Screening method for identification of β-lactams in bovine urine by use of liquid chromatography and a microbial inhibition test

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Objective—To develop a multiple-residue screening method for the detection of β-lactams in bovine urine.

Animals—6 clinically normal Holstein cows and 6 calves.

Procedure—Pooled urine obtained from cows was used as a negative-control sample or spiked with varying concentrations of 6 β-lactam antibiotics. Urine samples were prepared for liquid chromatography by diluting 1 ml of urine with 9 ml of 0.01 M KH2PO4, 0.01 M Na2PO4, and filtering. Filtrate (2,000 ml) was eluted with a mobile phase in a gradient program. A fraction corresponding to each β-lactam of interest was collected and evaporated to < 1 ml, and water then was added to achieve a 1 ml volume. The collected fraction was tested, using a microbial inhibition test. Then, calves were fed milk spiked with a mixture of 5 β-lactam antibiotics at a concentration 40× the FDA tolerance in milk. Three hours following the feeding, urine samples were obtained from the calves and tested, as described for the urine samples for the cows.

Results—The lowest concentrations of amoxicillin, ampicillin, cepharpin, cloxacillin, desfuroylethifucysteine, and penicillin G that were consistently detected in urine were 100, 10, 100, 250, 1,000, and 10 ng/ml, respectively. Amoxicillin, ampicillin, cepharpin, cloxacillin, desacetylcloxacillin, and penicillin G were detected in urine samples of 6/6, 6/6, 0/6, 6/6, 0/6, and 2/6 calves respectively, fed antibiotic-spiked milk.

Conclusions and Clinical Relevance—The integrated method described can be used to detect or identify β-lactam antibiotics in bovine urine. This method can be used to test cattle for β-lactam residues. (Am J Vet Res 2001:62:326-330)

Detection and identification of β-lactams in urine could contribute substantially to information on antibiotic residues in animals raised for food. Various methods of liquid chromatography (LC) have been used for the determination of residues of numerous β-lactams in milk, serum, and tissue of cattle. Liquid chromatography has been integrated with other assays to determine antibiotic residues in milk. To the authors’ knowledge, none of the methods for detection and identification of β-lactams in urine uses an integrated approach of LC and a residue screening test designed to detect residues in milk.

The study reported here describes results for a simple 1-step sample preparation procedure for use in LC with fraction collection. Fractions were subsequently analyzed, using a microbial inhibition test, for the determination and identification of β-lactams in bovine urine.

Materials and Methods

Stock solutions of β-lactams—Stock solutions (1 mg/ml of water) were prepared for each of several β-lactams (amoxicillin, ampicillin trihydrate, cephabaprin, cloxacillin, desfuroylethifucysteine [DFCC], and penicillin G sodium salt). Standard solutions were stored refrigerated and diluted as required to make working solutions. The β-lactam working solution contained a mixture of sodium penicillin G, amoxicillin, ampicillin, cephabaprin, cloxacillin, and DFCC at concentrations of 1, 1, 1, 1, and 0.2 µg/ml, respectively. Desacetylcloxacillin (DAC) was an impurity in the cephabaprin standard that increased as the solution aged, especially at room temperature.

Urine samples—Urine from clinically normal Holstein cows was obtained from cattle at a USDA dairy research facility or at our veterinary medical facility. The cows’ medical history indicated that they had not been treated with antimicrobial or anti-inflammatory drugs for ≥ 2 weeks prior to collection of urine samples. Furthermore, urine from each cow was tested, using a commercially available swab-specimen test kit, for the evidence of antimicrobials as an objective method of confirmation. These samples were used as negative-control samples or were spiked with β-lactams for assay validation. The β-lactam working solution was added to these urine samples to produce varying concentrations of β-lactams in urine.

