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# LC/MS/MS measurement of penicillin G in bovine plasma, urine, and biopsy samples taken from kidneys of standing animals

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## Abstract

Methods for the measurement of penicillin concentration in bovine plasma, kidney and urine were developed and validated. Detection was based on liquid chromatography/tandem mass spectrometry (LC/MS/MS). Phenethecillin was used as an internal standard. Plasma was extracted with acetonitrile using a method with a calculated limit of quantitation (LOQ) of 12 ng/mL. Kidney samples were homogenized in water and acetonitrile, then cleaned up on C18-bonded silica SPE cartridges. The LOQ of this procedure was 10 ng/g. Urine samples were diluted, filtered, and analyzed directly. The LOQ of this procedure was 63 ng/mL. The overall accuracy for plasma was 103% with coefficient of variation (CV) of 3%; for kidney, 96% and 11%, respectively, and for urine, 98% and 4%, respectively. These methods were applied to the analysis of plasma, urine, and kidney biopsy samples taken from standing animals that had been dosed with penicillin.
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*Keywords:* Penicillin; Liquid chromatography–tandem mass spectrometry; Residue analysis; Quantification; Plasma; Urine; Kidney

## 1. Introduction

The US Food and Drug Administration, Center for Veterinary Medicine (FDA/CVM) sets standards for the safe use of antibiotics in treatment of animals raised for food. For example, if penicillin is used to treat beef cattle, CVM calls for the residue concentration in kidney to deplete below 50 ng/g in order for edible tissues to be considered safe to eat. Enforcement of this tolerance depends on the availability of appropriate analytical tests for kidney concentration of penicillin. Tests that are administered post-mortem sometimes reveal violative drug levels, leading to condemnation of the carcass. This loss might be avoidable if a procedure was available for testing easily-accessible fluids pre-slaughter that can predict whether the kidney concentration will be below tolerance post-slaughter. Fewer animals would be slaughtered and later found unfit for consumption.

Our laboratory has been engaged in research to evaluate two new approaches to addressing this problem. First, we are investigating whether a correlation can be established between drug residue concentrations in various bovine fluids and tissues. This tissue-fluid correlation may mean that residue concentration in kidney can be predicted based on the residue concentration found in fluids such as urine or plasma. A model for this approach to predicting drug concentration has recently been published [1]. Second, we are using biopsy samples taken surgically from the kidneys of standing animals [2,3]. These small biopsy “bites” can be used to determine kidney concentration over multiple time points from a single animal, thereby reducing the number of animals needed for studies on tissue-fluid correlation. This would also enable differences between individuals to be tracked and distinguished.

To date, animal studies have been conducted with two drugs: penicillin G [4] and gentamicin [5]. The drugs differ widely in their depletion profile and so provided a good test for both tissue-fluid correlation studies and biopsy surgeries. Penicillin is excreted or metabolized completely over a few days while gentamicin is sequestered in kidney for many months.

Analytical methods were required for the assay of each drug residue in various tissues. Methods based on liquid chromatography/tandem mass spectrometry (LC/MS/MS) were developed and validated for the gentamicin study [6]. Now we report a similar set of methods for measuring the concentration of penicillin in bovine plasma, urine, and kidney. The target concen-

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tration ranges were based on existing knowledge of the recommended dosage and depletion profiles of penicillin G. The initial goal was to develop simple LC/MS/MS methods with working ranges of 10–10,000 ng/mL in plasma, 100–100,000 ng/mL in urine, and 10–10,000 ng/g in kidney. The kidney samples from biopsy surgery were expected to weigh approximately 100 mg.

There have been numerous methods published for extraction of beta-lactam antibiotics from bovine tissues (primarily milk) followed by LC/MS/MS detection. One of the earliest publications described single-stage LC/MS analysis for five penicillins in muscle, kidney, and milk [7]. Analytes were extracted into acetonitrile and cleaned up using liquid/liquid extraction into dichloromethane. Detection was in negative ion mode and nafcillin was used as an internal standard.

A simple extraction method was reported for seven beta-lactams in milk, including penicillin G [8]. The extraction consisted only of acidification and centrifugation followed by detection in positive ion mode and calibration by external standard. Recoveries ranged from 30% to 80% at 20 ng/mL. Five penicillins were extracted from liver and egg into water and cleaned up on C-18 solid phase extraction cartridges (SPE) [9]. Phenethicillin was added as an internal standard and analytes were detected in negative ion mode. This method was applicable in the range of 10–250 ng/g.

Ion trap LC/MS/MS was used to assay seven penicillins and cephalosporins down to 2.5 ng/mL in milk [10]. Antibiotics were extracted with acetonitrile and hydrophilic polymer SPE. Detection was based on the sodium adduct of penicillin G. Eleven penicillins and cephalosporins were extracted from milk with acetonitrile and the extract was injected directly on a C-18 LC column [11]. Detection was carried out in positive ion mode and nafcillin was used as an internal standard (added after extraction). Quantitative limits for penicillin G were reported at 7 and 4 ng/mL, for raw or retail milk, respectively.

An LC/MS method for 10 beta-lactams in milk made use of a carbonized SPE cartridge with detection of penicillin G in negative ion mode to overcome a significant matrix effect on ionisation [12]. Penicillin V was used as internal standard to quantitate at 5 ng/mL with LOQ of 0.5 ng/mL. A deuterated penicillin G internal standard was used in combination with centrifugation and C-18 SPE cleanup to measure penicillin in milk from 0.8 to 16 ng/mL [13]. Earlier work in our laboratory showed that ion trap LC/MS/MS could be used for qualitative analysis of seven beta-lactams in milk [14]. Analytes were extracted in acetonitrile and cleaned up on C-18 SPE. The confirmation limit for penicillin G was about 5 ng/mL, although this method was not used for quantitation.

