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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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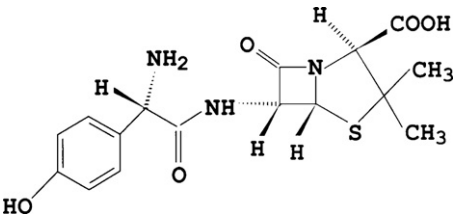


**Fig. 1.** Chemical structure of amoxicillin (α-amino-*p*-hydroxybenzylpenicillin).

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:

435 nm) determination of peak heights (in mV s), whereas retention and run times were 2.36 and 4.00 min, respectively.

### 2.7. Sample clean-up and derivatization procedure

For sample extraction and clean-up, 0.5 mL of blood serum was diluted with 2 mL of deionised water. Protein precipitation was achieved by addition of 1.5 mL of a 10% (w/v) trichloroacetic acid solution. The sample was immediately centrifuged at 4500 rpm for 5 min, at 4 °C. A 3-mL aliquot of the clear supernatant was transferred into a screw-cup 12-mL glass tube and 0.5 mL of a 2 M sodium hydroxide solution was added. The mixture was left at room temperature for 10 min, before the addition of 0.5 mL of a 2 M hydrochloric acid solution and 2 mL of a 0.5 M $Na_2HPO_4$ solution, containing mercury dichloride (0.002%, w/v). The glass tubes were placed in a water bath (50 °C) for 35 min and were subsequently cooled in fresh running water. The fluorescent amoxicillin derivative was extracted with 4 mL of ethyl acetate. Phase separation was achieved by centrifugation at 3000 rpm for 10 min, at 18 °C, and 3 mL of the organic supernatant were evaporated to dryness under a gentle nitrogen stream, at 40 °C. The dried residue was reconstituted in 500 μL of mobile phase and was left overnight at 4 °C. Injection volume was 50 μL.

The same as above procedure was followed for STCF pretreatment with initial sample volume in this case set at 0.25 mL, as a sole differentiation.

### 2.8. Validation

Validation of the present analytical method was performed to ensure its compliance with specific performance criteria [61], such as selectivity, sensitivity (limit of detection and limit of quantification), linearity of response within the specified concentration range, trueness and precision, derivatization yield and extraction recovery, stability and applicability in samples obtained from a biological experimentation.

## 3. Results and discussion

### 3.1. LC chromatography development itinerary

It was apparent, almost from the onset of this study, that straightforward UV (210–230 nm) amoxicillin determination, in both SBS and STCF, presented unsurpassable intricacies. Protein precipitation is typically performed by addition into the sample of strong acid solutions or organic solvents. Use of trichloroacetic and perchloric acid, acetonitrile, methanol, acetone and 2-propanol was successful in this part. Attempts to extract amoxicillin in organic solvents, such as dichloromethane and ethyl acetate (in various combinations with precipitating agents), with or without sample pH adjustment, yielded minimal recoveries, thus confirming the ineffectiveness of usual liquid–liquid extraction techniques in the case of amphoteric penicillins [17,19,20,24,27,32,56]. The remaining amoxicillin-containing aqueous component was deemed unsuitable for reliable analysis, not only because of the known instability of amoxicillin in acidic and organic media [12,16,17,19,21–24,33,50,56,58], but mainly due to the presence of a plethora of endogenous compounds, which not only do they have, as mentioned, deleterious effects on the chromatograph components, but they also increase the background chemical noise, especially at such low wavelengths [19,20,23,24,32,35,52,56], thus significantly affecting method sensitivity.

SPE was also considered, but, apart from being relatively time consuming, it significantly increases the cost, and, at occasions, the labour of analysis, especially when dealing with more than a few hundreds of samples, such as during the conduct of our pharmacokinetic study. Sample filtration or ultrafiltration and SPS methods suffer from mediocre sensitivity [22,33,38–40], whereas column switching techniques require additional, complex automated instrumentation, all combined with long chromatography run times of ~20–40 min [27,39].

Amoxicillin derivatization assays have offered increased sensitivity and the prospect of employing liquid–liquid extraction techniques, thus suppressing the cost and, on occasion, the time needed for analysis. Time needed for the derivatization reaction is vital to the practicality of a pre- or post-column derivatization method, especially for the latter, which also require extra equipment, such as pumps, reaction coils, mixing tees, etc. [15,16,20,23,51,52]. Therefore, a decision was taken to focus exclusively on pre-column methodologies.

