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Journal of Chromatography A, 812 (1998) 237–247

JOURNAL OF CHROMATOGRAPHY A

Multiresidue determination of β-lactam antibiotics in milk and tissues with the aid of high-performance liquid chromatographic fractionation for clean up

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Abstract

Screening of milk shipments for β-lactam antibiotic residues is mandatory in the USA and is widely used in other countries. Interpretation of positive screening test results has been difficult. Only six β-lactam antibiotics are approved for use in food-producing animals in the USA but many others are used in other countries. A multiresidue procedure was developed for identification and quantitation of unknown β-lactam antibiotics. The residues were extracted with acetonitrile and tetraethylammonium chloride. The extract was concentrated by evaporation and filtered. The concentrated extract was then loaded onto an HPLC column in 100% 0.01 *M* KH_2PO_4 and eluted with an acetonitrile gradient. Fractions corresponding to analytes of interest were collected and tested for antibiotics using rapid milk screening tests. Fractions testing positive were analyzed by HPLC. The identity of β-lactams was confirmed by treating a replicate with β-lactamase.

Keywords: Milk; Food analysis; Multiresidue methods; Lactams; Antibiotics; Penicillins; Cephalosporins

1. Introduction

The β-lactam group of antibiotics is widely used for the treatment of farm animals. In the USA, only six β-lactam antibiotics, amoxicillin, ampicillin, ceftiofur, cephapirin, cloxacillin, and penicillin G, are approved for use with food-producing animals [1]. Many additional β-lactams are approved for use in other countries [2]. There is also the possibility of extra-label use of unapproved β-lactams.

In the USA, testing of incoming shipments of milk for β-lactam antibiotics is now mandatory [3]. A number of screening tests have been approved for this purpose. For the year ending 30 September, 1996, the National Milk Data Base developed by the US Food and Drug Administration reported that 4 480 530 tests for β-lactam antibiotics in milk were run. Of these, 6148 (0.14%) were positive [4]. None of the positive tests were confirmed by more specific analytical methods.

Tissues are also tested for antibiotics using various screening tests usually based on inhibition of microbial growth [5–8]. US Department of Agriculture's Food Safety and Inspection Service uses a seven-plate microbial assay in which all but one of the plates contain the enzyme, penicillinase, to differentiate penicillins from other antibiotics [5]. However, many β-lactams are resistant to degradation by penicillinase and may be reported as unidentified microbial inhibitors (UMIs).

When products test positive for antibiotics by screening tests, the questions arise ''What is it?'', ''How much is there?'', and ''How did it get into the

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0021-9673/98/$19.00
PII: S0021-9673(98)00387-2

product?''. To answer these questions, sensitive and specific confirmatory procedures are needed. The confirmatory procedures should be able to determine all antibiotics detected by the screening tests and should equal or exceed the sensitivity of the screening tests. There are many published determinative procedures for residues of various β-lactam antibiotics in milk [9–41] and tissues [30,42–54] but most fall short of these requirements in one or more respects.

Our laboratory recently described a multiresidue procedure suitable for determination in milk of the six β-lactams approved for use in the USA [32,33]. The antibiotics were separated using HPLC fractionation for clean up. The fractions were tested for antimicrobial activity and those testing positive were analyzed by HPLC. The identity of residues was confirmed by treating a replicate with β-lactamase. The present paper describes application of this approach to determination of additional β-lactams and to residues in tissues. The structures of the β-lactams and metabolites considered in the present study are shown in Figs. 1 and 2.

2. Methods and materials

2.1. Chemicals and reagents

Acetonitrile was HPLC grade (EM Omnisolv or equivalent). Tetraethylammonium chloride (Et_4NCl), 1-decanesulfonic acid (sodium salt, 98%), and dodecyl sulfate (sodium salt, 98%) were obtained from the Aldrich Chemical (Milwaukee, WI). Ceftiofur, desfuroylceftiofurcysteine and pirlimycin were gifts from the Pharmacia and Upjohn (Kalamazoo, MI). The other antibiotics were purchased from Sigma Chemical (St. Louis, MO). Other chemicals were reagent grade from several sources. 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, dispensed in 0.1-ml portions into minicentrifuge tubes and stored frozen at −20°C until needed. Delvotest P-mini kits (Gist-Brocades) were obtained from Eastern Crown (Vernon, NY).