These existing methods pointed to a number of promising possibilities for method development. Our goals were to work with the relatively small sample size obtained during biopsy surgery, to develop a method for urine, and to validate the methods for all tissues. It was not clear at the outset how low the ultimate detection capability of the methods needed to be, especially in plasma.

## 2. Experimental

### 2.1. Apparatus

The instrument was a Waters Quattro Micro-LC benchtop triple quadrupole mass spectrometer. The liquid chromatograph was an Agilent 1100 binary pump and autosampler with refrigerated sample tray. The LC column was a Zorbax SB-C18 Rapid Resolution, 2.1 mm × 30 mm, with 3.5 μm silica (Agilent). The method made use of three benchtop centrifuges: Centra GP8-R, with 218A rotor capable of 5000 rpm (Thermo IEC); Spectrafuge microfuge capable of 14,000 rpm for 1.5 mL tubes (Labnet); and Z230-A MKII with fixed angle rotor capable of 5500 rpm with 15 mL tubes (Hermle). Eppendorf 1.5 mL centrifuge tubes (Brinkmann) were used with the microfuge. Graduated polypropylene centrifuge tubes of 15 and 50 mL volume were used for storing samples and carrying out extractions. All liquids were measured and transferred with calibrated variable pipetters fitted with disposable polypropylene pipet tips (Eppendorf). An Omni TH tissue homogenizer fitted with disposable saw-tooth plastic generator probes, 7 mm × 110 mm was used for blending kidney samples. Each sample was processed with a dedicated plastic probe tip. These probe tips are designed to be disposable; in this study, they were washed in a dishwasher and rinsed in methanol before reuse. Disposable PVDF syringe filters in polypropylene housings, 0.2 μm, 13 mm diameter were used (Whatman). The SPE cartridges were Bond-Elut C-18, 500 mg, 3 mL (Varian).

### 2.2. Reagents and standards

HPLC grade methanol and acetonitrile were obtained from Burdick and Jackson. Water processed with the Milli-Q system to give resistivity >18 MΩ cm (Millipore) was used for all subsequent references to water. Phenethecillin was obtained from Sigma Chemical. Penicillin G from U.S. Pharmacopeia (USP) was used for method validation. Penicillin G standard was also obtained from Sigma for use in method development, as were nafcillin, penicillin V, and cephalexin. The product ion mass spectra for penicillin G and phenethecillin were studied in positive ion mode (Figs. 1 and 2).

### 2.3. Solutions

Penicillin G sodium (USP) stock solution was prepared at 1 mg/mL nominal concentration. Approximately 50 mg were dried for 3 h at 60 °C at <5 mm Hg. After cooling, approximately 30 mg were accurately weighed in a glass weigh boat and transferred to a 25 mL volumetric flask with water rinses. After dilution to the mark with water, concentration was calculated by converting to concentration of free acid as mg/mL (weight in mg/25 mL) × (334.4/356.4). Stock solutions of phenethecillin, nafcillin, cephalexin and penicillin V were prepared in water at roughly 1 mg/mL.

Dilutions of penicillin in water were prepared at 100, 10, 1, 0.1 and 0.01 μg/mL. If the stock solution was not exactly 1 mg/mL, the volume of the first dilution was adjusted so the

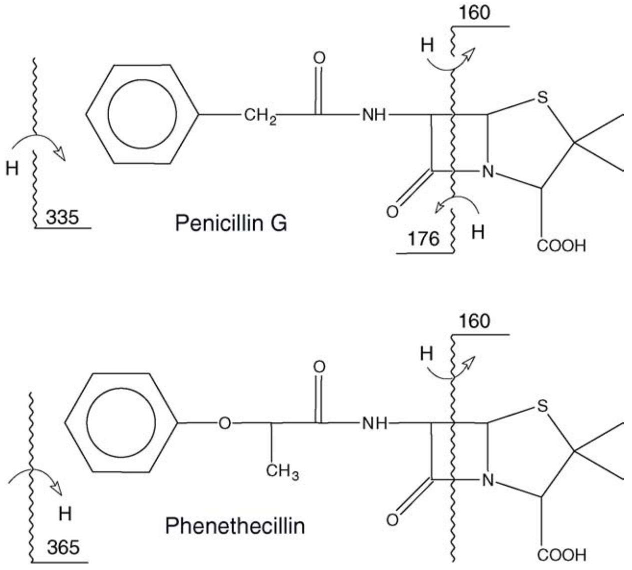

Fig. 1. Precursors and product ions from penicillin and phenethecillin.

first dilution was exactly 100.0 μg/mL. These solutions were subdivided and stored −80 °C. The frozen solutions were stable for 6 months under these conditions. Thawed standards were used once and discarded. Phenethecillin (and other compounds tested as internal standards) were diluted in water to 10 and 0.5 μg/mL, subdivided, and stored <−10 °C. Internal standard solutions were not used for more than five freeze–thaw cycles.

### 2.4. Sample storage

All tissue and fluid samples were stored at −80 °C unless analyzed the day they were obtained, in which case they were kept in the refrigerator pending extraction. Penicillin is known to degrade in tissue on long-term storage, even at −80 °C, so samples were analyzed as soon as possible after collection (see Section 3).

