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High-performance liquid chromatographic determination of penicillin G, penicillin V and cloxacillin in beef and pork tissues

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ABSTRACT

The objective was to develop confirmatory high-performance liquid chromatographic methods for penicillin residues in animal tissues with detection limits of $\leqslant 10$ ng/g. A previously described procedure was modified by using a larger sample size and isocratic analysis. Tissues (15 g) were blended with 45 ml of water and 20 ml of homogenate were mixed with 40 ml acetonitrile and filtered. The filtrate (30 ml) was mixed with 10 ml of 0.2 M H_3PO_4 and extracted with methylene chloride. The combined methylenc chloride layers were mixed with acetonitrile and hexane, washed with two 4-ml portions of water and then extracted with four 1-ml portions of 0.01 M phosphate buffer (pH 7). The combined buffer extracts were concentrated to 1 ml under reduced pressure. Analysis was isocratic during 0.01 M phosphate buffer (pH 7)–acetonitrile with proportions 85:15 (penicillin G), 82:18 (penicillin V) or 78:22 (cloxacillin). A polystyrene–divinylbenzene copolymer column, 150 × 4.6 mm I.D. (Polymer Labs. PLRP-S), was used with a flow-rate of 1 ml/min and detection at 210 nm. The presence of penicillins was confirmed by treating a duplicate sample with penicillinase. Recoveries were >90% in most instances. Detection limits were 5 ng/g in muscle and higher in liver and kidney. The procedure is a simple and sensitive method for confirming the presence of penicillins in animal tissues.

INTRODUCTION

Penicillins, especially penicillin G, are widely used in food animal production, both therapeutically and as growth promotants in feeds. Although penicillins are relatively non-toxic, residual amounts in foods have caused allergic reactions in sensitive individuals [1].

Although penicillins can be detected in tissues by microbiological assays, they cannot be distinguished from one another. Some progress has been made in the development of specific chromatographic methods for the determination of penicillin G and other β-lactam antibiotics [2–7] in tissues. However, the development of chromatographic methods of adequate sensitivity has been difficult and it is only recently that methods capable of detecting <10 ng/g of penicillin residues in tissues have been reported. Meetschen and Petz [7] described a sensitive gas chromatographic method capable of determining β-lactam antibiotics with neutral side-chains at levels of $\leqslant 3$ ng/g in a variety of substrates. Their procedure requires lengthy clean-up and derivatization. Boison *et al.* [2] recently described a high-performance liquid chromatographic (HPLC) method for penicillin G sensitive to 5 ng/g in tissues using tungstic acid precipitation, solid-phase extraction and derivatization.

The method described in this paper is a modification of that described previously [3], using a simple partitioning clean-up. By use of larger samples, a different type of HPLC column and isocratic elution at neutral pH, the sensitivity was increased about tenfold. The procedure is simpler than other reported procedures in that no derivatization is required. The use of the procedure with three penicillins with neutral side-chains is described.

EXPERIMENTAL

Chemicals and materials

Acetonitrile, hexane and methylene chloride were of Omnisolv grade (EM Science, Gibbstown, NJ, USA) or equivalent. The penicillins were obtained from Sigma (St. Louis, MO, USA), and used as received. Stock solution of 1 mg/ml were prepared in distilled water and diluted as appropriate. Stock solutions of penicillin G were prepared fresh weekly and others were prepared biweekly and stored refrigerated. Other chemicals were of analytical-reagent grade from various sources.

To prepare 0.2 *M* H_3PO_4, 13.6 ml of concentrated phosphoric acid was diluted to 1 l. To prepare 0.01 *M* buffer of pH 7, 1.36 g of KH_2PO_4 and 2.84 g of Na_2HPO_4 were dissolved in 3 l of water. The β-lactamase preparation used was Bacto penase concentrate obtained from Difco Labs. (Detroit, MI, USA).

Final filtration was done with 13-mm Acrodisc LCPVDF filter cartridges, 0.45-μm pore size, obtained from Gelman Sciences (Ann Arbor, MI, USA).

