Trace levels of amoxicillin residues were determined in animal tissues by liquid chromatography (LC) with fluorescence detection. An improved solid-phase extraction (SPE) procedure requiring less flammable solvent (diethyl ether) was developed for sample preparation. Muscle samples of beef, pork, chicken, and tilapia were extracted with a phosphate buffer followed by the modified SPE procedure for cleanup and concentration prior to the LC–fluorescence analysis. Average recoveries of fortified amoxicillin at 5, 10, and 20 μg/kg ranged from 83.9 to 85.8% in beef, 86.1 to 88.1% in pork, 81.7 to 82.9% in chicken, and 92.5 to 95.4% in tilapia. Relative standard deviations were <4%.

Amoxicillin has been widely used in human and veterinary medicines for controlling bacterial infections. However, excessive use of antibiotics may lead to the occurrence of these residues in food chains and to the development of new strains of bacteria resistant to these drugs. The U.S. official tolerance for amoxicillin residues is 0.01 ppm (10 ppb or 10 μg/kg) in milk and uncooked edible tissue of cattle (1). Highly sensitive, determinative methods are important for monitoring amoxicillin residues at this tolerance level.

Microbial inhibition assays are the conventional methods used to screen for antibiotic residues in animal tissues and/or milk. Recently, several liquid chromatographic (LC) techniques have been developed to determine residues in foods. A few LC methods have been reported for β-lactam antibiotics in pharmaceutical preparations, body fluids, and milk. However, information on the analysis of trace amounts of amoxicillin in animal tissues is limited. The major problem encountered in these matrices is the presence of numerous interfering substances.

Snippe et al. (2) developed an automated on-line dialysis and solid-phase extraction (SPE) method followed by LC with ultraviolet (UV) detection at 260 nm for amoxicillin residues in bovine muscle. However, this method was not sensitive enough for trace levels of the residues, i.e., the limit of detection (LOD) was reported as 0.2 μg/g (200 μg/kg). Rose et al. (3) reported an LC method using cation exchange and porous graphitic carbon SPE for sample cleanup for analysis of amoxicillin residues in animal muscle and liver. However, recoveries of fortified drugs at the maximum residue limit of 50 μg/kg in fortified cattle muscle were <60%. Ang et al. (4) developed an LC method using fluorescence detection which provided good sensitivity, accuracy, and precision for determination of amoxicillin residues in catfish and salmon muscle. The method’s LODs were ≤0.8 μg/kg, recoveries were ≥75% at 5–20 μg/kg fortification levels, and relative standard deviations (RSDs) were ≤6%. This method was further validated by comparison with the conventional microbial inhibition method for determining fortified and incurred amoxicillin residues in catfish muscle (5); however, it has not been applied to other animal tissues. A less desirable feature of the method was its use of diethyl ether. The objective of the present study was to develop an improved SPE method to reduce the use of diethyl ether and to validate the modified method for determination of amoxicillin residues in muscle tissues of various animal species.

METHOD

All reagents, chemicals, and apparatus were essentially the same as reported previously (4).

Reagents

(a) Amoxicillin.—Amoxicillin trihydrate containing 861 μg amoxicillin/mg; USP reference standard (U.S. Pharmacopeial Convention, Inc., Rockville, MD).

(b) Water.—Distilled, deionized (DD), >1.5 million ohm/cm and passed through a carbon filter (MilliQ water purification system, Waters Corp., Milford, MA) or equivalent.

(c) Chemicals.—Granular potassium phosphate, monobasic (KH₂SO₄) and KOH (reagent grade, Fisher Scientific Co., Fairlawn, NJ); NaCl and citric acid (J.T. Baker, Inc., Phillipsburg, NJ); and trichloroacetic acid (TCA; Aldrich Chemical Co., Milwaukee, WI), reagent grade, or equivalent.
Solvents.—Methanol, acetonitrile, and ethyl ether, LC grade (J.T. Baker); formaldehyde (Aldrich Chemical Co.), reagent grade, or equivalent.

0.01M Phosphate solution.—Dissolve 1.36 g KH$_2$SO$_4$ in 1 L water and adjust pH to 4.5 if needed.

TCA solutions.—Dissolve appropriate amounts of TCA in water to make 2, 20, and 75% (w/v) solutions. Stopper and mix thoroughly. Store at room temperature.

Formaldehyde solution.—Prepare 7% (w/v) formaldehyde by diluting concentrated formaldehyde (37%) with 0.4M citric acid. Store at room temperature.

