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DETERMINATION OF PENICILLIN G AND CLOXACILLIN RESIDUES IN BEEF AND PORK TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Tissues were homogenized and then deproteinized with acetonitrile. The acetonitrile extract was partitioned between dichloromethane and pH 2.2 buffer and then extracted with pH 7 buffer. After addition of ammonium sulfate to the aqueous solution, it was mixed with acetonitrile. The acetonitrile extract was separated and evaporated, and the residue was taken up in water. The aqueous solution was analyzed by high-performance liquid chromatography (HPLC) on a C_{18}, 10-μm particle size, reversed-phase column using gradient elution with 0.01 *M* orthophosphoric acid–acetonitrile (from 80:20 to 40:60 in 20 min) at a flow-rate of 1 ml/min and UV absorbance detection at 220 nm. Recoveries were generally greater than 90% with all tissues. Data on incurred residues from a treated cow showed recoveries of penicillin which were frequently several times higher by HPLC than by bioassay. Sensitivity limits in muscle were about 0.05 ppm for both penicillin G and cloxacillin, but higher in liver and kidney because of interferences. The method is suitable for other monobasic penicillins but not for dibasic or amphoteric penicillins.

INTRODUCTION

Antibiotic residues in animal tissues are ordinarily detected by microbiological assays. Procedures described include assays of drip from tissue collected separately[1], by insertion of swabs[2] or filter paper discs[3] into tissue, or by placing sections of frozen tissue directly on culture plates[3–5]. Other methods include assays of tissue homogenates, prepared in buffers, which are usually centrifuged prior to testing[6–9]. Solvent extracts of tissues have also been used[10–12]. These methods can be very sensitive for detection of some antibiotics, especially β-lactams[2–6]. Tissue extracts sometimes contain natural substances inhibitory to bacteria[4,5]. Penicillins can be distinguished from other antibiotics by inactivation with β-lactamase but cannot be distinguished from one another. Some semisynthetic penicillins, such as cloxacillin, are designed to be resistant to β-lactamases and are not completely inactivated by the usual test procedures[13].

Chromatographic methods offer a promising approach to detection and identification of specific antibiotic residues. High-performance liquid chromatography

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(HPLC) has been used for determination of a number of antibiotics in biological fluids, mainly blood serum, in clinical applications[14]. The application of HPLC procedures to analysis of antibiotics residues in foods has been more limited. Residues analysis generally requires both greater sensitivity than clinical applications and isolation from more complex substrates. HPLC procedures have been described for chloramphenicol in milk, eggs, and meat[15–19], tylosin in milk, blood and tissues[20], lasalocid in tissues[21], and penicillin G and cloxacillin in milk[22]. The present paper describes an adaptation of the method for penicillins in milk to the determination of penicillin G and cloxacillin in tissue.

METHODS AND MATERIALS

Chemicals and equipment

Equipment. Vortex mixer; Buchler Rotary Evapomix*; blender; graduated cylinders, separatory funnels; 15-ml conical centrifuge tubes graduated to 0.1 ml. All glassware was cleaned in special detergents designed for critical cleaning, and rinsed with 1% hydrochloric acid and distilled water before use.

Chemicals. Acetonitrile, UV-grade (O.D. 220 nm $\leqslant$ 0.02); dichloromethane; light petroleum (b.p. 30–60°C), residue analysis grades. Other chemicals, reagent grade.

Procedure

Preparation of tissue homogenates. About 25 g of tissue was blended for 2 min with 3 volumes (v/w) of distilled water for muscle or 0.2 *M* phosphate buffer (pH 2.2) for liver and kidney.

Deproteinization. A volume of 5 ml of blood serum was mixed with 1 ml 0.2 *M* disodium phosphate in a 125-ml conical flask, and 3 volumes (18 ml) of acetonitrile was added with switling. After standing for 1 min, the supernatant liquid was decanted through a plug of glass wool in the stem of a funnel and one-half volume (12 ml) of filtrate was collected. This is equivalent to 2.5 ml of original serum.

Tissue homogenates. A volume of 32 ml of acetonitrile was added to 8 ml of tissue homogenate as described for blood serum, and one-half volume (20 ml) of filtrate was collected. This is equivalent to 1 g of original tissue.

