obtained from Sigma (St. Louis, MO, U.S.A.), penicilinase from Calbiochem (San Diego, CA, U.S.A.), sodium alkylsulphonates from Tokyo Kasei (Tokyo, Japan), 18-crown-6 ether from E. Merck and methanol (HPLC grade) from Wako (Osaka, Japan). A Sep-Pak C_{18} cartridge was purchased from Waters Assoc. (Milford, MA, U.S.A.).

The phosphate buffer was prepared from 0.2 *M* potassium dihydrogen phosphate by titration to the required pH with 0.2 *M* phosphoric acid or 0.2 *M* sodium monohydrogen phosphate.

All the water used was purified with a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.).

Chromatographic procedure

Mobile phases were prepared immediately before use by dissolving the calcu-

lated amount of sodium alkylsulphonate in methanol–water–0.2 *M* phosphate buffer. The operating conditions are given in the figure captions.

Sample preparation

A 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.

A milk sample was filtered through a glass-wool plug, then *ca.* 30 g of it was weighed accurately in a 50 ml beaker and poured into the Sep-Pak C_{18} cartridge at a rate of 2 ml/min. After all of the sample had been added to the cartridge, the beaker was rinsed with 10 ml of water. The rinsing solution was poured into the cartridge at a similar rate to above, then the cartridge was washed with 5 ml of water and 10 ml of methanol–water–20% sodium chloride solution (1:8:1) containing 20 m*M* 18-crown-6 ether. The cartridge was then attached to another glass syringe and the β-lactam antibiotics were eluted with 10 ml of 15% (v/v) methanol. Aliquots (100 μl) of the eluate were subjected to HPLC.

Quantitation and confirmation

Quantitation was carried out using calibration graphs obtained from a standard solution containing 15% (v/v) of methanol.

When the peaks coinciding with PCG, PCV and ABPC appeared on the chromatogram, a confirmation test using penicillinase was carried out in the following manner. An aqueous solution of penicillinase (1000 U/ml, 0.2 ml) was added to 5 ml of eluate from a Sep-Pak C_{18} cartridge and the mixture allowed to stand at room temperature for at least 5 min. Then the solution (100 μl) was subjected to HPLC again and the disappearance of each peak on the chromatogram was confirmed.

RESULT AND DISCUSSION

For chromatographic separations of β-lactam antibiotics, ion-exchange or reversed-phase chromatography are usually chosen. However, ion-exchange chromatography provides separations of low efficiency and reversed-phase chromatography is unsuitable for monobasic penicillins and more polar amphoteric penicillins simultaneously. Recently, ion-pair reagents and crown ethers have been employed to increase the retention time of polar β-lactam antibiotics.

Fig. 1 shows the relationship between the capacity factor (k') of PCG, PCV and ABPC and carbon number of sodium alkylsulphonate added to the mobile phase as an ion-pair reagent, and Fig. 2 shows the effect of the concentration of sodium 1-heptanesulphonate added as an ion-pair reagent on the k' of PCG, PCV and ABPC. The k' of ABPC increased with increasing carbon number and/or concentration of ion-pair reagent, whereas the k' of PCG and PCV decreased. These results suggest that alkylsulphonates exert opposite effects on the retention of β-lactam antibiotics, giving an increasing and a decreasing effect on k'. The former effect predominates in the retention of ABPC by ion-pair formation by the amino group of ABPC and the alkylsulphonates. The latter effect is observed in the retention of PCG and PCV, which have no amino groups in the structures, owing to competition between the β-lactam antibiotics and the alkylsulphonates in binding to the stationary phase.