Stock solutions of the antibiotic standards were prepared at 1 mg/ml or less and stored frozen at −20°C until needed. Working dilutions of 100, 10, and 1 μg/ml were prepared biweekly or as necessary, depending on the stability of the compounds in water.

2.2. Glassware and other equipment

Glassware required included graduated cylinders, 25 and 50 ml; conical graduated centrifuge tubes, 15 ml, calibrated to 1 and 4 ml; glass-stoppered side-arm flasks, 250 ml; and conical flasks, 125 ml. All glassware was cleaned in special detergent (MICRO, International Products, Trenton, NJ, USA or equivalent) at about 60°C for 30 min (longer may etch glassware), then rinsed in deionized water, then rinsed 5 min or longer in a dilute acid bath (ca. 0.01 *M* HCl or H_2SO_4), then again with deionized water.

Other equipment required included a blender, Waring type, base, with 100- or 300-ml stainless steel jars with covers; a Vortex evaporator (Buchler, Ft. Lee, NJ, USA); a thermostated hot plate with shallow tray; and plastic-coated lead rings (I^2R, Cheltenham, PA, USA) to weight flasks during evaporation.

2.3. Extraction/deproteinization

2.3.1. Milk

Milk (10 ml) was measured into a 125-ml conical flask and mixed with 2 ml of 0.1 *M* Et_4NCl. Then 40 ml of acetonitrile was added slowly with continual stirring (final volume=50 ml). After standing for 10 min, the supernatant was decanted through a plug of glass wool in the stem of a funnel and 40 ml of filtrate (=8 ml of milk) was collected and transferred to a 250-ml glass-stoppered side-arm flask and 2 ml of 0.01 *M* pH 6 buffer (5:1 KH_2PO_4–Na_2HPO_4) was added. The flasks were connected to a water pump vacuum. After the contents had stopped boiling, the flasks were weighed with lead rings and placed in a shallow (1–2 cm) water bath heated to 40–50°C. The contents were evaporated to 1–2 ml but not to dryness and were rinsed into graduated tubes with several small portions of water to a final volume of 4 ml. This was filtered through a 25-mm 0.45-μm PVDF syringe filter into a 4-ml autosampler vial.