LC mobile phase.—Prepare 0.05M KH$_2$SO$_4$ solution (6.8 g/L) and adjust pH to 5.6 with 1M KOH solution. Mix 800 mL phosphate solution with 200 mL acetonitrile, filter through 0.45 µm pore nylon filter, and degas.

Apparatus

(a) LC system.—Solvent delivery system, Model 600E (Waters Corp.), or equivalent; injector, Model 7125 (Rheodyne, Cotati, CA) with fixed volume loops, or equivalent; detector, Model 470 scanning fluorescence detector (Waters Corp.) or equivalent; data processing and system monitor software, Millennion 2010 Chromatogram Manager (Waters Corp.), or equivalent; LC column, Spherisorb S5 ODS2, 25 cm x 4.6 mm (Phase Separations, Inc., Norwalk, CT), Prodigy 5 mm, ODS-3, 25 cm x 4.6 mm (Phenomenex C., Torrance, CA), or equivalent.

(b) Food processor.—Model 702 R (Hamilton Beach/Proctor-Silex, Inc., Washington, NC), or equivalent.

c) Tissue homogenizer.—Model Ultra-turrax T25 (Ika-Labortechnik, Janke & Kunkel GMBH & Co. KG, Staufen, Germany) with S25N-18G probe, or equivalent.

(d) Centrifuge.—Damon IEC refrigerated Model PR-J (Damon/IEC Div., Needham Heights, MA) with rotor and inserts capable of holding 50 and 15 mL tubes, or equivalent.

e) Vortex mixer.—Model M-37615 (Barnstead/Thermoline, Dubuque, IA), or equivalent.

(f) SPE system.—Vacuum manifold (Waters Corp.), or equivalent; Sep-Pak Vac 3 cc C$_{18}$ cartridges containing 500 mg sorbent (Waters Corp.), or equivalent. Recoveries from SPE cartridges should be at least 80%. Because SPE cartridges may vary from lot to lot, each lot should be evaluated. Fortified extracts rather than standard solutions are recommended for the evaluation.

g) Evaporator.—N-Evap (Organomation, Inc., South Berlin, MA), or equivalent.

(h) Water bath.—To maintain temperature at 100°C, Buchi Model 481 (Brinkmann Instrument, Inc., Westbury, NY), or equivalent.

(i) Glassware and pipettor tips.—50 mL disposable, graduated, polyethylene centrifuge tubes; 15 mL screw-capped glass tubes; and disposable pipet tips of various sizes (100–1000 µL). All glassware for standard solutions and/or samples must be washed with detergent solution and rinsed with, in succession, tap water, DD water, and acetonitrile. To avoid cross contamination, it is recommended that a microliter pipettor be reserved for high concentration amoxicillin stan-
standard solutions only. Disposable pipet tips are used throughout the experiment.

**Preparation of Standard Solutions**

(a) **Stock amoxicillin standard solution (1 mg/mL).**—To the nearest 0.01 mg, quantitatively weigh 58.07 mg reference standard and transfer to 50 mL volumetric flask; dissolve and dilute to volume with water. This solution is stable for 1 month in the dark at 4°C.

(b) **Intermediate standard solution (5 μg/mL).**—Dilute 250 μL stock solution with water in 50 mL volumetric flask. Store refrigerated for up to 1 week.

(c) **Working standard solutions (50 ng/mL).**—Dilute 1 mL intermediate standard with water in 100 mL volumetric flasks. Prepare fresh daily as needed.

(d) **Analytical standards for preparation of standard curve (2, 5, 10, 25, 50, 100, and 500 ng/mL).**—Dilute 40, 100, 200, and 500 μL as well as 1, 2, and 5 mL intermediate standard with water in individual 100 mL volumetric flasks. Prepare fresh as needed.

**Sample Preparation**

Samples of lean beef, pork, chicken (broiler breast and thigh), and tilapia (*Oreochromis aureus*, a snapper type fish of the Cichlid family) were purchased from a local supermarket. Remove and discard skin and excess fat. Cut the prepared lean muscle samples into small pieces (ca 2×2×1 cm), place in plastic bags, seal, and store at −70°C for <7 days. At the time of assay, partially thaw frozen samples at room temperature (23°C) for ca 30 min and blend in a food processor 4 times for 20–30 s at high speed. Stir the material after each intermittent blending to obtain a uniform paste-like consistency. Weigh aliquots of 5.0 g prepared tissue into 50 mL centrifuge tubes, cap, and store at −70°C until analyzed within 30 days. Prepare 5 replicate subsamples for each fortification level of each species.

