Journal of Chromatography B, 879 (2011) 1980–1986

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of a liquid chromatography/tandem mass spectrometry method for quantitative determination of amoxicillin in bovine muscle

B. Lugoboni, T. Gazzotti, E. Zironi, A. Barbarossa, G. Pagliuca*

Department of Veterinary Medical Sciences, Alma Mater Studiorum, University of Bologna – Via Tolara di Sopra, 50 I 40064 Ozzano dell'Emilia, Italy

ARTICLE INFO

Article history:
Received 22 November 2010
Accepted 24 May 2011
Available online 1 June 2011

Keywords:
Amoxicillin
LC–MS/MS
Bovine muscle

ABSTRACT

A simple, quick and economical liquid chromatographic/tandem mass spectrometry (LC–MS/MS) method for the quantitative determination of amoxicillin in bovine muscle was developed and validated. The sample preparation procedure involved a liquid extraction with water, followed by a protein precipitation step with acetonitrile. The extract was purified by a liquid–liquid partition with dichloromethane and the upper aqueous layer was directly injected into the LC–MS/MS system. Chromatographic separation was achieved on a reversed phase column, using a mixture of acetonitrile, water and 0.005% formic acid in water as mobile phase. Gradient elution was performed at a flow rate of 0.2 mL min^{-1}. Amoxicillin was detected using positive electrospray ionization in selected reaction monitoring (SRM) mode and was quantified using terbutaline as internal standard. The responses for standards prepared in solvent and in matrix were equivalent and additionally the absence of signal suppression was confirmed by the post column infusion technique. Amoxicillin stability in standard solution and in matrix was investigated at different times and storage conditions. Amoxicillin standards prepared in water were stable on storage up to 20 days at −20 °C. Amoxicillin stability in matrix (spiked bovine muscle samples) was assessed up to 15 days at −20 °C. The method was validated according to the parameters requested by European Commission Decision 2002/657/EC in terms of specificity, linearity, trueness, precision, decision limit (CCα) and detection capability (CCβ). All the trueness values fell within a range between 14.5% and 6.3%. Precision values for all levels of concentration tested were lower than the relative limit calculated by the Horwitz equation. The amoxicillin MRL is set at 50 μg kg^{-1} and the CCα and CCβ of the method were 61.2 μg kg^{-1} and 72.4 μg kg^{-1}, respectively.

1. Introduction

Amoxicillin is a broad-spectrum β-lactam antibiotic frequently used both in human and veterinary medicine for the treatment of bacterial infections caused by Gram-negative and Gram-positive organisms. Amoxicillin shows a bactericidal action, inhibiting the synthesis of peptidoglycans, which are part of the bacterial cell-wall.

The widespread use of this antibiotic may give rise to bacterial resistance, resulting in a constantly increasing potential risk for human and animal health. In addition, amoxicillin residues in food of animal origin can be a health hazard, especially for hypersensitive individuals [1].

The European Union included amoxicillin in Group B of Annex I of Council Directive 96/23/EC [2]. To protect consumer's health, a maximum residue limit (MRL) for this compound has been set at 50 ppb in tissues of all food-producing animals (muscle, liver, kidney, fat) and at 4 ppb in milk [3].

The quantitative determination of amoxicillin has always been hampered by analytical and technical difficulties. This analyte shows instability towards temperature and pH variations, and its amphoteric nature and high polarity make its extraction and chromatographic separation particularly critical. Due to these chemical properties the analysis of amoxicillin remains a challenge, especially in complex matrices like tissues samples [4,5].

For these reasons, few LC methods have been described for amoxicillin determination in tissue samples [1,4–15] and they generally require laborious and time-consuming sample treatment. Moreover most methods use ultraviolet (UV) or fluorescence (FL) detection [11–15], while just a few employ spectrometric detection [1,4–10].

Aim of this work was the development of a method for the quantitative determination of amoxicillin in bovine muscle by

* Corresponding author at: Department of Veterinary Medical Sciences, CABA-Lab, Via Tolara di Sopra, 50 I 40064 Ozzano dell'Emilia (BO), Italy.
Tel.: +39 0512097323; fax: +39 0512097323.
E-mail address: giampiero.pagliuca@unibo.it (G. Pagliuca).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.05.038

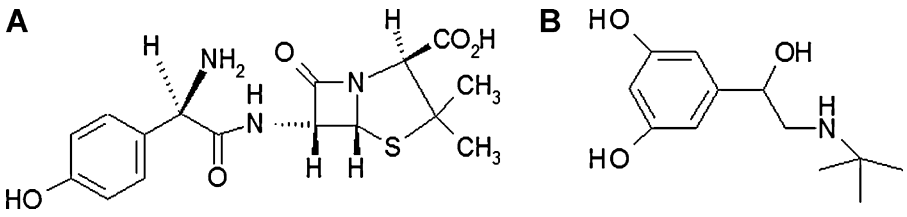

Fig. 1. Amoxicillin (A) and terbutaline (B) structure.

LC–MS/MS, a technique yielding excellent performances both in terms of quantification and unambiguous identification in the field of drug residue analysis. In particular, the proposed method requires a very simple and fast sample preparation, still proving to be reliable and efficient according to the validation parameters requested by European Commission Decision 2002/657/EC [16].