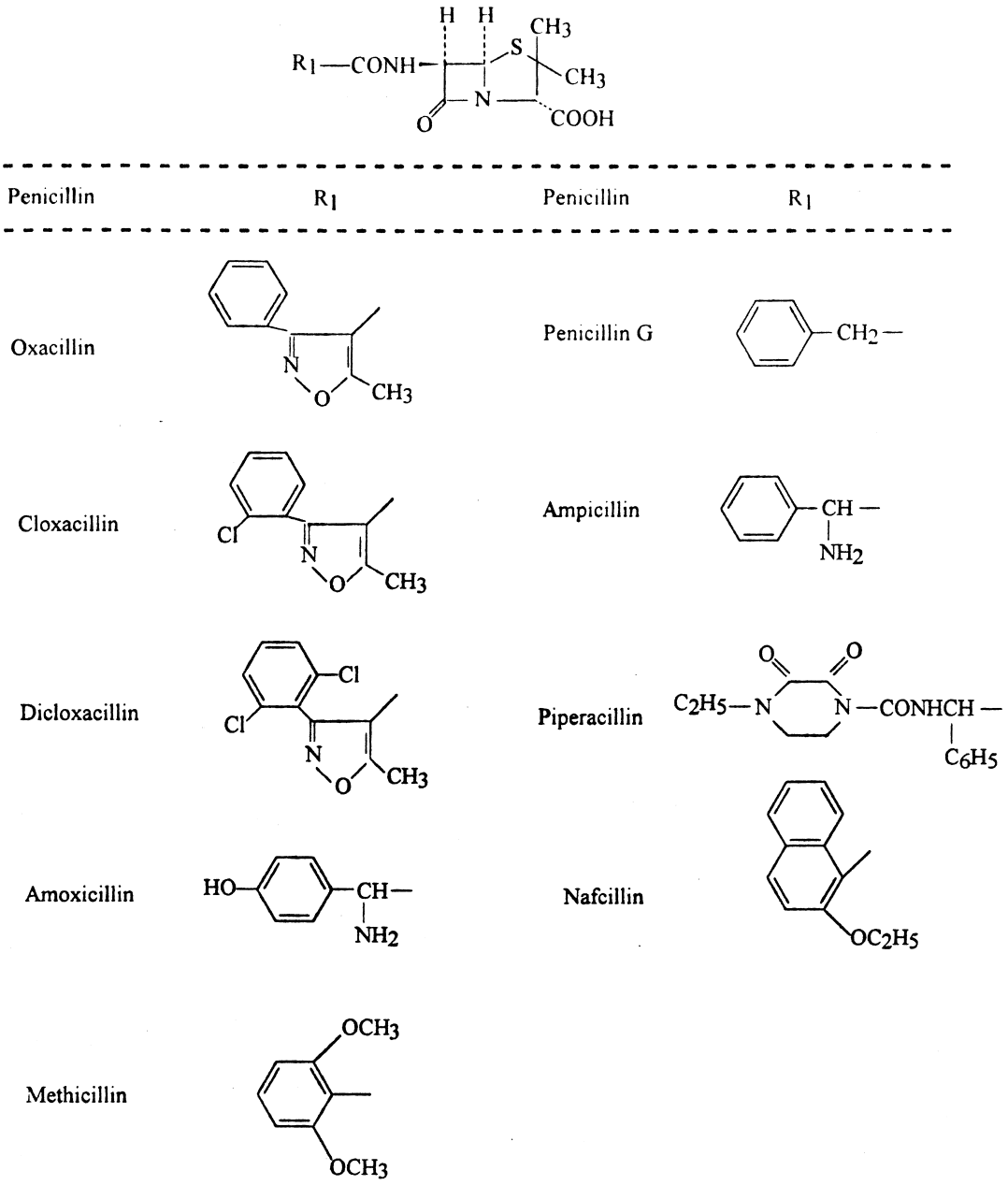
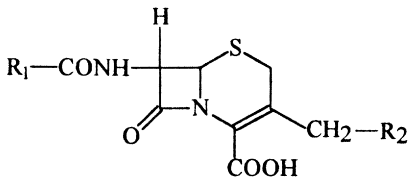


Fig. 1. Structures of some common penicillins.

2.3.2. Tissue—Procedure I

Tissue was cut into small pieces and 5 g was transferred to a small (100–300-ml) blender jar. Then 5 ml of water, 2 ml of 0.1 *M* Et_4NCl (for liver and kidney, 1 ml of 0.2 *M* Et_4NCl and 1 ml of 0.005 *M* KH_2PO_4), and 40 ml of acetonitrile were added and the mixture was blended for 1 min at one/half full power as measured by a variable resistance transformer (final volume=50 ml). After standing for 10 min, the supernatant was decanted through a



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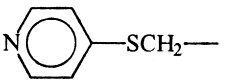
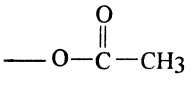
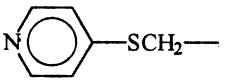
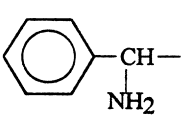
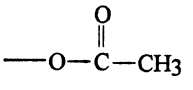
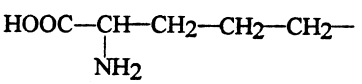
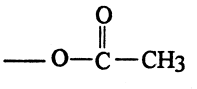
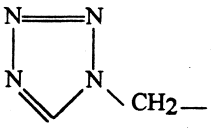
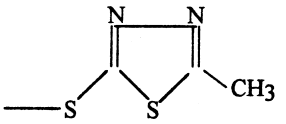
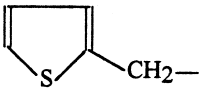
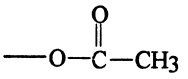
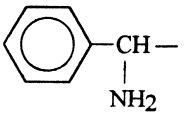
Cephapirin		
Desacetylcephapirin		—OH
Cephaloglycin		
Cephalosporin C		
Cefazolin		
Cephalothin		
Cephalexin		—H

Fig. 2. Structures of some common cephalosporins and metabolites.