### 2.5. Plasma extraction

Thawed samples were vortexed thoroughly at room temperature. One milliliter of each plasma was transferred to 15 mL centrifuge tubes. Control plasma was used for preparation of blanks, fortified samples (QCs), and standards in the range 10–10,000 ng/mL. For example, a 100 ng/mL standard was prepared by adding 100 μL of a 1 μg/mL solution. All samples received 100 μL of phenethecillin 10 μg/mL internal standard to yield a tissue equivalence of 1000 ng/mL. Lastly, water was added if necessary to bring the total volume of added liquid to 300 μL. Test samples received 200 μL additional water. Standards also received appropriate amounts of added water. For example, the 100 ng/mL standard described above received 100 μL. The additional water was necessary to equalize sample volumes, but it also contributed to more consistent protein precipitation (less clumping).

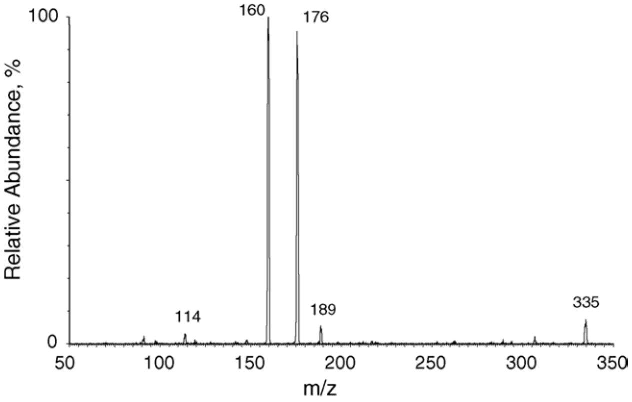

Fig. 2. Product ion spectrum of penicillin G, positive ion ESI-LC/MS/MS.

Samples were vortexed briefly and 3 mL acetonitrile were added. After vortexing for 30 s, samples were centrifuged for 10 min at 2000 RCF (g). The supernate was transferred to clean 15 mL centrifuge tubes. Acetonitrile was evaporated under nitrogen flow in a 40 °C water bath until volume was reduced to less than 0.4 mL. In this and other evaporation steps, it was critical to not let the extract go to dryness, to avoid losses. Volume was adjusted to 2.0 mL with water. The extracts were filtered through 0.22 μm PVDF filters into amber glass autosampler vials. Extracts were analyzed immediately if possible, but after no more than 48 h storage at 5 °C. Injection volume for LC/MS was 5 μL (Fig. 3).

### 2.6. Kidney extraction

This method was designed for assay of penicillin in biopsy samples taken by minimally-invasive surgery from the surface of bovine kidney. This tissue would consist exclusively of kidney cortex, so control tissue was prepared from a control animal whose kidney cortex had been previously homogenized and stored <−60 °C. The method was also applied to 100 mg subsamples of cortex homogenate taken from dosed animals postmortem. Samples were analyzed fresh if possible. Frozen samples were thawed before weighing. Any apparent fat or connective tissue was removed with clean razor blades before weighing. Samples were weighed as close to 0.1 g as possible into Falcon tubes (polypropylene centrifuge tubes, 10 mL capacity, with a snap cap). The exact weights were recorded. Tubes were centrifuged at 1000 rpm briefly to press all tissue to the bottom.

Controls, standards and fortified samples were prepared from the control kidney cortex homogenate. Standards were prepared in the range 5–10,000 ng/g, or a segment of this range with at least six points. For example, to prepare a 100 ng/g sample, 100 μL of a 0.1 μg/mL solution was added. All samples received 100 μL of phenethecillin 0.5 μg/mL (equivalent to 500 ng/g). Lastly, water was added if necessary to bring the total volume of added water to 1000 μL. Tubes were vortexed briefly to mix and 1 mL acetonitrile was added. The samples were homogenized 1 min using a different plastic Omni homogenizer probe tip for each one. Samples were centrifuged 10 min at 3000 × *g*. The supernates were poured into clean 15 mL graduated centrifuge tubes. Acetonitrile was evaporated under nitrogen stream in a 40 °C water bath to reduce volume to 0.4–0.5 mL. Extracts were diluted to 2 mL by addition of water.