Sample preparation procedure

Tissue (15 g) was blended with 45 ml (60 ml for liver and kidney) of water in 300-or 500-ml blender jars for 2 min at half full-power (or less to reduce foaming) as controlled by a variable-resistance transformer. A 20-ml aliquot of the homogenate was measured into a 125-ml conical flask and mixed with 40 ml of acetonitrile. After 5 min, the supernatant was decanted through a plug of glass-wool in the stem of a funnel and 30 ml (= 2.5 g of muscle or 2.0 of liver and kidney) of filtrate were collected. The filtrate was transferred to a separating funnel and 10 ml of 0.2 *M* H_3PO_4 and 20 ml of methylene chloride were added with vigorous shaking. The methylene chloride layer was drawn off into a flask and the water layer in the separating funnel was extracted with 10 ml methylene chloride (and 10 ml of acetonitrile for liver and kidney) and combined with the first extract. The water layer was discarded and the separating funnel was rinsed with water. The combined methylene chloride layers were returned to the separating funnel and 15 ml of acetonitrile and 40 ml of hexane were added. The mixture was washed twice with 4-ml portions of water, which were discarded. In the partitioning steps, the layers ordinarily separated quickly without formation of emulsions. If significant emulsion was present, a few milliliters of acetonitrile were added and the mixture was shaken again. The organic layer was then extracted with four successive 1-ml portions of 0.01 *M* buffer pH 7 and the extracts were combined in a calibrated 15-ml centrifuge tube.

A few drops (0.1–0.2 ml) of *tert.*-butanol were added to each tube to suppress foaming and they were placed in a Buchler (Fort Lee, NJ, USA) Rotary Evapomix. Vacuum was applied cautiously without heating. After the contents had become cold, the water-bath was warmed to a final temperature of 50°C with the tubes rotating under vacuum. The contents were concentrated to $\leqslant 1$ ml, adjusted to a final volume of 1 ml and filtered into autosampler vials through a Gelman Acrodise LCPVDF filter. Evaporation can also be done under a stream of air or nitrogen, but this was slower and required more heating of the sample solution.

HPLC analysis

For HPLC analysis a Varian (Sugarland, TX, USA) Model 5000 pump and a Varian Model 9090 autosampler were used with either a Beckman (Fullerton, CA, USA) System Gold diode-array detector or a Hewlett-Packard (Rockville, MD, USA) Model 1050 UV–visible detector with a Varian Model 650 data system. A Polymer Labs. (Amherst, MA, USA) PLRP-S polystyrene–divinylbenzene copolymer HPLC column 150 mm × 4.6 I.D., 5 μm particle size, 100 Å pore diameter) was used with a matching guard cartridge. The mobile phase was 0.01 *M* phosphate buffer (pH 7) (A)–acetonitrile (B). Analysis was isocractic with the proportions (A:B) adjusted to give a retention time of 9–11 min for each compound; these were 85:15 for penicillin G, 82:18 for penicillin V and 78:22 for cloxacillin. The injection volume was 200 μl with a flow-rate of 1 ml/min and detection at 210 nm. After 12 min, a flushing program was started to A–B (65:35) at 15–20 min and then to the starting conditions at 21 min. After 30 min, the next sample was injected. After use, the column was flushed for 5 min with water and then for 10 min with water–acetonitrile (40:60) for storage. Quantification was based on comparison with a 1 μg/ml standard injected in the same sample series. Peak area was more accurate

than peak height, but either was linear with concentration up to at least 2 μg injected. The blank (if any) found after penicillinase treatment was subtracted for more accurate quantification, especially at low levels.

Spiked samples

The indicated amount of the pencillin was added in 150 μl of solution to the tissue in a blender jar and equilibrated for 30 min before blending with water. An equivalent amount of 0.01 *M* buffer (pH 7) was added at the same time to serve as a standard. In some instances, a larger amount of tissue was homogenized and the 20-ml aliquots were spiked.

Penicillinase treatment

The time required to reduce or eliminate the penicillin peak was determined by adding 0.2 ml of penicillinase concentrate to 20 ml of spiked water and incubating at room temperature for 15 min for penicillin G, 1 h for penicillin V and 3 h for cloxacillin. A 20-ml aliquot of sample homogenate was mixed with 0.2 ml of penicillinase concentrate and incubated for the indicated time before adding acetonitrile.

