Journal of Chromatography, 507 (1990) 177–185
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1714

Determination of penicillin G in milk by high-performance liquid chromatography with automated liquid chromatographic cleanup

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SUMMARY

Specific confirmatory tests are needed to identify and quantify β-lactam antibiotic residues detected in milk at levels of < 10 parts per 10^9 (ppb) by screening tests. A liquid chromatographic method for penicillin G was developed using the liquid chromatography system for cleanup as well as analysis. Milk was deproteinized with two volumes of acetonitrile. The acetonitrile was extracted with hexane–methylene chloride (1:1) and the remaining water layer was concentrated by evaporation. The water layer (2 ml = 5 ml milk) was injected onto a Polymer Laboratories PLRP-S column using a WISP autosampler with the solvent, 0.01 *M* pH 7.0 phosphate buffer (A). Penicillin G was eluted with acetonitrile (B) gradient A–B (100:0) (0–3 min)–(40:60) (25 min). Penicillin G eluted as a narrow band in < 0.5 min. A narrow fraction containing penicillin G was collected and rechromatographed on the same type of column at low pH (1.96). This effectively separated penicillin G from interferences. Recoveries were 92 ± 9% with a sensitivity limit near 2 ppb. The approach used is applicable to determination of other β-lactam antibiotics but specific conditions for analysis must be determined for each one. The cleanup procedure can be automated using an autosampler, gradient controller, and fraction collector.

INTRODUCTION

A number of sensitive screening tests have been described for detection of β-lactam antibiotic residues in milk. These include microbiological tests[1], immunoassays[2,3], competitive binding[1,4,5], and enzyme inhibition (Penzym)[6]. These are all capable of detecting residues at levels of 10 ppb[a] or less. With he possible exception of immunoassays, none of the screening procedures can distinguish β-lactam antibiotics from one another. False positive tests may occur. Specific physico-chemical confirmatory tests for β-lactam antibiotics are needed for identification and quantitation of

[a] Throughout this article, the American billion (10^9) is meant.

suspect residues. However, development of methods of adequate sensitivity has proven difficult. The author described a procedure suitable for determination of β-lactam antibiotics with neutral side-chains at about 5–10 ppb in milk which used a partitioning cleanup with UV detection[7]. Meetschen and Petz[8] described a method using gas–liquid chromatography sensitive to < 1 ppb for β-lactams with neutral side-chains which required a lengthy partitioning cleanup and derivatization. Wiese and Martin[9] described a high-performance liquid chromatography (HPLC) procedure for penicillin G in milk sensitive to < 1 ppb which used electronic subtraction of chromatograms before and after treatment with β-lactamase. This procedure also used a partitioning cleanup and derivatization and required very precise reproducibility of chromatograms. Other published chromatographic methods do not achieve the required sensitivity[10–12].

Many β-lactam antibiotics cannot be partitioned between buffers and organic solvents. Studies in our laboratory have demonstrated that analytes can be concentrated directly on an analytical column from filtrates and eluted with a solvent gradient. This approach was used successfully for determination of novobiocin[13], virginiamycin[14] and tetracyclines[15]. If too much interference is present for direct determination, a narrow fraction containing the analyte of interest can be taken, using a procedure sometimes termed "heart-cutting"[16], and rechromatographed under different conditions. The application of this approach to determination of penicillin G in milk is described in the present paper. The approach is applicable in principle to other β-lactam antibiotics and has been successfully used for determination of lincomycin residues in milk and tissue[17].

EXPERIMENTAL[a]

Chemicals and reagents

Acetonitrile, HPLC grade, other chemicals, analytical-reagent grade. The sodium salt of penicillin G was obtained from Sigma (St. Louis, MO, U.S.A.). A stock solution of 1 mg/ml of sodium penicillin G was prepared fresh weekly in deionized water. Working solutions of 10, 5, and 1 μg/ml were prepared by diluting the stock solution with deionized water or 0.01 *M* pH 7.0 buffer as required.

Apparatus

Glassware required included 125-ml conical flasks, 50-ml graduated cylinders, 250-ml separatory funnels with PTFE stopcocks, 250-ml glass-stoppered side-arm flasks, 75-mm funnels, and 15-ml graduated conical centrifuge tubes. All glassware was cleaned by soaking overnight at room temperature or a few minutes at 50–70°C in special detergent (Micro International Products, Trenton, NJ, U.S.A.). The glassware was rinsed in deionized water, then in *ca.* 0.01 *M* HCl and then in deionized water again.