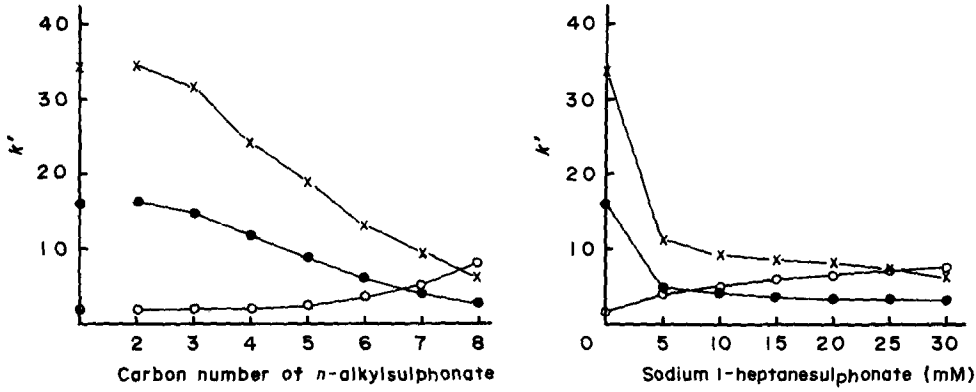


Fig. 1. Relationship between the k' of PCG, PCV and ABPC and carbon number of the sodium alkylsulphonate added to the mobile phase. Column, LiChrosorb RP-18 (5 μm) (15 cm × 4.3 mm I.D.); mobile phase, methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 10 m*M* sodium alkylsulphonate; flow-rate, 1.0 ml/min; column temperature, 45°C; detection, UV (210 nm). ●, PCG; ○, ABPC; ×, PCV.

Fig. 2. Effect of the concentration of sodium 1-heptanesulphonate on the k' of PCG, PCV and ABPC. Mobile phase, methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 0–30 m*M* sodium 1-heptanesulphonate. Other conditions as in Fig. 1.

Fig. 3 shows the effect of the pH of the phosphate buffer added to the mobile phase on the k' of PCG, PCV and ABPC. The k' of ABPC decreased with increasing pH, whereas at pH below 5 the k' of PCG and PCV decreased sharply with increasing the pH but at pH above 5 k' increased slightly, with a maximum at pH 7.

Fig. 4 shows the effect of the concentration of phosphate buffer (pH 4.0) added to the mobile phase on the k' of PCG, PCV and ABPC. The k' of PCG and PCV

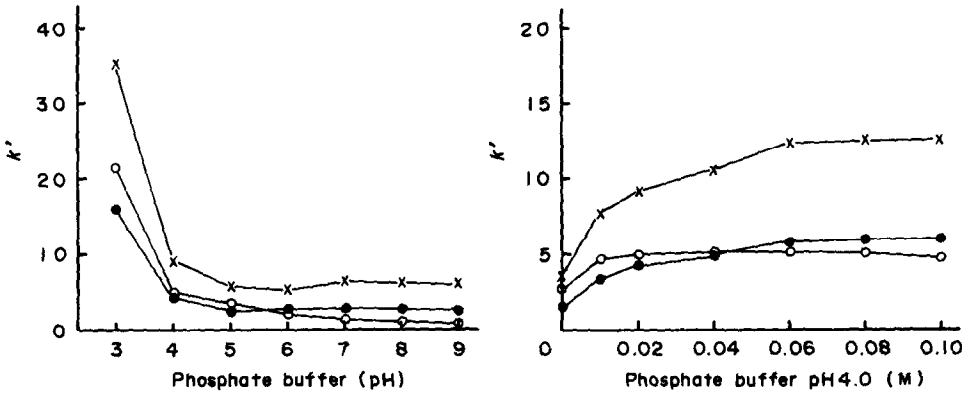
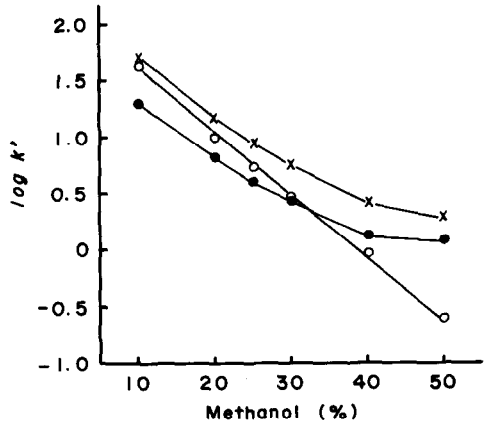


Fig. 3. Effect of the pH of the phosphate buffer added to the mobile phase on the k' of PCG, PCV and ABPC. Mobile phase, methanol–water–0.2 *M* phosphate buffer (pH 3.0–9.0) (5:13:2) containing 10 m*M* sodium 1-heptanesulfonate. Other conditions as in Fig. 1.

