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Rapid method for the quantification of amoxicillin and its major metabolites in pig tissues by liquid chromatography-tandem mass spectrometry with emphasis on stability issues

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Abstract

A fast method for the quantitative determination of amoxicillin (AMO), amoxicilloic acid (AMA) and amoxicillin diketopiperazine-2′,5′-dione (DIKETO) in pig edible tissues (kidney, liver, fat and muscle) with liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS) is presented. The method uses a simple liquid–liquid extraction of the tissue matrix with a 10 mM potassium dihydrogen phosphate buffer (pH 4.5) as extraction solvent. After deproteinisation by ultrafiltration, the tissue extract was directly injected onto the LC column. Chromatographic separation of the components was performed on a PLRP-S polymeric column using 0.1% of formic acid in water and acetonitrile. The mass spectrometer was operated in the positive electrospray MS/MS mode. The method was fully validated according to EU requirements (linearity, precision, trueness, quantification limit, detection limit and specificity). The stability of the components was evaluated over the pH range from 1.2 to 8.0. Biological samples of pigs medicated with AMO and AMO/clavulanic acid were analyzed using the developed method.

Keywords: Amoxicillin; Amoxicilloic acid; Amoxicillin diketopiperazine-2′,5′-dione; Liquid chromatography/electrospray ionization-tandem mass spectrometry; Stability; Pig tissues

1. Introduction

Amoxicillin (AMO) is an α-amino-substituted β-lactam antibiotic frequently used in human and veterinary medicine because of its broad spectrum and low cost [1,2]. AMO has a bactericidal action and inhibits the bacterial cell-wall biosynthesis by binding to the enzymes which produce the protein cell wall [3]. However, the use of penicillins in food producing animals may lead to the emergence of penicillin-resistant bacterial strains and the residues in milk and tissues are potential risks for individuals who are hypersensitive to penicillins [4]. Some cases of allergic reactions after consumption of foods containing penicillin residues are reported in the literature [5]. The two major metabolites of AMO are amoxicilloic acid (AMA) and amoxicillin diketopiperazine-2′,5′-dione (DIKETO). These metabolites have lost the antibacterial activity of the parent component [6], but the AMA metabolite could have potential allergic properties [7]. The strongest allergic effect is produced by the penicilloyl moiety, which is formed by a reaction between the β-lactam carbonyl group and the amino groups of proteins [7]. To protect the consumers' health, the European Union (EU) has set a maximum residue limit (MRL) for AMO at 50 $\mu g\,kg^{-1}$ in animal tissues (kidney, liver, muscle and fat) [8,9], however the AMA metabolite is not included in the MRL.

In the present study, we report a simple and fast method for the quantitative analysis of AMO and its metabolites in pig tissues. The starting point were two previously published methods of De Baere et al. [10,11]. A revision of these methods was performed to simplify the sample clean-up procedure. A further advantage was that sample analysis time was significantly reduced. Firstly, during routine analysis of AMO and its metabolites in animal tissues, some problems occurred due to the poor stability of AMO and AMA during the acidic deproteinization with trichloroacetic acid (TCA). Secondly, the amount of samples that could be analyzed each day was limited due to the solid-phase extraction step that followed the ultracentrifugation step.

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The revised method has been validated for the analysis of AMO, AMA and DIKETO according to the European Guidelines (EMEA/CVMP/573/00-FINAL). The method was used to monitor the concentrations of AMO and its metabolites in edible tissue samples from pigs treated with AMO alone and with AMO co-administered with clavulanic acid.

2. Experiment

2.1. Biological samples

Known penicillin-free tissue (kidney, liver, muscle, fat) samples were obtained from pigs which had not received any medication. Incurred tissue samples originated from 56 pigs which were treated with a single oral or intravenous dose of AMO (20 mg kg^{-1}) or AMO/clavulanic acid (20/5 mg kg^{-1}). The pigs were sacrificed at different time points after dosing (12, 36, 48, 60, 72 and 84 h). About 100 g of muscle and fat, 300 g of liver and the two whole kidneys were sampled within 1 h after slaughtering. The different tissues were minced and homogenized using a Robot-coupe® mixer (Robot-coupe, Mont-Ste-Geneviève, Belgium) at ambient temperature, immediately transferred into plastic bags and frozen at $\leq -70\,^{\circ}C$ until analysis.

2.2. Chemicals and standards

Amoxicillin sodium salt (AMO) and ampicillin (AMPI), used as internal standard (IS), were chemical reference substances (CRS) purchased from the European Pharmacopoeia (Council of Europe, Strasbourg, France). Amoxicilloic acid sodium salt (AMA) and amoxicillin diketopiperazine-2′,5′-dione (DIKETO) were obtained from LGC Promochem SARL (Molsheim, France). Solvents used for mobile phase, i.e. water, formic acid and acetonitrile, were of LC–MS grade (Biosolve, Valkenswaard, the Netherlands). All products and solvents used for extraction and preparation of the buffer solutions were of analytical grade and obtained from Merck (potassium *di*-hydrogen phosphate or KH_2PO_4, potassium chloride or KCl, sodium hydroxide or NaOH, hydrochloric acid-37% or HCl, Darmstadt, Germany) and Sigma–Aldrich (phosphoric acid-85% or H_3PO_4, Bornem, Belgium). Amicon Microcon® YM-30 Centrifugal Devices (molecular weight (MW) cut-off: 30 kDa) and IC Millex®-LG 0.20 μm filters were both from Millipore (Bedford, MA, USA).

