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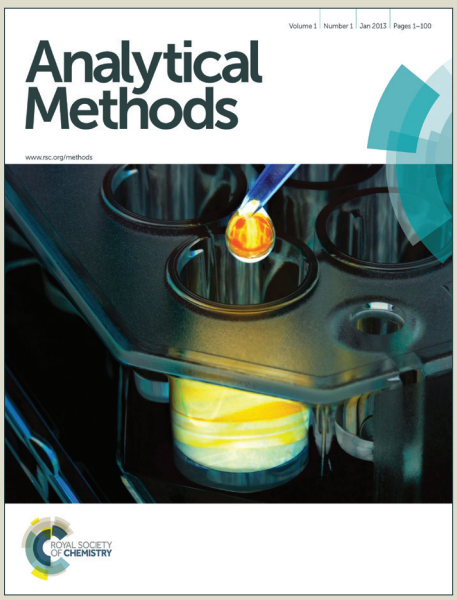

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Determination of tulathromycin in swine tissues by liquid chromatography–tandem mass spectrometry

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A liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method for determination of tulathromycin in swine tissues has been developed. Prior to the instrumental analysis, sample preparation technique involved extraction with a solution of meta-phosphoric acid followed by solid-phase extraction clean-up using polymeric mixed-mode strong cation-exchange sorbent. The chromatographic separation was performed on C8 chromatographic column with a gradient elution mode. Mass spectral acquisition was performed under selective multiple reaction monitoring mode by triple quadrupole mass spectrometer. The method have been characterized by providing the parameters of precision, accuracy, specificity, decision limit (CCα) and detection capability (CCβ) according to the Commission Decision 2002/657/CE. The calibration curves were linear in the range of 10-9000 μg kg^{-1}. The recoveries ranged from 92.9% to 102.1%. The repeatability was below 8.0% and within-laboratory reproducibility was lower than 9.2%. The limits of detection ranged from 2 to 4 μg kg^{-1} and the limit of quantification was 10 μg kg^{-1}. The presented analytical method can be used for measuring the tulathromycin depletion in swine tissues (kidney, liver and muscle) over time.

Introduction

Tulathromycin is a semi-synthetic triamilide, broad spectrum antimicrobial agent, which belongs to the macrolide group of antibiotics. Triamilides are semi-synthetic derivatives of the natural product, erythromycin, and are characterized by the presence of three polar amine groups that distinguish them structurally from other macrolides.[1] Tulathromycin exist either as one isomer in crystallised form, isomer A (CP-472,295) or as a stable mixture of two structural isomers in aqueous solution in a 9:1 ratio of a 15-member lactone ring (isomer A, tulathromycin A, CP-472,295) and 13-limb member ring lactone (isomer B, tulathromycin B, CP-547,272).[2,3] The equilibrated mixture of the two structural isomers is also referred as CP-472,295(e) (Pfizer code). The same ratio of isomers in biological matrix also remain in equilibrium.[1] Because equlilibrium in aqueous solutions is reached within 48 h, only isomer A is used as an analytical reference[2] and quantitatively analysed in this study. Both isoforms contain three amine groups at different location on the molecule. One amine group is included on the azalide ring and the other two exist on the two sugar moieties: desosamine and modified cladinose, attached to the macrolide core.[1]

Tulathromycin shows high efficacy against the common bacterial causes of respiratory disease in cattle and swine, and is used for treatment and prevention of bovine respiratory disease (BRD) caused by *Mannheimia haemolytica* and *Pasteurella multocida* in cattle, and swine respiratory disease (SRD) caused by *Pasteurella multocida, Actinobacillus pleuropneumoniae*, and *Mycoplasma hyopneumoniae.*[1,4,5] The dose is a single-dose of 2.5 mg kg^{-1} body weight (bw) by subcutaneous injection to cattle and intramuscular injection to pigs.

The mechanism of antimicrobial activity of tulathromycin, like other macrolides, rely on binding to the 50 S subunit of bacterial ribosomes, which leads to the inhibition of protein synthesis and consequently to inhibition of cell division. Although this drug is classified as a macrolide, which are generally regarded as bacteriostatic, it can also exhibit bactericidal activity at higher concentrations.[1]

According to the European Medicines Agency[6] tulathromycin is metabolised to a low extent and eliminated primarily as the unchanged drug in swine. For monitoring and surveillance purposes, the residue definition for tulathromycin is set as the sum of tulathromycin and its metabolites that are converted by acid hydrolysis to marker residue (CP-60,300), expressed as tulathromycin equivalents.[6] The European Commission regulation 37/2010/EU[7] has set maximum residue levels (MRLs) in porcine kidney and liver at the level of 3000 μgkg^{-1}. No MRL was appointed for muscle tissues. The marker residue is also one of the parent drug metabolites, but none of metabolites where the desoamine moiety or macrocyclic ring has been modified can be transformed by hydrolysis to the marker residue. Because tulathromycin is slightly metabolised in the body, and not every metabolite of tulathromycin can be hydrolyzed to the marker

residue[8] it seems not to be sufficiently approach proposing for routine analytical method, in which drug content refers to the marker residue expressed as tulathromycin equivalents.