**Sample Fortification and Extraction**

Thaw frozen samples in centrifuge tubes in warm water bath (30°–40°C) for ca 30 min. Fortify 5 g samples with 250, 500, or 1000 μL 100 ng/mL working standard solutions to yield final concentrations of 5, 10, or 20 μg/kg, respectively. Allow fortified samples to equilibrate at room temperature (23°C) for 30–60 min before homogenization and initial extraction. For initial extraction, add 20 mL 0.01M phosphate buffer solution (pH 4.5). Homogenize mixture at 10 000 rpm for 90 s. Centrifuge 10 min at 4500 rpm and decant supernatant into another 50 mL centrifuge tube. Homogenize residue with another 20 mL buffer solution, centrifuge as before, and combine supernatant with first extract. Filter through glass wool plug. Total volume of filtrate is ca 40 mL. For deproteinization, add 1 mL TCA solution (75%) into filtrate and mix 30 s on Vortex mixer. Centrifuge 20 min at 4500 rpm and filter supernatant through glass wool plug.

**Solid-Phase Extraction and Concentration**

Attach C18 SPE cartridges to vacuum manifold. Condition each cartridge with 5 mL methanol. Wash cartridge with 2 mL
water and 2 mL TCA solution (2%). Transfer sample filtrate onto cartridge and adjust flow to ca 1–2 mL/min. After all filtrate passes through, wash cartridge with 2 mL TCA (2%), wait until all TCA solution passes through cartridge, and then wash cartridge with 2 mL water. Discard all effluent collected. Apply 2.0 mL 50% acetonitrile in water to cartridge and control flow rate to ca 0.7 mL/min. Collect all eluate into 15 mL screw-capped glass centrifuge or culture tube. Store eluate under refrigeration overnight.

**Derivatization and Extraction**

Add 0.2 mL TCA solution (20%) into 15 mL screw-capped tube and mix on Vortex mixer for 15 s. Add 0.2 mL formaldehyde solution and mix on Vortex mixer for 30 s. Heat in boiling water bath for 30 min, and then cool to room temperature in cold water bath. The derivative mixture is further cleaned up by using liquid–liquid partition. Add ca 0.5 g NaCl to each tube containing derivatives and mix briefly. Extract fluorescent amoxicillin derivative from reaction mixture with 3 mL ethyl ether 3 times. Mix on Vortex mixer for 1 min, centrifuge for 3 min at 2000 rpm, and remove ether layer using Pasteur pipet each time. Combine ether extracts in 15 mL glass tube and evaporate tube contents to dryness under stream of nitrogen using an N-Evap with water bath set at 40°C. Reconstitute by adding 0.5 mL mobile phase to each tube and mix on Vortex mixer thoroughly. To establish a calibration curve, pipet 1 mL analytical standard solution (Standard Solution d, above) in duplicate into 15 mL glass centrifuge tubes. Follow procedure as described above.

**LC Analysis and Quantitation**

The LC analyses were the same as reported previously (4). The fluorescence detector is set at 358 nm for excitation and 440 nm for emission. Aliquots of 50 μL of the final extract are injected onto the LC and analyzed at 1.0 mL/min. The flow rate is increased to 1.5–2 mL/min after 10 min to facilitate elution of late-eluting compounds. A standard curve is constructed by plotting peak area vs concentration of amoxicillin. Once a standard curve is established and the linearity is found to be satisfactory (with correlation coefficient > 0.99 and negligible interception), a one-point standard can be used for daily calibration. During routine operations, duplicate 1 mL aliquots of working standard solution (50 ng/mL) are derivatized and analyzed along with samples for calibration. Amoxicillin derivative is identified by matching peak retention time with standard retention time. Residues are quantitated by external standard method. Values are corrected for appropriate dilution and calculated as μg/kg of tissue.

**Results and Discussion**

This method is a modification of a technique developed previously for determination of amoxicillin residues in catfish and salmon muscle tissues (4). The major difference is in the SPE procedure; the modified technique involves the use of 50% aqueous acetonitrile solution instead of 100% acetonitrile as in the original procedure. This modification gives a cleaner sample eluate which can be used directly in the derivatization step. Thus, it eliminates the need for an ether extraction step for removal of interfering substances. This im-
proved procedure requires less sample preparation time and uses less flammable solvent.