RESULTS AND DISCUSSION

The approaches developed in our laboratory for determing low levels of penicillin G [8,9] and other penicillins with neutral side-chains in milk were not satisfactory with tissue. When the acetonitrile filtrates were evaporated directly, a precipitate, almost certainly phospholipid, formed when the acetonitrile was driven off and recovery of the penicillins in the water layer was poor, indicating that penicillins were bound to the precipitate. When methylene chloride and hexane were added to the acetonitrile filtrate to separate the water layer, the recoveries of pencillins in the water layer were also poor. Penicillins were evidently complexed with something in the filtrate, probably phospholipids. The positively charged phospholipid molecules could act as ion pairs with the anionic penicillins. The penicillins were present in the acetonitrile filtrate and could be recovered by the partitioning technique described previously [3,4] and in this paper.

The partitioning clean-up described previously [3,4] proved adequate for isocratic analysis in pH 7 buffer. Some minor modifications in the earlier procedure together with improved detection systems increased the sensitivity about ten-fold with detection limits lowered from 0.05 to 0.005 ppm. A larger sample size was used. This required more acid to overcome the buffering action of the sample extract so that the pencillins could be converted to the acid form for extraction into methylene chloride. Addition of 10 ml of 0.2 *M* buffer (pH 2.2) was not satisfactory, pH 2 buffer gave a partial recovery and 0.2 *M* phosphoric acid gave essentially quantitative recoveries. Wiese and Martin [10] observed that penicillin G deteriorated rapidly in aqueous solutions below pH 3. However, it is probably more stable when organic solvents are present. No noticeable degradation occurred in 0.01 *M* phosphoric acid–acetonitrile during a run time of up to 20 min for HPLC analysis [3,4]. In the present instance, the effective pH of the methylene chloride extract was not known. However, there was no evidence of significant deterioration of penicillin G during the extraction procedure. The methylene chloride extracts were partitioned back into pH 7 buffer without undue delay, usually within 1 h.

The solvent composition used during partitioning usually gave rapid and clean separations of the layers. If stable emulsions were present, 5-ml portions of acetonitrile were added with shaking until the layers separated quickly.

Some samples, especially from liver and kidney, were turbid after final concentration and required filtration. Nylon 66 filters retained the penicillins quantitatively from water and, indeed, show promise for solid-phase extraction of these compounds. The PVDF filters specified were satisfactory. Some very fine turbidity sometimes passed through the filter but did not affect the chromatographic system. Isocratic analysis in pH 7 buffer improved the separation of penicillins from interferences over that achieved previously by gradient elution in 0.01 *M* H_3PO_4. Isocratic analysis had the disadvantage that retentions of the penicillins were so different that a different mobile phase was required for each penicillin. It also had the disadvantage that the column was not flushed between samples and late-eluting peaks were sometimes a problem, particularly with liver and kidney. A column flush was used af-

ter each sample to eliminate this problem.

Extraction/deproteinization with acetonitrile was rapid and effective. However, others [3,6] have successfully used tungstic acid. The partitioning clean-up used is simple, effective and reproducible. Reproducibility has sometimes been a problem when solid-phase extraction techniques were used [3,4,8]. Derivatization using imidazole or triazole and mercury (II) chloride has been used to form a derivative with a UV absorption maximum of 325 nm, where fewer interferences absorb than at the lower end of the UV range where penicillins have significant UV absorption. Wiese and Martin [10] observed that the molar absorptivity of penicillin G at 193 nm at acid pH was greater (38 000/1 mol · cm) than that of the derivative at 325 nm (20 000/1 mol · cm). Derivatization is therefore neither necessary nor advantageous if an adequate chromatographic separation of the parent compound can be achieved. At pH 7, use of absorbance at 210 nm was more satisfactory as the baseline noise was less than at shorter wavelengths.

Although the sensitivity of both UV–visible and diode-array detectors has improved considerably in recent years, the former are still better with respect to sensitivity. The present procedure was used successfully to measure incurred residues of penicillin G at levels <0.01 ppm in muscle and spiked levels of 0.01–0.02 ppm in muscle using a UV–visible detector connected to a data system. Detection at these low levels in liver and kidney and less certain because more interferents were present. However, residues were readily determined at 0.1 ppm in these tissues. Quantification was based on one or more external standards run at the same time as the samples and was generally based on peak area.