Clean-up

The acetonitrile filtrate was transferred to a separatory funnel and 10 ml 0.2 *M* phosphate buffer (pH 2.2) was added. The filtrate was then extracted with 20 and 10 ml of dichloromethane and the combined dichloromethane extracts were collected in a clean separatory funnel. Then 40 ml of light petroleum (b.p. 30–60°C) and 15 ml of acetonitrile were added to the dichloromethane extract and the mixture was washed twice with 2-ml portions of distilled water, which were discarded. The organic layer was then extracted with four successive 1-ml portion of 0.01 *M* phosphate buffer (pH 7) which were combined in 15-ml graduated conical centrifuge tubes. The pH 7 extract was washed by Vortex mixing for 10 sec with 2 ml of light petroleum and centrifuging for 1 min at low speed to separate the layers. The light petroleum was

* Mention of specific items or names is for identification purposes and does not imply endorsement by the U.S. Department of Agriculture over similar products not specifically mentioned by name.

removed with a pipet as completely as possible, and the remaining light petroleum was evaporated under reduced pressure in the Buchler Rotary Evapomix. Then, 1 ml of saturated ammonium sulfate and 2 ml of acetonitrile were added and mixed for 10 sec on a vortex mixer. The tube was centrifuged at low speed for 1 min, and the acetonitrile layer was carefully transferred with a pipet to a clean centrifuge tube. The aqueous layer was extracted as above with an additional 2 ml of acetonitrile which was combined with the first extract. The acetonitrile was evaporated in the Buchler Rotary Evapomix to a volume of <0.5 ml of residual water, and the volume was adjusted to 0.5–1.0 ml with distilled water.

An alternative procedure was to acidify the combined pH 7 extracts with 5 ml of 0.2 *M* phosphate buffer (pH 2.2) in a separatory funnel and to extract twice with 2-ml portions of dichloromethane. The combined dichloromethane extracts were evaporated to dryness in a centrifuge tube in the Buchler Rotary Evapomix. The residue was taken up in 1 ml of 0.01 *M* phosphate buffer (pH 7) and 2 ml light petroleum (b.p. 30–60°C), and mixed for 10 sec on a vortex mixer. The light petroleum layer was removed it a pipet and the residual light petroleum ether was removed under reduced pressure in the Buchler Rotary Evapomix.

HPLC procedure

A Varian Model 5000 liquid chromatograph was used with a Varian UV-50 variable-wavelength detector, set at 220 nm, and a Valco automatic loop injector with a 200-μl loop. The column was a Varian Micropak MCH-10 C_{18} reversed-phase column, 10-μm particle size, 30 cm × 4.6 mm I.D. Solvents were (A) 0.01 *M* or 0.02 *M* phosphoric acid, (B) acetonitrile. A gradient from 80A–20B to 40A–60B in 20 min was used with a flow-rate of 1 ml/min. Quantitation was based on peak height, which was linear up to 6 μg of penicillin G and 3 μg cloxacillin, injected onto the column.

Recovery of added penicillins

The penicillins were added at the indicated levels (based on original tissue weight) to tissue homogenates or serum and carried through the procedure. Quantitation was based on linear extrapolation from standards.

RESULTS AND DISCUSSION

The procedures used were adapted from those previously described for the determination of monobasic penicillins in milk[17]. Tissues were blended with 3 volumes (v/w) of water or, in the case of liver and kidney, 3 volumes of 0.2 *M* phosphate buffer (pH 2.2), which gave a final pH of about 3.4. Liver and kidney extracts somewhat cleaner after blending in pH 2.2 buffer instead of water. For deproteinization, 3 volumes of acetonitrile was satisfactory for blood serum and 4 volumes for tissue homogenates, while best results for milk were obtained with 2 volumes[22]. The remainder of the clean-up procedure was similar to that used for milk. With extracts from tissues, more acetonitrile was added to the organic phase to prevent formation of emulsions when the penicillins were reextracted into the pH 7 buffer. Two approaches to final clean-up were evaluated: (1) partitioning into acetonitrile from ammonium sulfate, and (2) partitioning into methylene chloride from pH 2.2 buffer and then back into pH 7 buffer. Method 1 was simpler, omitted buffer salts, and, after the acetonitrile was evaporated, allowed the final volume to be adjusted as needed

with distilled water. Extracts prepared by Method 2 were cleaner but gave a final volume of 1 ml in 0.01 *M* phosphate buffer (pH 7), which could not be concentrated without causing interference in the HPLC analysis. The amount of interfering materials with retention times near the penicillins was identical in both procedures. Attempts at further separation of the penicillins from interfering material were unsuccessful.

