COMPARISON OF MICROBIOLOGICAL PLATE-FOUR LEVEL ASSAY AND LIQUID CHROMATOGRAPHY ANALYSIS FOR THE DETERMINATION OF AMOXICILLIN IN MEDICATED FEEDINGSTUFF

MONIKA SZYMAŃSKA-CZERWIŃSKA, KATARZYNA PIETRUSZKA¹, AND DARIUSZ BEDNAREK

Department of Cattle and Sheep Diseases, ¹Department of Pharmacology and Toxicology
National Veterinary Research Institute, 24–100 Pulawy, Poland
monika.szymanska@piwet.pulawy.pl

Received for publication May 21, 2007

Abstract

The experiment was purposed to compare amoxicillin determination in medicated feedingstuff by a microbiological method (microbiological plate-four level test) and a liquid chromatography with fluorescence detection (LC–FLD) with pre-column derivatisation with formaldehyde. Amoxicillin was determined in the medicated feedingstuff at the level of 250 mg/kg. The results indicated that the microbiological method might provide adequate data suitable for amoxicillin determination in the medicated feedingstuff. These results were confirmed by the LC method. Therefore, for practical use, the values obtained with two methods can be considered equivalent.

Key words: amoxicillin, medicated feedingstuff, microbiological method, LC–FLD.

In the veterinary medicine, antibiotics are used for both the prevention and treatment of infectious diseases. At present, antibiotics as feed additives can be used only in the form of medicated feedingstuffs. Basing on the Council Directive 90/167/EEC, the manufacturing process of medicated feedingstuffs is strictly controlled in all European Communities (2). A fundamental aim of the medicated feedingstuff control is the determination of antibiotic concentrations and the products’ homogeneity. The products are examined both within the internal (during the production process) and external quality control which is supervised by veterinary inspection.

Recently, the following antibiotics were used in medicated feedingstuffs: tylosin, tiamulin, chlortetracycline, and amoxicillin (AMOX). AMOX belongs to the β-lactam antibiotics, which inhibit the biosynthesis of the bacterial cell wall. It is an effective antimicrobial agent both for Gram-positive and Gram-negative microorganisms. This antibiotic is widely used in medicated feedingstuffs for the treatment of bacterial infections in farm animals. Usually, AMOX is added to the medicated feedingstuffs at a level from 250 mg/kg to 500 mg/kg (10).

For AMOX determination, the microbiological method plays an important role because it is easy to perform, cheap, and well suited for the screening use in laboratories. Traditionally, AMOX is determined by the microbiological assay for screening (1, 8). However, because of its low specificity and semi-qualitative status, there is a need for confirmatory methods, such as liquid chromatography.

A few methods are available today for the identification and quantitative analysis of AMOX in feedingstuffs. Capillary electrophoresis is one of the more complicated techniques used for the determination of AMOX in feed (3, 9). In the literature, there are presented many chromatographic methods e.g. with diode array (10), electrochemical detection (7), sometimes after derivatisation with fluorescence detection (6), or liquid chromatography with tandem mass spectrometric detection (4).

The aim of the study was to compare two methods (microbiological assay and liquid chromatography) and the evaluation of their usefulness for AMOX determination in the medicated feedingstuff.

Material and Methods

Microbiological Plate-Four Level Assay
This procedure was adopted from Polish Pharmacopoeia (11) and modified according to own practical laboratory experiences.
**Materials.** The study was performed on the feed sample, which was spiked with AMOX at the level of 250 mg/kg. The medicated feedingstuff was prepared basing on a premix containing AMOX (Suramox 5%).

**Reagents.** The AMOX standard was obtained from the Sigma–Aldrich Chemical Company (Germany); methanol and sodium chloride were purchased from P.O.Ch. Gliwice (Poland); premix Suramox 5% was purchased from Virbac (France); potassium buffer (pH 8.0 and 7.0) was prepared with dipotassium hydrogen phosphate (K₂HPO₄), and potassium dihydrogen phosphate (KH₂PO₄), which was purchased from P.O.Ch. Gliwice (Poland). The 65.6 g of K₂HPO₄ and 3.0 g KH₂PO₄ were dissolved in distilled water to make 4000 ml of phosphate buffer (pH 8.0).

**Extraction.** 10 g of the feed sample was used for the extraction. It was weighed exactly to 0.001g using 250 ml flasks. Into each sample, 50 ml of potassium phosphate buffer (pH 8.0) was added and stirred vigorously for 10 min. The most adequate results were obtained when the samples were extracted using this buffer heated up to 80°C. Addition of heated buffer is important particularly when the feed is in a granulated form or contains enzymes. Then 50 ml of methanol was added into the extractions, which was then mixed for 5 minutes. After this procedure, the samples in the flasks were shaken again and their contents was transferred into the individual 100 ml test tubes and centrifuged (3000 rpm/10 min) at the room temperature. The top layer of the extract was taken for analysis. The supernatant was diluted using the phosphate buffer (pH 8.0) to obtain a series of AMOX concentrations: 0.3 (U₁), 0.15 (U₂), 0.075 (U₃), and 0.0375 (U₄) µg/mL.