Liquid chromatography method—In a 10-ml plastic tube, aliquots (1 ml) of urine were diluted with 9 ml of a 1:1 (vol/vol) solution of 0.01 M KH2PO4 and 0.01 M Na2HPO4. Samples were mixed by inverting the test tube 10 to 15 times, and 4 ml of the sample solution was poured into a 10-ml syringe fitted with a 0.45-µm polyvinylidene fluoride membrane, 25-µm syringe filter. The solution then was filtered into a 4-ml autosampler vial for the LC procedure.

β-lactamase treatment—We reconstituted β-lactamase in water, in accordance with manufacturer's instructions, and 0.1-ml portions were stored frozen (–20 C) until needed. β-lactamase (0.1 ml) was added to 5 ml of urine sample. Samples were incubated for ≥ 1 hour at room temperature (24 ± 5 C) to allow for full degradation of the antibiotics. Following incubation, the sample was processed similar to other samples.
Collection of fractions—Separation of fractions during LC was performed by use of a mobile phase in a gradient program as follows: 100% 0.01M KH₂PO₄ for 3 minutes, 40% 0.01M KH₂PO₄ and 60% acetonitrile for 40 minutes, and 100% 0.01M KH₂PO₄ for 5 minutes. The mobile flow rate was 1 ml/min with an injection volume of 2,000 μl. Chromatograms were acquired at 210 nm. The LC equipment consisted of a pump; autosampler with a 2,000-μl loop; 3 μm, 150 × 4.6 mm (ID) column with matching guard cartridge; photodiode array detector data system; and fraction collector.

The system was put through a complete cycle to condition and stabilize the column before determining the retention time of the antibiotic standard. Using the β-lactam working solution, the fraction collector was set to collect a 1.8-ml sample that was centered on the retention time of each β-lactam with a 0.1-minute delay; multiple fractions corresponding to each β-lactam were collected. Fractions were collected in 15-ml conical graduated centrifuge tubes. To aid in stability, 0.2 ml of 0.01M Na₂HPO₄ was added to tubes containing fractions for penicillin G and cloxacillin.

After evaporation, water was added to adjust the final volume to 1 ml.

Microbial inhibition screening test—A microbial inhibition test was used. Collected fractions of urine were used in place of milk samples. Negative (did not contain β-lactams) and positive (contained β-lactams) control samples were analyzed concurrently on each day and for differing lots of the test kit.

Urine from calves fed milk that contained antibiotics—Six clinically normal dairy calves, 3 weeks old, from our veterinary medical facility were used. Prior to the study, urine samples were obtained from the calves and tested, using a commercially available swab-specimen test kit and a microbial receptor assay. Samples had to have negative results for a calf to be included in the study. Amoxicillin, ampicillin, cloxacillin, cephalolin, and penicillin G were added to reconstituted milk replacer to achieve concentrations of 400, 400, 400, 800, and 200 ng/ml, respectively, which corresponded to a concentration 40 times the FDA-established tolerance for each drug in milk. The antibiotic-containing milk replacer was fed to calves at a rate of 6% of each calf’s body weight. Urine samples were collected before and 3 hours after consumption of the milk replacer; urine was collected during natural voiding. Urine was immediately stored on ice, allowed to settle, and decanted into plastic conical test tubes. The urine sample was divided into 3 aliquots: 1 was tested by use of the swab-specimen test kit, 1 was tested by use of the microbial receptor assay, and 1 was stored at –75°C until assayed by use of the aforementioned integrated LC method.

Results

Concentrations of β-lactams in urine samples—Using the β-lactam working solution, β-lactams were separated by use of the gradient elution LC procedure (Fig 1). Retention times for the β-lactam standard solutions were approximately 12.5, 15.5, 16.5, 18.0, 19.0, 23.9, and 27.9 minutes for amoxicillin, DAC, DFCC, ampicillin, cephalolin, penicillin G, and cloxacillin, respectively. Chromatograms were obtained for urine to which we added amoxicillin, ampicillin, cephalolin, cloxacillin, DFCC, and penicillin G (Fig 2).