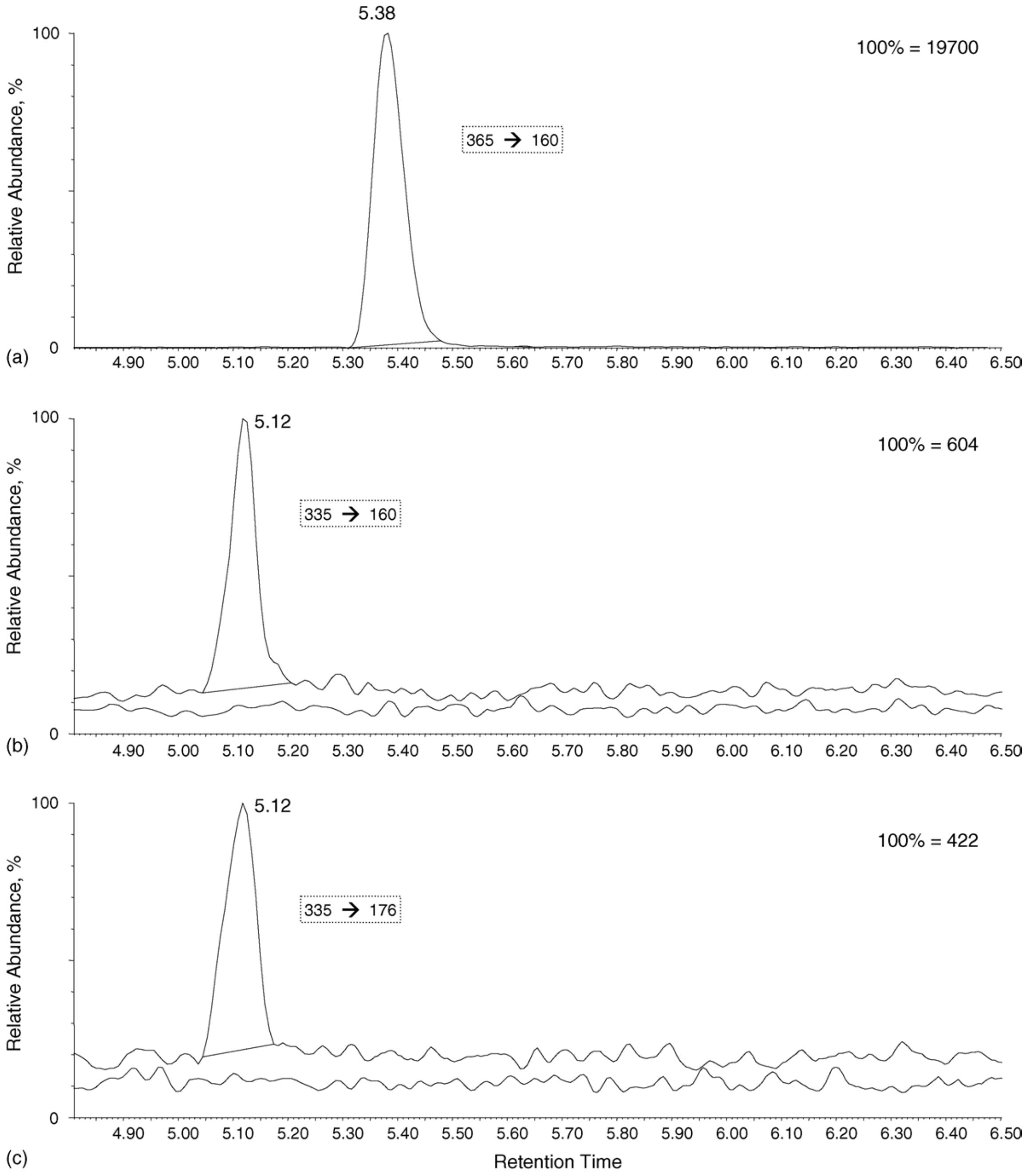

Fig. 3. Product ion chromatograms from a plasma extract found to contain 22 ng/mL penicillin G: (a) $m/z$ 365 → 160 (phenethecillin) from the incurred sample; (b) $m/z$ 335 → 160 (penicillin G) overlaid with corresponding trace from control plasma; (c) $m/z$ 335 → 176 (penicillin G) overlaid with corresponding trace from control plasma.

Further cleanup was carried out using C-18 SPE. The extract was passed through a Varian Bond-Elut C-18 SPE cartridge, 3 mL reservoir and 500 mg sorbent. The cartridge was previously conditioned with 2.5 mL methanol followed by 2.5 mL water. After loading, the cartridge was rinsed with 2.5 mL water. Analytes were eluted into clean 15 mL centrifuge tubes with 3 mL acetonitrile. One milliliter water was added and the volume was reduced to less than 0.4 mL under nitrogen flow in a 40 °C water bath. Final volume was adjusted to 1 mL and the extracts were filtered. If the extracts appeared cloudy after adjusting final volume to 1 mL, they were centrifuged 10 min at 5500 rpm in the Hermle centrifuge before filtering through 0.22 μm PVDF filters into amber glass autosampler vials. Extracts were analyzed immediately if possible, but after no more than 48 h storage at 5 °C. Injection volume was 25 μL (Fig. 4).

### 2.7. *Urine extraction*

Early attempts at analysis of urine were complicated by wide variation in the concentration of dissolved compounds and salts. Extraction by solvent dilution, pH adjustment with acid, or SPE cleanup were unsuccessful because of highly variable matrix effects on LC/MS ionization. Concentrations in urine