The 10-μm particle size C_{18} reversed-phase columns (Varian Micropak MCH-10) were rugged and trouble free, showed little deterioration after several months of use under the specified conditions, and were useable with unfiltered samples. Columns with smaller particle sizes gave little if any improvement in resolution and tended to develop excessive back pressures.

The best separation from interfering compounds was obtained with 0.01 or 0.02 *M* phosphoric acid in acetonitrile. Gradient elution maintained sharpness of penicillin peaks. Fig. 1 shows a chromatogram of 2 μg of each penicillin G and cloxacillin. The recorder response to cloxacillin is about three times that of penicillin G. A typical chromatogram from beef muscle with no known exposure to penicillins is shown in Fig. 2. Retention times of penicillin G and cloxacillin are indicated. A small interfering peak with a retention time near that of cloxacillin is present. Separation of cloxacillin from interfering material can be improved by using a buffer of pH 2.7. Fig. 3 shows a chromatogram of the same sample, spiked with 1 ppm penicillin G.

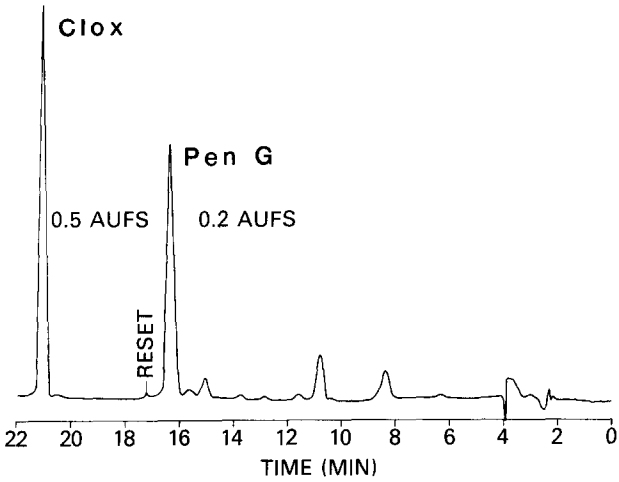

Fig. 1. Penicillin G (Pen G) and cloxacillin (Clox), 2 μg each. Injection volume, 200 μl. Varian Model 5000 liquid chromatograph, UV absorbance detector set at 220 nm, 0.2 AUFS (Pen G) reset to 0.5 AUFS (Clox), Varian Micropak MCH-10 column, 30 cm × 4.6 mm I.D. Solvent gradient, 0.01 *M* phosphoric acid–acetonitrile (from 80:20 to 40:60 in 20 min), Flow-rate, 1 ml/min.

About 0.05 ppm penicillin G is the lowest concentration which will give a clearly discernable peak above baseline noise with muscle. The recorder response is greater with cloxacillin, but the presence of small interfering peaks in muscle with elution times near cloxacillin also limits reliable detection to about 0.05 ppm. Levels of interference were greater with liver and kidney, thus reducing sensitivity. Results with pork tissue were similar to beef.