Fig. 4. Effect of the concentration of phosphate buffer (pH 4.0) on the k' of PCG, PCV and ABPC. Mobile phase, methanol (25%, v/v)–water–phosphate buffer (pH 4.0) containing 10 m*M* sodium 1-heptanesulphonate. Other conditions as in Fig. 1.



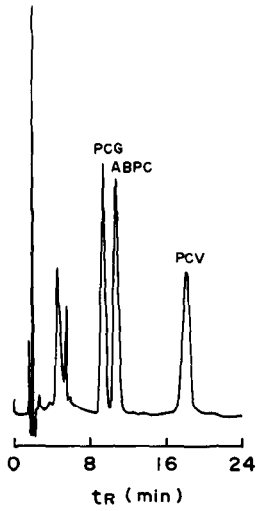
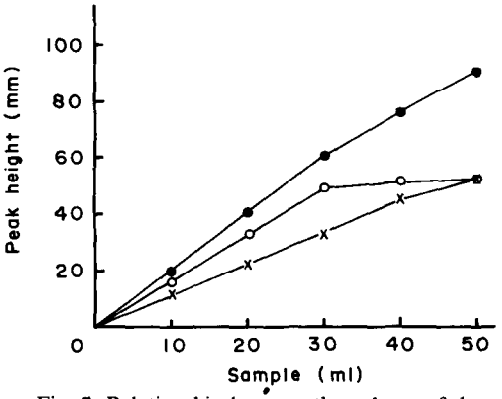


Fig. 5. Effect of the concentration of methanol on the k' of PCG, PCV and ABPC. Mobile phase, methanol–water containing 0.02 *M* phosphate buffer (pH 4.0) and 10 m*M* sodium 1-heptanesulphonate. Other conditions as in Fig. 1.

Fig. 6. Typical liquid chromatogram of PCG, PCV and ABPC. Mobile phase, methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 11 m*M* sodium 1-heptanesulphonate. Other conditions as in Fig. 1.

increased with increasing concentration, whereas the k' of amphoteric ABPC decreased slightly with increasing the concentration above 0.02 *M*.

Fig. 5 shows the effect of the concentration of methanol on the log k' of PCG, PCV and ABPC. Increasing methanol concentration caused them to elute earlier, but amphoteric ABPC was more affected than PCG and PCV and an approximately linear relationship existed between the log k' of ABPC and the methanol concentration.



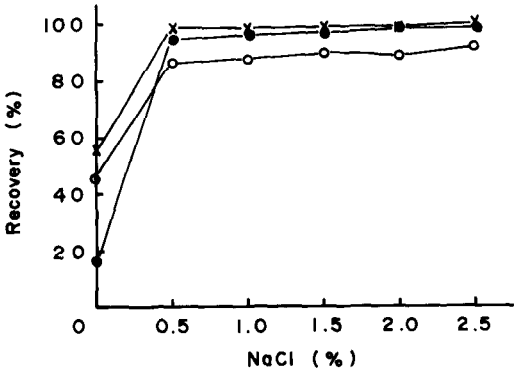


Fig. 7. Relationship between the volume of the sample poured into the Sep-Pak C_{18} cartridge and the peak height.

Fig. 8. Effect of sodium chloride concentration in the rinsing solution [containing 20 m*M* 18-crown-6 ether and 10% (v/v) methanol] for the Sep-Pak C_{18} cartridge on the recoveries of PCG, PCV and ABPC.