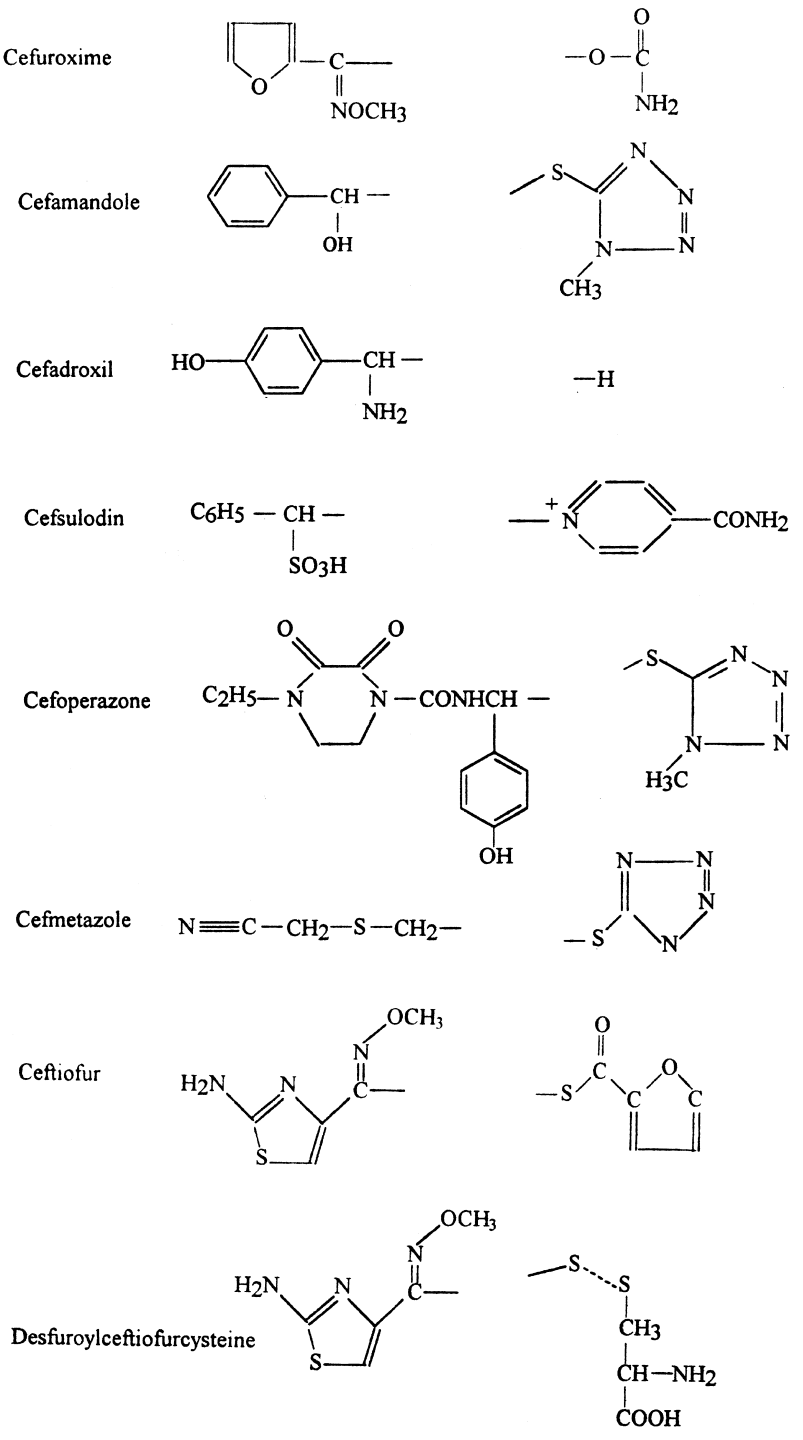


Fig. 2. (*continued*)

small plug of glass wool in the stem of a funnel and 40 ml of filtrate (20 ml for liver and kidney) was collected which was equivalent to 4 g of tissue (2 g for liver and kidney). The filtrate was transferred to a 250-ml side-arm flask and 2 ml of 0.01 *M* pH 6 buffer, 5 ml of water and 5 ml of *tert*.-butanol (to suppress foaming) were added. The filtrate was concentrated by evaporation as described for milk. If foaming persisted, more *tert*.-butanol was added, always with an equal volume of water.

