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Application and validation of a LC/fluorescence method for the determination of amoxicillin in sheep serum and tissue cage fluid

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ABSTRACT

A LC method with fluorescence detection after pre-column mercury dichloride derivation was developed and validated for the quantitative determination of amoxicillin in sheep blood serum and tissue cage fluid at levels down to 100 and 200 ng/mL, respectively. Spiked blood serum and tissue cage fluid samples were deproteinized, derivatized with mercury dichloride and extracted prior to reversed phase LC analysis with fluorescence spectrophotometric detection at an excitation wavelength of 355 nm and an emission wavelength of 435 nm. Separation was carried out on a C_{18} column with a mobile phase consisting of phosphate buffer, octanesulphonate sodium (OCT), and acetronitrile. A regression model using 1/concentration weighting was found the most appropriate for quantification. The intra-day precision for serum was 1.65–8.74% and for tissue cage fluid was 2.48–6.27%. The inter-day precision for serum was 0.39–3.57% and for tissue cage fluid was 0.44–2.54%. The overall precision over 3 days for blood serum using of 108 replicates was 1.70–9.44% and for tissue cage fluid using of 54 replicates was 2.51–6.76%. Studies of amoxicillin stability in blood serum and tissue cage fluid indicated that amoxicillin was stable after 4 weeks storage at −85 °C. The method was successfully applied for the determination of amoxicillin in blood serum and tissue cage fluid samples collected from rams after intravenous administration.

1. Introduction

Since its introduction in the field of antibacterial chemotherapy, in the early 1970s [1], amoxicillin, a semi-synthetic α-amino-*p*-hydroxybenzylpenicillin derivative (Fig. 1), has known widespread use both in human and veterinary medicine, mainly for the treatment of severe respiratory, gastrointestinal, urinary and skin infections, due to its effectiveness against a broad spectrum of both gram-positive and gram-negative microorganisms [2–5]. During more than 30 years of systematic amoxicillin administration, the emergence of (mostly β-lactamase-induced) resistance amongst targeted pathogens has been effectively dealt with by the concomitant administration of a β-lactamase inhibitor, such as clavulanic acid [6,7]. Yet, amoxicillin is still formulated as a sole active substance and continues to be successfully administered, both in humans and animal species [8,9].

As antimicrobial therapy in farm animal medicine is increasingly employing the principles of pharmacokinetic/pharmacodynamic integration, scrupulous pharmacokinetic studies are needed to determine the disposition of drugs in the body, involving their quantitative determination not only in blood, but also in several body tissues and fluids, such as tissue cage fluid (TCF) [10,11]. To this goal, the development and validation of reliable analytical methods comprises a key factor.

Amoxicillin is a polar, amphoteric, hydrophilic compound, non-extractable with usual liquid–liquid extraction procedures, unstable in strongly acidic or alkaline media and organic solvents, lacking fluorescent chromophores and absorbing at wavelengths around 210 nm [12–26], and it is this chemical profile that has principally determined the experimental approaches in developing LC methods for its quantitative determination in biological samples. For years, amoxicillin determination in biological fluids and tissues has offered a challenge among investigators [27].

Methods involving direct UV detection of amoxicillin after sample pretreatment with strong organic acids [19,28,29] or organic solvents [24,25,30–32] generally report minor sensitivity and induce the risk of severe damage to the chromatographic system due to extreme sample pH and/or build-up of endogenous compounds onto the column [15,17,19,33]. This issue has been addressed to, by application of SPE [14,17,34–37] and ZIC-HILIC SPE [26], sample filtration or ultrafiltration [22,33,38,39], column switching [27,39], chromatography with use of specific semi-permeable surface (SPS) columns [40], and pre-column [18,41–50] and post-column [15,16,23,51,52] derivatization methodologies.

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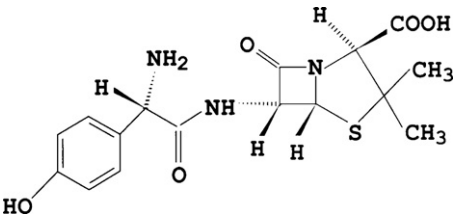

Fig. 1. Chemical structure of amoxicillin (α-amino-*p*-hydroxybenzylpenicillin).

On the other hand, MS is constantly emerging as a very powerful analytical technique, in terms of sensitivity and selectivity [53] and amoxicillin could not have constituted an exception [54–60]. The increased cost of respective instrumentation, though (where not readily available) is not always counterbalanced by MS comparative advantages, which are primarily brought out in applications such as the separation and individual quantification of chemically related substances (i.e. parent compound and metabolites), the determination of the chemical structure of detected compounds by observing their fragmentation, analysis of particularly complex samples, etc.

In quest of a LC assay to be employed in the analysis of blood serum and tissue cage fluid samples, during the conduct of a pharmacokinetic study of amoxicillin in sheep, we reviewed previously published methods and evaluated their performance characteristics, as well as their applicability in our laboratory. The proposed methodology has been validated and successfully applied for the quantitative determination of amoxicillin in experimental sheep blood serum (SBS) and tissue cage fluid (STCF) samples.