2. Materials and methods

2.1. Materials and reagents

Amoxicillin trihydrate (AMO) and terbutaline hemisulphate (TERB), used as internal standard (IS), were purchased from Sigma–Aldrich (St. Louis, MO, USA). The AMO standard reference material was stored at 4 ± 3 °C, whereas TERB standard reference material was stored protected from light at room temperature (20 ± 4 °C). The chemical structures of AMO and TERB are shown in Fig. 1.

Acetonitrile and formic acid, used as mobile phases, were specific for LC–MS analysis and were obtained from Riedel-de Haën (Seelze, Germany). Acetonitrile and dichloromethane, used as extraction solvents, were of analytical grade and purchased from Merck (Darmstadt, Germany).

RO (Reverse Osmosis) and UP (Ultra Pure) water, used as extraction solvent and chromatographic mobile phase respectively, were produced by a Human Power® I apparatus from Human Corporation (Seoul, Korea).

2.2. Preparation of standard solutions

Standard stock solution of AMO was prepared at a concentration of 100 μg mL^{-1} by dissolving the pure substances in a volumetric flask with UP water. Intermediate standard solution (5 μg mL^{-1}) was prepared from the stock standard solution. For spiking purposes, five AMO working solutions (0.25, 0.375, 0.5, 0.75 and 1 μg mL^{-1}) were prepared by appropriate dilution of the intermediate standard solution in UP water.

Standard stock solution of TERB was prepared at a concentration of 100 μg mL^{-1} by dissolving the pure substances in UP water and the IS working solution (1 μg mL^{-1}) was obtained by appropriate dilution of stock solution in UP water.

All the standard solutions were stored at −20 °C in darkness until use and according to the data reported in Fig. 2 these solutions were stable up to 21 days. The AMO working solutions were divided into small vials and each analysis day a new portion was used to prevent amoxicillin deterioration due to the repeated freeze-thaw procedure.

Tuning standard solutions of AMO and TERB, at a concentration of 1 μg mL^{-1}, were made in UP water containing 0.1% formic acid.

2.3. Equipment

The liquid chromatographic device was an Alliance 2695 system consisting of a quaternary pump, solvent degasser, auto sampler and column heater from Waters Corporation (Milford, USA). Separation was performed on an Atlantis T3 Waters column (3 μm, 2.1 mm × 150 mm) in combination with a protecting guard column of the same type (3 μm, 2.1 mm × 10 mm) (Waters Corporation, Milford, USA).

The mass spectrometer was a Quattro Premier XE triple quadrupole instrument equipped with an ESCI™ Multi-Mode Ionization Source (Waters Corporation, Milford, USA).

High purity nitrogen was produced by nitrogen gas generator Mistral-4 from DBS Instrument (Padua, Italy).

Data acquisition and processing was performed using Mass Lynx 4.1 Software (Waters Corporation, Milford, USA).

2.4. Samples and pretreatment

The matrix used for validation consisted of a mixture of four bovine muscle samples (20 ± 0.4 g each one) purchased from a retail market. Preliminary analyses showed that the four individual muscle tissues were analyte-free. Tissues were minced together and homogenized for 2–3 min at 28,000 rpm after dilution with water (1:1, w/w) using an IKA A11 basic analytical mill (Staufen, Germany) at room temperature. Aliquots of 600 ± 12 mg of homogenized muscle (equivalent to 300 mg of tissue) were transferred into individual 10 mL plastic tubes and stored at −20 °C until being thawed for analysis.

For reliable analysis of incurred samples it is strongly recommended to pretreat and homogenize, as described above, a representative amount of muscle tissue (at least 80–100 g).

2.5. Preparation of calibration curve samples and quality control samples

For preparation of calibration curves 30 μL of each of the five AMO working solutions (0.25, 0.375, 0.5, 0.751 and 1 μg mL^{-1}) were added to 600 mg of blank bovine homogenate (equivalent to 300 mg of tissue) to obtain the following AMO concentrations: 25, 37.5, 50, 75 and 100 μg kg^{-1}.

Quality control samples (QC) were prepared in a similar way at concentrations of 25, 50 and 75 μg kg^{-1} corresponding to 0.5 MRL, MRL and 1.5 MRL, respectively.

After adding the AMO working solutions the samples were mixed using a Vortex Wizard (Velp Scientifica, Milano, Italy) for 15 s

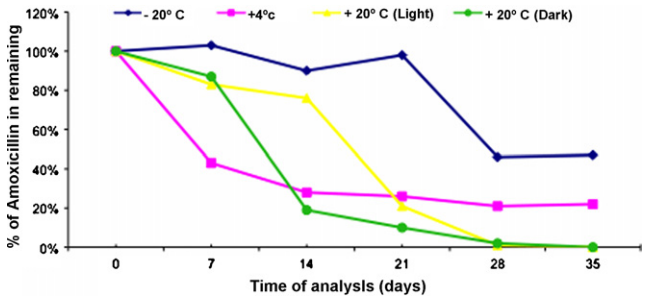

Fig. 2. Graphical representation of the standard solution stability experiments (mean, $n = 3$).