The eluate fractions corresponding to ampicillin and penicillin in urine samples supplemented with 10 ng/ml of those antibiotics had positive results when tested by use of the microbial inhibition test. The lowest concentration of amoxicillin, ampicillin, cephalolin, cloxacillin, DFCC, and penicillin G added to urine that yielded consistently positive results (100% of tested samples at a specific concentration) for the microbial inhibition test were 100, 10, 100, 250, 1,000, and 10 ng/ml of urine, respectively (Table 1).

Concentrations of β-lactams in urine of calves fed antibiotic-containing milk replacer—Using the swab-specimen test kit, of 6 urine samples collected from calves 3 hours after drinking antibiotic-containing milk replacer had positive results. Using the microbial receptor assay, β-lactams were detected in all of these same urine samples.

Using the integrated LC method, at least 1 of the β-lactams was detected in the urine samples of all calves. Specifically, amoxicillin and cloxacillin were detected in the urine of all 6 calves 3 hours after consumption of antibiotic-containing milk replacer. Ampicillin was detected in urine samples of 5 calves, and penicillin G was detected in urine samples of 3
Results of the microbial inhibition screening test on urine samples to which a mixture of β-lactams was added. Results within each concentration are for separate urine samples. Results of the microbial inhibition test on samples treated with β-lactamase were negative for all β-lactams.

Discussion

Analysis of results for the β-lactam working solutions revealed good separation of the fractions of interest, using gradient elution (Fig 1). Results were highly reproducible on successive tests. This LC environment (with modifications) and fraction collection procedure is routinely used by the USDA, Agricultural Research Service for analysis of milk and tissue.7-9 It requires approximately 1 h/sample; however, with automated equipment, the procedure can be performed throughout the night without the need for personnel to monitor the process.

After appropriate conditioning of the column, a standard solution should always be used to assure retention times of various fractions. Approximate time for each peak should not vary greatly from previously stated or observed times; when time for each peak varies considerably, the column and conditions should be examined. A standard solution does not need to be used between successive samples, because retention times are highly constant and reproducible during the course of successive tests. Use of a standard solution between samples would greatly increase time and materials needed while increasing the chances for memory effect.1 Examination of peaks from various urine samples and retesting a standard solution after the final urine sample can reveal whether conditions have changed and altered retention times. When uncertainties arise, the standard solution and samples should be tested again.

The LC environment is not optimal for detecting the β-lactams used in the study reported here. The compounds of interest usually are not discernible on the chromatogram because of background noise caused by urine constituents (Fig 2), with the exception of cloxacillin, which may be detected at high concentrations. The initial use of the β-lactam standard solution allowed for the determination of retention times for the various β-lactams; thus, when we analyzed the urine samples, all fractions were collected without prior knowledge of the β-lactams that were in each sample. Collecting and testing eluate fractions must be conducted to ascertain detection of the β-lactams.

Collecting a 1.8-ml fraction centered on the retention time of each β-lactam necessitates collecting material 0.9 minutes before and after peak retention. The amount of time separating peak retention for DAC and DFCC (15.5 and 16.5 minutes, respectively) was insufficient for complete collection of each fraction. The interval between peaks was divided equally; thus, fraction collection continued for 0.5 minutes after the DAC peak retention, then collection of the DFCC fraction was begun (0.5 min prior to its peak retention). Ampicillin and cephapirin had a similar situation. Penicillin G and ceftiofur have similar retention times with the LC method used here (ie, approx 24 minutes). Ceftiofur is rapidly metabolized in ruminants without any parent compound eliminated in the urine.12 Consequently, penicillin G was the most likely antibiotic to be involved if the fraction collected at 24 minutes yielded positive results when tested by use of the microbial inhibition test. When involvement of ceftiofur needs to be ascertained, a fraction corresponding to a metabolite of ceftiofur (ie, DFCC), should be collected (retention time, approx 16.5 minutes).