**Preparation of Micrococcus luteus suspension and test agar plates.** *Micrococcus luteus* (ATCC 9341) was prepared in the agar slant culture from the special culture medium. It was incubated over night at 37°C. The culture prepared in this way was stored in a refrigerator and re-inoculated systematically using a new agar slant culture every fourth weeks.

A freshly prepared culture of *Micrococcus luteus* on a nutrient agar slant culture was harvested into sterile glass beads containing 3 ml of 0.8 % sodium chloride. Before the proper analyses, a preliminary test was made on the assay plates using the assay medium in order to determine the inoculum amount needed to obtain the largest possible clear zones of inhibitions of the test bacterium growth at the concentration of 2 µg/mL AMOX used. The agar medium consisted of: agar (15 g), yeast extract powder (3 g), peptone (6 g), and beef extract powder (15 g) at pH 8.0. It was inoculated with 0.03 ml of suspension of *Micrococcus luteus*. The described above composition and compound proportion was used for 100 ml of the medium. The assay medium was inoculated at 50°C. Then, 26 ml of the inoculated agar was pipetted into each Petri dish.

**Preparation of standard solutions.** 20 mg of AMOX standard was dissolved in potassium phosphate buffer (pH 7.0) to a concentration of 1 000 µg/mL and shaken until completely dissolved. AMOX standard is poorly soluble in methanol and ethanol. Stock solution can be stored no longer than 3 days at 4–6°C. Using the stock solution, a standard solution was prepared. The most adequate results were obtained when the stock solution was diluted using potassium phosphate buffer (pH 8.0) to the AMOX concentrations of 0.3 (S₁), 0.15 (S₂), 0.075 (S₃), and 0.0375 (S₄) µg/mL.

**Determination.** Eight metallic cylinders (diameter of 6 mm) were put into each plate with inoculated agar and suitably situated on its surface. Equal volumes (265 µl) of standard solutions (S₁, S₂, S₃, S₄) and dilutions of extract (U₁, U₂, U₃, U₄) were pipetted into cylinders. The plates were incubated at 37°C for approximately 18 h. After the incubation, the diameter of the growth inhibition zone of the test culture was measured. The microbiological method depends on a comparison of AMOX diffusion from the standard solutions and feed samples into the special agar medium (pH 8.0). AMOX activity was determined by measuring its diffusion in the agar medium inoculated with *Micrococcus luteus*. The test strain of the bacteria is recommended for AMOX determination using a microbiological method. The diffusion is shown by the formation of inhibition zones in the presence of the microorganism. The inhibition zones were measured using the electronic caliper (Sylvac). The diameters of these zones are taken to be in direct proportion to the logarithm of the antibiotic concentration.

**Validation.** The following validation parameters were used for quality evaluation of the method: Recovery at the level of 94.8%, limit of quantification (LoQ), defined as the lowest amount of AMOX, which can be determined, i.e. 10 mg/kg, and limit of determination (LoD) defined as the lowest concentration of AMOX that still inhibits the bacterial growth, i.e. 375µg/kg.

**Liquid Chromatography Method**

**Reagents.** AMOX standard and trichloroacetic acid (TCA, minimum purity 99%) were obtained from the Sigma–Aldrich Chemical Company (Germany); formaldehyde 36 – 38% and sodium acetate trihydrate were purchased from P.O.Ch. Gliwice (Poland); acetonitrile gradient grade for analysis was obtained from Merck (Germany); water was purified using Milli-Q system (Millipore, France).

**Standard solutions.** Stock standard solution (1 mg/mL) was prepared by weighing 10.0 ± 0.1 mg of standard substances and dissolving in 10 ml of 20% acetonitrile (stable for 6 months when stored at the temperature below -16°C in amber glass). Working standard solution (100 µg/mL) and mixed standard solution (10 µg/mL of each compound) were prepared by diluting suitable aliquot of stock standard (stable for 1 month when stored at 2–8°C in amber glass). Working standard solution (1 µg/mL) was prepared by diluting suitable aliquot of stock standard (stable for 2 weeks when stored at 2–8°C in amber glass).

**Sample preparation.** The feed sample was pulversised using a domestic grinder to obtain a homogeneous powder. A 5 g sample was weighed in a 250 ml polypropylene jar and 100 ml of water/acetonitrile solution (75:25, v/v) was added. The mixture was shaken in a horizontal shaker for 45 min,
and then the mixture was centrifuged for 5 min at 3500 rpm. The 1 ml of the supernatant was diluted 1:5 (v/v) with water/acetonitrile (75:25, v/v). To 0.5 ml of the diluted extract, 0.5 ml of water, 0.5 ml of formaldehyde, and 0.3 ml of 20% TCA were added. The tube was vortexed during 20 s and then heated for 30 min at 100°C. The whole of solution was analysed by LC–FLD.

