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Liquid chromatography with fluorescence detection of amoxicillin and ampicillin in feeds using pre-column derivatization

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Abstract

A rapid analytical procedure for the routine identification and quantification of two penicillins, amoxicillin (AMOX) and ampicillin (AMPI), in feeds by liquid chromatography (LC) was developed and tested. AMOX and AMPI are normally the only penicillins added to medicated feeds because of their good resistance to gastric juice. The ground feed samples were extracted using water–acetonitrile (75:25, v/v) and derivatized, without any clean-up, with a formaldehyde solution in acidic medium at 100 °C for 30 min. The fluorescent derivatives were analysed by reversed-phase on an ODS column (150 mm × 4.6 mm, 5 μm) with a gradient between acetonitrile, methanol and phosphate buffer containing thiosulfate as mobile phase. Fluorescence detection was carried out at excitation and emission wavelengths of 358 and 440 nm, respectively. The recoveries of both penicillins from spiked samples were, in a concentration range of 200–500 mg kg⁻¹, >80% with repeatabilities below 15% R.S.D. The limits of detection of AMOX and AMPI in feed, based on a detector signal-to-noise ratio of 3, were 5 mg kg⁻¹.

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1. Introduction

Amoxicillin (AMOX) and ampicillin (AMPI) are two β-lactam antibiotics that belong to the group of penicillins. The basic structure of penicillins, 6-aminopenicillanic acid, consists of a thiazolidine ring fused to a β-lactam ring with a side chain where AMOX and AMPI present a primary amine group in all other penicillins except epicillin and becampicillin. AMOX and AMPI are extremely active against both gram-positive and gram-negative organisms, including several pathogenic enteric organisms. AMPI is used to treat infections caused, for example, by Escherichia coli, Salmonella, Proteus and Klebsiella. It is not resistant to penicillinase and is similar to other penicillins in its hypersensitivity properties. It is distributed to liver, bile, muscle, kidney, crop, and fat following absorption from the gastro-intestinal system or injection site [1]. AMOX differs from AMPI by the presence of a hydroxyl group in the side chain. It has greater resistance to gastric juice than AMPI and its antimicrobial activity is similar to that of the AMPI. Apparently AMOX is more completely absorbed than AMPI, as demonstrated by a 70% urine recovery in 6 h as compared to 40–50% for AMPI [1]. Because of such activity, AMOX and AMPI are widely used in veterinary practice for the treatment of gastro-intestinal and systemic infections. They are normally the only penicillins added to medicated feeds, at the level of 250–500 mg kg⁻¹, because of their resistance to gastric juice [2].
When these antibiotics are used in food-producing animals for treatment of disease, they are present in feed at therapeutic levels; however AMOX and AMPI may become harmful when present at cross-contamination levels. For these reasons, the importance of a regular control of finished feeds for AMOX and AMPI concentrations is quite clear.

Traditionally, penicillins are determined by microbiological assay [3]. However, it is very difficult to distinguish AMOX from AMPI using microbiological methods.

Capillary electrophoresis is one of the techniques used for the determination of penicillins in feeds [4–6]. In the literature, there are many chromatographic methods with diode array and fluorimetric detection for the determination of penicillins in food of animal origin [7–10]. To our knowledge, only one article deals with the analysis for penicillins in chicken feed by liquid chromatography, in this method pulsed electrochemical detection is used [11].

The purpose of this study was to develop a method for the simultaneous determination of AMOX and AMPI in finished swine feeds at their normal added concentration as well as at sub-therapeutic levels as a result of a possible cross-contamination at the point of manufacture.

2. Experimental

2.1. Reagents and equipment

Trichloroacetic acid (TCA) and citric acid were purchased from Carlo Erba (Milan, Italy); formaldehyde was purchased from J.T. Baker (Deventer, Holland); sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate dihydrate and acetonitrile for analysis were obtained from Merck (Darmstadt, Germany); Sodium thiosulfate pentahydrate was purchased from Fluka (Milan); acetonitrile hypersolv and methanol hypersolv for LC were obtained from BDH (Poole, UK). Water for LC analysis was prepared with a Barnstead Nanopure Ultrapure Water system from International PBI (Milan).

Amoxicillin trihydrate and ampicillin trihydrate were certified and purchased from Riedel-de-Haen (Seelze, Germany).

The aqueous phosphate buffer for LC analysis consisted of 3.4 mM Na₂HPO₄·2H₂O (0.605 g l⁻¹), 6.5 mM NaH₂PO₄·2H₂O (1.014 g l⁻¹), and 15.7 mM Na₂S₂O₃·5H₂O (3.896 g l⁻¹).

The formaldehyde solution was formaldehyde (7%, v/v) in 0.4 M aqueous citric acid solution.

The stock standard solutions were prepared in water/acetonitrile (75:25, v/v) at a concentration of 1 mg ml⁻¹. The working standard solutions in water/acetonitrile (75:25, v/v) at 100 μg ml⁻¹ were prepared on the day of use.