Unlike ceftiofur, the retention time of cephapirin does not overlap with any of the β-lactams approved and commonly used in food animals. A positive result on the microbial inhibition test for the cephapirin fraction is indicative of cephapirin content. However, it is advisable to collect the fraction corresponding with its metabolite (ie, DAC) when the use of cephapirin in cattle is to be determined by monitoring residues in urine. Cephapirin is metabolized to DAC; DAC and its parent compound can be found in urine and milk after administration of cephapirin.13,14 The DAC is microbiologically active, although to a much lesser extent than the parent compound.15

The use of β-lactamase treatment on a duplicate sample confirmed the β-lactam content. The original and β-lactamase-treated samples were tested consecutively on the LC, and fractions were collected. Fractions of the β-lactamase-treated sample were refrigerated pending results of the microbial inhibition test of the original (nontreated) sample. When the nontreated fraction had a positive result for the microbial inhibition test, then the corresponding fraction that was treated with β-lactamase was tested. Only refrigerated fractions corresponding to a nontreated

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fraction that has positive results need to be tested. This procedure confirms β-lactam content in the sample while allowing efficient use of resources.

A crucial step in the integrated method reported here was the evaporation of the collected fraction to < 1 mL in volume. This removed acetonitrile from the collected fraction; residual acetonitrile in the fraction can interfere with the microbial inhibition test, causing false-positive results. Evaporation was not accomplished under a stream of air or nitrogen, because these procedures cause slower evaporation and do not effectively remove the acetonitrile. The mobile-phase buffer (0.01M KH₂PO₄) does not interfere with the microbial inhibition test.

The integrated method described here was most sensitive for the detection of ampicillin and penicillin G in urine; urine with a concentration of ampicillin or penicillin G of 10 ng/ml yielded positive results when tested. The LC method was most sensitive for DFCC and cloxacillin, whereas the integrated screening method was least sensitive for these 2 compounds (Fig. 1, Table 1). Sensitivity of the LC method for DFCC and cloxacillin was serendipitous, because the LC method is used predominately for the separation of β-lactams contained in the sample. The integrated method was not as sensitive for amoxicillin, cephalin, and cloxacillin, and it was least sensitive for DFCC, the metabolite of cefapirin. Determining the lowest concentration of DAC that could be detected by the microbial inhibition test was not evaluated during this study. Because DAC in the cephalin standard was an impurity caused by the breakdown of cephalin, and concentrations of DAC increased as the solution aged, the concentration of DAC was not known.

We documented the usefulness of this integrated LC method by feeding calves milk replacer containing a mixture of β-lactams at 40× the FDA-established tolerance in milk. Following consumption of the antibiotic-containing milk replacer, the swab-specimen test identified β-lactams in urine samples of 4 of 6 calves, and the microbial receptor assay detected β-lactams in urine samples of all 6 calves. However, neither test could be used to determine the number of β-lactams in the urine samples, nor could the tests be used to identify which β-lactams were in the urine samples. Use of the integrated method described here enabled us to detect amoxicillin and cloxacillin in the urine of all calves 3 hours after the consumption of the antibiotic-containing milk replacer; ampicillin was detected in urine samples of 5 of 6 calves. Cephalin was not detected in urine samples of any of the calves, but urine samples of 2 calves had detectable amounts of DAC. This finding was expected because of the rapid metabolism of cephalin to its metabolite, DAC. An unexpected finding was the detection of penicillin G in urine samples of 3 calves.

We evaluated the integrated LC method by using 6 β-lactams, or a metabolite thereof, approved for use in animals raised for human consumption. These β-lactams are commonly used in veterinary medicine. In the part of the study that involved feeding milk replacer to calves, cefotiofur was not fed, because when it is used properly, the parent drug is not excreted in milk; therefore, only the metabolite should be detectable in waste milk fed to calves.

A LC assay that included a simple 1-step preparation of samples combined with a commercially available rapid screening test for antibiotic residues in milk may be used for the determination and identification of β-lactams in bovine urine. The integrated method could be used in studies involving administration of multiple or unknown β-lactams to cattle. It also may be used to assist in the identification of specific β-lactams in urine to complement studies on depletion of concentrations in blood, milk, or tissue.

References
14. Tyczkowska KL, Voykener RD, Aronson AL. Development