**Apparatus.** The instrumental analysis was performed using Shimadzu CLASS−10VP liquid chromatography equipped with quaternary pump, autosampler, column, and fluorescence detection, with excitation wavelength $\lambda = 358$ nm and emission wavelength $\lambda = 440$ nm. The whole analyse process was controlled by CLASS VP Workstation software. The separation was performed on Interstil ODS - 3 column (5 µm; 150 mm x 4.6 mm) at room temperature, with mobile phase consisting of acetonitrile and 0.03 M sodium acetate, pH 6.0 (20:80, v/v) with flow rate 1.0 ml/min. The injection volume was 10 µl.

**Validation.** Standard calibration curve was prepared by injection of standard solution on three levels over the range 1, 2, and 4 µg/mL. The feed samples were spiked with AMOX at the level of 250 mg/kg. The repeatability and reproducibility of the assay were determined as the coefficient of variation calculated for one concentration level. Recoveries were calculated by comparing peak area of fortified sample to the corresponding standard. The recovery was at the level of 96%–97%. For the quality evaluation of the method, LoD and LoQ were estimated. They were 0.125 and 0.5 µg/kg, respectively.

**Results**

The results of the comparative evaluation of microbiological and liquid chromatography methods for AMOX detection in the medicated feedingstuff are summarised in Table 1. The results obtained showed that the statistical parameters used for their evaluation are satisfactory, and the procedures are comparable and suitable for the determination of AMOX in medicated feedingstuffs.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Procedure</th>
<th>Microbiological Mean, mg/kg, Day 1 (n=12)</th>
<th>LC-FLD</th>
<th>SD, mg/kg</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Repeatability, CV, % (n=6)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Reproducibility, CV, % (n=12)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Recovery, %</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>237.0</td>
<td>240.1</td>
<td>20.3</td>
<td>24.4</td>
<td>8.6</td>
<td>9.6</td>
<td>8.7</td>
<td>8.8</td>
<td>94.8</td>
<td>111.8</td>
<td>96.1</td>
<td>97.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As it was presented in Fig. 1, the Petri dish with characteristic inhibition zones for *Micrococcus luteus* growth was caused both by the standard and extract solutions of AMOX with 0.3 (S₁, U₁), 0.15 (S₂, U₂), 0.075 (S₃, U₃), and 0.0375 (S₄, U₄) µg/mL of AMOX concentrations. Two kinds of zones are usually comparable when extraction and dilution procedures are correctly performed.

![Fig. 1](image1.png)

**Fig. 1.** The inhibition zones of *Micrococcus luteus* growth on the agar plate in presence of AMOX solutions added into the suitable cylinders (AMOX standards: S₁, S₂, S₃, S₄; and AMOX extractions of feed samples U₁, U₂, U₃, U₄).

![Fig. 2](image2.png)

**Fig. 2.** Liquid chromatograms of the feedingstuff: a) blank; b) blank spiked with AMOX at 250 mg/kg.

**Confirmation of the identity of AMOX in feed** was performed by liquid chromatography with fluorescence detection using pre-column derivatisation with formaldehyde in acidic solution. Fig. 2 shows the chromatograms of a blank feed (a) and of the same feed spiked with AMOX at the level of 250 mg/kg (b). The figure shows that there were no interfering peaks at the retention time of analyte and the AMOX peak was well resolved.
Discussion

In the literature, there are a few analytical procedures described for the detection and determination of AMOX in medicated feedingstuffs. The most of them use microbiological test for the qualifying and LC technique for the quantification of AMOX in the analysed material (1, 5, 6, 8). Therefore, the aim of our study was to compare the results of AMOX determination in feeds obtained by the microbiological method with those received by LC.

The isolation of analyte from the feedingstuffs is a crucial part of analytical procedure. In microbiological assay, the best results were obtained when the sample of the feedingstuff was extracted by using methanol and phosphate buffer at pH 8.0, which was heated up to 80°C. This procedure eliminates false positive results caused by the presence of different natural substances (e.g. enzymes), which can appear in matrix and interfere with detected active substances. The most adequate results were obtained when the AMOX determination was performed in four concentrations of the component for standards and sample extracts i.e. 0.3, 0.15, 0.075, and 0.0375 µg/mL (Fig. 1). These dilutions were very important because the high concentrations of AMOX gave too large inhibition zones and the lower concentrations of AMOX gave too small and not clear inhibition zones. No significant differences were found between analytical sample series in different days of analysis (day 1 and day 2).

The presented LC procedure is an efficient and reliable method for the determination of AMOX in a medicated feedingstuff. Using formaldehyde derivatisation of the analyte made the method more sensitive and selective. It also allows preparing sample without any clean up of the extract.

The results obtained in our study by the microbiological method were confirmed by LC techniques. As it was found, that the developed microbiological procedure allowed receiving comparable and adequate results for AMOX analysis in medicated feedingstuffs and it can be used routinely in monitoring of the compound in the animal feeds. It can be useful, especially as a screening test. However, in questionable cases, confirmatory methods e.g. LC or LC-MS should be used additionally.

References