The Waters chromatographic system consisted of an automatic gradient controller, two Model 510 pumps, a WISP autosampler with a 2000-μl loop and either a Model 481 UV detector or a Model 990 diode array detector (Waters, Milford, MA,

[a] Mention of specific items or trade names is for identification purposes only and does not imply endorsement by the U.S. Department of Agriculture over similar items not specifically mentioned.

U.S.A.) with an ISCO (Lincoln, NE, U.S.A.) FOXY fraction collector. A Varian system (Varian, Sugarland, TX, U.S.A.) consisting of an LC-5000 chromatograph, a 9090 autosampler and a Waters Model 481 UV detector was used for analysis.

Chromatographic columns used were, with matching guard cartridges a Supelcosil LC-18-DB, 150 × 4.6 mm, 5 μm particle size (Supelco, Bellefonte, PA, U.S.A.), and a Polymer Labs (Amherst, MA, U.S.A.) PLRP-S column, 150 × 4.6 mm, 5 μm particle size, 100 Å pore diameter.

A Buchler Rotary Evapomix® (Buchler, Ft. Lee, NJ, U.S.A.) was used to evaporate samples under reduced pressure in centrifuge tubes.

Extraction and cleanup procedures

A 20-ml volume of milk was measured into a 125-ml conical flask and 40 ml of acetonitrile was added slowly with vigorous swirling. After standing for 5 min, the clear suprenatant was decanted through a plug of glass wool in the stem of a funnel and 30 ml filtrate collected. The filtrate was transferred to a separatory funnel, 30 ml methylene chloride and 30 ml hexane or light petroleum (b.p. 30–60°C) were added and the mixture was allowed to separate 5 min. The water layer was collected in a 250-ml side-arm flask. The organic layer was washed with 5 ml water. The combined water layers were evaporated under reduced pressure in the side-arm flask in a 40–50°C water bath to 1–2 ml. The residue was rinsed into a 15-ml graduated centrifuge tube with small (0.5 ml) portions of water. The sample solution was diluted to 4 ml and filtered through a very small plug of glass wool in the stem of a funnel to remove any coarse particles. Slight turbidity if present was not a problem. The filtrate was transferred to 4-ml autosampler vials.

HPLC cleanup

Sample and standards were loaded into 4-ml vials in the WISP. The column was equilibrated with 0.01 *M* pH 7.0 phosphate buffer, flow-rate 1 ml/min. The autosampler automatically started the gradient and the fraction collector. A gradient program of buffer (A)–acetonitrile (B), (100:0) (0–3 min)–(40:60) (25–30 min)–(100:0) (31 min) was used. Loading of the next sample was started 40 min after injection of the previous sample. Two 2-μg standards were injected first, one in 200 μl and one in 2000 μl, to establish the retention time and to make sure that the operation was stable. Fractions were collected in 15-ml conical graduated centrifuge tubes (calibrated in the 0.5–1 ml range). The fraction collector was set to collect a 1.2-min (1.2-ml) fraction centered on the retention time of penicillin G with 0.1 min delay. The gradient controller was programmed to flush the column and shut itself off after elution of the last sample was completed. The fraction was concentrated to $\leq$0.5 ml in the Buchler Rotary Evapomix under reduced pressure and the volume was adjusted to 0.5 ml and transferred to autosampler tubes (1 ml inserts for the WISP).

HPLC analysis

Analysis was done under isocratic conditions using a Polymer Labs, PLRP-S column identical to that used for cleanup, flow-rate 1 ml/min, solvent 0.01 *M* pH 1.96 phosphate buffer–acetonitrile (66:34) using the Varian system. The injection volume was 100 μl (1 ml original milk). Detection was at 200 or 210 nm using either a UV or diode array detector (Waters 990). The response was linear at least to 1 μg. Standards

of 0.1, 0.4 and 1 μg were run with each set of samples to correct for minor changes in response.

Spiked samples and confirmation with penicillinase

An appropriate amount of penicillin G solution was added to the milk prior to carrying out the extraction procedure. For confirmation, 0.2 ml of penase concentrate Bacto (Difco Labs., Detroit, MI, U.S.A.) was added to 20 ml of milk and allowed to stand 15 min before carrying out the procedure.

RESULTS AND DISCUSSION

For the present procedure, the approach adopted was to determine penicillin G by UV absorption thus avoiding lengthy derivatization procedures. Sensitivity was improved over the previous procedure[7] by using absorption at 200 or 210 nm rather than at 220 nm (Fig. 1). Baselines were less noisy at 210 nm. In general, 5–10 ng were required for adequate quantitation although less could be detected. For quantitation at 5–10 ppb, it was therefore necessary to inject the equivalent of 1 ml of milk. This required considerable concentration and cleanup. The practical limit of cleanup which could be achieved by partitioning between organic solvents and buffers was achieved in a procedure described previously[7].