2. Experimental

2.1. Standard substances, reagents and chemicals

Standard amoxicillin (acid form) was obtained from Sigma–Aldrich Co. (St. Louis, Missouri, USA). Several other chemicals, reagents and solvents were as well used during method development. Trichloroacetic acid, perchloric acid 70%, potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were from Riedel-de-Haën GmbH (Seelze, Germany). Octanesulphonic acid sodium salt monohydrate (OCT), 2-propanol, acetone and dichloromethane were from Fluka Chemie GmbH (Buchs, Germany), HPLC-grade acetonitrile was from J.T. Baker (Deventer, The Netherlands) and 1,2,4-triazole was from Sigma–Aldrich Co. All other chemicals (sodium hydroxide pellets, potassium chloride, hydrochloric acid 37%, *ortho*-phosphoric acid 85%, mercury dicloride, methanol, hexane, ethyl acetate and formaldehyde 37%) were from Merck KGaA (Darmstadt, Germany).

2.2. Instrumentation

Chromatography was carried out on a Shimadzu LC-10A system (Shimadzu Corporation, Kyoto, Japan). The system comprised a Model CBM-10A controller unit, two Model LC-10AD piston pumps, a Model SIL-$10A_{XL}$ autosampler, a Model CTO-10A column oven and a Model RF-551 spectrofluorometric detector. Data were processed with the use of the Class-LC10 software (version 1.41, Shimadzu). Helium (He) gas, delivered by a Model DGU-2A degasser unit, was used for the removal of soluted air from the mobile phase before use.

Instrumentation also included a Model Elix 3 water purification system (Millipore SA, Mansheim, France), a Model AX-105 analytical balance (Mettler-Toledo Inc., Greifensee, Switzerland), a Model Genie-2 vortex mixer (Scientific Industries Inc., Bohemia, NY, USA), a Model Centra-CL3R refrigerated centrifuge (Thermo IEC, Needham Heights, MA, USA), a Model Accumet Basic pH meter (Fisher Scientific, Manchester, UK), a Model WB14/SV1422 water bath (Memmert GmbH + Co. KG, Schwabach, Germany) and a Model Reacti-Therm III evaporation unit (Pierce Chem., Rockford, IL, USA).

2.3. Biological materials

Hollow, perforated tissue cages were prepared from silicon rubber tubing (EVO Enterprises S.A., Athens, Greece) and were subcutaneously implanted in the lateral neck area of young adult rams, equidistantly between the trachea and the cervical vertebrae. A 5-week period was allowed to ensure a complete surgical wound healing and an ample proliferation of granulation tissue around and inside the tissue cage cavity which was then after filled with tissue cage fluid. Amoxicillin-free STCF samples (1-mL each) were obtained by percutaneous aspiration. A subsequent centrifugation at 3000 rpm for 5 min, at 4 °C removed potentially present cellular debris.

Blood samples were also collected by aspiration from the jugular vein. Soon after a clot was formed, centrifugation at 3000 rpm for 15 min, at 4 °C yielded SBS.

2.4. Stock and working standard solutions

An amoxicillin stock solution was prepared at a nominal concentration of 1 mg/mL in phosphate buffer saline (PBS) (pH 6.0; 0.1 M). Thorough vortexing and overnight stay of the stock solution at 4 °C, protected from light, ensured complete solubility before subsequent aliquoting. Working solutions at three concentration levels (25, 50 and 500 μg/mL) were prepared daily by successively diluting the stock solution in water.

2.5. Calibrators and validation control samples

2.5.1. Sheep blood serum (SBS)

Calibration curves were prepared in respective media. For SBS, 12 levels of calibrators, covering a concentration range from 0.10 to 40 μg/mL, were prepared by adding 20–100 μL of working solutions in pooled blank SBS. Following equilibration, fortified SBS was divided in 0.5 mL aliquots. Validation control (VQ) samples were prepared at six concentration levels (0.10, 0.30, 0.50, 1, 5 and 20 μg/mL).

2.5.2. Tissue cage fluid (STCF)

The concentration range covered by the seven calibrators likewise prepared in STCF was significantly narrower (0.20–4 μg/mL), and in direct correlation with amoxicillin levels expected to be attained during a biological experimentation with the usual dosage (7.5–15 mg/kg body weight). Amoxicillin levels at VQ samples were set at 0.20, 1 and 4 μg/mL.

2.6. Chromatography

The mobile phase was a mixture of acetonitrile–KH_2PO_4 (pH 3.5; 50 mM, containing OCT 5 mM) (35:65, v/v). Adjustment of pH was performed by use of an *ortho*-phosphoric acid solution 1 M. Prior to use, the aqueous component of the mobile phase was filtered through 0.2 μm, Nylon 47 mm filters (Alltech Ass. Inc., Deerfield, IL, USA) and degassed as mentioned. Isocratic operation at 1.0 mL/min delivered the sample for separation on a MZ-Analytical (MZ-Analysentechnik, Mainz, Germany) Spherisorb ODS-2 (250 mm × 4 mm i.d., 5 μm particle size), C_{18} RP analytical column, maintained at 40 °C. Quantification was performed by fluorometric (excitation wavelength: 355 nm, emission wavelength: