DETERMINATION OF AMOXICILLIN IN PIG PLASMA
BY LIQUID CHROMATOGRAPHY
WITH FLUORESCENCE DETECTION

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Abstract

The concentrations of amoxicillin in pig plasma were determined by HPLC technique. Separation was achieved on a Lichrocart 250 x 4 mm Purospher column and detection was performed with a fluorescence detector. The mobile phase consisted of a mixture of acetonitrile with buffer solution (0.05 M KH₂PO₄, pH 5.6) delivered by an isocratic programme. For amoxicillin assay, the plasma samples were exposed to reaction with formaldehyde under acidic and heating conditions. The limit of detection and quantification, as well as the accuracy and precision of the method were evaluated from spiked plasma samples at concentration levels ranging from 10 to 500 ng·mL⁻¹. Recovery of spiked amoxicillin was >91%, with coefficient of variation equals 0.12%. This method offers a rapid, repeatable, and accurate procedure and has been useful for determination of pharmacokinetic parameters of amoxicillin in pigs¹ and poultry plasma.

Key words: pigs, amoxicillin, HPLC, validation.

Amoxicillin (AMX) - is a semisynthetic penicillin belonging to aminopenicillin group with a wide range of bactericidal activity against G-negative and G-positive pathogens (4). Mycoplasma, Klebsiella sp., Proteus sp., Pseudomonas aeruginosa, and Bacteroides fragilis belong to amoxicillin-resistant G+ and G- bacteria producing β-lactamase enzymes (1). Fig. 1 shows the structure of amoxicillin.

A valuable feature of amoxicillin is its very low toxicity to an animal organism in comparison with other antibacterial preparations. The mechanism of its activity is connected with the blockage of alaninotranspeptidase synthesis, which is vital for bacteria and is not produced in mammalian cells (3). According to the recommendations of international organisations involved in the registration of veterinary medicines (FDA and the WHO Expert Committee/FHo), analytical methods for determining residues of veterinary medicines must meet several requirements proving their usefulness, and they must be validated. The parameters that characterise the usefulness of the method are: specificity, limit of detection and quantitation, linearity, accuracy, sensitivity, and repeatability. Over the years, many methods have been described for the analysis of penicillins in various matrices, including feeds (5).

Fig. 1. Structure of amoxicillin (p-hydroksyampicylina).
To stabilise penicillins during sample processing, several analytical methods apply derivatisation with fluorescamine (11), 1,2,4-triazole mercury (II) reagent (10), or formaldehyde with trichloroacetic acid (2, 15). An additional aim of derivatisation is the introduction of chemical moieties that improve detection by liquid chromatography-fluorescence (11, 16). HPLC methods, using an ultraviolet (7), MS (6), or DAD (18) detection, were reported for the determination of amoxicillin and other penicillins in milk (17), fish (2), urine (9), and human (18) and animal plasma (8, 12, 14, 19).

The presented study aimed at modification of the extraction method of amoxicillin from plasma of pigs, adapting the modified technique for determination of amoxicillin by HPLC with fluorescence detection, and validation of the prepared method.

**Material and Methods**

**Biological samples.** Known amoxicillin-free blood samples were obtained from pigs that previously did not receive any medication. Incurred samples were taken from healthy pigs, which received a commercial amoxicillin preparation via the drinking water. Blood was sampled at different time points after drug administration and centrifuged. The plasma was decanted and stored at -30°C until the day of analysis.

**Chemicals and reagents.** All chemicals were HPLC or analytical grade. Acetonitrile (ACN) and trichloroacetic acid (TCA) were obtained from Merck, formaldehyde (HCHO, 36%), citric acid, and potassium trichloroacetic acid (TCA) were obtained from Merck, HPLC or analytical grade. Acetonitrile (ACN) and chemicals and reagents.

An analytical standard AMX (amoxicillin·3 H2O with 0.4 M solution of citric acid) (0.2 : 0.4 ; (w/v) HCHO was received by diluting concentrated make 70% (w/v) solution) and HCHO solution (7% (dissolved appropriate amounts of TCA in water to reagent was obtained by mixing up of the TCA solution (Poland) at the highest purity available. Derivatis ed decanted and stored at -30°C. The plasma was sampled at different time points after drug administration and centrifuged. The plasma was decanted and stored at -30°C until the day of analysis.