Table 1
Retention times and mass spectrometric details of amoxicillin and terbutaline.

Analyte	Retention time (min)	Cone voltage (V)	Dwell time (ms)	Quantification transition (collision energy, eV)	Confirmation transition (collision energy, eV)	Ion ratio
AMO	8.1	14	100	366.08 > 113.90 (21)	366.08 > 207.90 (12)	1.8
TERB (IS)	7.9	26	100	226.09 > 151.90 (17)	226.09 > 106.80 (32)	4.5

and then kept at room temperature for 10 min before the sample extraction procedure.

2.6. Sample preparation procedure

Bovine muscle tissue samples, pretreated as described in Section 2.4, were thawed at room temperature and spiked with 30 μL of IS working solution (corresponding to a concentration of 100 μg kg^{-1}). After mixing using a vortex for 15 s, 700 μL of water was added. The sample was homogenized with an Ultra-Turrax, T25 digital IKA (Staufen, Germany), at 24,000 rpm for 2 min. The homogenized sample was transferred into a 1.5 mL Eppendorf tube and centrifuged at 15,000 rpm for 5 min at 4 °C in a refrigerated centrifuge Hettich 320 R (Beverly, MA, USA). Then 500 μL of the supernatant were transferred into a 1.5 mL Eppendorf tube together with 500 μL of acetonitrile; the sample was then vortex mixed for about 2 min. After centrifugation for 5 min at 15,000 rpm at 4 °C, 700 μL of the supernatant was transferred to a new 1.5 mL Eppendorf tube and 700 μL of dichloromethane were added. The sample was vortex mixed for about 2 min before being centrifuged for 5 min at 15,000 rpm at 4 °C. Approximately 150 μL of the upper aqueous layer were put into an autosampler vial and 10 μL were directly injected into the LC–MS/MS system.

2.7. LC–MS/MS conditions

Chromatographic separation was achieved using reversed phase chromatography with gradient elution. Mobile phase A was acetonitrile, mobile phase B was water containing 0.005% formic acid and mobile phase C was water.

The following gradient program (time, %A–%B–%C) was applied: (0 min, 0–10–90), (3 min, 25–10–65), (4 min, 25–10–65), (5 min, 0–10–90), (9 min, 0–10–90). Between each sample the column was allowed to equilibrate at initial conditions for 3 min. The flow rate was 0.2 mL min^{-1} and the column temperature was maintained at 35 °C. The chromatographic eluent was directed to the ionization source between minutes 7 and 9, while before and after this period the flow was diverted to the waste.

The mass spectrometer interface was an electrospray ionization source operating in positive ion mode (ESI+) with the following conditions: capillary voltage 2.75 kV, source and desolvation temperature 120 °C and 300 °C, desolvation and cone gas (nitrogen) flow 700 L/h and 90 L/h, respectively.

The mass spectrometer operated in SRM (selected reaction monitoring) mode by monitoring two specific transitions for each compound, with a dwell time of 100 ms. Argon was used as collision gas for collision-induced dissociation. The analyte-dependent MS/MS parameters were optimized via direct infusion of tuning standard solution into the mass spectrometer. The selected values of cone voltage, collision energy and the two main transitions monitored for each compound are given in Table 1.

2.8. Stability experiments

The stability of the amoxicillin in standard solution was studied. A 50 μg L^{-1} amoxicillin solution in water was prepared and analyzed to measure the initial concentration. This standard solution was then divided into small aliquots that were stored in different conditions: at −20 °C, +4 °C and room temperature (around +20 °C) in darkness and at room temperature under light exposure. Every week, for a total of 5 weeks, one aliquot was analyzed to ascertain degradation phenomena. The aliquots of amoxicillin standard were fortified with 10 μL of IS working solution (1 μg mL^{-1}) just prior to LC/MS-MS analysis. The IS working solution was freshly prepared each analysis day for the standard stability experiment.

Amoxicillin stability in matrix was also investigated. Different storage conditions were tested in order to simulate real conditions in which samples could undergo in a laboratory before analysis. Amoxicillin stability in matrix was determined using groups of 6 QC samples fortified at the MRL (50 μg kg^{-1}). In particular short-term or bench-top stability was evaluated analyzing samples stored at room temperature for 6 h. Stability at 4 °C for 24 h and at −20 °C for 15 days was also tested. The freeze/thaw stability was assessed after two and three freeze/thaw cycles (during each cycle samples were maintained for at least 24 h at −20 °C, then thawed for 2–3 h before being refrozen).

Post preparative stability was assessed analyzing extracts of QC samples at the MRL after 24 h of storage in the autosampler at room temperature.

The results obtained from the analysis of the QC samples stored under the different described conditions were compared with those obtained from a set of 6 QC freshly prepared and CV% was measured.

2.9. Method validation

The proposed LC–MS/MS method for amoxicillin quantification in bovine muscle tissue was validated in-house according to European Commission Decision 2002/657/EC [16]. The following parameters were evaluated: specificity, linearity, trueness, precision, decision limit (CCα) and detection capability (CCβ).