2.3. Preparation of buffer solutions

A 10 mM KH_2PO_4 extraction buffer was prepared by diluting 1.36 g of potassium *di*-hydrogen phosphate in 900 ml of water. The pH was adjusted to 4.5 with concentrated hydrochloric acid, thereafter the volume was completed to 1000 ml.

The buffer solutions used for the stability study (pH ranging from 1.2 to 8.0) were prepared according to the United Stated Pharmacopoeia (USP) [12]. Buffer solutions of pH 1.2, 1.5 and 2.0 were prepared with 0.2 M HCl and 0.2 M KCl in water. The other buffer solutions were obtained by adjusting 0.01 M KH_2PO_4 and 1 M NaOH with 85% H_3PO_4 to the required pH. All buffer solutions were stored in the dark at 4 °C.

2.4. Sample preparation

2.4.1. Calibration curve and quality control

Separate stock solutions of AMO, AMA and AMPI of 1 mg ml^{-1} were prepared in HPLC-water. A stock solution of DIKETO was prepared in methanol/water (50/50, v/v). All stock solutions were divided into amber coloured Eppendorf cups (Novolab, Geraardsbergen, Belgium) and stored at $\leq -70\,^{\circ}C$. They were found to be stable for at least 6 months. On each analysis day, cups were thawed for the preparation of the working solutions. The stock solutions of AMO, AMA and DIKETO were combined and diluted with HPLC-water to obtain working solutions containing 100 μg ml^{-1}, 10 μg ml^{-1} and 1 μg ml^{-1} AMO, AMA and DIKETO. By adding 50 μl of the working solution of 100 μg ml^{-1}, 25 μl of the working solution of 100 μg ml^{-1}, 100, 50 and 25 μl of the working solution of 10 μg ml^{-1} and 100, 50 and 25 μl of the working solution of 1 μg ml^{-1} to 1 g of blank tissue sample, AMO, AMA and DIKETO concentrations of respectively 5000, 2500, 1000, 500, 250, 100, 50 and 25 ng g^{-1} were obtained. The stock solution of the IS was diluted in HPLC-water to a final concentration of 10 μg ml^{-1}. All working solutions were discarded after use. Quality control (QC) samples were prepared in a similar way at a concentration of 50 and 100 ng g^{-1}. These QC samples were analysed at the beginning and the end of the analytical batch to examine the post-preparative stability.

2.4.2. Tissue extraction

One gram of tissue homogenate was transferred into a 50-ml polypropylene centrifuge tube and spiked with 25 μl of the IS (= 10 μg ml^{-1}). After vortex mixing for 15 s, 7 ml of a 10 mM KH_2PO_4 phosphate buffer solution pH 4.5 were added. The sample tube was homogenized by vortex mixing for 15 s and rotated for 20 min on a homemade apparatus for extraction. After centrifugation for 10 min at 1800 × *g* at 4 °C, 1.5 ml of the upper aqueous layer was centrifugated for 10 min at 10,000 × *g* at 4 °C. A 500 μl aliquot of the supernatant was transferred into a Microcon® YM-30 Centrifugal Filter Device and centrifugated at 10,000 × *g* for 30 min at 4 °C. The filtrate was poured through a 0.20 μm filter into an autosampler vial and a 10 μl aliquot was directly injected onto the LC column.

2.5. LC–MS/MS analysis

The LC–MS/MS analysis was performed using an Alliance separations module with column heater and cooling device (all type 2695) and a Quattro Ultima® triple quadrupole mass spectrometer from Waters (Milford, MA, USA), run by Masslynx software (version 4.0). For chromatographic separation, a reversed-phase PLRP-S polymeric column (150 mm × 2.1 mm i.d., 100 Å) from Polymer Laboratories (Shropshire, UK), protected with a guard column of the same type (10 mm × 2 mm) was used. A gradient elution was performed with a mobile phase of 0.1% formic acid in water (A) and acetonitrile (B), at a flow

rate of 0.2 ml min^{-1}, i.e.: 0–1.9 min, 2% B; 2–5 min, 20% B; 5.1–12 min, 50% B; 12.1–20 min, 2% B. The autosampler was set at 5 °C. Operating conditions for the ESI source used in the positive ionization mode were optimized by direct infusion of AMO, AMA, DIKETO and AMPI at 3 μl min^{-1}, in combination with the mobile phase using a T-piece. The following tune parameters were used for AMO, AMA, DIKETO and AMPI: capillary voltage, 3.50 kV; cone voltage, 25 V; source temperature, 120 °C; desolvation temperature, 250 °C; cone gas flow ±50 l h^{-1}; desolvation gas flow ±850 l h^{-1}; resolution (LM1, HM1, LM2, HM2), 15.0; ion energy 1, 1.0; ion energy 2, 3.0; entrance, −1; exit, 1; multiplier, 650 V; collision gas: argon (Pirani pressure, $\pm 3.4 \times 10^{-3}$ mbar); dwell time, 0.2 s. The optimal settings for collision energy, corresponding to a (nearly) 100% fragmentation of the molecular ion (or precursor ion), were 15 eV for AMA and AMPI, 14 eV for AMO and 13 eV for DIKETO.

3. Stability study

To simulate the *in vivo* stability of AMO, AMA and DIKETO in the gastro-intestinal tract at the body temperature of pigs, *in vitro* experiments were carried out using different USP buffers with pH values ranging from 1.2 (∼stomach) to 8 (∼colon).