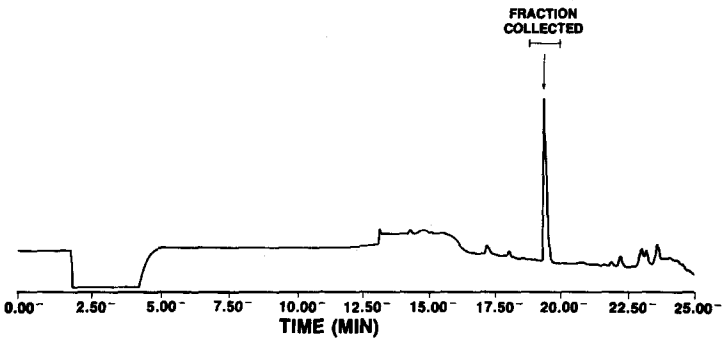

Fig. 1. Penicillin G, 2-μg standard, in 2000 μl, gradient elution, 0.01 *M* pH 7.0 phosphate buffer (A)–acetonitrile (B), 100:0 (0–3 min)–40:60 (25 min); flow, 1 ml/min, detection, UV at 200 nm; Polymer Labs. PLRP-S column, 150 × 4.6 mm, 5-μm packing, 100 Å pore size.

Our previous studies[7] demonstrated that extraction/deproteinization of milk with two volumes of acetonitrile was rapid and gave essentially quantitative recoveries of penicillins in the filtrate. Since the concentration in the filtrate was the same as in the mixture before filtration, an aliquot of the filtrate could be taken as representative. Therefore, no tedious washing of precipitates was required. We explored concentration and cleanup approaches using solid-phase extraction on short laboratory-packed colums and also pre-packed absorbent cartridges widely sold for cleanup. Our labaratory-packed columns somtimes worked well but we were unable to reproduce our results with different lots of the same absorbent. This approach could therefore not

be recommended. The results with pre-packed absorbent cartridges were scarcely more promising. Some disadvantages of the pre-packed cartridges were:

(1) They usually required tedious prewashing with solvents to "activate" them.

(2) Contaminants were frequently eluted from the cartridges.

(3) Analytes either were not fully retained or did not elute sharply. This is well illustrated by data of Terada *et al.*[12] and Terada and Sakabe[11] who found that 10–20 ml of eluent were required to recover penicillins from Sep-Pak™ C_{18} cartridges.

We therefore concluded that a more rigorous approach using an analytical HPLC column would be required to achieve the concentration and rigorous fractionation necessary for determination at <10 ppb.

If the analyte is immobile under the chromatographic conditions used to load it on the column, then the shape of the peak obtained by subsequent gradient elution is not affected by the volume in which the sample is injected. Penicillin G is immobile on reversed-phase packings when injected in water at pH 7 but is readily eluted by acetonitrile. For concentration by solid-phase extraction, it was therefore first necessary to get rid of the organic solvents from the filtrates prepared with acetonitrile. The water layer was separated by adding methylene chloride and petroleum ether to the filtrate. The penicillins were essentially quantitatively recovered in the water layer. This layer was concentrated to <4 ml under reduced pressure and diluted to 4 ml prior to concentration by solid-phase extraction and fraction collection. When penicillin G is loaded on the column in water and eluted with a gradient, the peak height and shape are not affected by the volume of solvent in which the sample is injected.

The Waters WISP autosampler with a 2-ml loop will inject a larger amount of sample than other available autosamplers. However, it took 27 min to load the loop. Injection of an even larger amount of more dilute sample would be preferable. As it is, the sample extract must be concentrated considerably by evaporation in order to load the desired amount onto the analytical column. A polymeric HPLC column (Polymer Labs., PLRP-S), 5 μm particle size, was used with the pH 7 buffer. We found that the polymeric columns were more stable than silica-based reversed-phase columns in the pH range 7–8. Column efficiencies of the PLRP-S columns closely approached those of bonded silica packings of comparable particle size. We found that at least under our conditions the polymeric packing was very unstable above pH 8. The columns developed excessive back pressures indicating that the packing swelled. This could result in permanent damage to the packing even after the column was flushed with buffer of lower pH. This is contrary to the manufacturer's claims that these packings are stable to pH 13. Penicillins eluted as a sharp band (Fig. 1) in <0.5 ml (0.5 min). However, a slightly wider (1.2 ml) fraction was collected to allow for slight variation in retention in successive runs. Fig. 2 shows the chromatogram of a milk sample containing 1 ppm of penicillin G using the fraction collection procedure. The penicillin peak is visible at this concentration.