The specificity was evaluated checking the ion chromatograms of 21 blank samples extracted and analyzed with the above-mentioned method for potential co-eluted interfering compounds which can impair interpretation at the specific retention time of the analytes.

The linearity of the method was assessed with calibration curves freshly prepared each analysis day. The calibration curves were obtained spiking blank tissue samples with amoxicillin at the following concentrations: 0, 25, 37.5, 50, 75 and 100 μg kg^{-1} (for details, see Section 2.5).

Peak area ratios between the amoxicillin and the IS were plotted against their concentration ratios, then a linear regression study and the analysis of variance (ANOVA) were performed. To check the validity of the regression model an *F*-test was also performed, while the linearity of the calibration curves was investigated by using the Lack-of-Fit test (LOF) [17].

Since no certified reference material is available, the trueness and the precision were determined by analyzing blank samples fortified (QC samples) at three different concentrations (0.5 MRL, MRL and 1.5 MRL). In particular, 18 spiked samples for each level were processed individually on three different days (six samples per day).

2.10. Matrix effect

Some experiments were conducted to evaluate the matrix effect, which can greatly affect the analyte response in biological

matrices. Seven point standard and matrix-matched calibration curves were compared and a *t*-test was applied to statistically quantify the slope difference between these two calibration curves. The solutions for standard calibration curve were prepared in water at different amoxicillin concentrations (0, 12.5, 18.75, 25, 37.5, 50, 75 and 100 ng mL^{-1}), while a pool of blank bovine muscle samples (obtained following the pretreatment, extraction and clean-up procedures described above) was spiked at the same concentrations to prepare a matrix-matched calibration curve. A fixed amount of IS working solution (1 μg mL^{-1}) was added to all standard and matrix-matched solutions.

The *t* value was calculated using the following formula described by Soliani [18]:

$$t_{(n-4)} = \frac{b1 - b2}{es_{(b1-b2)}}$$

where $n-4$ are the degrees of freedom, $b1$ and $b2$ the slopes of the two calibration curves to be compared and $es_{(b1-b2)}$ the standard error of the difference between the two slopes.

Moreover the post column infusion technique was carried out injecting a blank tissue sample while a standard solution containing amoxicillin and terbutaline at 1 μg mL^{-1} was continuously infused into the mass spectrometer interface through a T-connection system [19].

3. Results and discussion

3.1. Optimization of the sample preparation procedure

While developing the proposed method special attention was paid to optimization of the sample preparation procedure. Different parameters were considered and tested, namely: matrix amount (1000, 500 and 300 mg), type of extraction solvent (10 mM KH_2PO_4 solution at pH = 7, 10 mM KH_2PO_4 solution brought to pH = 4.5 with formic acid, water:methanol (30:70, v/v) and water), volume of extraction solvent (7, 5, 2.5 mL and 700 μL), extraction technique (ultrasonication, vortex mixing and homogenization with ultraturrax), clean-up procedure (SPE, ultrafiltration and liquid–liquid partition).

Although the use of 10 mM KH_2PO_4 solutions [1,4,6,20] and water:methanol (30:70, v/v) [7,8] as extraction solvents yielded satisfactory results (amoxicillin recovery ranging from 62% to 78%), the best extraction solvent in this study was water (amoxicillin recovery of 86%). Indeed, penicillin antibiotics are easily extracted from animal tissues with water, as reported by Ito et al. [21] and Goto et al. [22].

Good results, in terms of recovery and sensitivity, were gained pretreating 300 mg of bovine muscle and extracting with 700 μL of water. Since there was no need to extract a larger sample or increase the volume of the extraction solvent the whole sample preparation procedure could be miniaturized with an evident saving of solvents, consumables and time.

Simple vortex mixing or the ultrasonication of the sample proved ineffective to extract the analytes effectively, while homogenization with ultraturrax enhanced the matrix–water interaction with a good extraction efficiency.

A purification of the aqueous extracts on a Oasis® HLB solid phase extraction (SPE) column (Waters Corporation, Milford, USA) was tried. According to some authors [1,6,23], the results of the SPE clean-up procedure were not always reproducible (amoxicillin recovery varying from 46% to 83%), thereby affecting the repeatability, reproducibility and robustness of the method.

To avoid the time-consuming SPE clean-up step, ultrafiltration was tried to deproteinize the samples [1,5]. Satisfactory results were obtained centrifuging water extracts into a Microcon® YM-30 Centrifugal Filter Device (Molecolar weight (MW) cut-off: 30,000 Da) (Millipore, Bedford, MA, USA) at 14,000 rpm, but at least 40 min were required to ensure the passage of the entire sample extract through the filter membrane.

In the present work, a protein precipitation step with acetonitrile followed by a liquid–liquid partition with dichloromethane was chosen to purify the extract. This procedure takes just 20 min and was highly effective, rendering a clean extract in just two quick and easy centrifugation steps. A similar procedure has already been described for amoxicillin extraction from human plasma [24] and the novelty of the present work consists in the application of a very easy, cheap and fast extraction and clean-up procedure to a tissue matrix.