The LC system consisted of a Hewlett-Packard (HP) 1100 series quaternary pump, a HP1100 series fluorescence detector, and a HP1100 series autosampler, all controlled by a HP computer using HP chemstation software. Separation was carried out on a 5 μm Supelcosil LC-18 (150 mm × 4.6 mm) column, at room temperature, with a 5 μm Supelguard LC-18 (20 mm × 4.6 mm) guard column (both from Supelco, Bellefonte, PA). The LC eluents were: (A) methanol, (B) acetonitrile, (C) phosphate buffer. The gradient initiated with A/B/C (2:12:86, v/v), continued with a linear increase to A/B/C (4:18:78, v/v) over 15 min followed by a linear increase to A/B/C (6:24:70, v/v) over 5 min and constant A/B/C (6:24:70, v/v) for 4 min. The flow rate was initially 0.5 ml min⁻¹ over 15 min; it continued with a linear increase to 1 ml min⁻¹ over 5 min and was constant at 1 ml min⁻¹ for 4 min. The excitation wavelength was 358 nm, the emission wavelength was 440 nm. The injection volume was 50 μl. The PT-GAIN was 14 for the first 16 min and after was 10.

2.2. Spiked samples

For the recovery studies, swine feed samples spiked with AMOX and AMPI in the concentration range 200–500 mg kg⁻¹ were prepared on the day of use by spiking aliquots of blank control swine feed with the stock standard solution at 1 mg ml⁻¹ and were processed as described in the Section 2.4.

2.3. Linearity

Aliquots of 100, 200, 400, 500 and 600 μl of the working standard solution (100 μg ml⁻¹) were placed in graduated flasks and the volumes adjusted to 10 ml with diluted blank feed extract. The 500 μl of each
solution was derivatized as described in the Section 2.4 below.

2.4. Sample preparation

The feed sample was pulverised using a domestic grinder to obtain a homogeneous powder. A portion (5 g) was weighed into 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, then the mixture was centrifuged for 5 min at 4200 rpm. The 2 ml of supernatant was diluted 1:5 (v/v) with water/acetonitrile (75:25, v/v), and 500 μl of diluted extract was transferred to a 10-ml centrifuge tube equipped with a screw-stop; 500 μl of water, 300 μl of aqueous TCA solution (50%, w/v) and 500 μl of formaldehyde solution were added. The tube was tightly capped, vortex-mixed for 20 s and heated for 30 min at 100 °C. The derivatized solution was cooled in a ice/water/NaCl bath; the solution was transferred in a graduated flask and adjusted to exactly 2 ml with water/acetonitrile (75:25, v/v). The derivative was injected into the LC system operated as described above.

3. Results and discussion

In the literature, there are very few articles about the LC determination of AMOX and AMPI in feeds. The present analytical procedure is suitable for determination and quantification of AMOX and AMPI in medicated swine feed. The procedure developed provides, after extraction, a derivatization already utilized for the determination of penicillins in animal tissues [7–9]; the derivatization can be done without any clean-up of the extract. The fluorimetric response of AMPI is much greater than that of AMOX; a change of PT-GAIN during the analysis is therefore needed.

The linearity of the fluorimetric detector responses of both penicillins were verified in the range 1–6 ng μl⁻¹ by injecting 50 μl of the derivatized working solutions. All the responses, in the range 12.5–75 ng injected (100–600 mg kg⁻¹ of both penicillins)

![Figure 1](Fig. 1. Liquid chromatograms of swine feed. (a) Blank; (b) blank spiked with AMOX and AMPI at 400 mg kg⁻¹ each.)
Table 1
AMOX and AMPI recoveries and relative standard deviation (R.S.D.) of replicates \((n = 9)\) of spiked samples of blank swine feed

<table>
<thead>
<tr>
<th>AMOX added (mg kg(^{-1}))</th>
<th>AMOX Recovery (%)</th>
<th>R.S.D. (%)</th>
<th>AMPI Recovery (%)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>89</td>
<td>4</td>
<td>87</td>
<td>9</td>
</tr>
<tr>
<td>300</td>
<td>89</td>
<td>4</td>
<td>86</td>
<td>9</td>
</tr>
<tr>
<td>400</td>
<td>86</td>
<td>4</td>
<td>83</td>
<td>7</td>
</tr>
<tr>
<td>500</td>
<td>91</td>
<td>9</td>
<td>92</td>
<td>15</td>
</tr>
</tbody>
</table>

were found to be linear with a correlation coefficient of \(\geq 0.999\).

Fig. 1 shows the chromatograms of a blank swine feed (a) and of the same feed spiked with 400 mg kg\(^{-1}\) of both penicillins (b). The figure shows that there were no interfering peaks in the chromatogram of the blank feed extract and that the AMOX and AMPI peaks were well resolved.

The limit of detection (LOD) for each penicillin, calculated \((S/N = 3)\) from six different chromatograms of blank feed extracts, was 5 mg kg\(^{-1}\) and the lowest concentration tested in spiked feed samples was 200 mg kg\(^{-1}\). To evaluate the method performance, blank swine feeds were spiked at 200, 300, 400 and 500 mg kg\(^{-1}\) and processed as described. The mean recovery from spiked feed samples was 89% (R.S.D. 6%) for AMOX and 87% (R.S.D. 11%) for AMPI. The results are summarized in Table 1.

The data reported show that the method is suitable for routine determination of AMOX and AMPI in swine feeds with good recovery and with acceptable specificity and repeatability. We observed that it is also possible to apply this procedure to the analysis of feed for rabbits and cattle.

References