Over the past years, LC-MS/MS methods have been developed for analysis of tulathromycin in different biological matrices, such as muscle[9-11], lung[2,4,5], plasma[2,4,5,9,12-14], milk[10], bovine liver and porcine kidney.[3] These methods have focused mostly on the determination of the parent compound[2,4,5,9-14] or on the determination of tulathromycin marker residue by means of acid hydrolysis conversion of parent compound and selected metabolites.[3]

The aim of this work was to develop a reliable LC-MS/MS method for the analysis of tulathromycin, which can be useful in depletion study of drug in swine tissues over time. This paper describes a new method with an SPE clean-up step and LC-MS/MS measurement for the determination of tulathromycin A (CP-472,295) in swine muscle, kidney and liver. One of the novelties of this method is use of meta-phosphoric acid as an extraction solvent followed by solid-phase extraction clean-up using a polymeric mixed-mode strong cation-exchange sorbent Strata-X-C. Another novelty is use of azithromycin as internal standard, because of structural similarities between tri-basic tulathromycin and di-basic with 15-membered lactone ring – azithromycin. The method was validated in accordance with the Commission Decision 2002/657/EC[15] and demonstrates to be suitable for detection and quantitation of tulathromycin residue in food of animal origin. Additionally, applicability of the presented method was tested during analysis of incurred swine tissue samples. Thus, this method is useful for studying of tulathromycin drug depletion in swine tissues.

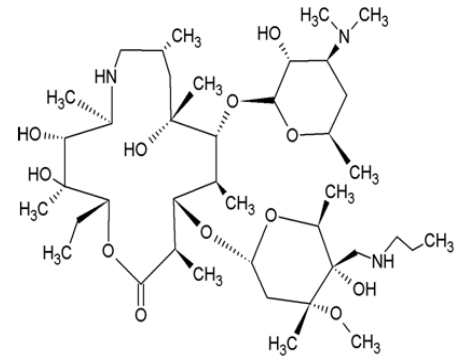
Materials and methods

Reagents and chemicals

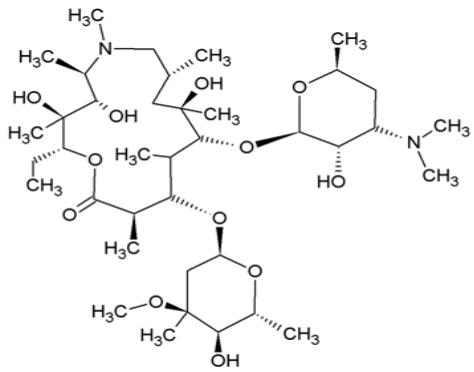
All of the solvents used were of analytical grade. Acetonitrile and methanol were obtained from J.T. Baker (Germany). Meta-phosphoric acid was from Sigma–Aldrich (USA). Ammonium acetate was purchased from LGC Standard (UK). Ammonium hydroxide and dipotassium hydrogen phosphate were purchased from POCh (Poland). Formic acid was obtained from Fluka (USA). Ultra-pure water (resistance > 18 mΩ) was obtained from a Milii-Q system (Millipore, France). The cartridges used for solid-phase extraction (Strata-X-C, 200 mg, 3ml) were obtained from Phenomenex (USA). The analytical reference standard of tulathromycin A (CP-472,295) and Draxxin® medicinal product were obtained from Pfizer (USA). The internal standard, azithromycin (IS) was purchased from Sigma – Aldrich (USA). Chemical structures of analytes are shown in Fig. 1.