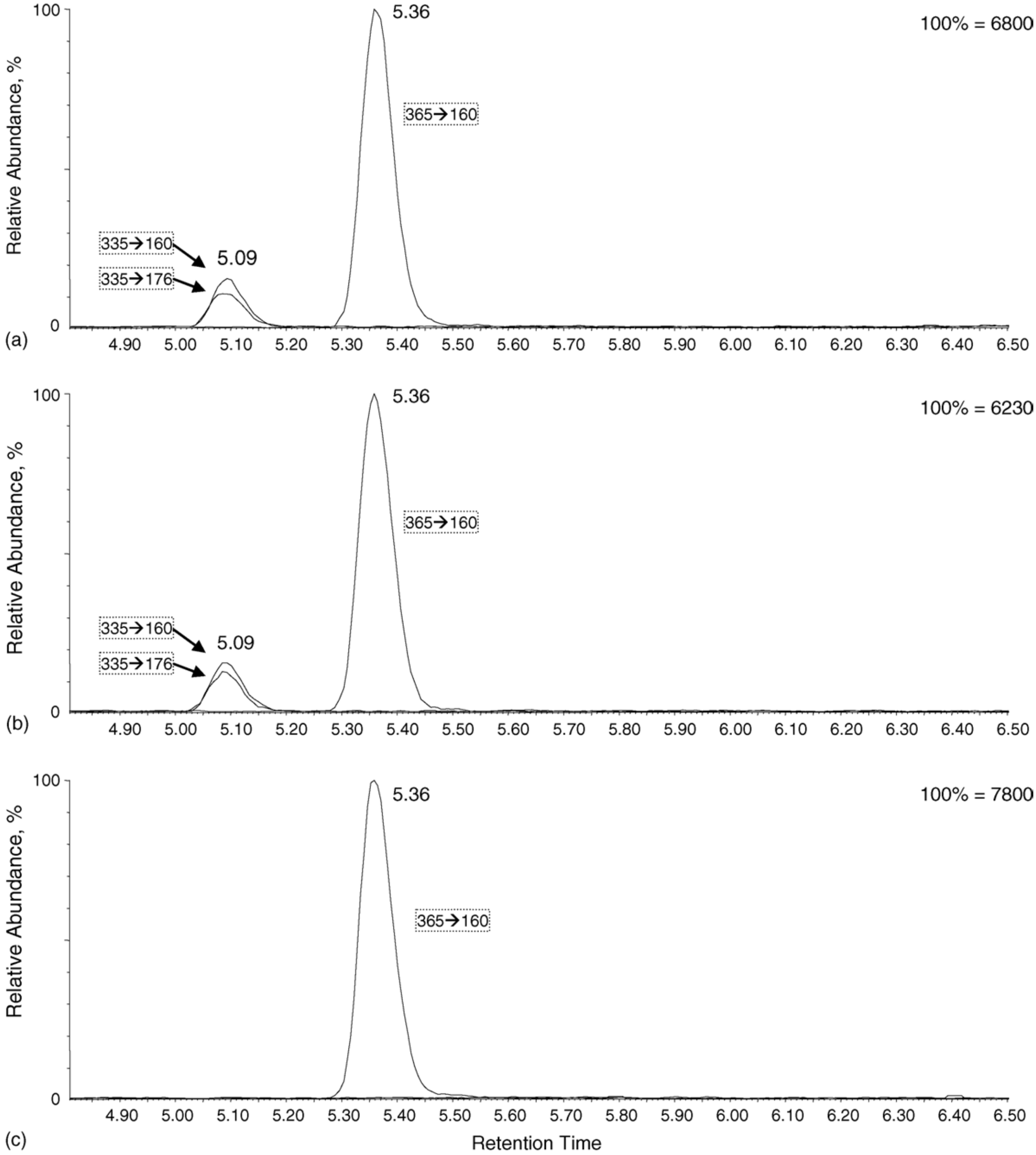

Fig. 4. Product ion chromatograms from kidney procedure: (a) 50 ng/g matrix standard, (b) assay of kidney biopsy sample, measured at 58 ng/g, (c) control kidney cortex homogenate. All contain phenethecillin internal standard equivalent to 500 ng/g.

were expected to be fairly high because penicillin is excreted rapidly into the urine. The high concentrations meant that penicillin could be detected directly following a simple dilution and filtration. Unfortunately the matrix effects were still highly variable among various urine samples after 1:100 dilution with water. The problem was caused by highly variable urine concentration, ranging from dilute (pale yellow) to concentrated (deep yellow). Dilution with water was not sufficient to eliminate differences between the samples.

We successfully controlled the matrix effects by using control urine from a fasting steer that had not been dosed with penicillin to “damp out” the natural variations among the samples. The matrix effects were thus matched among various samples and standards. It was possible to quantify by diluting 10-fold with control urine, then 10-fold again with water. The same source of control urine was used for each day’s batch. The control urine had a leveling effect on matrix suppression, so samples behaved more similarly to one another and to the standard curve (prepared only in control urine).

Frozen urine was thawed the day of analysis and thoroughly vortex-mixed. An aliquot of 0.5 mL was transferred to a 50 mL centrifuge tube. This was diluted with 4.5 mL of control urine and vortex-mixed. Standards and fortified samples were prepared in 5 mL of control urine. Standards were prepared at 100, 200, 400, 1000, 4000, 10,000, 40,000 and 100,000 ng/mL. For example, a 1000 ng/mL standard was prepared with 50 μL of

10 μg/mL penicillin standard. QC samples were similarly prepared at 2000 and 20,000 ng/mL.

The internal standard was added (200 μL of 10 ppm phenethecillin, equivalent to 4000 ng/mL) and the tubes were vortexed again. Samples were diluted to 50 mL with water, then shaken and vortex-mixed. About 1 mL was filtered into an autosampler vial. Injection volume for LC/MS/MS was 5 μL (Fig. 5).

### 2.8. Liquid chromatography

The extracts were stored at 5 °C in the autosampler tray until analyzed. Analysis was carried out within 48 h (although usually right after extraction). Separation of analytes was carried out with a binary gradient. Initial conditions were held for 0.5 min at 0.3 mL/min: 90% solution A (0.1% formic acid in water) + 10% acetonitrile. The mixture was ramped to (40 + 60) over the next 1.5 min and held there for another 2.0 min. The mixture was ramped back to initial conditions (90 + 10) over 1 min and held for 4 min before starting the next injection cycle. Column temperature was held at 30 °C. Column backpressure was roughly 75 bar for new columns. If backpressure rose above 125 bar the column was replaced. The divert valve was programmed to send LC effluent to waste except from 3.5 to 6.5 min after injection. Penicillin retention time was 5.1 min and phenethecillin retention time was 5.4 min under these conditions.