2.3.3. Tissue—Procedure II (β-lactamase)

Tissue was cut into small pieces and 15 g was weighed into a 100–300-ml blender jar and blended with 45 ml of water for 1–2 min at low power until the tissue was thoroughly broken up. Ten ml of homogenate was treated as described for milk. For evaporation, *tert*.-butanol and water were added to the filtrate to suppress foaming.

2.3.4. β-lactamase treatment

Before beginning the extraction procedure, 0.1 ml of the reconstituted β-lactamase was added to 10 ml of milk or tissue homogenate (Procedure II) and the mixture was incubated for 1 h at room temperature.

2.4. HPLC fractionation

The HPLC system used for clean up consisted of a Varian (Sugarland, TX, USA) Model 9012 pump, a Waters (Milford, MA, USA) WISP 712 autosampler with a 2000-μl loop, an ISCO (Lincoln, NE, USA) FOXY fraction collector, a Waters 990 diode array detector and a Supelcosil LC-18 column (150×4.6 mm, 5-μm particle-size) (Supelco, Bellefonte, PA). When a sample was injected, the autosampler started the other components of the system. Two ml of sample extract were loaded onto the HPLC column with a flow of 100% 0.01 *M* KH_2PO_4, flow-rate of 1 ml/min. After 3 min, an acetonitrile gradient was started to 60% acetonitrile at 40 min. The column was returned to starting conditions at 41 min and was ready to load another sample at 55 min. Standards of analytes of interest were run initially to determine retention times and the fraction collector was set to collect 1.5–2.0 time windows centered on the retention time of these analytes. For the penicillin G and cloxacillin fractions, 0.1 ml of 0.01 *M* Na_2HPO_4 was added to the tubes prior to fraction collection.

2.5. Analysis of fractions

The fractions were evaporated to <1 ml under reduced pressure in the vortex evaporator and the volume was adjusted to 1 ml with water. Unknowns were tested for antimicrobial activity using the Delvotest P-mini as described for milk (other milk screening tests may be satisfactory). Those testing positive were analyzed by HPLC. Prior to HPLC analysis, 0.2 ml of 0.01 *M* KH_2PO_4, 0.01 *M* H_3PO_4, 0.01 *M* sodium decanesulfonate was added to the amoxicillin, ampicillin, desfuroylceftiofurcysteine, and cephapirin fractions. With knowns, this was done prior to adjusting the volume to 1 ml. The HPLC system used for analysis consisted of a Varian Model 9012 pump, a Varian 9090 autosampler with a 200-μl loop, a Waters 481 UV–VIS detector and a Varian Model 654 data system, with a flow-rate of 1 ml/min. For HPLC analysis, conditions different than those used for clean-up were used. Some successful analysis conditions are summarized in Tables 1 and 2. The buffer–acetonitrile combinations were premixed and were stable indefinitely.

3. Results and discussion

The use of HPLC fractionation for sample clean up was initially explored when it became evident that other approaches were not adequate to achieve the sensitivity needed for determination of β-lactam antibiotic residues. In this approach, residues from a sample extract were concentrated on-line from pure buffer and then eluted with an acetonitrile gradient. With the column packing used, there was no loss in performance when 100% buffer was used. In fact, polar analytes such as amoxicillin were poorly retained if even traces of acetonitrile were present. Fractions corresponding to each analyte of interest were collected and analyzed under different conditions. Fig. 3 shows gradient elution of six β-lactam antibiotics approved for use in the USA as well as some metabolites. Fig. 4 shows gradient elution of some other antimicrobials. The gradient elution procedure worked with most antibiotics except for

