Determination of Penicillin G in Beef and Pork Tissues Using an Automated LC Cleanup

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A procedure previously described for determination of β-lactam antibiotics in milk was modified for use with tissues. Tissues were extracted directly with acetonitrile (procedure I) to avoid degradation of penicillin G in water homogenates. Recovery of penicillin G in acetonitrile extracts of tissues was improved by addition of tetraethylammonium chloride and, in some cases, pH adjustment. The acetonitrile extracts were concentrated by evaporation and filtered. For cleanup, an automated high-performance liquid chromatography (HPLC) fractionation was used. The fraction corresponding to penicillin G was tested by a rapid screening test and, if positive, was analyzed by HPLC. For analysis, an Inertsil ODS-2 column was used with a mobile phase of 0.0067 M KH_2PO_4, 0.0033 M H_3PO_4−acetonitrile (68 + 32) with UV detection at 215 nm. For confirmation by treatment with β-lactamase, tissues were homogenized in water. An aliquot of the water homogenate was treated with β-lactamase prior to extraction with acetonitrile and tetraethylammonium chloride (procedure II). Recoveries were 66−95% in the 0.1−1.0 ppm range, depending on the tissue, with a detection limit of about 5 ng/g.

Keywords: *Penicillin G; determination; high-performance liquid chromatography; tissue; beef; pork; liver; kidney; muscle*

INTRODUCTION

Antibiotics, including the β-lactam group, can be detected in animal tissues by a variety of screening tests. The β-lactams have traditionally been differentiated from other antibiotics by degradation with the enzyme penicillinase (Katz, 1986). The screening tests cannot identify specific β-lactam antibiotics or tell how much is present. Specific determinative procedures have been described for determination of penicillin G in tissues (Terada et al., 1985; Boison et al., 1991; Aoyama et al., 1992; Tarbin et al., 1996). Multiresidue procedures suitable for simultaneous determination of several penicillins with neutral side chains including penicillin G have also been described (Moats, 1992; Meetschen and Petz, 1991; Blanchflower et al., 1994; Gee et al., 1996; Igualada et al., 1996). More recently, procedures have been expanded to include amphoteric penicillins (Hong et al., 1995; Boison and Keng, 1996). There is, however, a need for multiresidue methods capable of determining any β-lactams detected by screening tests including those of the cephalosporin group.

Our laboratory recently described a sensitive multiresidue procedure for determination in milk of the six β-lactams approved for use with food-producing animals in the United States (Moats and Harik-Khan, 1995; Harik-Khan and Moats, 1995). The compounds were separated by using high-performance liquid chromatography (HPLC) fractionation for cleanup of sample extracts. The application of this approach to residues in tissues has required some modification of the procedures. This paper describes the application of this approach to determination of penicillin G in animal tissues.

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MATERIALS AND METHODS

Chemicals and Reagents. Acetonitrile, HPLC grade, EM Omnisolv, or equivalent, was purchased from EM Industries, Gibbstown, NJ. Tetraethylammonium chloride (Et_4NCl) was purchased from Aldrich Chemical Co., Milwaukee, WI. Other chemicals were reagent grade from various sources. Penicillin G was purchased from Sigma Chemical Co., St. Louis, MO. A stock solution of 1 mg/mL (corrected for purity) was prepared in water and diluted to working standards of 100, 10, and 1 μg/mL biweekly. The stock solution was stored frozen at −20 °C. β-Lactamase (RP-BLASE-R) was purchased from Charm Sciences, Malden, MA. The dry powder was reconstituted in water according to the manufacturer's instructions and was dispensed in 0.1 mL portions into minicentrifuge tubes and stored frozen until needed. The Delvotest P-mini (Gist-Brocades) was purchased from Eastern Crown, Vernon, NY.

Glassware and Equipment. Glassware required included graduated cylinders, 25 and 50 mL, 15 mL conical graduated centrifuge tubes (calibrated to 1 and 4 mL), 250 mL glass-stoppered sidearm flasks, and 125 mL conical flasks. All glassware was cleaned in special detergent (MICRO, International Products, Trenton, NJ) or equivalent and rinsed in deionized water, then ca. 0.01 N HCl or H_2SO_4, and deionized water again. Other equipment included a Vortex Evaporator (Buchler Instruments, Ft. Lee, NJ), a thermostated hot plate with a shallow tray containing 1−2 cm of water, plastic-coated lead rings to weight sidearm flasks during evaporation, and blender jars, 100−300 mL, glass or metal.