**Apparatus.** Quantitative measurements of the AMX content in the samples were performed using a Gilson liquid chromatography system (Gilson, USA) equipped with a fluorescence detector Shimadzu (WL-1, Bio-mix, Poland), a centrifuge (Sigma 2-16), an analytical balance (Sartorius BP 61S, Germany), Vortex (WL-1, Bio-mix, Poland), a centrifuge (Sigma 2-16), water bath (MILL547, A.L. Electronics, U.K.), and Milli Q Plus 185 system (Milipore-Waters) to produce deionized water, were also used.

**Chromatographic conditions.** The mobile phase was prepared by mixing up acetonitrile (15%) with buffer solution (0.05 M KH2PO4, pH 5.6, 85%), and degassed by sonication in an ultrasonic bath before usage. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min. After an acceptable stable baseline was achieved, the standards, and then the samples were analysed. The concentrations were scanned by a fluorescence detector at excitation wavelength of 355 nm and emission wavelength of 450 nm and the injection volume was 20 µl. All analyses were performed at ambient temperature.

**Standard solutions.** The standard of AMX was dissolved in deionised water at a concentration of 25 µg·L-1 to obtain the stock solution. The working solutions (10, 25, 50, 100, 250, and 500 ng·mL-1) were prepared by appropriate serial dilution of the stock solution with a deionised water. These solutions were then injected in order to obtain the calibration curve for the determination of concentration of AMX in the pig plasma.

**Extraction procedure.** Frozen plasma samples were thawed to room temperature prior to extraction. A sample of 0.5 ml of test plasma was transferred into a vial and mixed with 1 ml of phosphate buffer KH2PO4 (pH 4.5), and then mixed for 1 min in Vortex with 0.5 mL of 70% TCA. After centrifugation for 15 min at 5,500 x g, the supernatant layer was transferred into a clean vial and derivatised using HCHO with TCA reagent. The sample was mixed for 20 s in Vortex and next heated in a boiling water bath for 30 min. After derivatised step, the sample was cooled in a water bath (at 20°C) for 10 min and diluted to 10 mL using a deionised water. The sample was filtered using 0.45 µm PTFE filter (Sartorius) and a 20-µl volume of the elute was injected into the HPLC system.

**Accuracy/recovery.** The accuracy of the method was determined by the recovery of AMX from the control pig plasma samples. The spiked level was prepared by adding 50 µL of standard solution of AMX (50 µg·mL-1) to 0.7 ml portions of the sample. The extraction of AMX was made in accordance with Section Extraction procedure. To appoint procedure recoveries, the detector responses for AMX in fortified plasma samples subjected to extraction, clean-up and LC analysis, were compared with the corresponding internal standard solution. Recovery was assessed as:

\[ R(\%) = \frac{S}{S_x \cdot 100\%} \]

where R is the recovery of the fortified plasma sample, S is the peak area of the fortified plasma sample, and Sx is the peak area of the corresponding standard solution.

**Results**

The method was validated for linearity, specificity, solution stability, accuracy, precision, limit of detection, and limit of quantification. The results obtained for the method validation are shown in Table 1. The high value of the coefficient (R) indicated good correlations between drug concentrations and peak heights. The linear range experiments provided the necessary information to estimate the LOD and the LOQ limits based on the peak of lowest concentration in the linear range with a signal-to-noise ratio, S/N of 3.3 for
LOD and 10 for LOQ. The LOD and LOQ results were satisfactory. Precision of the method has been assessed for within-day and between-day variations. The operator carried out three trials each day for three concentrations and during three days.

In regard to the specificity of the method, the chromatogram corresponding to the extract of the blank plasma sample revealed no peak interfering with AMX (Fig. 2). Typical chromatogram of pig plasma spiked with an AMX is shown in Fig. 3. The stability of AMX was determined in two different ways: in solvent (working solutions) and in plasma samples containing AMX. The working solutions were analysed every week and the instrumental responses were compared with peak areas obtained on the day of solution preparation. No degradation phenomenon was observed during the 2 month storage period at 4°C. The stability of AMX in plasma samples was determined from blank matrices spiked at recovery level. Samples were frozen at −30°C and analysed after 7, 14, and 21 d, evaluating the amounts of AMX present. AMX was stable throughout the freezing process. No differences were observed between the amount of AMX spiked and that observed in the samples stored during different periods.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
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<tbody>
<tr>
<td><strong>Compound</strong></td>
<td>Amoxicillin</td>
</tr>
<tr>
<td>Linearity (working range) ng·mL⁻¹</td>
<td>10-500</td>
</tr>
<tr>
<td>Limit of detection (LOD) ng·mL⁻¹</td>
<td>17.10</td>
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<tr>
<td>Limit of quantification (LOQ) ng·mL⁻¹</td>
<td>57.10</td>
</tr>
<tr>
<td>Recovery (%±SD) a, CV (%)</td>
<td>91.67±0.11, 0.12</td>
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<tr>
<td>The retention time (min±SD)</td>
<td>7.64±0.06</td>
</tr>
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<tr>
<th>Level of spiked samples plasma ng·mL⁻¹</th>
<th>50</th>
<th>100</th>
<th>150</th>
</tr>
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<tbody>
<tr>
<td>C⁰ CV (%)</td>
<td></td>
<td></td>
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<tr>
<td>Day 1</td>
<td>52.24±0.43</td>
<td>0.83</td>
<td>98.47±0.45</td>
</tr>
<tr>
<td>Day 2</td>
<td>52.00±0.44</td>
<td>0.85</td>
<td>98.29±0.47</td>
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<tr>
<td>Day 3</td>
<td>51.79±0.45</td>
<td>0.86</td>
<td>98.12±0.48</td>
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*Mean results (n=5), †mean concentration found (n=3) (ng·mL⁻¹ ± SD).*