Use of the HPLC system for cleanup offers a number of advantages over the use of cartridges:

(1) Reproducibility —since the same column was used repeatedly, results were reproducible and were not dependent on the quality control of the manufacturer.

(2) No special washing or activation was required since the column was flushed and reequilibrated between each run.

(3) Fractionation was sharper. Analytes were usually completely recovered in 0.2–0.5 ml.

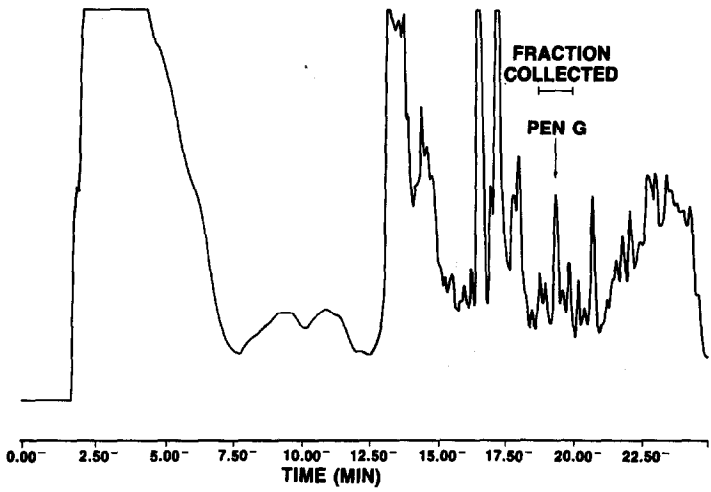

Fig. 2. Milk filtrate, 5 ml equiv. concentrated to 2 ml, 1 ppm penicillin (PEN) G. Conditions as in Fig. 1.

(4) The system could be automated with standard HPLC equipment.

The cleanup required about an hour per sample but was fully automated. Samples were loaded into the autosampler in the afternoon and the machine was run overnight. pH 7-Buffer was used because penicillin G is most stable at that pH and showed little deterioration during holding of samples prior to final analysis.

For analysis, the fraction collected was rechromatographed under conditions which would separate penicillin G from interferences in the fraction. There are several ways of changing the retention of an analyte both absolutely and relative to other compounds:

(1) Change the concentration of organic modifier. This usually does not change the relative retentions very much.

(2) Change the organic modifier. In the present case, only acetonitrile could be used with UV determination at 200 nm.

(3) Add ion-pairs such as quaternary ammonium compounds or alkyl sulfonates.

(4) Change the pH to convert the compound from the salt (ionized) to acid (non-ionized) form. This produce a much larger change in retention on a reversed-phase column than ion-pairing.

(5) Use a different chromatographic mode such as ion-exchange or normal-phase chromatography on silica.

Table I shows the effect of pH and ion-pairs (tetraethylammonium and heptanesulfonate) on retention of penicillin G on polymeric and bonded reversed-phase packings of comparable particle size. An identical solvent gradient was used in all cases to facilitate comparison. At pH 7.0 and 4.6, addition of tetraethylammonium chloride increased retention of penicillin G. The tetraethylammonium chloride had little effect in 0.01 *M* orthophosphoric acid as would be expected. The acid form of penicillin G was much more strongly retained than the salt form and this is the basis of the separation used in the present procedure. The heptanesulfonate anion interfered

TABLE I

EFFECT OF pH, ION-PAIR AND COLUMN PACKING ON RETENTION OF PENICILLIN G

Gradient elution: buffer (A)–acetonitrile (B), 100:0 (0–3 min)–40:60 (25 min). Column packings 5 μm particle size, columns 150 × 4.6 mm. SHS = Sodium heptane sulfonate.

Buffer	*Retention time (min)*	
	Column type	
	Styrene–divinylbenzene (Polymer Labs. PLRP-S)	*Bonded C_{18} (Supelco LC-18-DB)*
0.01 *M* Phosphate (pH 7.0)		
Buffer only	18.97	19.80
0.005 *M* $(C_2H_5)_4NCl$	19.47	20.15
0.005 *M* SHS	16.97	17.13
0.01 *M* $NH_4H_2PO_4$ (pH 4.6)		
Buffer only	19.30	19.98
0.005 *M* $(C_2H_5)_4NCl$	19.86	20.69
0.005 *M* SHS	20.78	21.42
0.01 *M* H_3PO_4 (pH 1.6)		
Buffer only	24.53	25.25
0.005 *M* $(C_2H_5)_4NCl$	24.52	25.21
0.005 *M* SHS	21.48	21.47

with retention of the anion of penicillin G at pH 7 but not at pH 4.6. Retention was also reduced at low pH relative to buffer alone.