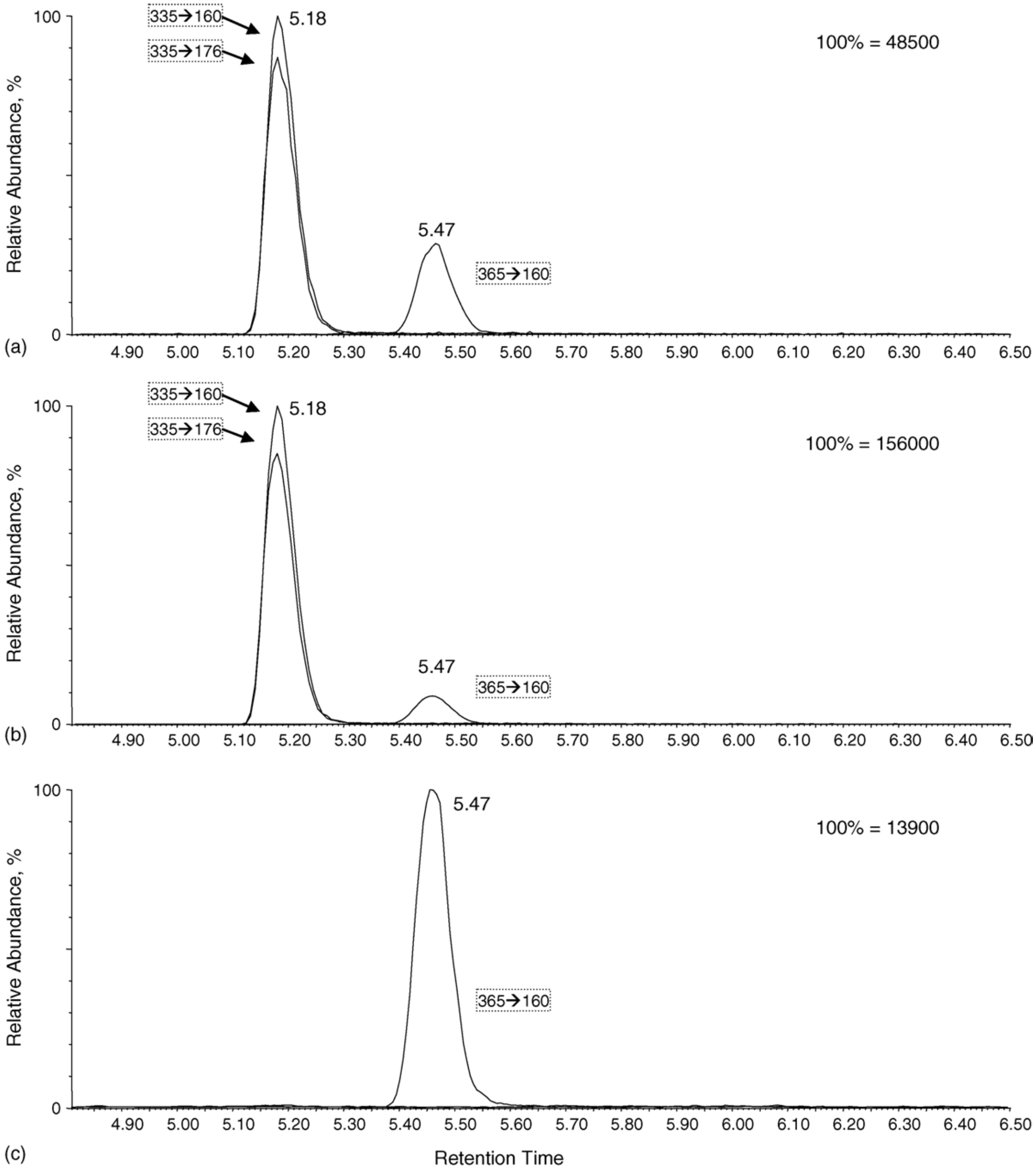

Fig. 5. Product ion chromatograms from urine procedure: (a) 10,000 ng/mL matrix standard; (b) incurred urine sample, measured at 30,700 ng/mL and (c) control urine. All contain phenethecillin internal standard equivalent to 4000 ng/mL.

### 2.9. Data acquisition and processing

The LC/MS data system was MassLynx 4.0, with SP3 and the TargetLynx quantification package. The mass spectrometer used the electrospray source in positive ion mode. The tune was optimized in positive ion MS/MS mode for the three SRM transitions: penicillin G, $m/z$ 335 → 160 and $m/z$ 335 → 176, and phenethecillin, $m/z$ 365 → 160. Optimization was carried out while infusing 1 μg/mL solutions into mobile phase of 50:50 acetonitrile:0.1% aqueous formic acid flowing at 0.3 mL/min. Low mass resolution was set in both MS1 and MS2 (LM = 12). Argon collision cell pressure was set at approximately 3.2 mTorr. Normally two injections of control samples were made at the beginning of each run to allow the system to equilibrate and stabilize.

The tuning conditions used for validation of these methods were ESI capillary 3 kV, cone 15 V, extractor 3 V, Rf lens 0.4 V, source temperature 120 °C, desolvation temperature 400 °C, desolvation gas flow 700 L/h, cone gas flow 10 L/h, entrance lens-5, exit lens-1, collision energy 14 V. The acquisition dwell time was 100 ms per transition.