Typical liquid chromatograms showing controls and fortified samples are presented in Figures 1–4. No interference was encountered in analyzing these samples. The retention time shifted slightly from day to day, possibly due to the variation in mobile phase composition, room temperature, and column conditions (variation could occur from column to column even when columns from the same manufacturer are used). Detector response factors also changed slightly from day to day. Therefore, standard solutions were derivatized and analyzed daily along with samples. Acetonitrile (20%) is suggested as a starting point as it gives adequate separation. A small change in the mobile phase modifier can cause a shift in the retention time of amoxicillin. Users need to optimize the separation of amoxicillin on their columns and make any appropriate adjustments to the acetonitrile concentration of the mobile phase to maximize the resolution. Sample preparation should be completed in 1 workday, i.e., amoxicillin is eluted off the SPE cartridge within 1 day. The eluate can be stored at 4°C overnight. After derivatization and/or liquid–liquid partition and concentration, the solutions can be stored at –10°C for 1 week provided that standards and samples are derivatized and handled in a similar manner in the same run.

Table 1. Recoveries of amoxicillin from fortified animal muscle tissues

<table>
<thead>
<tr>
<th>Fortification, µg/kg (ppb)</th>
<th>Beef</th>
<th>Pork</th>
<th>Chicken</th>
<th>Tilapia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>RSD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>85.8</td>
<td>1.5</td>
<td>1.74</td>
<td>86.1</td>
</tr>
<tr>
<td>10</td>
<td>84.2</td>
<td>3.0</td>
<td>3.56</td>
<td>88.1</td>
</tr>
<tr>
<td>20</td>
<td>83.9</td>
<td>2.5</td>
<td>2.98</td>
<td>86.2</td>
</tr>
<tr>
<td>Average</td>
<td>84.6</td>
<td>2.3</td>
<td>2.76</td>
<td>86.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standard deviation (n = 5).

<sup>b</sup> Relative standard deviation, % (SD/mean × 100%).

<sup>c</sup> Nondetectable (<0.8 µg/kg).
Table 1 shows the amoxicillin recovery data of samples fortified at 5, 10, and 20 μg/kg. The fortification levels were equivalent to ½, 1, and 2 times the recommended tolerance level for method evaluation of residue analysis in animal tissues (6). The amoxicillin level in control tissues was below the limit of detection (0.8 μg/kg) in each of the 4 species. Mean recoveries for the 3 fortification levels ranged from 83.9 to 85.8% for beef; 86.1 to 88.1% for pork; 81.7 to 82.9% for chicken; and 92.5 to 95.4% for tilapia. The variability (expressed as RSDs) between subsamples ranged from 1.26 to 3.56%.

According to the FDA guidelines (6) for the analysis of residues at levels <0.1 ppm (100 μg/kg), within laboratory variation (RSD) should not exceed 20%, and average recovery should be within 60–110% (n ≥ 5). The present data showed that the LC–fluorescence method with the modified SPE technique met the FDA criteria in precision and accuracy for analysis of amoxicillin residues in these animal tissues.

Sensitivity and recovery aspects are the most difficult problems in trace analysis of residues in animal tissues. Earlier studies have attempted to enhance the sensitivity of UV detection at 260 nm by using a post-column addition of 0.1M NaOH (2) or by using precolumn derivatization with acetic anhydride followed by triazole/mercuric chloride prior to reversed-phase LC analysis with UV detection at 325 nm (3). However, none of these techniques provided the sensitivity and accuracy recommended for residue analysis in tissue. The fluorescence detection of amoxicillin derivative reported earlier (4) and in the current study apparently overcame the difficulties for determination of trace amounts (<20 μg/kg) of amoxicillin residues in fish and other animal species.

Additional investigations were conducted to test potential interference from other animal drugs which may be used in livestock farming and/or aquaculture. Antibiotics tested included other β-lactam antibiotics (ampicillin, penicillin G, cloxacillin, and cephapirin), erythromycin, lincomycin, and streptomycin. No interference peaks were observed that would be of any concern in performing the amoxicillin determination. Thus, it is clear that the present method is specific for amoxicillin; other β-lactam antibiotics are not detected as interference in the presence of amoxicillin. The present study focused on the methodology for fortified tissues. This modified procedure has not been applied to incurred tissues. Further studies should include incurred tissues and microbiological assays for comparison.

Conclusions
An improved SPE procedure was developed for the LC–fluorescence determination of amoxicillin residues in beef, pork, chicken, and tilapia tissues. Recoveries of fortified drugs at 5, 10, and 20 μg/kg were >80%, with RSDs < 4%. The method’s sensitivity, accuracy, and specificity were satisfactory for analysis of trace amoxicillin residues in 4 animal tissues.

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References