The fractions collected at pH 7.0 were rechromatographed in acid buffers under isocratic conditions. The best separations from interferences were obtained with pH 1.96 buffer with a mobile phase composition buffer–acetonitrile (72:28). Better separations were obtained if the same type of column was used for both fraction collection and analysis. Both bonded and polymeric columns were stable at least to the pH of 0.01 *M* orthophosphoric acid. Although penicillin G is unstable at low pH, there was no evidence of decomposition during the analysis procedure or even when longer gradients were used with 0.01 *M* orthophosphoric acid. No suitable internal standard is known for this procedure because of the rigorous fractionation used. In practice use of an external standard in the same sample series is adequate to correct for any variation in instrument performance.

Fig. 3 shows a milk blank and milk spiked with 10 ppb and 100 ppb penicillin G. Penicillin was readily quantitated at 10 ppb. There is little noise in the baseline at the sensitivity used. Table II shows recoveries which averaged 92% at three levels of spiking. Results at 10 ppb were less precise as would be expected. The detection limit at which the penicillin peak can be clearly detected usually above baseline noise is about 2 ppb, comparable to the most sensitive screening procedures.

The procedure is intrinsically simple although not particularly fast, requiring 4–5 h for an individual sample. For multiple samples run, add 1 h per sample since cleanup must be done sequentially. In practice, we ran the chromatographic cleanup unattended overnight so that the time factor was irrelevant.

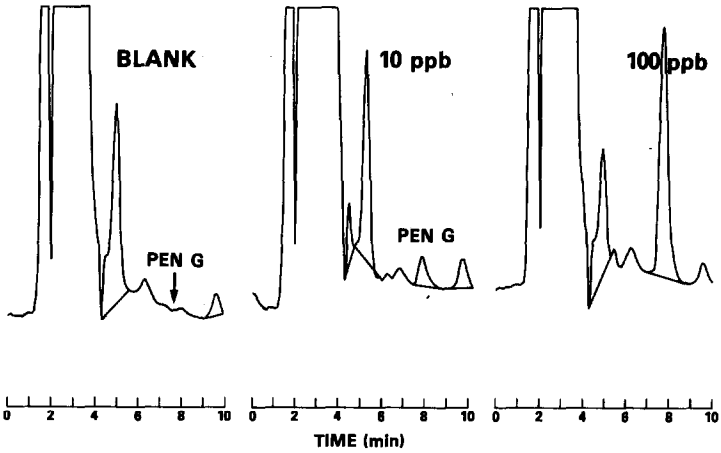

Fig. 3. Analysis of penicillin G, 100 μl injected = 1 ml milk. Blank, 10 ppb and 100 ppb. Isocratic elution, 0.01 *M* pH 1.96 phospate buffer–acetonitrile (66:34). PLRP-S column; detection, UV at 210 nm. Arrow indicates retention time of penicillin G.

TABLE II

RECOVERIES OF PENICILLIN G FROM MILK

Amount added (ppb)	*Found (ppb)*			
	1	*2*	*3*	*Mean ± S.D.*
10	8.9	7.8	11	9.2 ± 1.4
100	98	93	86	92 ± 5
1000	940	960	850	920 ± 48
Mean recovery from all (%)				92 ± 9
Milk from treated cow (32 h)	93	109		

Since the penicillin G was well isolated from interfering peaks in the blank milk, the presence of a peak with the retention time of penicillin G gave a presumptive test for its presence and provided good quantitation. The absence of any peak clearly established that penicillin G was not present above the sensitivity limits of the procedure. Further confirmation of the penicillin G may be based on the UV spectrum obtaind by using a diode array detector and/or by repeating the analysis after treating the sample with penicillinase.

The general approach of using the LC system for cleanup should be applicable to determination of low levels of other β-lactam antibiotics or other residues where rigorous cleanup is required. Conditions for the cleanup and analysis steps which give good separation of analytes from interferences must be established. The approach can be automated to considerable extent.

ACKNOWLEDGEMENTS

The author thanks Miau Huang for technical assistance and Mike Thomas, FDA/CVM, Beltsville, MD, U.S.A., for providing milk samples from treated cows.

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