Treatment of the sample with penicillinase is a simple and effective method of confirming that a suspect chromatographic peak is indeed a penicillin [8,11]. Wiese and Martin [11] used a "digital subtraction" technique for samples with and without penicillinase treatment to determine low levels of penicillin G in milk. Pencillin G was rapidly inactivated by pencillinase. However, many other penicillins such as cloxacillin are resistant to pencillinase and require several hours for complete inactivation.

The application of this method to meat samples revealed unsuspected low levels of penicillin G residues. The compound was so ubiquitous that it was

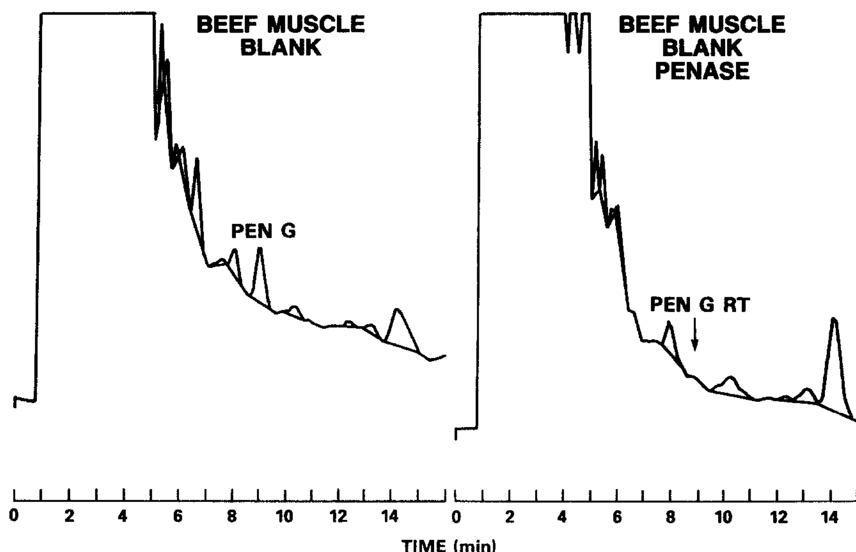

Fig. 1. Beef muscle, isocratic analysis: 0.01 *M* phosphate buffer pH 7.0–acetonitrile (85:15); flow-rate, 1 ml/min; Polymer Labs. PLRP-S column (150 × 4.6 mm I.D.); UV detection at 210 nm, 0.5 g equivalent injected, 0.0078 a.u.f.s., Hewlett-Packard Model 1050. UV–visible detector, Varian Model 650 data system. (Left) before and (Right) after treatment with penicillinase. The presence of penicillin G equivalent to 0.022 ppm of penicillin G sodium salt was confirmed. PEN G = Penicillin G; RT = retention time.

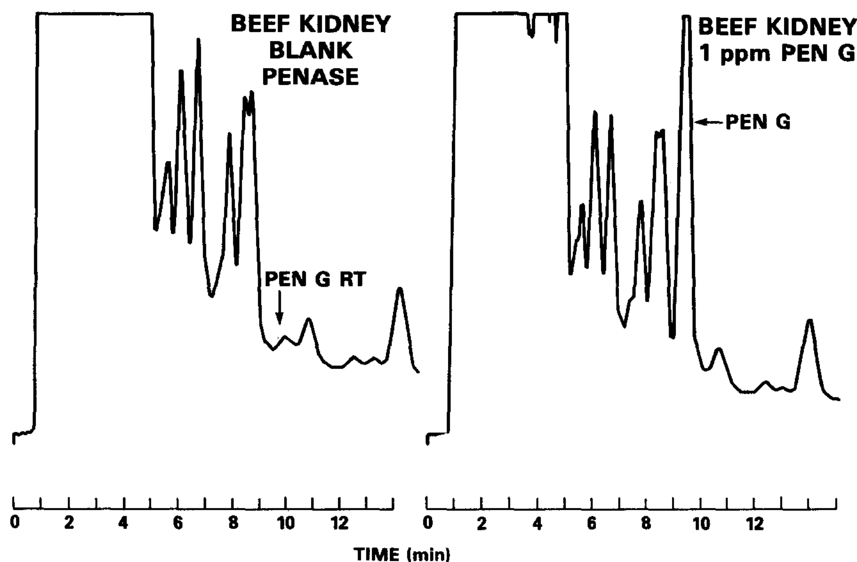

Fig. 2. Beef kidney: Conditions as in Fig. 1; 0,4 g equivalent injected; blank treated with penicillinase and spiked with 1 ppm of sodium penicillin G.