A separate working solution (10 μg ml^{-1}) of AMO, AMA and DIKETO was prepared in the different buffer solutions and mixed on a horizontal roller (CAT, Staufen, Germany) for 24 h in screw-capped tubes, kept at 39 °C in a heater (Heraeus, Hanau, Germany). At different time points (0, 5, 10, 15, 30, 45, 60 min and 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 7 and 8 h) a 100 μl aliquot of the working solution was diluted in 900 μl buffer solution of pH 6.0, 25 μl of IS (1 μg ml^{-1}) was added and the sample was immediately stored at 5 °C in the autosampler. The injection volume was 2.5 μl.

All experiments were performed in triplicate, mean and standard deviations were calculated.

4. Validation criteria

The proposed method for the quantitative determination of AMO and its major metabolites was validated by a set of parameters, which are in compliance with generally used recommendations and as defined by the EU [13–16].

- *Linearity*: determined on calibration curves using spiked blank tissue samples (for levels, see Section 2.4.1). Peak area ratios between AMO, AMA, DIKETO and the IS were plotted against their concentration and a linear regression was performed. Each time the correlation coefficient (r) and the goodness-of-fit (g) were determined and should fall within the ranges specified ($r \geq 0.99$, $g \leq 10\%$) [13–15].
- *Trueness*: determined by analyzing six independently spiked blank tissue samples at 25, 50 and 100 ng g^{-1}, a concentration level corresponding to half the MRL, MRL and double the MRL of AMO. The trueness, expressed as the difference between the mean found concentration and the spiked concentration (in %) had to be within −20 to +10% [15].
- *Precision*: expressed as the relative standard deviation (RSD, in %), being the ratio between the standard deviation (SD) and the mean found concentration. The RSD (%) had to fall within 2/3 of the values calculated according to the Horwitz equation: $RSD_{max} = RSD \times 2/3$, with $RSD = 2^{(1-0.5*\log c)}$, and c the analyte concentration in g g^{-1}. The precision was determined on the same samples as used for the trueness evaluation [15].

  The inter-day precision was evaluated using samples with the same spike levels, but prepared and analyzed on different days. Pig tissue samples were fortified at 50 and 100 ng g^{-1}. The RSD should be lower than the $RSD_{max} = 2^{(1-0.5*\log c)}$ [15].
- *Limit of quantification* (*LOQ*): defined as the lowest concentration for which the method is validated with a trueness and precision that fall within the ranges recommended by the EU [15]. The LOQ was set at 25 ng g^{-1}, since the guideline requires a value corresponding to at least half the MRL [16].
- *Limit of detection* (*LOD*): defined as the lowest measured concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty. In this study, the criterion of a signal-to-noise (S/N) ratio of 3/1 was used [14].
- *Specificity*: evaluated with respect to endogenous interferences by extracting and analyzing blank samples with the above-mentioned method. Known β-lactam free tissue samples were obtained from pigs which did not receive any medication [14].
- *Stability*: post-preparative stability was investigated by the analysis of QC samples spiked at 50 and 100 ng g^{-1}, and run at the beginning and the end of the batch. Trueness should fall within the range of −20 to +10%. Long-term stability of the components in the matrix during storage at ≤−70 °C was investigated by spiking blank tissue samples at 50 ng g^{-1}.
- *Ion suppression*: for the study of ion suppression a post column infusion technique was used. A blank tissue sample was extracted and injected onto the LC-MS instrument. A standard solution containing AMO, AMA, DIKETO and AMPI at 1 μg ml^{-1} was continuously infused through a T-coupling device into the LC eluate. This allowed to visualize sections in the chromatogram were ion suppression occurs.

5. Results and discussion

5.1. Sample clean-up

There are methods reported to extract AMO from animal tissues followed by HPLC with UV [17–21], fluorimetric [22–25] and mass spectrometric detection [4,10,11,26-32], but only one LC–MS/MS assay is involved with the simultaneous determination of AMO and its major metabolites [10].

The β-lactams are relatively unstable in aqueous solutions. Their degradation is catalyzed both by acids and bases [33]. De Baere et al. [11] used a 20% TCA solution for deproteinisation of the tissue extract. The use of solutions at that low pH implicated that further analysis should be performed within 20 min. A short mean half-life of degradation was indeed measured for AMO and AMA, i.e. 1.49 and 2.07 h, respectively ($n = 3$), indicating

the poor stability of the components in solutions containing 20% TCA. In the literature combinations of sulphuric acid and sodium tungstate solutions [17,21] are reported for deproteinisation of tissue samples. Others used high volumes of organic solvents like acetonitrile [4,20,29,30], methanol [19,32] or iso-octane [24] (up to >25 ml). Moreover, it is reported that the use of organic solvents should be avoided during extraction of penicillins due to degradation [33]. The use of ion-pairing reagents, like tetraethylammonium chloride [20] for extraction was not advisable in combination with LC–MS/MS analysis, since they can produce unacceptable levels of chemical noise on the mass spectrometer using electrospray ionization [29]. Bogialli et al. [31] used water of 65 °C as extractant at a pH of 3 and stated a recovery of almost 90% for AMO and AMPI in tissue samples. Their sample pre-treatment included furthermore some time-consuming steps, which hamper the analysis of many samples within 1 day.

In the present study, an extraction buffer at a pH value of 4.5 was chosen, followed by a centrifugation step using a Microcon® filter (cut-off: 30 kDa) for the removal of proteins. The latter ultrafiltration step can be a valuable alternative to overcome extreme pH circumstances or the use of organic solvents for deproteinisation. A centrifugation time of only 30 min was necessary to pass enough sample through the filter for a direct injection. Experiments with the Microcon® filter showed that no binding occurred on the filter, since high recoveries of all the components were obtained, when the sample extract was directly injected onto the LC-column. Although no concentration step was performed, a 10 μl injection volume is yet enough for quantifying all components at 25 ng g^{-1}.