Standard solutions

Stock standard solutions (1000 μg ml^{-1}) of analytes were prepared by weighting of 10 mg of reference standard and dissolving in 10 ml of methanol. These solutions were stored in the dark glass bottles at < -18 °C and were stable for at least 6 months. The working standard solutions, used for sample fortification were prepared by the dilution of these solutions with 10 mM ammonium acetate buffer, pH 3 and were stored in the dark at 2-10 °C for at least one month. Working solution of internal standard (10 μg ml^{-1}) in 10 mM ammonium acetate buffer, pH 3



Tulathromycin A (CP-472,295)



Azithromycin (IS)

Fig. 1 Chemical structures of tulathromycin A and azithromycin

was prepared separately and stored in the dark at 2-10 °C for at least one month.

A 1% meta-phosphoric acid solution in water was prepared by dissolving 10 g of meta-phosphoric acid in a 1000-ml volumetric flask and adjusting the volume with water. A 10 mM ammonium acetate, pH 3 solution was prepared by dissolving 0.78 g of ammonium acetate in 1000 ml of water. The pH of the ammonium acetate solution was adjusted to 3 by addition of formic acid. A 5% ammonium hydroxide solution in methanol was prepared by diluting 5 ml of ammonium hydroxide to 100 ml of methanol. A 5% ammonium hydroxide solution in acetonitrile was prepared by diluting 5 ml of ammonium hydroxide to 100 ml of acetonitrile.

Sample preparation procedure

A 1 g of homogenised pig tissue was placed in a 15 ml polypropylene centrifuge tube and fortified with 50 μl of 10 μg ml^{-1} working internal standard solution. Then, 5 ml of 1% meta-phosphoric acid was added into the tube and the sample was shaken for 10 min. After the centrifugation at 4500 rpm for 10 min at 4 °C, 0.5 ml of supernatant was taken and placed in a fresh 15 ml polypropylene tube and diluted to 5 ml with water for further solid-phase clean-up. The Strata-X-C SPE cartridge was preconditioned with 5 ml of methanol and 5 ml of water. The diluted sample was passed through the cartridge under the vacuum at a flow rate of 1-2 ml min^{-1}. Further, the cartridge was

washed with 5 ml of water and 5 ml of methanol and vaccum-dried for 10 min. After the drying step analytes were eluted with 3 ml of 5% ammonium hydroxide in methanol and 3 ml of 5% ammonium hydroxide in acetonitrile into 10 ml tube. The extract was evaporated to dryness under a weak stream of nitrogen at 45 °C. The dry residue was reconstituted in 0.5 ml of 10 mM ammonium acetate buffer, pH 3 and transferred into HPLC vial for LC-MS/MS analysis.

Liquid chromatography–tandem mass spectrometry analysis

An Agilent Series 1200 HPLC system (Agilent Technologies, Germany) was connected to QTRAP 5500 mass spectrometer (AB SCIEX, Canada). The Analyst 1.5 software controlled the LC-MS/MS system and processed the data. The mass spectrometer was operated in electrospray positive ionisation mode (ESI+) and multiple reaction monitoring (MRM) mode was used to quantify the analytes. The mass spectrometer settings were optimised and following parameters were used: resolution Q1 and Q3: unit; curtain gas: 20, ion spray voltage: 5500 V; temperature of ion source: 400 °C.

The chromatographic separation was performed on a BDS Hypersil C8 column (3 µm, 2.1 x 50 mm, Thermo, USA) coupled with BDS Hypersil C8 precolumn (3 µm, 2.1 x 10 mm, Thermo, USA). The mobile phase consisted of a solvent A: acetonitrile and solvent B: 10 mM ammonium acetate buffer, pH 3. The elution was performed in a gradient mode. The starting conditions for the mobile phase were 90% of eluent B and then decreased to 50% within 7 min and in 7.01 min increased to 90%. With the following equilibration time of 6 min, the resulting total run was 13 min. The column was operated at 30 °C with a flow rate of 0.25 ml min^{-1}; the injection volume was 5 µl.

Method validation

The method was validated according to the recommendations of the Commission Decision 2002/657/EC[15] on the basis of an in-house validation concept. The validation study was performed in terms of linearity, specificity, selectivity, accuracy, precision (repeatability and within-laboratory reproducibility), stability, matrix effect and robustness. The decision limit (CCα), detection capability (CCβ), limit of detection (LOD) and limit of quantification (LOQ) were also estimated. Matrix-matched calibration curves were prepared at eleven concentrations by plotting the response of analyte/internal standard peak area ratio versus the analyte concentration. To evaluate possible interferences encountered in the method, the specificity was verified by analysing 20 different kidney, liver and muscle blank samples. To demonstrate whether another analytes from macrolide group of antibacterials potentially present in the sample could affect the results, blank samples of swine liver, kidney and muscle were fortified with macrolide standard solution containing tylosin, erythromycin, spiramycin, tilmicosin, josamycin and roxythromycin in order to evaluate the method's selectivity.

In the precision and accuracy study blank muscle, kidney and liver samples were spiked with tulathromycin at three different levels. Kidney and liver matrices were fortified at 0.5, 1 and 1.5 times of the MRL level. Since MRL for tulathromycin in muscle has not been fixed fortified levels studied for this compound were done close to LOQ, at 10, 30 and 50 µg kg^{-1}. In the repeatability study, three series were analysed under the identical conditions (six samples for each fortification level). Standard deviation (SD) and coefficient of variation (CV, %) were calculated for each level. The within-laboratory reproducibility was obtained by analysis of two additional series (on three levels) under reproducibility conditions (two different occasions, another technician) and overall SD and CV were calculated. The overall mean concentrations obtained in the reproducibility study were used to calculate accuracy expressed as percent. Decision limits (critical concentration at alpha-error, CCα) and detection capability (critical concentration at beta-error, CCβ) were calculated using within-laboratory reproducibility results, in accordance with the procedure described in the Commission Decision 2002/657/EC.[15] Limit of detection (LOD) and limit of quantification (LOQ) were calculated on the basis of signal to noise ratio (S/N = 3 for LOD and S/N = 10 for LOQ) on the chromatograms of 20 blank kidney, liver and muscle samples. To verify LOQ values, kidney, liver and muscle samples were spiked with tulathromycin at the levels of 10 µg kg^{-1}. The robustness of the method was investigated using the Youden approach as described in the Commission Decision 2002/657/EC.[15] The influence of following parameters on the results were tested: time of shaking (12 min and 8 min), time of centrifugation (12 min and 8 min), volume of water added to the extract (5.5 ml and 4.5 ml), time of vaccum-drying of the SPE cartridges (12 min and 8 min), volume of 5% ammonium hydroxide in methanol for the elution (3.3 ml and 2.7 ml), volume of 5% ammonium hydroxide in acetonitrile for the elution (3.3 ml and 2.7 ml) and the temperature of evaporation (50 °C and 40 °C). Eight experiments were carried out for the evaluation of seven factors by determination of 8 spiked blank kidney and liver samples at 3000 µg kg^{-1} and muscle samples at 50 µg kg^{-1}. The results were statistically evaluated by *t*-test and comparison of standard deviation of differences (SD_i) and standard deviation of within-laboratory reproducibility.

The mixed standard solutions in methanol and in 10 mM ammonium acetate buffer, pH 3 were used for the verification of stability of the analytes. The solutions were stored in the refrigerator (2-10 °C) and below -18 °C up to 6 months. After 1, 3 and 6 months, the concentration of tested solutions were compared to the standard solution kept in the reference condition (< -70 °C).

To assess the matrix effect for kidney, liver and muscle matrix, 6 blank samples of each matrix were spiked after the sample preparation step at level 3000 µg kg^{-1} for kidney and liver and at level 50 µg kg^{-1} for muscle by addition of appropriate amount of standard solution. Simultaneously a standard solution at the same concentration level was also prepared. Spiked extracts and standard solution were injected and analysed by LC-MS/MS. The following equation was used for matrix effect (ME) calculation:

$$ME(\%) = \frac{B}{A} 100$$

where B is the average analyte peak area in extract of blank sample spiked after the extraction and A is the average analyte

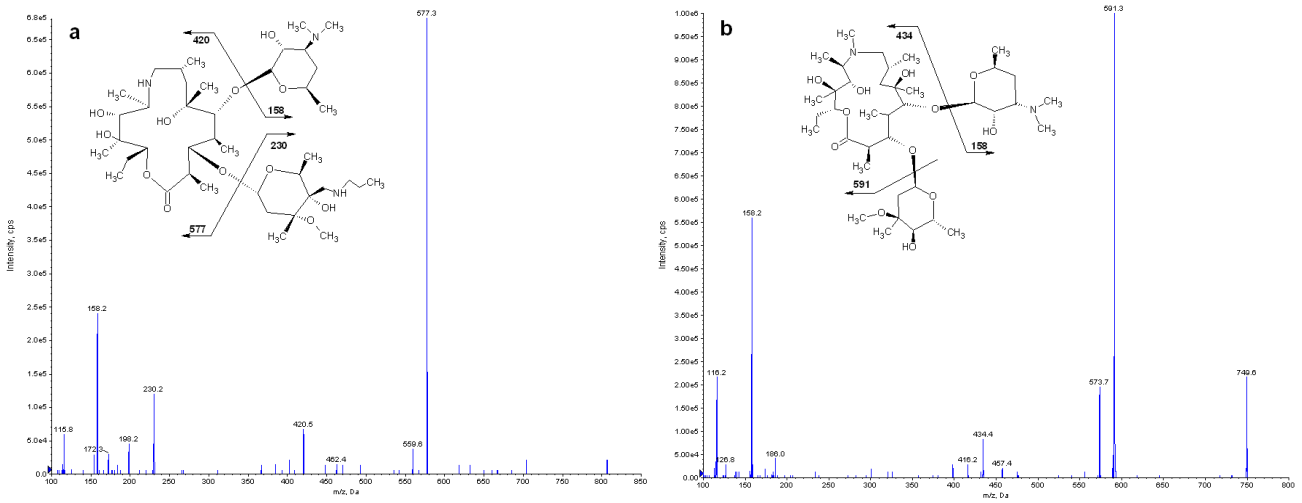


Fig. 2 The mass spectra with chemical structures of: (a) tulathromycin (parent ion *m/z* 806.6) and (b) internal standard (parent ion *m/z* 749.4)

peak area in standard solution. Signal enhancement is observed when ME value is greater than 100% and signal suppression is observed when the ME value is less than 100%.

In order to verify method application to incurred samples the experiment was conducted on three pigs. One pig, never treated with tulathromycin, was used as a control (blank samples). Two other animals were treated individually with single intramuscular injection at 2.5 mg kg^{-1} bw of tulathromycin (Draxxin®, Pfizer). Muscle, kidney and liver tissue samples were collected at 24 h and 48 h after administration of tulathromycin. Collected samples were stored frozen at approximately -20ºC until analyses.

Results and discussion

Sample preparation optimization

In order to assess the occurrence of tulathromycin in tissue samples, sensitive analytical methods are required which enable to determine these drug residues at quite low concentration levels. One important requirement of the extraction method was that it should be fast and robust enough to allow the rational analysis of a large number of samples.

Solid-phase extraction (SPE) is one of the most successful extraction techniques due to its efficiently enrich and purify analytes from sample matrices. The selectivity of SPE can provide the advantage of a cleaner sample extract and, therefore, less interference and suppression from matrix components in LC-MS/MS analysis. Several methods reported for the analysis of tulathromycin in biological matrices involve SPE for the clean-up purposes[2-5,12-14]. In those studies isolation was performed using aqueous solvents such as 0.04 M phosphoric acid[2,4], 0.02 M oxalic acid buffer (pH 4)[14] or 2 N hydrochloric acid[3], followed by solid-phase extraction.

Various types of SPE, involving weak cation exchange Bond Elut CBA[2,4], mixed-mode cation exchange Oasis MCX[3,12,13] and polymeric Strata-X[14] sorbent have been used. Extraction with acetonitrile prior to the clean-up of the extracts by liquid-liquid extraction with hexane has also been reported.[9] Juan *et al.*[10] have reported extracting tulathromycin by using pressurised liquid extraction (PLE) procedure with acetonitrile as the extracting solvent in milk and meat.

The extraction procedure of our method, as described above, was optimized after evaluating the performance of different mixtures of solvents as well as different clean-up procedures. Biological matrices like muscle, kidney and liver contain many possible interfering substances that need to be removed selectively. Understanding the chemistry of the compound under analysis such as its hydrophilicity or ionizability can be useful in designing appropriate conditions to obtain efficient extraction recovery. In this study, the efficiency of isolation of analytes after the use of different extraction solvents like meta-phosphoric acid, trichloroacetic acid and hydrochloric acid were compared. After the comparison study, meta-phosphoric acid, which is a strong precipitating agent for water-soluble proteins, was chosen as an extraction solvent. This property, analytically, serves to distinguish it from the other phosphoric acids but does not cause hydrolysis of tulathromycin and IS how it happens when trichloroacetic acid or hydrochloric acid are used. Experimentally studying the impact of the concentration of meta-phosphoric acid on the degree of isolation of analytes from tissue matrices shown, that the best recoveries were obtained when a solution of 1% meta-phosphoric acid in water was used.

Due to the presence of three amine groups in chemical structure of tulathromycin and two amine groups in IS, which are positively charged in acid environmental conditions, polymeric mixed-mode strong cation exchange SPE cartridges (Strata-X-C) were selected for further optimization steps. The optimal conditions for SPE clean-up procedure were obtained after extensive optimization of conditioning, washing and elution steps to ensure sufficient separation of the analytes from endogenous compounds of matrix. In this procedure, two washing steps with 5 ml of water and 5 ml of methanol were used. As it was found, the use of that combination of reagents were highly effective in removing retained matrix components form sorbent bed. In order to completely retrieve analytes from Strata-X-C cartridge 3 ml of

Table 1 LC-MS/MS parameters used for determination of tulathromycin

Analyte	Retention time (min)	Ion transition (m/z)	DP (eV)	CE (eV)	CXP (eV)	MRM ratio
Tulathromycin	8.1	806.6 → 577.4[a]	20	32	35	22.9
		806.6 → 158.1	20	55	16	
Azithromycin (IS)	9.1	749.4 → 591.3	116	41	26	

[a] Ion transition used for the quantification.

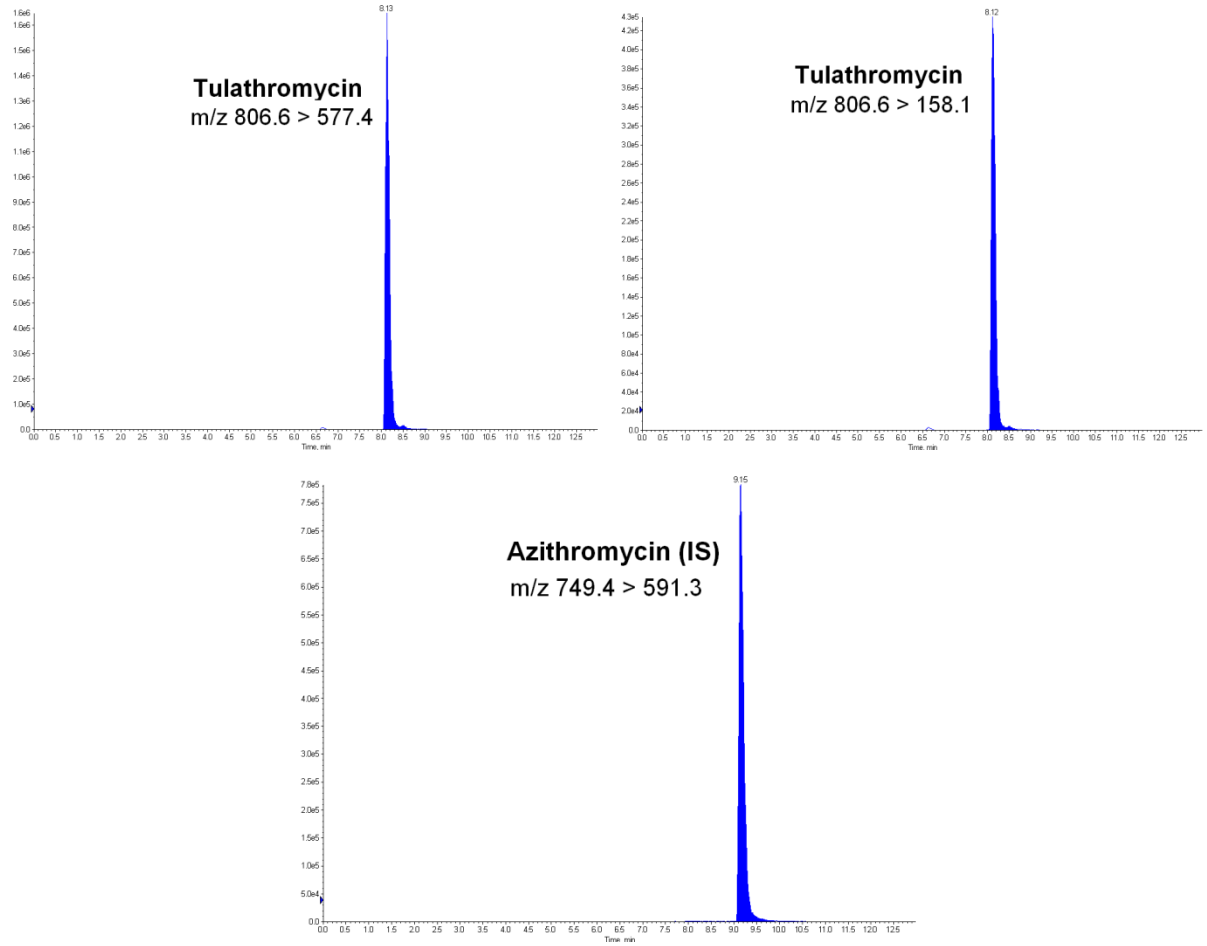


Fig. 3 LC-MS/MS chromatograms in MRM mode of porcine kidney sample spiked with tulathromycin and IS at 3000 μg kg^{-1}

5% ammonium hydroxide in methanol and then 3 ml of 5% ammonium hydroxide in acetonitrile were used. This combination of solvent system provided the optimum levels of analyte recovery.

Liquid chromatography-mass spectrometry

The fragmentation reactions used for monitoring of tulathromycin and IS were selected on the basis of their significance in product ion spectra during the optimisation by injecting working standard (100 ng ml^{-1}) into a mass spectrometer. The positive ion ESI–MS/MS spectra of tulathromycin and IS are shown in Fig. 2. During ionization, both tulathromycin and IS form positively charged molecular ions $[M+H]^+$. The main ions visible in the MS/MS spectrum of tulathromycin corresponded to the sequentional elimination of sugars from the molecule (Fig. 2a). The loss of the modified cladinose moiety (229 Da) gave an ion at *m/z* 577. Further elimination of desosamine (157 Da) gave an ion at *m/z* 420. The ion at *m/z* 230 corresponds to modified cladinose moiety and the ion at *m/z* 158 corresponds to desosamine sugar moiety. For IS similar routes of fragmentation are observed (Fig. 2b). The loss of the cladinose moiety (158 Da) gave an ion at *m/z* 591. Further elimination of desosamine (157 Da) gave an ion at m/z 434. The ion at *m/z* 158 corresponds to desosamine sugar moiety. Two multiple reaction monitoring (MRM) transitions of tulathromycin and one transition (*m/z* 749.4 > 591.3) for IS were monitored in order to comply with the criteria needed for a confirmatory method. First transition

Table 2 Validation results of the method for the determination of tulathromycin in swine tissues

Parameter	Kidney			Liver			Muscle		
Spiking level (μg kg^{-1})	1500	3000	4500	1500	3000	4500	10	30	50
Accuracy (%)	99.1	94.1	92.9	101.7	100.5	94.2	96.0	102.1	102.1
Repeatability (CV, %)	2.7	2.5	2.2	2.3	1.8	3.2	8.0	7.2	4.4
Within-lab Reproducibility, (CV, %)	7.7	7.9	7.6	4.0	3.9	4.6	9.2	7.3	6.3
CCα (μg kg^{-1})		3347			3293			11.9	
CCβ (μg kg^{-1})		3836			3560			13.6	
LOD (μg kg^{-1})		3			4			2	
LOQ (μg kg^{-1})		10			10			10	

Table 3 Statistical evaluation of robustness test results (7 factors, 8 experiments)

	Kidney	Liver	Muscle
SD_{WLR}[a]	223.2	116.7	3.23
SD_i[b]	201.3	105.5	2.86
t_{crit}[c]		2.109	
	t[d]	t[d]	t[d]
Time of shaking	0.336	0.386	0.681
Time of centrifugation	1.187	1.157	1.703
Volume of water	1.053	0.900	1.765
Vacuum-drying of the SPE cartridges	0.650	1.071	0.495
Volume of 5% ammonium hydroxide in methanol	1.366	1.585	1.115
Volume of 5% ammonium hydroxide in acetonitrile	1.949	1.928	1.486
Temperature of evaporation	1.635	1.328	0.898

[a] SD_{WLR} – standard deviation of within-laboratory reproducibility. [b] SD_i – standard deviation of differences. [c] t – experimental value of the t-test. [d] t_{crit} – critical value of t-test (95%).

(806.6 > 577.4) was chosen for the quantification, and the second (*m/z* 806.6 > 158.1) was used for the confirmation. Identification of analyte was carried out by retention time and relative ion ratio of selected MRM transition. Declustering potential (DP), collision energy (CE) and cell exit potential (CXP) were established. The optimal conditions for detection of tulathromycin by LC-MS/MS with retention time and MRM ion ratio of analyte are reported in Table 1.

Chromatographic separation of analytes was performed on C8 column after optimization of mobile phase composition and gradient elution program. A BDS Hypersil C8 column was chosen based on previous method for the analysis of tulathromycin.[2] During chromatographic separation different pH (3, 4, 5 and 6) of 10 mM ammonium acetate as a mobile phase buffer was tested. The obtained chromatograms shown that 10 mM ammonium acetate at pH 3 provided sharp, symmetrical chromatographic peaks with minimum band boadering than ammonium acetate buffer with higher pH values. The optimal separation of analytes was obtained with the column BDS Hypersil C8 (3 μm, 2.1 x 50 mm) coupled with mobile phase, consisting of acetonitrile and 10 mM ammonium acetate, pH 3. Using these conditions, the compounds of interest were eluted with retention time for tultahromycin = 8.1 min, IS = 9.1 min, and complete running time was 13 min. Ion chromatograms obtained from kidney sample spiked with tulathromycin and IS are presented in Fig. 3.