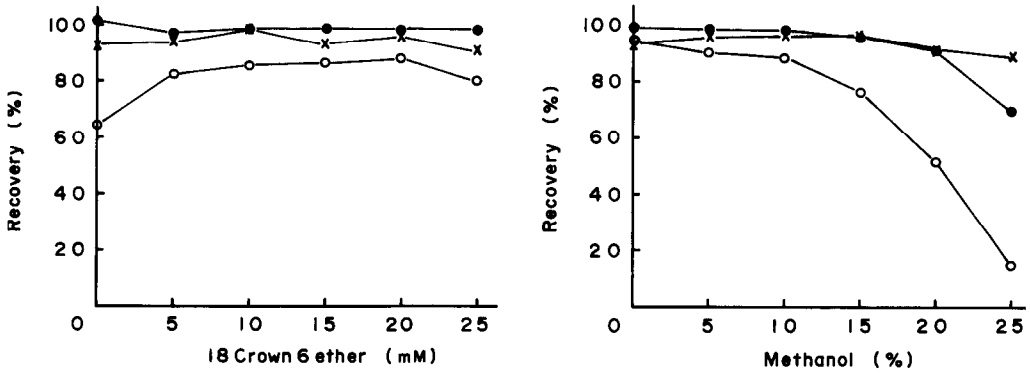


Fig. 9. Effect of 18-crown-6 ether concentration in the rinsing solution [containing 2% (w/v) sodium chloride and 10% (v/v) methanol] for the Sep-Pak C_{18} cartridge on the recoveries of PCG, PCV and ABPC.

Fig. 10. Effect of methanol concentration in the rinsing solution [containing 20 m*M* 18-crown-6 ether and 2% (w/v) sodium chloride] for the Sep-Pak C_{18} cartridge on the recoveries of PCG, PCV and ABPC.

On the basis of these results, methanol–water–0.2 *M* phosphate buffer (pH 4.0) (5:13:2) containing 11 m*M* sodium 1-heptanesulphonate was chosen as the mobile phase. Fig. 6 shows the typical chromatogram obtained from a standard mixture. A good separation was attained in only 20 min, even without gradient elution.

The use of a Sep-Pak C_{18} cartridge for the pre-treatment led to a rapid and effective sample preparation. PCG, PCV and ABPC were absorbed in the cartridge from a milk sample injected directly. Fig. 7 shows the relationship between the volume of sample poured into the Sep-Pak C_{18} cartridge and the peak height obtained with the overall procedure. It indicates that the volume of sample should be less than 30 ml to ensure stable recoveries of PCG and ABPC. Consequently, 30-ml volumes (about 30 g) of sample were used.

Optimal clean up conditions for the Sep-Pak C_{18} cartridge could be chosen by reference to the investigation of chromatographic behaviour in HPLC, because the packing material of the Sep-Pak C_{18} cartridge had similar properties to those of the analytical column.

Figs. 8–10 show the effect of sodium chloride, 18-crown-6 ether and methanol concentration, respectively, in the rinsing solution for a Sep-Pak C_{18} cartridge on the recoveries of the β-lactam antibiotics. PCG and PCV showed good recoveries independent of the concentration of 18-crown-6 ether when the sodium chloride concentration was above 0.5%, but the recovery of ABPC fell to 64% when 18-crown-6 ether was not added to the rinsing solution. Nakagawa *et al.*[18] reported that the addition of crown ethers to the mobile phase in reversed-phase HPLC enhanced the k' of β-lactam antibiotics that had primary amino groups in the structure. Therefore, the absorption efficiency with respect to ABPC was enhanced by the addition of 18-crown-6 ether.

The background readings on the chromatogram decreased with increasing methanol content in the rinsing solution, but above 15% for ABPC and 20% for PCV the peak height decreased sharply. On the basis of these results, methanol–