The C18 solid-phase extraction (SPE) procedure of a previous report [10] showed a varying recovery of the components ranging from 40 to 90%. In addition, the recoveries of AMPI were not always reproducible, resulting in a lower robustness of the method for routine analysis.

The omission of a time consuming SPE extraction step is one of the major advantages of the present method in comparison to other publications [4,10,11,17,19,21–24,27,30]. As a consequence, the analytical protocol is simplified and the analysis time is shortened.

5.2. Mass spectrometry

The structures of AMO, AMA, DIKETO and AMPI are shown in Fig. 1, together with their MS and MS/MS traces, obtained by infusing of a standard solution of 1 μg ml^{-1} in combination with the LC-flow. Most publications for the quantitative determination of AMO with LC-MS/MS also used the positive ionization mode [4,10,11,25–31]. Yoon et al. [34] reported the negative ionization mode for AMO for the quantification of the antibiotic in human plasma with an LOQ of 120 ng ml^{-1}. Preliminary experiments showed that the negative ionization signal was insufficient for the AMO quantification around the MRL, moreover DIKETO gave only a signal in the positive ionization mode. Straub and Voyksner [26] also reported lower signal intensities in the negative ionization mode for AMO and other β-lactam antibiotics. In the MS mode the precursor ion for AMO, AMA, DIKETO and AMPI is the protonated molecular ion $[M+H]^+$ at *m/z* 366, 384, 366 and 350, respectively. In the MS/MS for AMO two most important product ions at *m/z* 208 and 349 were detected. The ion at *m/z* = 349 corresponds to the loss of NH_3 and was used as quantification trace [11].

For AMA, five predominant product ions were obtained at *m/z* 367, 349, 323, 189 and 160, respectively. The most abundant of the five ions (*m/z* = 323) was selected for quantification of AMA. This fragment can be assigned to the $[M+H–COOH–NH_3]^+$ fragment.

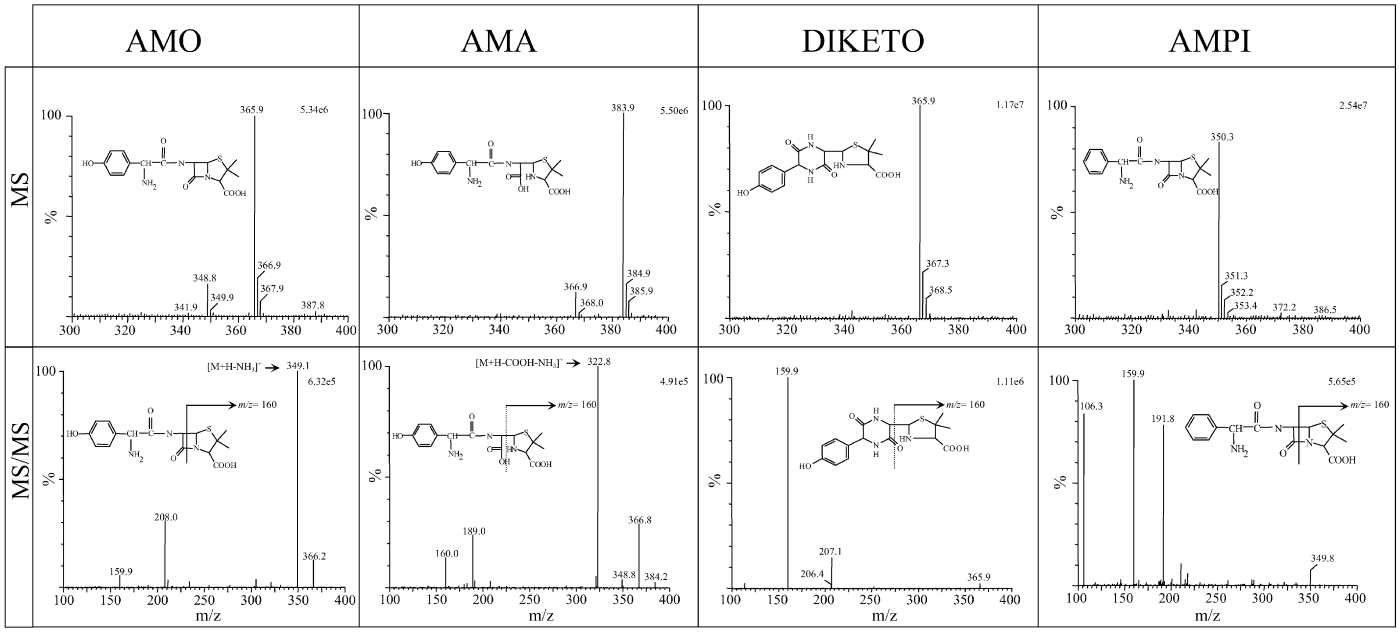

Fig. 1. Chemical structure, MS and MS/MS spectra of AMO, AMA, DIKETO and AMPI obtained after direct infusion of standard solutions of 1 μg ml^{-1} of each component in combination with the mobile phase.