The literature reports that amoxicillin degradation could be promoted by using not only a strong acid solution, but also organic solvents (like acetonitrile) to deproteinize tissue samples [1,6]. The present work used acetonitrile for deproteinization of sample extracts since its degradation effect seems to be negligible. This could be due to the short interaction time (around 5 min) between amoxicillin, present in the sample extract, and acetonitrile. The washing step with dichloromethane effectively removes acetonitrile that dissolves better in dichloromethane than in water [24]. The final upper layer was mainly composed of neutral water resulting in negligible amoxicillin degradation.

3.2. Choice of the internal standard

The use of an internal standard improves the accuracy and reproducibility of the method since it can compensate for variations in extract volume, sample losses and mass spectrometric response, due to suppression or enhancement of the matrix effect. Usually stable isotope analogues, isomers or homologues of the analyte are chosen as internal standard. While developing the present method, two different internal standards were compared: ampicillin and terbutaline. Even though ampicillin is chemically correlated to amoxicillin, terbutaline was chosen as internal standard on the basis of its solubility, mass spectrometric and chromatographic properties. In particular, terbutaline and amoxicillin showed similar solubility and recovery features through the sample preparation procedure and extremely close retention times (see Table 1). Moreover terbutaline proved to be more stable in both standard solutions and extracted samples [24].

3.3. Performance of LC–MS/MS analysis

Different mobile phase gradients were tested to obtain an optimal retention and a good separation of the analytes from the matrix interferent compounds in a reasonable analysis time. The chosen chromatographic conditions allowed a clear separation of the analytes in 9 min. After elution of the analytes, the column was equilibrated for at least 3 min to have a good reproducibility of retention times and sharp peaks.

The different influence of the mobile phase on ionization was studied. Ionization efficiency was decreased using methanol instead of acetonitrile, and was also affected by the formic acid concentration, so two different concentrations (0.005% and 0.1%, v/v) were tested in mobile phase B (water). In agreement with Becker et al. [23] the best sensitivity was achieved using the lowest concentration of formic acid.

The use of a divert valve ensured that early eluting matrix compounds were discarded into the waste thereby reducing contamination of the ion source and allowing a large number of samples to be analyzed without having to clean the source components.

The mass spectra information of AMO and TERB was acquired after direct infusion of the tuning standard solutions. The best sen-

Table 2
Results of the amoxicillin stability experiments in matrix.

	Storage conditions		Mean concentration ($\mu g\,kg^{-1}$) ($n=6$)	CV%
	Temperature	Time		
Matrix stability[a]	Room temperature	6 h	55.0	5.0
	+4 °C	24 h	52.1	−0.4
	−20 °C	15 days	46.5	−11.1
Freeze/thaw stability[a]	Room temperature/−20 °C	2 Cycles	50.4	−3.7
	Room temperature/−20 °C	3 Cycles	47.3	−9.6
Post preparative stability[b]	Room temperature	24 h	52.5	9.0

[a] Compared to a set of 6 QC samples freshly extracted with a mean concentration of 52.3 $\mu g\,kg^{-1}$.
[b] Compared to a set of 6 QC samples freshly extracted with a mean concentration of 48.1 $\mu g\,kg^{-1}$.

sitivity for both analytes was found using an electrospray interface (ESI) with positive ionization. The protonated molecular ion $[M-H]^+$ was chosen as precursor ion and the cone voltage was optimized in MS mode to gain the maximum signal of these ions. Then the collision energy was adjusted in MS/MS mode to obtain two main product ions.

Both analytes were identified on the basis of retention time, presence of two specific mass transitions for each compound and ion ratio of these product ions (Table 1). This fulfilled the identification criteria established by Commission Decision 2002/657/EC: even though a minimum of three identification points are required for the confirmation of substances listed in the Group B of Annex I of Council Directive 96/23/EC [2], four identification points were gained by measuring two product ions in addition to the precursor ion. Moreover the ion ratios observed in samples matched the ion ratios of the standards. The relative intensity of the two monitored transitions, expressed as a percentage of the intensity of the most intense transition, corresponded to the mean ion ratios of the calibration standard, within the maximum permitted tolerance (for ion ratios >0.50 the maximum permitted tolerance is ±20%).

3.4. Stability experiment

The stability of the standard solution was evaluated over 5 weeks at different storage conditions: −20 °C, +4 °C and +20 °C in the dark and +20 °C exposed to the light. Aliquots of the amoxicillin standard solution were analyzed weekly and the responses calculated by measuring the ratio between the peak areas of amoxicillin and the peak area of the internal standard prepared and added just before the analysis. The remaining amoxicillin concentrations were then expressed as a percentage of the initial amoxicillin concentration and the results are reported in Fig. 2. Degradation phenomena were observed in all the storage conditions and after 5 weeks no remaining amoxicillin concentrations were detected in standard solutions kept at +20 °C, whereas an acceptable stability was observed for 20 days in water standard solutions stored at −20 °C. These results are in agreement with those reported by Mastovska and Lightfield [9].

The amoxicillin degradation observed in this experiment could be explained by the presence of the β-lactam ring that is susceptible to hydrolysis in aqueous solution, especially when pH conditions are significantly different from amoxicillin's isoelectric point (pH 4.8) [25]. The standard solution used for the stability experiments was prepared in non buffered UP water and its pH, measured at the beginning of this test, was 7.6. This pH value could have promoted the degradation of the β-lactam ring over the 5 weeks of observation.