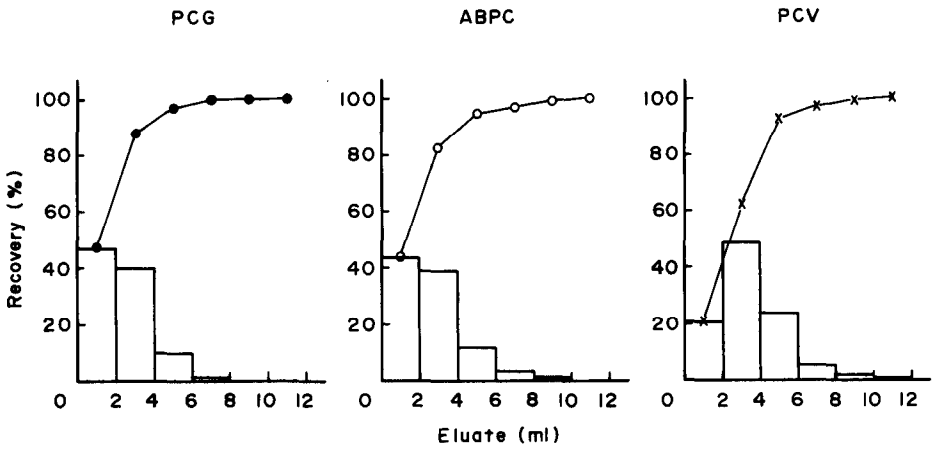


Fig. 11. Elution pattern of PCG, PCV and ABPC from the Sep-Pak C_{18} cartridge.

water–20% sodium chloride solution (1:8:1) containing 20 m*M* 18-crown-6 ether was used to rinse the Sep-Pak C_{18} cartridge.

Fig. 11 shows the elution pattern of the β-lactam antibiotics from a Sep-Pak C_{18} cartridge. Most part of the PCG, PCV and ABPC were eluted with 10 ml of 15% (v/v) methanol.

In a recovery test, the proposed method was applied to milk samples spiked with sodium PCG, potassium PCV and ABPC at levels of 0.5 and 0.1 μg/g. The reproducibility was determined by carrying out five identical analyses. The results are summarized in Table I.

TABLE I

RECOVERIES OF PCG, PCV AND ABPC IN MILK

Added (μg/g)	*PCG*		*PCV*		*ABPC*	
	Av. (%)*	*CV** (%)*	*Av.* (%)*	*CV** (%)*	*Av.* (%)*	*CV** (%)*
0.5	98.4	4.34	95.9	3.41	88.0	3.59
0.1	101.1	1.17	97.7	3.17	87.0	4.98

* Average of five determinations.
** Coefficient of variation.

Fig. 12 shows the chromatograms obtained in the recovery experiment (a) and from the sample solution treated with penicillinase (b). The peaks corresponding to PCG, PCV and ABPC disappeared. The detection limit of this method was 0.03 μg/g for each β-lactam antibiotic.

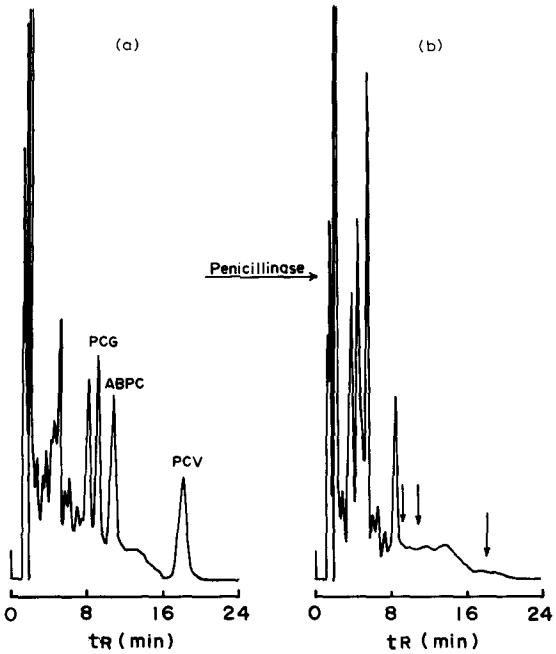


Fig. 12. Liquid chromatograms obtained from a milk sample by the overall procedure (a) and the sample solution treated with penicillinase (b). Sample spiked with 0.5 μg/g each of sodium PCG, potassium PCV and ABPC. Operating conditions as in Fig. 6.

CONCLUSION

A method for the HPLC determination of PCG, PCV and ABPC has been developed that has several advantages. The addition of sodium 1-heptanesulphonate to the mobile phase gives good separations, as PCG, PCV and ABPC can be completely separated in only 20 min without gradient elution. The use of a Sep-Pak C_{18} cartridge makes it possible to pre-treat the sample rapidly. It is applicable to unstable substances such as β-lactam antibiotics, which are liable to degrade on extraction and evaporation. The peaks corresponding to each β-lactam antibiotics are easily confirmed by treatment with penicillinase.

The proposed method, therefore, should be applicable to routine determinations of residues of β-lactam antibiotics in milk.

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