### 2.10. System suitability and quality control

Preliminary tests were used to qualify the LC/MS system for analyses according to these methods. The relative standard deviation for absolute response of penicillin G and phenethecillin in repetitive injections of 1000 ppb standard had to be <15%. A suitability test was applied to an injection of 0.5 ng penicillin (for example, 5 μL of a 0.1 μg/mL solution). The peak-to-peak signal-to-noise calculated by the MassLynx software was >100:1 for the transition $m/z$ 335 → 160.

Acceptance tests were applied as follows, in case of abnormal background in controls or highly variable response among extracts. The apparent phenethecillin peak area in chromatograms of penicillin G standard must be <0.2%. The apparent penicillin G peak area in chromatograms of phenethecillin standard must be <10% of the lowest standard injected in a given batch. For a given calibration curve to be acceptable, up to a 15% deviation by standards was acceptable above the lowest level, and a 20% deviation was acceptable at the lowest level. The coefficient of determination had to be >0.99. At least one QC sample at each level tested had to show a deviation <15%. The acceptability of recovery from extracts should be evaluated, to reject extracts if the phenethecillin peak area deviates >50% from the average phenethecillin response.

## 3. Results and discussion

Four candidates were tested for possible use as internal standards: cephalexin, nafcillin, penicillin V, and phenethecillin. Phenethecillin was selected as the most similar to penicillin G in terms of absolute recovery and response in electrospray ionization, as well as suffering the least ion suppression of the four candidates carried through the plasma extraction. Positive ion mode was selected over negative mode based on somewhat better limit of detection. The positive ion signal-to-noise for penicillin in plasma extracts was about four-fold better then in negative ion.

Table 1
Validation data for measurement of penicillin G in bovine tissues

| | Mean accuracy (%) | CV (%) | $n$ |
|---|---|---|---|
| Plasma (ng/mL) | | | |
| 30 | 102 | 4 | 18 |
| 300 | 104 | 3 | 17 |
| 600 | 102 | 4 | 5 |
| 3000 | 103 | 2 | 9 |
| Overall | 103 | 3 | 49 |
| Kidney (ng/g) | | | |
| 60 | 97 | 13 | 8 |
| 150–300 | 99 | 15 | 6 |
| 2000 | 93 | 4 | 6 |
| Overall | 96 | 11 | 20 |
| Urine (ng/mL) | | | |
| 2,000 | 96 | 5 | 8 |
| 20,000 | 98 | 3 | 4 |
| Overall | 98 | 4 | 12 |

### 3.1. Method performance

Method performance was characterized by assessing accuracy, precision (Table 1) and limit of quantitation (LOQ). Values for accuracy in Table 1 reflect the recovery correction inherent in internal standard calibration. The LOQs were calculated from the apparent measured value of blanks (mean + 10$x$ standard deviation) analyzed during method validation and study analyses. The resultant LOQ values were 12 ng/mL in plasma (number of blanks, $n = 22$)), 10 ng/g in kidney ($n = 20$), and 63 ng/mL in urine ($n = 8$).

Ion suppression effects were evaluated using the plasma method. Standard curves were prepared in plasma and in water at five levels from 10 to 200 ng/mL. The absolute response in extracts versus water (a combination of loss during extraction and ion suppression) was roughly 45% for penicillin and 35% for phenethecillin. Therefore preparation of standards in water would not be acceptable for plasma analysis, because the different ion suppression would cause a 30% bias in quantitation. Preliminary assessment of the plasma procedure suggested that both penicillin G and phenethecillin showed absolute recovery of almost 100%. In both the plasma and urine procedures, the internal standard response was very similar in samples and matrix-standards. We feel this provided evidence that matrix effects were under control in those procedures.

### 3.2. Solid phase extraction

The first attempt at kidney extraction was based only on homogenization in acetonitrile for protein precipitation. However, the extracts tended to clog the LC column and cause gradual loss of electrospray response. Solid phase extraction was found to be necessary for improved kidney cleanup. Solid phase cartridges based on either C-18 bonded silica or hydrophilic polymer were found to give acceptable results using this method. The method was validated and applied using the Varian C-18 SPE cartridge. Absolute recovery through the kidney extraction procedure was found to be approximately 80%. It was assumed that matrix effects could be overcome by (1) use of a structurally similar internal standard which gave similar absolute recovery to the penicillin analyte, and (2) use of calibration standards prepared in a similar matrix to the kidney biopsy samples.

During the animal phase of the study, two incurred plasma samples were found to contain less than 10 ng/mL penicillin. The SPE cleanup step was applied to the plasma extracts to further clean up and concentrate the analytes. To cover the lower range, standards were prepared with penicillin at 1–100 ng/mL. For example, a 2 ng/mL standard was prepared by adding 200 μL of 0.01 μg/mL penicillin. Phenethecillin internal standard was added at a fixed concentration of 50 ng/mL (100 μL of 0.5 μg/mL). The total added water was adjusted to 300 μL as above. Injection volume for LC/MS was 50 μL, representing 20-fold more equivalent plasma per injection compared to the high range procedure.