The stability of amoxicillin in matrix was investigated using QC samples fortified at the MRL and stored in different conditions; the results of these experiments are reported in Table 2. Stability in matrix is considered acceptable if the mean concentration obtained from the analysis of QC stability experiments samples agrees with the freshly prepared QC samples within ±15% [26]. In the tested conditions the results fulfilled this requirement being included in the range between −11.1% and +9.0%. In particular amoxicillin proved to be stable in bovine muscle samples spiked at the MRL and stored at −20 °C for at least 15 days. Moreover stability in matrix

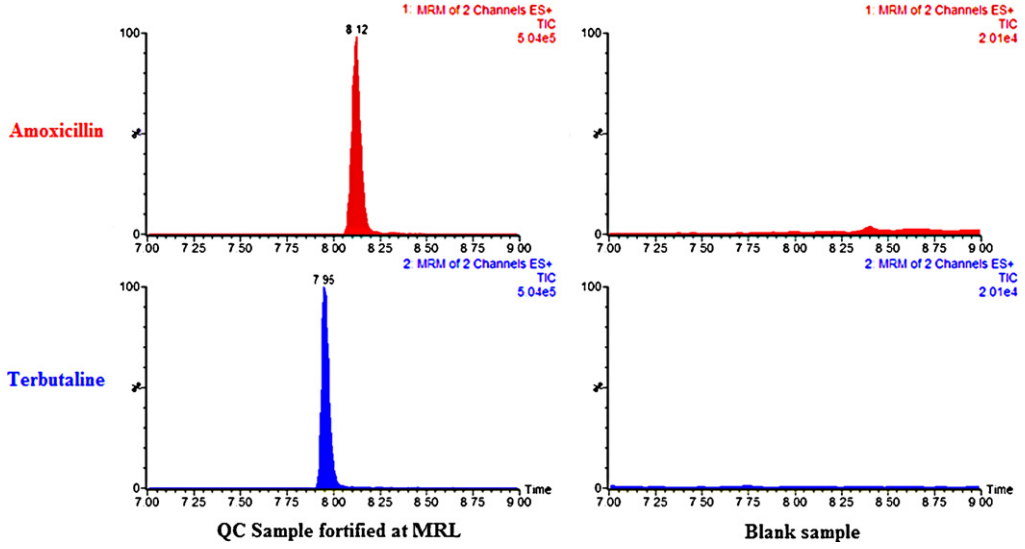

Fig. 3. Chromatograms of a blank bovine muscle sample and a bovine muscle sample fortified with amoxicillin 50 $\mu g\,kg^{-1}$ (MRL) and with terbutaline (100 $\mu g\,kg^{-1}$).

Table 3
Overview of the validation parameters in terms of calibration curves linearity.

Day of analysis	Slope ± SE	Intercept ± SE	r^2	F_{REG} (α = 0.05; df1 = 1; df2 = 10)	F_{LOF} (α = 0.05 df1 = 4; df2 = 6)
1	$5.08 \times 10^{-4} \pm 2.15 \times 10^{-5}$	$1.79 \times 10^{-3} \pm 1.25 \times 10^{-3}$	0.9824	558.08	16.39
2	$3.16 \times 10^{-4} \pm 1.32 \times 10^{-5}$	$9.41 \times 10^{-5} \pm 8.48 \times 10^{-4}$	0.9878	567.27	0.07
3	$4.14 \times 10^{-4} \pm 2.94 \times 10^{-5}$	$1.37 \times 10^{-3} \pm 1.80 \times 10^{-3}$	0.9845	508.10	3.81

was assessed after three freeze/thaw cycles and in sample extracts maintained at room temperature for 24 h.

3.5. Method validation

The specificity of the method was demonstrated by the absence of endogenous interferences at the specific retention time of amoxicillin. Fig. 3 shows typical chromatograms of a blank muscle sample and a muscle sample spiked with amoxicillin at MRL level (50 μg kg^{-1}) and terbutaline at 100 μg kg^{-1}.

During each day of analysis, calibration curves were obtained with duplicate measurement (at the beginning and end of the batch analysis) of samples fortified at 6 different concentrations: 0, 25, 37.5, 50, 75 and 100 μg kg^{-1}.

Each daily calibration curve was accepted when the accuracy value of the 75% of calibration samples fell within ±15% the nominal value (except for lower limit of quantification of the curve for which the acceptance criterion was ±20% the nominal value) [26].

The regression lines obtained were all satisfactory with a coefficient of determination (r^2) always higher than 0.9824. Table 3 lists the curves' equations, determination coefficients and the F_{REG} and F_{LOF} values calculated on the three different days of validation. F_{REG} was always consistently higher than the tabulated value of 4.96 (α = 0.05; df1 = 1; df2 = 10), proving the validity of the chosen model of regression; F_{LOF} did not exceed the reported value of 4.53 (α = 0.05; df1 = 4; df2 = 6) except for one case, showing a slightly significant lack of fit that did not influence the linearity of the calibration curves.