Extraction/Deproteinization. *Procedure I.* Tissue was cut into small pieces, and 5 g was transferred to a small blender jar. Then, 5 mL of water, 2 mL of 0.1 M Et_4NCl, and 40 mL of acetonitrile were added, and the mixture was blended for 1 min at half of full power. For kidney, 1 mL of 0.2 M Et_4NCl, 1 mL of 0.005 M KH_2PO_4, and 40 mL of acetonitrile were used. The supernatant liquid was decanted through a

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plug of glass wool in the stem of a small funnel, and 40 mL (=4 g tissue) of filtrate (20 mL for liver and kidney) was collected.

Procedure II (Used with β-Lactamase Treatment). Tissue was cut into small pieces, and 15 g was weighed and transferred to a small blender jar. Then 45 mL of water was added, and the mixture was blended 1 min at half of full power as controlled by a variable resistance transformer. A 10 mL aliquot of homogenate was transferred to a 125 mL conical flask, 0.1 mL of the β-lactamase solution was added with mixing, and the mixture was allowed to stand for 1 h at room temperature. For extraction/deproteinization, 2 mL of 0.1 M Et_4NCl and 40 mL of acetonitrile were added to the homogenate, stirring after each addition. For kidney, 1 mL of 0.2 M Et_4NCl, 40 mL of acetonitrile, and 1 mL of 0.005 M KH_2PO_4 were added in that order, mixing after each addition. After standing for 5 min, the supernatant liquid was decanted through a plug of glass wool in the stem of a small funnel, and 40 mL (=2 g tissue) of filtrate was collected.

Evaporation. The filtrate was transferred to a 250 mL glass-stoppered sidearm flask, and 2 mL of 0.01 M buffer (5:1 KH_2PO_4−Na_2HPO_4), 5 mL of *tert*-butyl alcohol (to suppress foaming), and 5 mL of water were added. The flasks were attached to a water pump without heating. The contents boiled briefly and then evaporated quietly. The flasks were then placed in a shallow water bath heated to 40−45 °C, and the contents were evaporated to 1−2 mL. If foaming continued, a few milliliters of *tert*-butyl alcohol and an equal volume of water were added and the process was restarted. The residue was rinsed into calibrated tubes with several small portions of water, and the volume was adjusted to 4 mL. The sample was then filtered through a 25 mm, 0.45 μm PVDF syringe filter into a 4 mL autosampler vial.

HPLC Fractionation. The cleanup system consisted of a Varian (Sugarland, TX) 9010 pump, a Waters (Milford, MA) Wisp 712 autosampler with a 2000 μL loop, a Waters diode array detector, an ISCO (Lincoln, NE) FOXY fraction collector, and a Supelcosil (Bellefonte, PA) LC-18 column, 4.6 × 150 mm, 5 μm particle size. The initial conditions were a mobile phase of 0.01 M KH_2PO_4 and a flow rate of 1 mL/min, with UV detection at 210 nm. A 2 mL aliquot of the sample extract was loaded onto the HPLC column, and, after 3 min, an acetonitrile gradient was started to 60% at 40 min. The system was returned to starting conditions at 41 min and was ready to load another sample at 55 min. The autosampler started the other components when the sample was injected. A penicillin G standard was run first, and the fraction collector was set to collect a 1.5 mL time window centered on the retention time of penicillin G. For the penicillin G fractions, 0.2 mL of 0.01 M Na_2HPO_4 was added to each fraction collection tube to improve stability. The fractions were concentrated to <1 mL under reduced pressure in a Buchler Vortex evaporator. Evaporation under a stream of air or nitrogen was slower and did not effectively remove acetonitrile. The volume was adjusted to 1 mL, and the sample was transferred to a 2 mL autosampler vial.

Screening HPLC Fractions. If the identity of the suspected β-lactam antibiotic was unknown, the HPLC fractions could be screened for antimicrobial activity. A 0.1 mL aliquot of the evaporated HPLC fraction was tested for antimicrobial activity using the Delvotest P-mini procedure as described for testing milk. For a control, 0.1 mL of 0.01 M KH_2PO_4 was used. The tubes were incubated 2.5−3 h at 64 °C. If the tube remained purple, the test was positive, while if it turned yellow, the test was negative. Fractions testing negative did not require further analysis by HPLC.