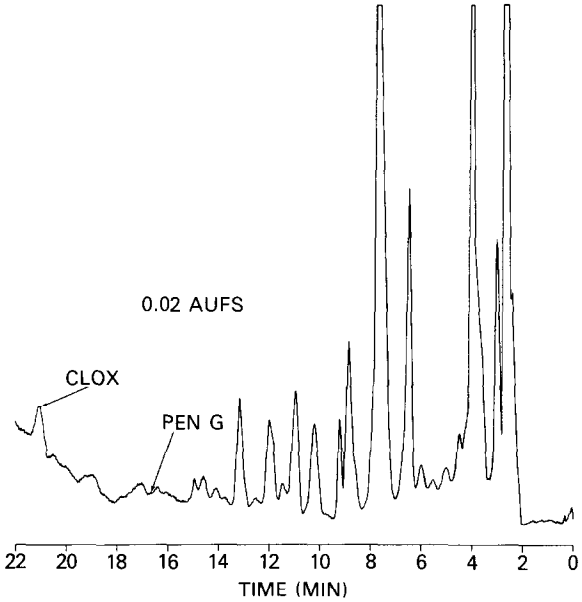

Fig. 2. Beef muscle, blank, ammonium sulfate–acetonitrile clean-up, blank, 0.2 g equivalent injected in 200 μl. Varian Model 5000 liquid chromatograph, UV absorbance detector set at 220 nm, 0.02 AUFS, Varian Micropak MCH-10 Column, 30 cm × 4.6 mm. I.D. solvent gradient 0.01 *M* phosphoric acid–acetonitrile (from 80:60 to 40:60 in 20 min), Flow-rate, 1 ml/min. Arrows indicate retention times of penicillin G and cloxacillin. A small interfering peak has a retention time near that of cloxacillin.

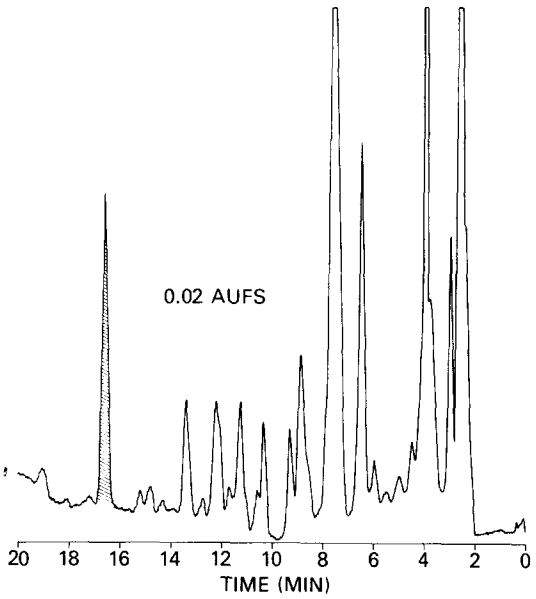

Fig. 3. Beef muscle spiked with 1 ppm sodium penicillin G. Conditions as in Fig. 1. Penicillin G peak is shaded.

TABLE I

RECOVERY OF PENICILLIN G FROM SPIKED PORCINE TISSUE SAMPLES

Tissue	*Penicillin G added (ppm)*	*Penicillin G found (ppm)*					*Mean % recovery ± S.D.*
		1	*2*	*3*	*4*	*N*	
Muscle	0.50	0.45	0.48	0.43	0.45	14	92.2 ± 5.5
	0.50	0.45	0.53				
	1.00	0.93	1.00	0.92	0.92		
	2.00	1.80	1.66				
	2.50	2.23					
	5.00	4.70					
Kidney	1.00	0.92				7	89.7 ± 7.0
	2.00	1.92	1.88	1.96			
	5.00	3.95	4.00	4.45			
Liver	1.00	0.93	0.84	0.90		11	93.2 ± 5.5
	2.00	2.00	1.88	1.92	1.94		
	5.00	4.65	4.85	4.10	4.95		
Blood (serum)	0.20	0.19				10	95.1 ± 5.0
	0.40	0.38	0.39				
	0.50	0.46					
	1.00	1.02	0.86	0.92	0.98		
	1.00	0.90					
	5.00	5.15					

Recoveries of penicillin G and cloxacillin added to tissue homogenates and blood serum are summarized in Tables I and II. Mean recoveries of both penicillins were 90% or higher with coefficients of variation ranging from 2.9 to 8.1%. At the levels added, interfering peaks were negligible.

In order to evaluate the procedure for residues in treated animals, a cull dairy cow, weighing approximately 600 kg was injected with 6,000,000 units of penicillin G and slaughtered 2 h after treatment. Homogenates of the tissues were prepared, and portions of the same homogenate were analyzed by HPLC and bioassay. The results (Table III) by the HPLC procedure were consistently higher for tissue samples. concentrations were more than 10 times higher for injected muscle and kidney and almost 40 times higher for liver. This is undoubtedly a result of the efficiency of acetonitrile in extracting penicillin from the tissues. It appears that penicillin is bound to proteins in tissues, and thus the response to bioassay procedures is lower when tissue homogenates are tested directly. When portions of the solutions prepared for HPLC analyses of liver and kidney were applied to the bioassay plates, results were much higher than those obtained by direct bioassay of homogenates. The penicillin concentrations in the solutions were too high for accurate quantitation, but the results do show that extraction with an organic solvent is beneficial for bioassay tests of tissues. Other investigations have shown that solvent extraction improved the sensitivity of microbiological assays[10–12] but the relative efficiency of extracting incurred residues from tissues was not reported. Portions of the tissue samples were also tested by the USDA Swab Test On Premises (STOP) procedure[2]. This test was