Perhaps the most often employed amoxicillin derivatization reaction, in biological samples, involves its conversion into α-acetamido-/α-benzoyl-amidobenzylpenicillenic acid mercuric mercaptide, in the presence of acetic/benzoic anhydride, mercury dichloride and 1,2,4-triazole (or, alternatively, imidazole) and UV detection at 323–328 nm, coupled with sample dilution [18] or SPE [45–48]. Pilot experimentations with the use of 1,2,4-triazole and attempts for liquid extraction of the derivatization product did not yield satisfactory results, in terms of selectivity and sensitivity. A similar pattern could be observed with the use of formaldehyde, as proposed for human blood serum and urine by Davidson [41], with amoxicillin fluorescent derivative detection unfeasible at levels below 1 μg/mL. Another potent derivatization procedure, proposed by Miyazaki et al. [42], involves a mercury dichloride-catalysed conversion of amoxicillin into a hydroxyphenylpyrazine derivative, followed by liquid–liquid extraction and fluorometric detection ($\lambda_{ex}$: 355 nm; $\lambda_{em}$: 435 nm).

Preliminary trials in our laboratory displayed the last methodology to possess a satisfactory degree of sensitivity. However, fine optimization required adjustments, notably in mobile phase composition. The initial use of methanol–water (55:45, v/v) as mobile phase yielded a blunt chromatographic peak and an insufficient separation from matrix coextractives. Attempts to solve this issue by adjusting the proportion of methanol or shifting to a different solvent (acetonitrile) were unsuccessful. Eventually, the combined use of OCT, as an ion-pairing agent, in an acidic, silanol-suppressing, acetonitrile-containing mobile phase significantly reduced background noise and yielded a sharp and symmetric, Gaussian peak. All matrix-originated polar compounds were drifted forwards in the chromatogram and an overall control over amoxicillin elution time was accomplished. A final selection of a 35% acetonitrile proportion allowed for a derivative elution at 2.36 min and a remarkable 4.00 min run time.

A higher baseline noise in STCF, as compared to SBS, was dealt with by diminishing the initial sample volume to its half, resulting in a respective loss in terms of sensitivity.

An overnight stay of the reconstituted (as mentioned in Section 2.7) samples at 4 °C, ensured a superior dissolution of the residue, as compared to immediate chromatography. This slow dissolution rate has also been reported by Ensink [62] who used a respective modification of the method of Miyazaki et al. for the determination of ampicillin in horse and rabbit blood plasma, rabbit tissues and horse tissue cage fluid.

### 3.2. Validation

#### 3.2.1. Selectivity

The ability of the analytical method to differentiate and quantify amoxicillin over other components present in the sample

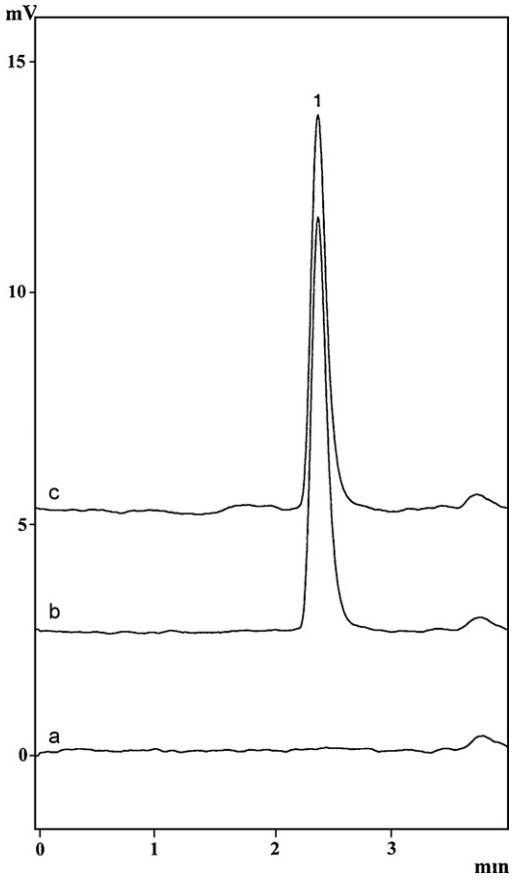


**Fig. 2.** Chromatograms of a SBS samples: blank sample (a), sample fortified at 10 μg/mL (b) and sample obtained 1 h after amoxicillin (peak 1) administration to a ram, at the dose of 15 mg/kg b.w. (c).

(i.e. endogenous substances or chemicals used in pretreatment) was explored by analysing blank SBS and STCF samples from six rams. Chromatography displayed excellent resolution of the peak attributed to amoxicillin fluorescent derivative and absence of disturbing interferences in both media (Fig. 2).

### 3.2.2. Sensitivity

The sensitivity of an analytical method is highly determined by its (lower) limits of detection (LoD) and quantification (LoQ). To establish the LoD in both media, the base line of chromatographs from six blank samples was recorded during the time window of amoxicillin elution, and the average value was superadded by three times its SD. LoDs for SBS and STCF were 0.06 and 0.10 μg/mL, respectively. LoQs, determined as the lowest standards of the calibration curves and the lowest fortification levels yielding acceptable validation results concerning trueness and precision (see below), were 0.10 μg/mL for SBS and 0.20 μg/mL for STCF.

### 3.2.3. Linearity of response—calibration curves

The required concentration range of amoxicillin in SBS (0.10–40 μg/mL), was considered too wide to allow for a reliable quantification with the use of only one calibration curve, especially at its lowest part. Therefore, it was decided that two separate curves would be simultaneously used: a "low curve" for determination of concentrations from 0.10 to 1 μg/mL (seven calibrators) and a so-called "high curve" for concentrations from 1 to 40 μg/mL (six calibrators). In the case of STCF, one calibration curve was used to cover the entire concentration range (0.20–4 μg/mL).

Furthermore, since pilot trials showed that SDs of peaks heights in both SBS and STCF were almost proportional to their mean, a weighted linear regression with a factor of $1/x$ ($x$: amoxicillin fortification level) was preferred to meet linear regression assumptions of homoscedasticity and normality. Runs for calibration curve preparation were performed in four experimentations. Slopes (mean value ± standard error of the mean (S.E.M.)), intercepts and determination coefficients ($R^2$) are presented in Table 1. Use of $t$-distribution yielded non-significant results for intercept terms.

### 3.2.4. Trueness and precision

For trueness and precision determination, SBS samples fortified at six concentration levels, namely 0.10, 0.30, 0.50, 1, 5 and 20 μg/mL were analysed in replicates ($n = 6$) and the procedure was repeated yet again on 2 additional days. For STCF study, three fortification levels were used (0.20, 1 and 4 μg/mL), also in replicates of six, on 3 separate days. One-way ANOVA with day considered a random effect was used for statistics acquisition [63]. Trueness was expressed as the percent deviation of the mean value (concentration found) determined for each fortification level, by use of the (daily prepared) respective calibration curve, from the theoretical value (concentration added) of the analyte. Precision (within-day and between-days), describing the closeness of replicate measures to each other, was expressed in terms of coefficient of variation (CV%). Total, within-laboratory repeatability was also determined as their resultant. In all cases, trueness and precision values fell well within acceptable limits of ≤20% at LoQ and ≤15% at higher fortification levels (Table 2). A noteworthy between days precision being lower in all cases than the respective within day value indicated a remarkable consistence in method day-to-day performance.

### 3.2.5. Yield of derivatization reaction and extraction recovery

The yield of the derivatization reaction and extraction recovery could not be absolutely calculated, due to the unavailability of standard fluorescent derivative to be used as an external standard. However, the exceptional linearity of the detector response and the adequate trueness and precision values clearly suggested consistence and repeatability, concerning both the rate of conversion of amoxicillin to its degradation product and the extraction efficiency of the sample clean-up procedure, at least within each medium.

**Table 1**
Coefficients and performance of amoxicillin calibration curves in SBS and STCF.

| Matrix | Mean (S.E.M.) | | Coefficient of determination ($R^2$)[a] |
|---|---|---|---|
| | Slope[a] | Intercept[a] | Range |
| SBS | | | |
| Low-calibration curve | 2.742 (0.07) | 11.126 (10.92) | 0.992–0.997 |
| High-calibration curve | 2.838 (0.05) | −294.894 (78.71) | 0.999–1.000 |
| STCF | 1.772 (0.04) | 30.328 (18.49) | 0.996–0.999 |

Construction of calibration curves was performed by least squares linear regression of amoxicillin peak height ($y$ in mV s) on amoxicillin calibrator concentration ($x$ in ng/mL).
[a] Four experimentations.

**Table 2**
Trueness and precision data of amoxicillin in SBS and STCF obtained over a 3-day validation procedure ($n = 6$ per fortification level, per day).

| Amoxicillin added (μg/mL) | Accuracy | CV (%) | | |
|---|---|---|---|---|
| | Mean (dev. from theoretical %) | Within-day precision | Between-days precision | Within-laboratory repeatability |
| SBS | | | | |
| 0.10 | 0.106 (5.82) | 8.74 | 3.57 | 9.44 |
| 0.30 | 0.309 (3.17) | 3.30 | 1.25 | 3.53 |
| 0.50 | 0.506 (1.16) | 4.63 | 1.42 | 4.84 |
| 1 | 1.026 (2.63) | 1.83 | 1.09 | 2.13 |
| 5 | 5.087 (1.74) | 2.81 | 1.29 | 3.10 |
| 20 | 20.427 (2.14) | 1.65 | 0.39 | 1.70 |
| STCF | | | | |
| 0.20 | 0.196 (−2.20) | 6.27 | 2.54 | 6.76 |
| 1 | 1.024 (2.41) | 2.48 | 0.44 | 2.51 |
| 4 | 4.045 (1.13) | 4.68 | 2.40 | 5.26 |

### 3.2.6. Amoxicillin stability

Although the stability of an analyte in a specific biological matrix during storage pending analysis, is obviously a function of the storage conditions, the inherent quality of the analyte and the container system (and therefore independent of the analytical method used for subsequent quantification), stability assay under various storage conditions has become an inextricable part of an analytical method validation procedure. On the other hand, post-preparative stability is a crucial aspect for reliable analyte quantification and in direct dependence on the procedure followed.

To assess amoxicillin storage stability in SBS, blank samples were fortified at 0.10, 1 and 20 μg/mL and were handled immediately, after 4 weeks storage at −85 °C, and after undergoing three freeze–thaw cycles. Furthermore, for the evaluation of post-preparative amoxicillin stability, samples likewise fortified were extracted, analysed and remained in the autosampler for extra 24 h before being re-quantified.

Amoxicillin stability assays in STCF were performed at two fortification levels, namely 0.20 and 1 μg/mL. Unfortunately, due to the limited availability of STCF, the three freeze–thaw experimentation was skipped.

Results, presented analytically in Table 3, demonstrate satisfactory amoxicillin stability under all investigational conditions. However, a rather marginal non-significance in the results of the three freeze–thaw cycles in SBS (i.e. $p = 0.056$ at 1 μg/mL) is recommendatory of an amplified caution when over-handling biological matrices containing amoxicillin. A plain freeze–thaw–analyze sample treatment is preferably advised.

### 3.3. Applicability of the method in samples from a biological experimentation

This method was designed for use in a pharmacokinetic study of amoxicillin in sheep. A pilot experimentation was deemed necessary to ensure its applicability. Amoxicillin (Amoxil®, ps.inj.sol.) was administered intravenously (i.v.) to four young adult rams of the Chios breed, at the dose of 15 mg/kg b.w. Blood and STCF samples were collected at predetermined time points after administration. SBS and debris-free STCF were harvested and stored at −85 °C, pending analysis. The method provided faultless chro-

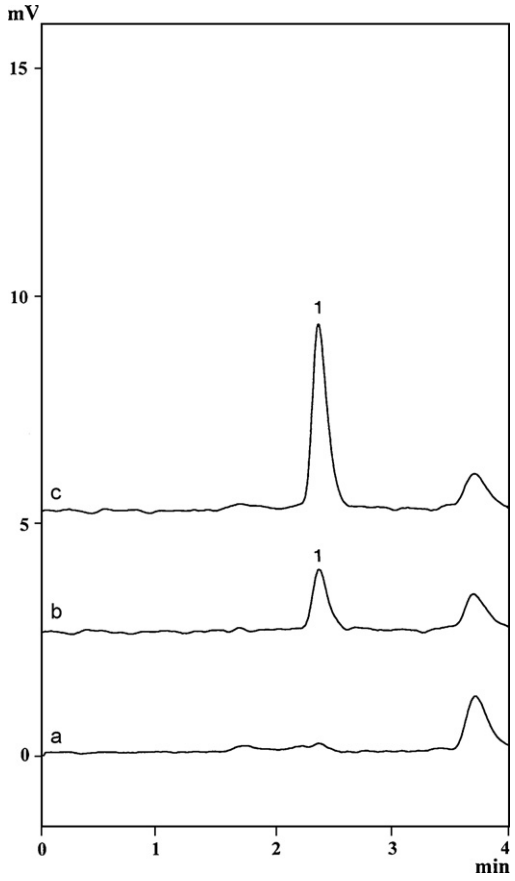


**Fig. 3.** Chromatograms of STCF samples: blank sample (a), sample fortified at 5 μg/mL (b) and sample obtained 3 h after amoxicillin (peak 1) administration to a ram, at the dose of 15 mg/kg b.w. (c).

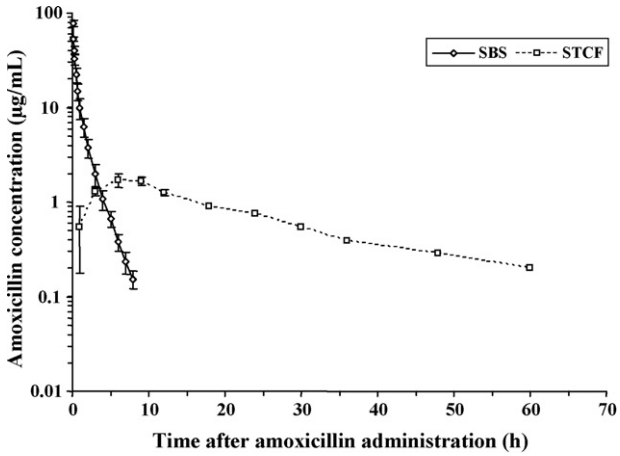


**Fig. 4.** Amoxicillin concentration vs. time curves in SBS and STCF after i.v. administration to four ($n = 4$) rams, at the dose of 15 mg/kg b.w.

**Table 3**
Stability data of amoxicillin in SBS ($n = 6$ per fortification level, per day).

| Amoxicillin added (μg/mL) | Mean concentration found (S.D.) (μg/mL) | | | |
|---|---|---|---|---|
| | Immediate analysis | 24 h in autosampler | 4 weeks at 85 °C | Three freeze–thaw cycles |
| SBS | | | | |
| 0.10 | 0.113 (0.01) | 0.108 (0.02) | 0.106 (0.01) | 0.101 (0.01) |
| 1 | 1.042 (0.01) | 1.050 (0.04) | 1.007 (0.05) | 0.988 (0.06) |
| 20 | 20.373 (0.52) | 20.733 (0.32) | 19.941 (0.77) | 19.710 (0.71) |
| STCF | | | | |
| 0.20 | 0.193 (0.01) | 0.195 (0.01) | 0.186 (0.01) | |
| 1 | 1.017 (0.03) | 1.004 (0.05) | 0.966 (0.06) | |
| 4 | 3.902 (0.08) | 3.914 (0.15) | 3.877 (0.16) | |

matograms (Fig. 3) and allowed the depiction of amoxicillin pharmacokinetic profile in both SBS and STCF (Fig. 4).

## 4. Conclusions

The LC method presented in this paper fulfills all performance criteria set by international guidelines [61], thus proving its value as a reliable analytical tool in amoxicillin pharmacokinetic studies, possibly to be extrapolated to other animal species, as well. To our knowledge, it is the first attempt to quantitatively determine amoxicillin levels in the TCF of sheep. The attained scale of sensitivity in both SBS and STCF has allowed full depiction of the area under the concentration vs. time curve ($AUC_{0-tLoQ}$) corresponding to at least 80% of the total AUC ($AUC_{0-\infty}$) in real samples, as required [64]. A daily one analyst throughput of 70–100 samples is considered absolutely feasible.

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