HPLC Analysis. The analysis system consisted of a Varian 9012 pump, a Varian 9090 autosampler, a Waters 481 UV detector, a Varian 654 data system, and an Inertsil-ODS-2 column (Metachem, Torrance, CA), 4.6 × 150 mm, 5 μm particle size. The mobile phase was 0.0067 M KH_2PO_4, 0.0033 M H_3PO_4−acetonitrile (68 + 32), premixed, with a flow rate of 1 mL/min, 200 μL sample injection, and UV detection at 215 nm. Concentrations were calculated from a 1 μg/mL standard run at the same time. Recoveries from spiked samples were made by comparison with concentrations in an equivalent amount of 0.01 M buffer (5:1 KH_2PO_4−Na_2HPO_4) spiked at the same level with appropriate corrections for the sample size injected.

Table 1. Degradation of Penicillin G (1 ppm) Added to a Veal Kidney Homogenate

time	% recovery
no delay	75
1 h	56
2 h	33

RESULTS AND DISCUSSION

Our initial approach (procedure II) was to blend tissue in water and to then use the extraction and cleanup procedure previously described for milk (Moats and Harik-Khan, 1995; Harik-Khan and Moats, 1995). The addition of Et_4NCl during extraction prevented penicillin G from complexing with substances in the acetonitrile extract as was observed previously (Moats, 1992). Blending initially in water had the advantage that an aliquot of the water homogenate could be treated with β-lactamase prior to starting the extraction procedure. This provided a simple and sensitive confirmatory procedure. Blending with water worked reasonably well with muscle. However, recoveries from liver and kidney were sometimes very poor by this procedure. We found that penicillin G was sometimes rapidly degraded in the liver and kidney homogenates, as illustrated in Table 1. This problem was avoided by blending the tissue directly with acetonitrile (procedure I). Direct blending with acetonitrile improved sensitivity since the sample was not diluted with water. However, procedure II was still used for confirmation by β-lactamase treatment where degradation of the β-lactam antibiotics is intentional.

The procedure requires evaporation of considerable amounts of solvent, and the development of practical methods for doing this was crucial. We evaluated a vacuum centrifugal evaporator, evaporation under a stream of air or nitrogen, rotary evaporators, and evaporation in sidearm flasks. Evaporation in sidearm flasks under reduced pressure was faster, required minimal heating of sample extracts, used inexpensive equipment, and required little bench space. Some extracts, especially those from liver and kidney, tended to foam under reduced pressure. Addition of a small amount of *tert*-butyl alcohol was effective in suppressing foaming. An equivalent amount of water was added to ensure complete removal of organic solvent in the evaporation procedure. Evaporation of 40 mL of solvent required less than 1 h by this procedure.

Penicillin G degrades slowly when dissolved in water. Schwarz and Buckwalter (1962) reported that penicillin G had a sharp peak of maximum stability at pH 6.5 and that stability dropped rapidly at higher or lower pH's. A small amount of buffer of about pH 6 (5:1 KH_2PO_4−Na_2HPO_4) was added to the filtrates to improve stability during evaporation. The KH_2PO_4 cleanup buffer with a pH of 4.6 was not optimal for stability of penicillin G and could be a problem when fraction collection was run overnight. A small amount (0.1 mL) of 0.01 M Na_2HPO_4 was therefore added to the tubes used for collection of the penicillin G fractions.

For analysis of fractions, HPLC conditions were changed from those used in the cleanup procedure in order to separate the analyte from interferences in the

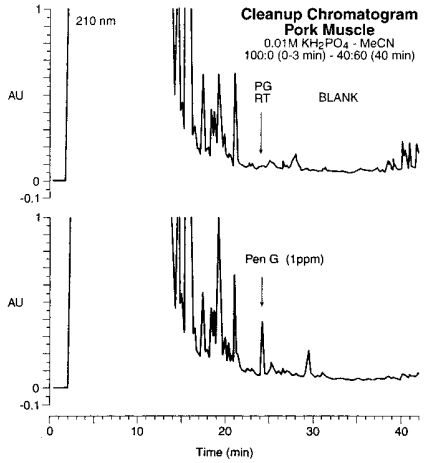

Figure 1. Cleanup chromatogram of a pork muscle extract.

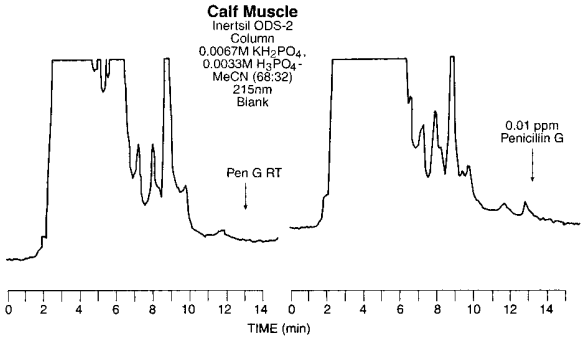

Figure 2. Penicillin G in calf muscle: a blank and a 0.01 ppm sample.

fractions collected. For penicillin G, both the column type and the pH of the buffer were changed. The Supelcosil LC-18-DB and the Inertsil ODS-2 columns both gave satisfactory results. Buffers prepared using various ratios of KH_2PO_4 and H_3PO_4 gave satisfactory separations from interferences. The Inertsil column gave better separation from interferences in liver. For this approach to be successful, the conditions and column types used both for cleanup and analysis must be rigidly followed. It is likely that other combinations of columns and mobile phases might be successful, but they were not discovered in the present investigation.

Figure 1 shows a cleanup chromatogram of pork muscle, a blank and a sample with 1 ppm of penicillin G. At this level, the penicillin G peak was clearly visible and could be determined directly without further cleanup. Figure 2 shows an analysis chromatogram of 0.01 ppm of penicillin G in calf muscle. Figure 3 shows an analysis chromatogram of 0.01 ppm of penicillin G in calf kidney. Recoveries are summarized in Table 2. As would be expected, there was more variation in apparent recoveries at the 0.01 ppm level than at higher levels. The apparent recoveries from calf liver were anomalously low at this level, and those from pork muscle were high and variable. These results were not corrected for background. At higher levels, the results were more consistent and background effects were less. The estimated detection limit which would give a clearly discernible peak above the background was about 0.005 ppm. Lower levels may be detected when the β-lactamase procedure is used to correct for background.

This method is intended to be used as part of a multiresidue procedure for identification and quantitation of β-lactam antibiotics detected by screening tests such as those previously described for analysis of milk samples (Moats and Harik-Khan, 1995; Harik-Khan and Moats, 1995). The automated LC fractionation is sequential, requiring 1 h per sample, and is ordinarily run overnight if more than four to five samples are run. The procedure requires fewer steps than other reported methods and achieves comparable sensitivity without the use of troublesome derivatizing agents. It can be readily adapted to the determination of additional β-lactam antibiotics including those of the cephalosporin group by collecting appropriate fractions. Further work is needed to establish optimum conditions for analysis of fractions from tissue samples containing other antibiotics.

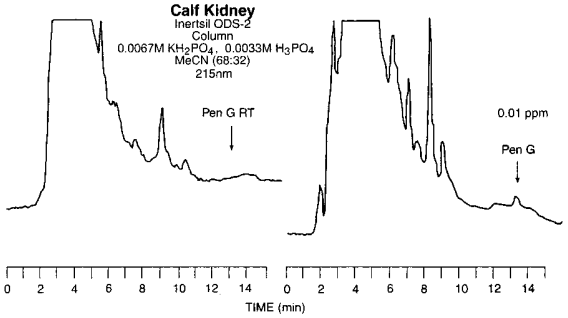

Figure 3. Penicillin G in calf kidney: a blank and a 0.01 ppm sample.

Table 2. Recovery of Penicillin G Added to Tissues

	mean % recovery ± sd ($N = 3$) for given amount added (ppm)		
tissue	0.01	0.1	1.0
pork muscle	114 ± 26	93 ± 7	89 ± 4
liver	88 ± 11	95 ± 11	82 ± 12
kidney	104 ± 8	91 ± 5	77 ± 9
calf muscle	88 ± 11	78 ± 7	80 ± 8
liver	47 ± 7	77 ± 13	68 ± 1
kidney	75 ± 17	66 ± 8	77 ± 9

If the identity of the suspect antibiotic is unknown, the HPLC fractions can be screened for the presence of antibiotics (Harik-Khan and Moats, 1995). This is particularly useful when multiple fractions are collected. The Delvotest P-mini was found to be a simple, inexpensive, and reliable test for this purpose. Other milk screening tests might also be suitable but would need to be evaluated for this application. Only samples testing positive require HPLC analysis for confirmation. Treament of a replicate with β-lactamase prior to starting the analytical procedure provides a simple and sensitive confirmatory test. If the suspect chromatographic peak disappears, the identity of the peak as a β-lactam is confirmed. Any remaining background can be subtracted, thus improving quantitation, especially at low levels.

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