The trueness was expressed as bias, difference between the mean value measured and the spiked concentration (in %), and had to be within −20 and +10%, according to the Commission Decision 657/2002/EC [16] for the considered concentration level.

The precision was measured as relative standard deviation to the mean (CV% or coefficient of variation). Values of CV% in within-laboratory reproducibility conditions (samples analyzed by different operators on different analysis days) had to be lower than the value calculated according to Horwitz equation: $CV\% = 2^{(1-0.5 \times \log c)}$, where c is the concentration of analyte expressed as a decimal fraction. In repeatability conditions (intra-day analysis) the CV% values had to be lower than two thirds of the values calculated according to Horwitz equation.

The results of the trueness and precision experiment, summarized in Table 4, show the good accuracy of the method. For all concentration levels tested, the trueness values fell within the range established by Commission Decision 657/2002/CE (−20 and +10%). The worst bias is −14.5% and was observed at the lowest level of fortification (25 μg kg^{-1}) but is still acceptable according to the above criteria.

The precision values, calculated both in repeatability conditions (intra-day analysis carried out by different operators) and within-laboratory reproducibility conditions (inter-day analysis), were below the maximum CV% values recommended by the Commission Decision 657/2002/CE. According to the Horwitz equation, the relative standard deviation for repeated analysis, under reproducibility conditions, for concentrations of 75 μg kg^{-1}, 50 and 25 μg kg^{-1} should not exceed 23.6%, 25.1% and 27.9%, respectively. The CV% values obtained in repeatability conditions should be two-thirds of the above values: 15.8%, 16.7% and 18.6% for concentrations of 75, 50 and 25 μg kg^{-1}, respectively. These maximum allowed CV% values were respected in all cases, as shown in Table 4.

The decision limit (CCα) and the detection capability (CCβ) were determined as described in Commission Decision 657/2002/EC [16].

CCα is defined as the concentration level above which a sample can be declared non-compliant with an error probability equal to α (=5%), while CCβ is defined as the concentration limit at which the method is able to detect permitted limit concentrations with a statistical certainty of $1 - \beta$ (β = 5%). The decision limit and detection capability were calculated following the calibration curve procedure described in the Commission Decision 657/2002/EC [16]. Blank samples, fortified for trueness and precision experiment at 0.5 MRL, MRL and 1.5 MRL levels (n = 18), were used to obtain a calibration curve and then CCα and CCβ were calculated in accordance with ISO standard 11843-2 [27].

Verdon et al. [28] described this procedure for calculating CCα and CCβ, both for non-permitted substances and for MRL substances. According to their equations, the CCα and CCβ values of the present method were 61.2 μg kg^{-1} and 72.4 μg kg^{-1}, respectively.

3.6. Matrix effect

The effect of matrix on the determination of amoxicillin in bovine muscle was evaluated comparing the matrix-matched

Table 4
Summary of results of validation of the method.

Fortification level	Parameter	Day 1	Day 2	Day 3	Inter-day
25 μg kg^{-1}	n	6	6	6	18
	Precision (CV%)	4.2[a]	6.0[a]	4.4[a]	10.4[b]
	Trueness (bias%)	−14.5[c]	0.5[c]	6.3[c]	−2.6[c]
50 μg kg^{-1}	n	6	6	6	18
	Precision (CV%)	3.7[a]	7.5[a]	14.7[a]	10.5[b]
	Trueness (bias%)	4.6[c]	−3.7[c]	−8.6[c]	−2.6[c]
75 μg kg^{-1}	n	6	6	6	18
	Precision (CV%)	10.5[a]	7.6[a]	14.4[a]	10.9[b]
	Trueness (bias%)	−12.8[c]	−8.0[c]	−7.6[c]	−9.5[c]

[a] Maximum CV% in repeatability conditions: 25 μg kg^{-1} = 18.6%; 50 μg kg^{-1} = 16.7%; 75 μg kg^{-1} = 15.8%.
[b] Maximum CV% in within-laboratory reproducibility conditions: 25 μg kg^{-1} = 27.8%; 50 μg kg^{-1} = 25.1%; 75 μg kg^{-1} = 23.6%.
[c] Acceptable trueness values range: −20% + 10%.

calibration curve with the standard solution calibration curve. The equation of the matrix-matched curve was: $y = 3.31 \times 10^{-4} + 1.67 \times 10^{-4}$; while the standard solution curve showed the following equation $y = 3.50 \times 10^{-4} + 7.68 \times 10^{-4}$. The absence of a significant difference between the slopes of the two calibration curves was confirmed by the *t*-test. With a significance alpha level of 0.05 and 12 degrees of freedom, the critical *t*-value is 2.18 and for *t*-values higher than 2.18 the slope difference is considered statistically significant, meaning that the matrix effect is present. In the present case the *t*-value, calculated according to Soliani [18], was 1.75 and hence no matrix effect was observed.

Finally, the absence of compounds likely to interfere with ionization of the analyte was confirmed with the post-infusion technique as described by Antignac et al. [19]. The ion current, recorded during the simultaneous injection of a blank sample and infusion of the standard solution, was stable and no interferences were recorded at the specific retention times of amoxicillin and terbutaline.

4. Conclusions

A simple and economical HPLC–MS/MS method for amoxicillin analysis in bovine muscle was developed. The fast sample preparation, based on extraction with water followed by protein precipitation and liquid–liquid partition, yielded samples ready for injection into the LC–MS/MS system in about 20 min.

The method was validated according to the main parameters recommended by Commission Decision 2002/657/EC (specificity, linearity, trueness, precision, decision limit (CCα) and detection capability (CCβ)) and all the performance data fell within the specified ranges.

Based on the reported stability experiments, amoxicillin in water standard solution proved to be stable up to 20 days at −20 °C and amoxicillin stability in matrix samples was confirmed up to 15 days at −20 °C. The easy and fast preparation steps proposed provide high sample throughput allowing to process 60–80 samples in 24 h (instrumental analysis included). This allows a laboratory with similar equipment to carry on effectively amoxicillin analysis in bovine muscle with the described performances.

The proposed method proved to be effective, sensitive, selective and reliable, and can be considered a useful tool for residue analysis.

References

[1] T. Reyns, M. Cherlet, S. De Baere, P. De Backer, S. Croubels, J. Chromatogr. B 861 (2008) 108.
[2] Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and decision 89/187/EEC and 91/664/EEC (Off. J. Eur. Union 1996, L125).
[3] Commission Regulation 37/2010/EC of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin (Off. J. Eur. Union 2010, L15).
[4] S. De Baere, M. Cherlet, K. Baert, P. De Backer, Anal. Chem. 74 (2002) 1393.
[5] S. Bogialli, V. Capitolino, R. Curini, A. Di Corcia, M. Nazzari, M. Sergi, J. Agric. Food Chem. 52 (2004) 3286.
[6] S. De Baere, P. Wassink, S. Croubels, S. De Boever, K. Baert, P. De Backer, Anal. Chim. Acta 529 (2005) 221.
[7] K. Granelli, C. Branzell, Anal. Chim. Acta 586 (2007) 289.
[8] J. Chico, A. Rubies, F. Centrich, R. Companyo, M.D. Prat, M. Granados, J. Chromatogr. A 1213 (2008) 189.
[9] K. Mastovska, A.R. Lightfield, J. Chromatogr. A 1202 (2008) 118.
[10] P.A. Martos, F. Jayasundara, J. Dolbeer, W. Jin, L. Spilsbury, M. Mitchell, C. Varilla, B. Shurmer, J. Agric. Food Chem. 58 (2010) 5932.
[11] M.D. Rose, J. Tarbin, W.H.H. Farrington, G. Shearer, Food Addit. Contam. 14 (1997) 127.
[12] M. McGrane, M. O'Keeffe, M.R. Smyth, Analyst 123 (1998) 2779.
[13] E. Verdon, P. Couder, J. AOAC Int. 82 (1999) 1083.
[14] L.K. Sørensen, H. Hansen, L. Snor, J. AOAC Int. 82 (1999) 1345.
[15] L.K. Sørensen, L. Snor, T. Elkær, H. Hansen, J. Chromatogr. B 734 (1999) 307.
[16] Commission Decision 657/2002/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of the analytical methods and the interpretation of results (Off. J. Eur. Union 2002, L221).
[17] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics. Part A, Elsevier, Amsterdam, 1997.
[18] L. Soliani, Manuale di statistica per la ricerca e per la professione, in: Capitolo XVII: Confronti tra rette, calcolo della retta con y ripetute, con verifica di linearità e introduzione alla regressione lineare multipla, 2005, Available: http://www.dsa.unipr.it/soliani/soliani.html (last access 23rd July 2010).
[19] J.P. Antignac, K. De Walsh, F. Monteau, H. De Brabander, F. Andre, B. Le Bizec, Anal. Chim. Acta 529 (2005) 129.
[20] S. De Baere, P. De Backer, Anal. Chim. Acta 586 (2007) 319.
[21] Y. Ito, Y. Ikai, H. Oka, T. Kagami, K. Takeba, J. Chromatogr. A 885 (1999) 247.
[22] T. Goto, Y. Ito, S. Yamada, H. Matsumoto, H. Oka, J. Chromatogr. A 1100 (2005) 193.
[23] M. Becker, E. Zittlau, M. Petz, Anal. Chim. Acta 520 (2004) 19.
[24] K.H. Yoon, S.Y. Lee, W. Kim, J.S. Park, H.J. Kim, J. Chromatogr. B 813 (2004) 121.
[25] P.O. Erah, A.F. Goddard, D.A. Barret, P.N. Shaw, R.C. Spiller, J. Antimicrob. Chemother. 39 (1997) 5.
[26] EMEA/CVMP/VICH/463202/2009. VICH Topic GL49. At step 4. Guidelines for the validation of analytical methods used in residue depletion studies.
[27] International Standard ISO 11843-2: 2000. Capability of Detection. Part 2. Methodology in the Linear Calibration Case, Geneva, Switzerland.
[28] E. Verdon, D. Hurtaud-Pessel, P. Sanders, Accredit. Qual. Assur. 11 (2006) 58.