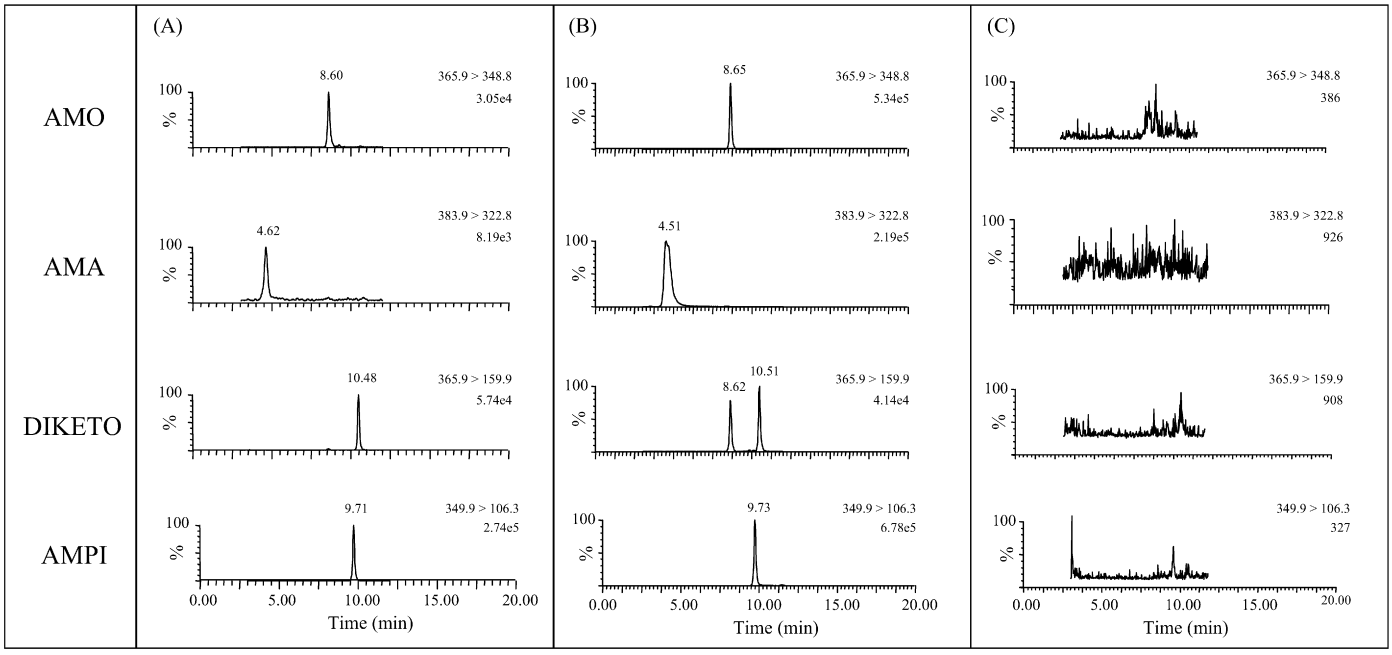

Fig. 2. MS/MS chromatograms of AMO (365.9 > 348.8), AMA (383.9 > 322.8), DIKETO (365.9 > 159.9) and AMPI (349.9 > 159.9), for (A) a blank pig kidney spiked at 25 ng g^{-1} (LOQ), of (B) an incurred pig kidney sample (AMO, AMA and DIKETO concentration: 3550 ng g^{-1}, 9690 ng g^{-1} and <LOQ, respectively) and of (C) a blank pig kidney sample.

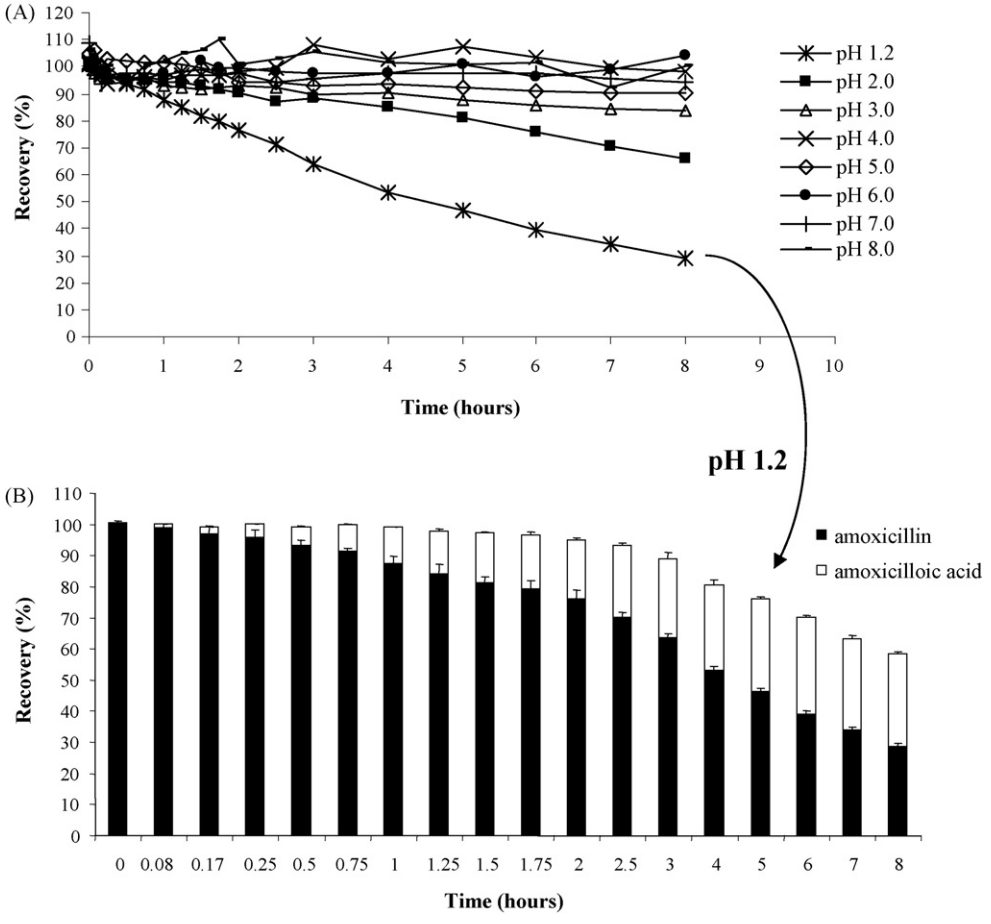

Fig. 3. Plot of the degradation of AMO (mean, $n = 3$) in the different pH levels at 39 °C (A) and the degradation profile of AMO into its AMA metabolite at pH 1.2 kept at 39 °C (B).

The molecular ion of DIKETO is nearly the same as for AMO ($m/z = 366$), so special attention was given to the separation between AMO and DIKETO (see Section 5.3). The MS/MS spectrum of DIKETO and AMPI shows one abundant product ion at $m/z = 160$. The ion at $m/z = 160$, a common cleavage product of the β-lactam ring, is present in the MS/MS spectra of the four components [27].

5.3. Chromatography

During preliminary experiments, special attention was paid to the chromatographic separation of AMO and DIKETO since they have the same molecular ion at m/z 366. Indeed, AMO shows a signal in the transition trace of DIKETO, but on the other hand, DIKETO does not have a signal in the transition trace of AMO. This can be attributed to the absence of the product ion at m/z 349 in the DIKETO spectrum [10]. In a previous publication, De Baere et al. [10] used ion-pair chromatography, 9.6 mM PFPA in water and 9.6 mM in a mixture of acetonitrile and water (50/50, v/v), as mobile phases. However, the metabolites were not completely separated on a reversed-phase Hypersil column from Chrompack (Middelburg, the Netherlands) and eluted early in the chromatography (<5 min). These authors also described that in incurred samples and in function of the column lifetime, the separation of the metabolites deteriorated, resulting at the end in a co-elution of all metabolites and AMO.

Moreover, the use of an ion-pairing reagent in the mobile phase could have a suppression effect on the MS/MS signal, due to the formation of neutral ion-pairs [35,36]. Therefore, 0.1% formic acid in water and acetonitrile were used as mobile phases in this study. The efficacy of formic acid in the chromatographic separation of penicillins was also mentioned by Becker et al. [30]. The polymeric type PLRP-S column overcomes some problems which appeared during the method development. The high robustness of this type of column gave no deterioration when used with an acidic mobile phase of 0.1% formic acid in water (pH ≈ 2.7). Moreover, no detrimental effect on the separation of the components was seen when incurred samples were injected onto this type of column. Even after more than 550 injections of tissue samples, the relative retention times of AMO, AMA and DIKETO showed a shift of only +3.1, +5.7 and +1.7%, respectively, between the first and last batch of samples. Different gradient programmes were tested for the separation of the four components. Due to the high polarity of the AMA metabolite, the gradient was started with only 2% of acetonitrile. To avoid carryover and to rinse of the column between two consecutive sample injections, a stronger gradient up to 50% of acetonitrile was run. Using the gradient elution as described in Section 2.5, all metabolites were completely separated and eluted later in the chromatographic run than with the original method [10]. The divert valve was used to send the first 3.5 and the last 8 min to the waste. This makes it possible to analyse a

Table 1
Results of the linearity, trueness and intra-day precision evaluation and limit of detection (LOD) for the various pig tissues

Parameter	Analyte	Conc. (ng g^{-1})	Kidney	Liver	Muscle	Fat
Linearity	AMO	0–5000	$r = 0.9998$ $g = 3.49\%$	$r = 0.9992$ $g = 3.50\%$	$r = 0.9990$ $g = 4.11\%$	$r = 0.9999$ $g = 2.96\%$
	AMA	0–5000	$r = 0.9994$ $g = 5.73\%$	$r = 0.9996$ $g = 2.78\%$	$r = 0.9985$ $g = 5.34\%$	$r = 0.9999$ $g = 2.47\%$
	DIKETO	0–5000	$r = 0.9996$ $g = 4.57\%$	$r = 0.9991$ $g = 5.83\%$	$r = 0.9991$ $g = 4.51\%$	$r = 0.9996$ $g = 4.36\%$
Precision (RSD, %) ($n = 6$)	AMO	25	18.3	7.8	5.3	2.2
		50	2.9	5.4	4.9	2.6
		100	7.1	11.1	3.1	2.7
	AMA	25	3.5	7.8	2.7	3.4
		50	4.1	5.4	4.0	3.1
		100	6.8	4.2	3.1	3.3
	DIKETO	25	5.0	5.3	6.2	3.1
		50	4.9	4.6	1.4	3.1
		100	3.1	3.0	4.5	2.6
Trueness (%) ($n = 6$)	AMO	25	+9.1	+1.6	+0.5	+3.1
		50	+5.4	−7.0	+2.0	+3.0
		100	−3.3	+7.0	+0.2	+5.1
	AMA	25	−2.8	+1.6	+6.5	−2.6
		50	−0.6	−0.2	+8.2	−0.4
		100	−2.1	−3.0	+3.8	+1.5
	DIKETO	25	−5.7	−2.6	−0.8	+1.9
		50	−12.0	+2.3	−6.6	+1.9
		100	−14.3	+4.8	−3.9	+3.6
LOD (ng g^{-1})	AMO		1.7	3.5	1.5	1.7
	AMA		7.1	14.2	11.1	11.6
	DIKETO		2.7	1.6	0.9	0.8

r: correlation coefficient; g: goodness-of-fit; RSD: relative standard deviation (the ratio between standard deviation and mean found concentration); trueness: difference between mean found concentration and spiked concentration; LOD: limit of detection (signal-to-noise ratio of 3/1).

high number of samples without cleaning the sample cone of the MS detector.

Fig. 2 shows the different MS/MS chromatograms for AMO, AMA, DIKETO and AMPI for a blank pig kidney sample spiked at 25 ng g^{-1} (LOQ), an incurred pig kidney sample and a blank pig kidney sample. Mass chromatograms for pig muscle, liver and fat tissue samples were similar to those shown for kidney. The chromatograms of blank pig tissue samples show that no interferences from endogenous components at the elution time zone of AMO, AMA, DIKETO and AMPI were present.

5.4. Stability study

Since AMO is known to be unstable in aqueous solutions with a low pH value, its stability in the gastric juice can be discussed. The instability in acidic conditions of AMO is involved at the intramolecular attack of the side-chain amide in the β-lactam moiety. The AMO levels, and moreover the extent of degradation in the intestinal tract, is important to ensure an effective pharmaco-therapeutic regimen [37–39].

Fig. 3 shows the stability of AMO in buffer solutions of different pH levels, kept for 8 h at 39 °C. A pH value of 1.2 (∼stomach) resulted in the degradation of AMO into its corresponding penicilloic acid (at least 10% of the initial AMO concentration within 1 h), without any formation of DIKETO, since the β-lactam ring of AMO is known to be more susceptible to hydrolytic degradation when the pH deviates significantly from its isoelectric point at pH 4.8 [40]. The overall comparison of the recoveries at the different pH values indicated that a decrease in pH resulted in a faster first-order degradation. For AMO, the lower the pH value the more significant degradation of AMO into AMA occurred. The same pH dependent phenomenon was obtained for AMA, while DIKETO remained more stable in the different buffer solutions. The degradation half-life of AMO in this acidic condition (pH 1.2) was very short (4.23 ± 0.05 h).

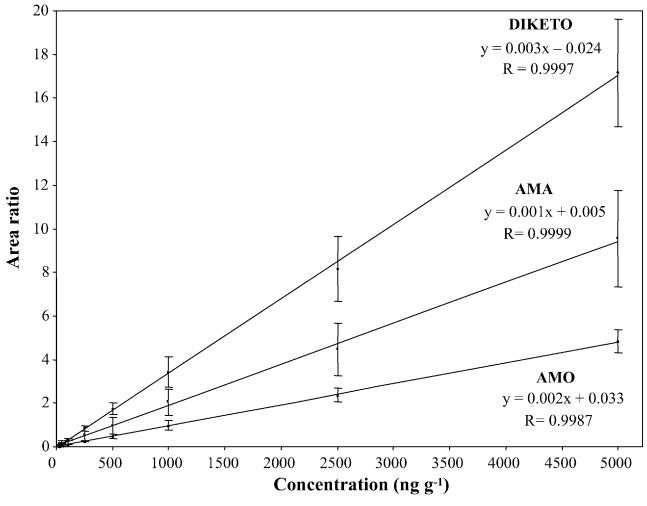

Fig. 4. Matrix matched calibration curves of AMO, AMA and DIKETO in pig liver, represented by the mean values of six calibration curves constructed of a period of 132 days, each individual calibration curve being a new set of extraction.

Table 2
Results of the trueness and inter-day precision evaluation for the various pig tissues

Analyte	Conc. (ng g^{-1})	Kidney			Liver			Muscle			Fat		
		Mean Conc. (ng g^{-1})	Precision (RSD, %)	Trueness (%)	Mean Conc. (ng g^{-1})	Precision (RSD, %)	Trueness (%)	Mean Conc. (ng g^{-1})	Precision (RSD, %)	Trueness (%)	Mean Conc. (ng g^{-1})	Precision (RSD, %)	Trueness (%)
AMO	50	50.2 (n = 10)	8.7	+0.3	52.1 (n = 10)	4.1	+4.2	50.1 (n = 6)	3.0	+0.1	53.0 (n = 6)	2.7	+5.9
	100	100.8 (n = 10)	5.4	+0.8	100.0 (n = 8)	4.5	–	101.8 (n = 6)	3.9	+1.8	106.3 (n = 4)	4.2	+6.3
AMA	50	52.0 (n = 10)	9.1	+4.0	53.9 (n = 10)	23.3	+7.8	50.1 (n = 6)	5.7	+0.2	53.5 (n = 6)	1.5	+7.0
	100	109.2 (n = 10)	11.5	+9.2	99.3 (n = 8)	5.8	−0.7	102.4 (n = 6)	5.1	+2.4	102.6 (n = 4)	3.3	+2.6
DIKETO	50	49.6 (n = 10)	5.1	−0.8	53.4 (n = 10)	3.3	+6.8	51.4 (n = 6)	8.2	+2.8	45.2 (n = 6)	11.5	−9.7
	100	102.3 (n = 10)	3.2	+2.3	101.9 (n = 8)	4.9	+1.9	101.2 (n = 6)	1.9	+1.2	88.7 (n = 4)	20.9	−11.4

RSD: relative standard deviation (the ratio between standard deviation and mean found concentration); trueness = difference between mean found concentration and spiked concentration.

The half-lives of AMO in the buffers with pH > 4.0 were all >100 h.

From the results, the poor stability of AMO in an acidic medium lower than pH 4 at a body temperature of 39 °C is confirmed. This could include a degradation of AMO in gastric juices. Indeed, a previous study showed already an increased stability of AMO, co-administered with omeprazole, a selective proton pump inhibitor which decreases acid production in the stomach [40].