Unfortunately this procedure was complicated by variable matrix effects: plasma from study animals showed different suppression effects from the control plasma that had been used successfully with the higher-range procedure. The cause of this problem was not identified. To work around this problem for two samples from one particular animal (the only study samples found to contain less than 10 ng/mL penicillin), concentration was measured by the method of standard addition. Five 0.5 mL aliquots of the sample were carried through the plasma extraction as described above, followed by SPE cleanup as described for kidney. Four aliquots were spiked with four different concentrations of penicillin G (2, 4, 20 and 40 ng/mL) and the fifth was not spiked. Linear regression analysis was applied to the penicillin:phenethecillin response ratios in the five extracts, and the concentration of the unknown samples were calculated accordingly.

Matrix effects were controlled in the urine analysis by dilution with one control urine lot per analytical batch. If the same lot of urine was used for each analytical batch, the results should be internally consistent. Also, multiple batches should be comparable to one another because the same standard and internal standard were used for all batches. The main evidence supporting the validity of the urine dilution was the very similar absolute response of the internal standard in the calibration standards (10% control urine) and the samples (9% control urine + 1% sample urine).

### 3.3. Penicillin stability in water, plasma and tissues

Aqueous penicillin standards were found to be stable for 6 months at −80 °C. Six stock solutions were prepared over a 6-month period during the course of this study. At the conclusion, 1 μg/mL solutions from each preparation were analyzed by LC/MS/MS. The absolute response from four replicate injections of each preparation was averaged. The relative standard deviation of these mean values was 2.6% and there was no pattern of loss or degradation over time.

A large volume (50 mL) of control plasma was fortified at 500 ng/mL and thoroughly mixed. The fortified plasma was then dispensed in 1 mL aliquots. Three samples were analyzed immediately and the rest were stored at either 5, −15, or −80 °C. Each day, some tubes were removed from their storage conditions and allowed to thaw on the bench at room temperature for 4 h before being replaced. Two tubes from each storage condition were thawed and refrozen once, another pair was thawed twice and a third pair was thawed three times over consecutive days. The remaining tubes were not thawed until analysis 29 days later.

Degradation was observed over long term storage under all three conditions. When stored at −80 °C for about a month, 6% of the penicillin was lost. At −15 °C, 32% was lost, and at 5 °C, nearly 75% of the penicillin was lost. All samples in the short-term freeze–thaw study dropped a few percent in penicillin content. Multiple freeze–thaw cycles increased the rate of degradation somewhat more.

Other studies have found degradation of beta-lactams on storage. The half-life of benzylpenicillin and cloxacillin in liver was only 62 days for spiked tissue and 71 days for incurred tissues when stored at −20 °C [15]. Ampicillin was found to be stable in porcine muscle at −75 °C over 8 months whether ground or in chunks [16]. However, storage at −20 °C led to 10% loss over 3 months followed by an additional loss of about 40% over the next 5 months.

Penicillin in plasma was found to be somewhat stable at −20 °C over 2 months, with a 10–20% loss. Degradation in kidney and liver was more rapid at −20 °C, up to 20% loss in only 10 days, and almost 50% loss from muscle after 10 days. However, incurred tissue samples stored at −75 °C remained stable [17]. On the other hand, penicillin was found to be stable in urine for up to 12 weeks at −15 °C, [18] suggesting that pH has an influence on penicillin stability. Based on these studies and reports, it was critical to store incurred tissues at −80 °C and to analyze the kidney and plasma samples as soon as possible. Urine is apparently the only tissue that can tolerate extended storage before analysis without running the risk of penicillin degradation.

### 3.4. Kidney biopsy samples

The minimally-invasive surgery procedure was used to remove samples ranging from 100 to 400 mg from the kidney cortex of eleven standing animals. In four cases, sufficient tissue was available to analyze four replicates per animal. The analytical variability within each set of four was somewhat greater than the overall CV of 11% found with fortified kidney cortex homogenate (Table 1).

It was not possible to directly compare biopsy concentrations to those found by conventional analysis post-mortem, because of the time lapse between surgery and slaughter (ranging from 30 min to 24 h). Nevertheless, the overall trend in results suggests similarity between the two sampling techniques within experimental error, although the biopsy sampling method may be slightly lower overall due to presence of bodily fluid not present in post-mortem tissue.

In those cases where animals were slaughtered within several hours of biopsy surgery, [3] the biopsy and post-mortem results, although similar, were not always the same. Also, in our work with gentamicin [4] we found that post-mortem concentrations tended to be about 1/3 higher on average than that predicted by the depletion trend of the biopsy samples.

It may simply be that the fluid content of fresh tissue is higher than in ex-sanguined carcasses, causing a dilution effect in the biopsy samples. Khor and co-workers suggested another possible explanation, that under certain circumstances it may be necessary to correct for the presence of drug in the residual blood remaining in the tissue [19]. This is an issue which should be addressed before making use of the biopsy technique for assessing depletion profiles. However, analytical methods alone cannot fully decide this question.

The analytical challenges resulting from small sample size should not deter future application of minimally-invasive surgery for drug residue studies in living animals. Rather, our experience to date shows that development of methods for this purpose must include testing of kidney samples prepared by a wide variety of sampling techniques from whole kidney. This would include sampling to various depths from the kidney surface, and using various starting weights for preparation of homogenized samples, including whole kidney. It will be important to consider the relationship between whole kidney concentration (on which regulatory tissue tolerances are based) and the concentration found near the surface, in the cortex tissue. There may have to be further study of the relationship between residue concentrations found in biopsy samples and in post-mortem tissues. At that point, researchers will be able to maximize the utility of the minimally-invasive surgery techniques to perform tissue residue studies on standing animals, while minimizing the number of animals required.

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