Table 1
Analysis of fractions from milk

Fraction	Column	Mobile phase and detection
Amoxicillin	Supelcosil LC-18[a]	0.015 *M* H_3PO_4, 0.0075 *M* sodium dodecyl sulfate–acetonitrile (68:32) UV 215 nm
Ampicillin, cephalexin	Supelcosil LC-18	0.01 *M* H_3PO_4, 0.005 *M* KH_2PO_4, 0.005 *M* sodium dodecyl sulfate–acetonitrile (65:35) UV 215 nm (ampicillin), 260 nm (cephalexin)
Cephapirin	Supelcosil LC-18	0.01 *M* H_3PO_4, 0.005 *M* KH_2PO_4, 0.005 *M* sodium dodecyl sulfate–acetonitrile (68:32) or 0.015 *M* H_3PO_4, 0.0075 sodium dodecyl sulfate–acetonitrile (65:35) (best) UV 290 nm
Penicillin, ceftiofur	Supelcosil LC-18-DB[a]	0.0033 *M* H_3PO_4, 0.0067 *M* KH_2PO_4 or 0.005 *M* H_3PO_4, 0.005 *M* KH_2PO_4–acetonitrile (72:28) UV 215 nm (Pen G), 290 nm (Ceft)
Penicillin V	Supelcosil LC-18-DB[a]	0.005 *M* H_3PO_4, 0.005 *M* KH_2PO_4, acetonitrile (67:33) UV 215 nm
Desacetylcephapirin	Polymer Labs. PLRP-S	0.01 *M* H_3PO_4, 0.01 *M* KH_2PO_4, 0.01 *M* sodium decanesulfonate–acetonitrile (82:18) UV 290 nm
Cloxacillin, Oxacillin, Nafcillin, Dicloxacillin	Supelcosil LC-18-DB	0.0020 *M* H_3PO_4, 0.0080 *M* KH_2PO_4,—acetonitrile (62:38) or 0.0025 *M* H_3PO_4, 0.0075 *M* KH_2PO_4–acetonitrile (60:40) UV 215 nm
Cefmetazole, Cefaperazone, Cefuroxime	Supelcosil-LC-18	0.02 *M* H_3PO_4, 0.01 *M* sodium decanesulfonate–acetonitrile (82:18) UV 260 nm

[a] Other C_{18} columns can probably be substituted for the LC-18. However, the LC-18-DB column has unique selectivity for some penicillins.

Table 2
Analysis of fractions from tissue

Fraction	Column	Mobile phase and detection
Amoxicillin, ampicillin	Supelcosil LC-18	Derivatize with HCHO, method of Ang and Luo [40] 0.0083 *M* KH_2PO_4, 0.0017 *M* Na_2HPO_4–acetonitrile, (82:18) (amoxicillin) or (78:22) (ampicillin), fluorescence detection
Cephalexin	Supelcosil LC-18	0.0067 *M* H_3PO_4, 0.0033 *M* KH_2PO_4, 0.0025 *M* sodium dodecyl sulfate–acetonitrile (67:33) UV 260 nm
Cloxacillin, dicloxacillin	Inertsil ODS-2	0.008 *M* KH_2PO_4, 0.002 *M* H_3PO_4–acetonitrile (60:40) UV 215 nm
Desacetylcephapirin	Polymer Labs. PLRP-S	0.0133 *M* KH_2PO_4, 0.0067 *M* H_3PO_4, 0.01 *M* sodium decanesulfonate–acetonitrile (85:15) UV 290 nm
Penicillin G	Inertsil ODS-2	0.0067 *M* KH_2PO_4, 0.0033 *M* H_3PO_4–acetonitrile (70:30) UV 215 mn
Desfuroylceftiofurcysteine	Supelcosil LC-18	0.015 *M* H_3PO_4, 0.0075 *M* sodium dodecylsulfate, acetonitrile (60:40) UV 270 nm

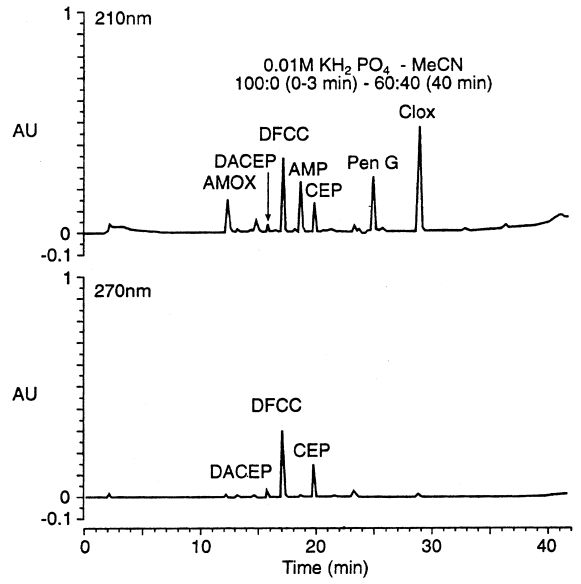


Fig. 3. Gradient elution of standards, 1 μg/ml. Injection volume, 2 ml. AMOX=Amoxicillin; DACEP=Desacetylcephapirin; DFCC=Desfuroylceftiofurcysteine; AMP=Ampicillin; CEP=Cephapirin; Pen G=Penicillin G; Clox=Cloxacillin.