6. Method validation

For the calibration curves good linearity was observed up to 5000 ng g^{-1} in all tissue samples for all components. The goodness-of-fit coefficients (g) of the individual curves were all <7.4% and the correlation coefficient (r) all >0.9919 for all components (Table 1). In Fig. 4 the calibration curves of AMO, AMA and DIKETO in pig liver are presented as the mean of six calibration curves made over a period of 132 days, each curve originating from a new set of extractions.

The results of the intra-day trueness and precision evaluation are summarized in Table 1. The trueness fell within the range of −20 to +10%, and the precision also fell within the maximum RSD values. The inter-day precision also fell within the ranges specified (Table 2).

The results of LOD and LOQ are also summarized in Table 1. The 25 ng g^{-1} level could be quantified fulfilling the criteria for trueness and precision, and was therefore set as LOQ of the method. For all tissue samples, the LOQ is at least half the MRL of AMO, as required by the EU guidelines [16].

Special attention was paid to the stability of the components during storage. The stability in the incurred tissue samples during storage at ≤ −70 °C was studied using blank tissue samples spiked at 50 ng g^{-1} (Table 3). The results confirmed that AMO, AMA and DIKETO were stable for at least 1 year when stored at ≤ −70 °C. The post-preparative stability evaluation showed that all components were stable in the extract of pH 4.5 for at least 24 h when stored at 5 °C in the autosampler (trueness fell within the range of −16.3 to 7.9%).

Using the post column infusion technique, no ion suppression was noticed at the elution time zones of all four analytes in each pig tissue.

Table 3
Results of long-term stability of AMO, AMA and DIKETO in spiked tissue samples (50 ng g^{-1}) at ≤ −70 °C for 12 months

Matrix	Analyte (n = 6)	Recovery at the 50 ng g^{-1} level (%)
Kidney	AMO	0.6
	AMA	8.2
	DIKETO	8.4
Liver	AMO	−17
	AMA	−4.8
	DIKETO	8.2
Muscle	AMO	3.6
	AMA	−1.6
	DIKETO	−7
Fat	AMO	−9
	AMA	−5
	DIKETO	−0.6

7. Biological sample analysis

The above-developed method was applied for a depletion study in pigs, after a single oral and intravenous administration of AMO and AMO/clavulanic acid. The pigs were slaughtered at different time points (12, 36, 48, 60, 72 and 84 h) after dosing and incurred tissue samples were taken (liver, kidney, muscle and fat), homogenized and stored at ≤ −70 °C pending analysis. A remarkable phenomenon was that the AMA metabolite remained much longer in the kidney and liver tissues, than the AMO molecule. From 36 h after dosing onwards, the AMO concentrations were all below the LOQ, while the AMA metabolite remained at concentrations much higher than the MRL of AMO (mean AMA concentration at 60 h: 231 ng g^{-1} in kidney tissue) [41]. Nevertheless, this metabolite is not included in the MRL of AMO, despite the potential risk for human health [7]. A mean residue depletion curve of AMA in pig kidney tissue is presented in Fig. 5.

To demonstrate further the practicability and applicability of the LC–MS/MS method, the following data can be mentioned. The total number of incurred pig tissue samples was 220. The total number of blank and spiked analyzed tissue samples was 375. All samples were analyzed using the same analytical column, without a replacement of the guard column. These findings indicate that a tissue extraction based on a simple deproteinisa-

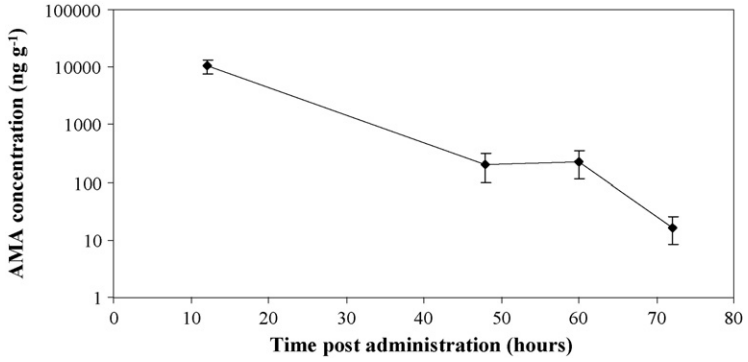

Fig. 5. Residue depletion curve (n = 4, mean ± SD) of AMA in pig kidney after oral administration of AMO (20 mg kg^{-1}).

tion by ultrafiltration does not necessarily result in a shorter half-life of the guard and/or analytical column.

8. Conclusions

A rapid, sensitive and specific method for the quantitative analysis of AMO, AMA and DIKETO in pig tissues is described. The point of interest was a previously published method with some difficulties in the acidic deproteinisation, solid-phase extraction step and chromatographic separation of the metabolites. The minimum sample preparation, consisting of only a simple liquid extraction and ultrafiltration step for deproteinisation, allows the extraction of many samples a day (up to 60), with a significant reduction of sample analysis time. The analytical circumstances do not result in degradation of AMO and metabolites in the matrix during storage and extraction. The method was successfully applied for residue depletion studies in pigs were high concentrations of AMA were detected in kidney and liver tissues, and this at time points when the AMO concentration was already below its MRL. Some questions raised regarding the allergic potential of those amounts of the AMA metabolite in liver and kidney tissues. The same methodology was also validated for bovine tissues and was used for the analysis of biological samples from pigs and cattle, which demonstrates the applicability and reliability of the presented method.

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