![Fig. 2. Representative HPLC chromatograms obtained from blank sample of pig plasma.](image)

![Fig. 3. Representative HPLC chromatograms obtained from pig plasma spiked with an amoxicillin.](image)
A critical aspect of drug residue analysis is the sample extraction and purification, required to isolate amoxicillin from pig plasma. The presented method is a modification of previously developed techniques for determination of amoxicillin residues in muscle tissues of cattle, swine, and chicken (15), and salmon and catfish (2). The major differences are in the extraction and derivatisation step. The modified technique ruled out the solid phase extraction. Instead of the SPE procedure, the liquid-liquid partition for the initial extraction by buffer phosphate solution was used. The buffer sulfuric solution was used in the original study. The solution of 70% TCA was selected to precipitate proteins from a plasma matrix and was similar to that published in the original paper (75% TCA) (15). The results showed that deproteinisation of the sample with this solution achieved significant purification. As reported in the literature, in protein precipitation step, many different solvents were used: cold methanol (20), acetonitrile (18), or perchloric acid (12). For amoxicillin assay, the plasma samples were derivatised using formaldehyde with trichloroacetic acid reagent, almost the same composition as described in the original paper (2, 15). Optimal concentrations of formaldehyde in citric acid buffer and TCA solutions were tested for formation of fluorescent product. Peak area increased with formaldehyde concentration in citric acid buffer solution and was stable during the changing of concentration of TCA solution. Maximal fluorescent response occurred with 2.5% to 10% formaldehyde solution and with 10% to 70% TCA solution. The composition of derivatised reagent, 7% formaldehyde and 70% TCA solutions were selected for further work. Luo et al. (15) used 20% TCA solution but their method required a new extraction of fluorescent amoxicillin derivative from reaction mixture with ethyl ether. After heating and cooling step, the diluted aliquot of the extract was analysed on LC-FL instrument according to our technique. These modifications gave a cleaner sample elute without a loss of the amount of amoxicillin, which was improved by high recovery (91.67%). The modified method was able to rapidly determine amoxicillin in plasma of pigs using HPLC without complicated extraction and cleanup procedures. Less organic solvent was needed in comparison to the technique offered by Luo et al. (15). Our extraction and derivatisation steps were also time-saving. The maximum excitation wavelength and maximum emission wavelength of amoxicillin were 355 and 450 nm, which was determined - using a spectrophotometer UV-Vis instrument. Both acetonitrile and methanol were tested as organic mobile phases for the LC separation. The injections resulted in the broadening of the peak when methanol was used, whereas in case of acetonitrile this effect was not observed. Therefore, acetonitrile was chosen as the organic solvent in the composition of the mobile phase. Various proportions of ACN and phosphate buffer solution in the mobile phase were tested. A mixture of 15 parts of acetonitrile and 85 of buffer (0.05 M KH₂PO₄, pH 5.6) was chosen as suitable, according to the peak shape and the run time as well (Fig. 3). The coefficient of retention under proposed chromatographic conditions had also a satisfactory value (k=2.25). The proposed method was used to determine pharmacokinetic parameters of amoxicillin in pigs and poultry plasma by our group (13).

In conclusion, amoxicillin analysis in plasma was performed by a sensitive high performance liquid chromatography method with FL detection, using extraction procedure already described for edible tissues of animals and adapted and validated for pig plasma. Sample preparation by initial extraction of amoxicillin and protein precipitation took only 5 min. HPLC analysis was completed within 10 min, using one of two simple solvent mixtures eluted on a single C18 reversed phase column. The sample preparation and extraction procedure was simple, rapid, and quantitative.

References

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