difficult to obtain suitable blank tissue for recovery experiments. The levels found from subsamples of the same tissue were variable. It was therefore necessary to prepare a uniform sample homogenate and spike subsamples of the homogenate to determine recoveries of penicillin G. The background levels were generally below the US tolerance limit of 0.05 ppm [12]. No background levels of penicillin V or cloxacillin were observed.

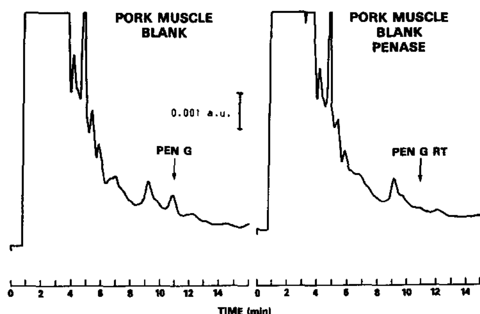

Fig. 3. Pork muscle; conditions as in Fig. 1, except mobile phase, 0.01 *M* phosphate buffer (pH 7)–acetonitrile (86:14). (Left) before and (right) after treatment with penicillinase. The presence of the equivalent of 0.019 ppm of sodium penicillin G was confirmed.

Fig. 1 shows a beef chuck sample with incurred penicillin G residue confirmed by penicillinase treatment. The residue is equivalent to 0.022 ppm of sodium penicillin G. Fig. 2 shows beef kidney blank and spiked with 1.0 ppm of penicillin G. There was no detectable penicillin G in the blank. Fig. 3 shows pork with an incurred pencillin G residue equivalent to 0.019 ppm of sodium penicillin G, confirmed by penicillinase treatment. With penicillinase treatment, penicillins can be differentiated from interferences with similar retention times.

Recoveries from spiked samples are shown in Table I. Recoveries from beef muscle were >90% for all three compounds. Recoveries of penicillin G were >90% except from beef kidney. Recovery of cloxacillin was lower from pork muscle and kidney. Cloxacillin was not recovered from beef liver.

This is a simple, specific and sensitive HPLC confirmatory test for penicillin G, penicillin V and cloxacillin. It should be applicable to any penicillin with a neutral side-chain. The use of penicillinase for confirmation enhances the sensitivity and makes the method suitable for regulatory confirmation of penicillin residues.

TABLE I

RECOVERY OF PENICILLINS ADDED TO TISSUES

Compound	Tissue	Amount added (μg/g) and recovery[a] (%)	Mean recovery ± S.D. (%)
Penicillin G	Beef muscle	1.5(98)[b], 1(86,100,92), 0.15(99)[b], 0.015(96)[b]	95 ± 5
	Beef kidney	1(71)	
	Pork muscle	1(101,95)[b], 0.1(92)[b]	
	Pork liver	1(102,89), 0.1(98)	
	Pork kidney	1(96)	
Penicillin V	Beef muscle	10(98), 1(97,105,98)	99 ± 3
	Beef kidney	1(67,76), 0.1(118)	
	Pork muscle	10(104)[b], 1(100), 0.1(97[b],93)	99 ± 4
Cloxacillin	Beef muscle	10(91), 2(89), 1(89,97,91), 0.2(92), 0.02(91)	91 ± 2
	Beef kidney	1(85), 0.1(86)	
	Beef liver	No recovery	
	Pork muscle	1.5(76)[b], 1(85)[b], 0.15(74)[b], 0.1(77)[b], 0.015(107)[b]	84 ± 12
	Pork kidney	10(69)	

[a] In parentheses.
[b] Spiked homogenate.

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