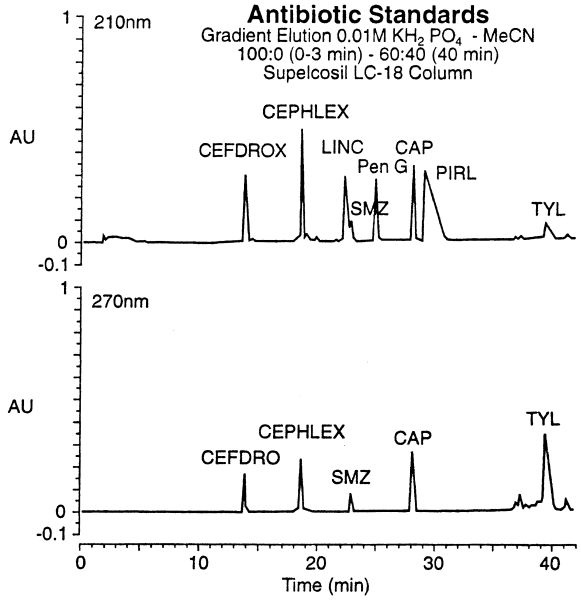


Fig. 4. Gradient elution of some antimicrobials. Injection volume, 2 ml. CEFDROX=Cefadroxil; CEPHLEX=Cephalexin; LINC=Lincomycin; SMZ=Sulfamethazine; Pen G=Penicillin G; CAP=Chloramphenicol; PIRL=Pirlimycin; TYL=Tylosin. CAP, LINC, PIRL=10 μg/ml, others=1 μg/ml.

the tetracycline group. It is thus potentially possible to determine a wide variety of antibiotics using a single clean up procedure. HPLC fractionation was used for determination of lincomycin in milk and tissues [55].

For single analytes, column switching has been used in which a fraction containing an analyte of interest is diverted into a second HPLC system for analysis. This approach has been successfully used for determination of amoxicillin in biological fluids [56]. Use of this approach for multiple analytes would be unwieldy since a separate analytical system would be required for each analyte.

The advantages of HPLC fractionation for cleanup are:

1. It is a simple one-step procedure which can be fully automated.
2. It is highly reproducible.
3. The operating cost is low. The same column can be reused hundreds of times. The only cost is a small amount of buffer and acetonitrile.
4. By collecting narrow fractions, separation from interferences is better than with methods based on solid-phase extraction with disposable cartridges or partitioning.

There are also some disadvantages.

1. High equipment cost. However, the HPLC system can be used for other purposes when not configured for clean up.
2. Each fraction must be analyzed individually. However, by testing fractions from unknown samples with rapid screening tests, only those testing positive require HPLC analysis.

Our initial approach to analysis of tissues (Procedure II) was to homogenize the tissues in water and then treat the tissue homogenates as previously described for milk [32,33]. However, we found that some β-lactams were degraded fairly rapidly in tissue homogenates, especially from liver and kidney. A procedure (Procedure I) was therefore developed using direct extraction with acetonitrile. Procedure II was still used for confirmation by treatment with β-lactamase where degradation of the antibiotics was intentional.

The extracts were buffered to about pH 6 using 5:1 KH_2PO_4–Na_2HPO_4 which improved the stability of some analytes. The peak shape of amoxicillin was markedly improved when it was injected in the

pH 6 buffer. Penicillin G and cloxacillin were slightly unstable in KH_2PO_4 and so a small amount of Na_2HPO_4 was added to the tubes used for fraction collection.

For analysis of unknowns, we usually collected six fractions from milk samples corresponding to amoxicillin, desacetylcephapirin (DACEP), ampicillin, cephapirin, penicillin G-ceftiofur, and cloxacillin. With tissues, a slightly broader fraction including desfuroylceftiofurcysteine (DFCC) and DACEP was collected and the cephapirin fraction was not collected. If the presence of other residues was suspected, additional or broader fractions could be collected. The approximate retention times of some other β-lactams in the gradient elution program are summarized in Table 3 to indicate the potential usefulness of the method with these compounds. Cephalexin coeluted with ampicillin. Five compounds eluted within 1 min of cephapirin.