TABLE II

RECOVERY OF CLOXACILLIN FROM SPIKED PORCINE TISSUE SAMPLES

Tissue	*Cloxacillin added (ppm)*	*Cloxacillin found (ppm)*					*Mean % recovery ± S.D.*
		1	*2*	*3*	*4*	*N*	
Muscle	0.50	0.44	0.51	0.51		11	91.5 ± 8.1
	1.00	0.98	0.95	0.93	0.93		
	2.00	1.58	1.72				
	2.50	1.90					
	5.00	4.75					
Kidney	1.00	1.04				7	98.4 ± 7.7
	2.00	2.02	2.02	2.16			
	5.00	4.60	4.35	4.55			
Liver	1.00	0.86	0.82	0.93		11	92.3 ± 5.7
	2.00	2.00	1.88	1.86	1.92		
	5.00	4.20	4.85	4.55	4.95		
Blood (serum)	0.20	0.19				10	95.3 ± 2.9
	0.40	0.37	0.40				
	0.50	0.48					
	1.00	0.98	0.96	0.92	0.99		
	1.00	0.93					
	5.00	4.55					

TABLE III

DETERMINATION OF PENICILLIN G RESIDUES IN TISSUES OF A DIARY COW SLAUGHTERED TWO HOURS AFTER INTRAMUSCULAR INJECTION OF 6.000.000 UNITS OF PENICILLIN G

Sample	*Swab**	*Penicillin G found (ppm)*		*Bioassay after clean-up for HPLC*
		*Bioassay***	*HPLC*	
Injection site muscle	+	0.14	2.0	
Shoulder (contra-lateral to injection site)	–	0.04	0.07	
Liver	+	0.58	21.7	2.28***
Kidney	+	0.55	6.1	4.56***
Neck	–	0.05	0.16	
Loin	–	0.03	0.18	
Inside round	–	0.03	0.06	
Blood serum	N.A.	0.79	0.95	

* USDA STOP procedure[2].

** *Sarcinia lutea* test on LabLine bioassay plates.

*** Bioassay plates were grossly overloaded.

positive only for the tissues containing higher levels of residues (injected muscle, liver and kidney).

The limits of detection at which penicillins can be distinguished clearly from small interfering peaks with similar retention times are about 0.05 ppm for penicillin G and cloxacillin in muscle and 0.1–0.5 ppm in liver and kidney. Reported detection

limits for penicillin G by microbiological assays are about 0.007 ppm in beef tissue by the method of Vilim and Larocque[9]; 0.015 IU/g in meat by the method of Bielecka *et al.*[6]; 0.03–0.06 IU/g muscle by the *B. subtilis* BGA and the "four-plate method"[4]; 0.006 ppm in muscle by a modified four-plate method[3] and 0.001 ppm using solvent extraction[1]. Detection limits for cloxacillin by microbiological assay are appreciably higher, 0.04 ppm by the method of Vilim and Larocque[9]; >0.1 ppm by the *B. subtilis* BGA test and the four-plate method[7] and 0.05 ppm in muscle by the modified four-plate method[3].

Microbiological assays are extremely sensitive to penicillin G and many other β-lactam antibiotics, and it is difficult to obtain comparable sensitivity with physico-chemical procedures. Microbiological assays are less sensitive for cloxacillin. The apparent better recoveries of penicillin from tissues using solvent extraction as used in the HPLC method more than offset the slightly lower sensitivity. Use of solvent extraction should be beneficial in improving the accuracy of microbiological assays for antibiotics in tissues. Chromatographic methods are necessary to distinguish β-lactam antibiotics from one another. This HPLC method should work for residues of other monobasic penicillins in tissues, but is not suitable for determination of dibasic or amphoteric penicillins.

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