Table 3
Approximate retention times of some β-lactam antibiotics with the gradient elution program[a]

Antibiotic	Retention time (min)
Amoxicillin	12.5
Cephalosporin C	13.5
Cefadroxil	14.8
Cefsulodin	15.5
Desacetylcephapirin	15.5
Desfuroylceftiofurcysteine	16.5
Ampicillin, Cephalexin	18.5
Cefuroxime	19.5
Cefmetazole	19.7
Cephapirin	19.8
Cefazolin	20.0
Cephaloglycin	20.3
Cefaperazone	22.1
Cefamandole	22.5, 25.2
Methicillin	23.0
Cephalothin	23.5
Penicillin G, Ceftiofur	24.5
Piperacillin	24.9
Oxacillin	27.0
Cloxacillin	28.6
Nafcillin	30.3
Dicloxacillin	30.5

[a] Ceftriaxone did not give a discernible peak with this program.

3.1. Analysis of HPLC fractions

1. Remove acetonitrile by evaporation under reduced pressure.
2. Test for the presence of antibiotics using the Delvotest P or other rapid screening test.
3. Analyze positive fractions by HPLC.

For HPLC analysis of fractions, conditions different from those used in cleanup must be used to separate analytes from interferences. Some approaches used were:

1. Change the pH of the mobile phase.
2. Add ion-pairs to the mobile phase.
3. Change column type.
4. Combinations of 1, 2 and 3.
5. Derivatize analytes in fractions.

Most analytes could be adequately separated from interferences by approaches 1–4 for analysis using UV detection. Ampicillin and amoxicillin in tissue required derivatization. The derivatization procedure of Ang and Luo [40] was simple and worked satisfactorily with the fractions. The analytical approaches used for different β-lactams are summarized in Tables 2 and 3 for milk and tissue, respectively. These are suitable for use with fractions prepared as described. Both cleanup and analysis conditions must be rigidly followed for satisfactory results. Other nominally equivalent HPLC columns may not give the same separations. Other combinations of cleanup and analysis conditions would likely prove satisfactory but they were not discovered in the present study. When the amphoteric β-lactams were injected in the KH_2PO_4 buffer for analysis, the antibiotic peaks were sometimes distorted or doubled. This could be counteracted by adding a small amount of a more acid buffer containing sodium decanesulfonate as an ion-pairing agent. This was done after testing the fractions by microbial screening tests.

The composition of the mobile phase used for analysis was adjusted to give retention times of the analytes of 10–15 min in most cases. This improved separation from interferences and any system peaks which might be present. With DFCC, the selectivity of the column appeared to change at higher acetonitrile concentrations. It was necessary to have a retention time of >15 min to separate this compound from interferences. For determination of amoxicillin in milk, a retention time of near 20 min was optimal.

It was essential to completely remove residual acetonitrile from both the sample extracts and the HPLC fractions prior to the next steps. Evaporation

under reduced pressure as described was faster, more effective in removing acetonitrile and required less heating than evaporation under a stream of air or nitrogen as is commonly done.

Treatment of a replicate with β-lactamase provided a simple and sensitive confirmatory test. Any residual background could be subtracted, thus improving the accuracy of the analysis, particularly at low levels of residue.

The method is intended for examination of the small percentage of samples testing positive for antibiotics by screening tests rather than primary screening for residues. For analysis of a single sample, the approximate times required for each step are:

1. Extraction and evaporative concentration – 2 h.
2. HPLC fractionation – 1 h.
3. Screening of fractions – Delvotest P, 2 1/2 h, others <1 h.
4. Analysis of positive HPLC fractions – 1 h for each positive fraction.

Since cleanup was sequential, one or two samples could be completed on the day they are started. For multiple samples, HPLC fractionation can be run overnight which is normal practice in our laboratory. As many as 12–15 samples per day can be run by this approach.

This procedure has been used successfully to identify unknown β-lactam antibiotics in a number of milk samples from commercial sources which tested positive by various screening tests. It has also been used to identify β-lactam antibiotics including DFCC in liver and kidney samples reported to contain unidentified microbial inhibitors by USDA's Food Safety and Inspection Service. The application of this approach to determination of penicillin G [57] and DFCC [58] in tissues has been described in more detail elsewhere.

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