Method validation

The whole procedure was validated in-house according to the Commission Decision 2002/657/EC.[15] Because isotope labelled tulathromycin was not available, we used azithromycin as an internal standard. Azithromycin is very similar in chemical structure and physicochemical properties to tulathromycin. Gàler *et al.*[2] presented the synthesis of heptadeutero tulathromycin derivate which was used as IS. However, response and stability experiments showed that deuterated tulathromycin did not parallel the chemical behavior of tulathromycin. The usage of roxithromycin containing 14-membered lactone ring as IS was also reported.[12,13]

The specificity of the method was checked by analysing 20 blank tissue samples and no peak was detected in these samples at the retention time corresponding to each analyte. The results of the selectivity studies indicated that none of the tested macrolide antibiotics affected on the detector response intensity in retention times of analyte. Matrix-matched calibration curves with 1/x weighting factor were used for quantification with acceptable linearity (correlation coefficient, r > 0.99) over the ranges 10-9000 μg kg^{-1}. Accuracy, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ), limit of detection (LOD) and limit of quantification (LOQ) were calculated and presented in Table 2. Validation results show that accuracy was high in the range of 92.9 - 102.1%. Results from assessment of repeatability and from the within-laboratory reproducibility study (CV = 1.8-8.0% and 3.9-9.2%, respectively) show that the precision of the determination of tulathromycin was acceptable. Calculated CCα and CCβ values were slightly higher than MRL value in kidney and liver, and than LOQ value in muscle. Sensitivity of the method was satisfactory, which can be confirmed by low LOD (2-4 μg kg^{-1}) and LOQ (10 μg kg^{-1}). Moreover, LOQ values in our method are lower than LOQ values in other described methods used for determination of

tulathromycin residues in tissues. Boner *et al.*[3] developed method for the determination of tulathromycin residues in porcine kidney and bovine liver with an LOQ of 7.50 and 2.75 μg g^{-1}, respectively. Juan *et al.*[10] calculated LOQ for tulathromycin in muscle at the level of 15 μg kg^{-1}, which is slightly higher than calculated in our method.

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small changes in method parameters ensuring that the analytical method is reliable during use. The robustness study indicated that evaluated factors had no significant effect on the results. Standard deviation of differences was lower than standard deviation of within-laboratory reproducibility for each matrix (Table 3). Moreover, experimental *t*-values were always lower than t_{crit} (2.109) what demonstrated the method's robustness for slight variations of selected parameters. Consequently, the method proved to be fairly robust and able to withstand minor fluctuation in the operating variables that may occur during the sample preparation. The test for the stability of analytes in solutions, showed that the standard solutions stored at < -18 °C were stable for at least 6 months. The stability of mixed standard solutions stored at refrigerator (2 - 10 °C) were maintained for at least 1 month.

Matrix effect means, that the matrix is co-extracted with the analyte and cause signal suppression or enhancement during the ESI ionization, and thus could result in a poor analytical accuracy and precision. Therefore, it is important to eliminate or compensate this effect to achieve reliable analytical results. The matrix effect was calculated for tulathromycin in muscle, liver and kidney tissues. It was observed that the matrix effect on the response of the analyte depends on the type of tissue. Generally, signal suppression takes place. The largest suppression occurs in liver (ME = 62.7%), a little less in kidney (ME = 65.5%) and the smallest in muscle (ME = 89.7%) tissues. Despite the selectivity of sample clean-up step using polymeric mixed-mode cation exchange SPE, matrix effect was not eliminated completely. To compensate this adverse effect, for the quantitative analyses of tulathromycin matrix-matched calibration curves were used.

Applicability of the presented method was tested in a pilot study, with muscle, kidney and liver tissue samples from swine administrated with tulathromycin at a dose rate of 2.5 mg kg^{-1} bw. The calculated concentrations of tulathromycin in muscle were 1680 and 1130 μg kg^{-1} at 24 and 48 h after treatment. At the same time points, concentrations of tulathromycin were 7330 and 6860 μg kg^{-1} in kidney; and 1440 and 1620 μg kg^{-1} in liver, respectively.

Conclusions

An analytical method for the determination of tulathromycin in porcine kidney, liver and muscle has been successfully developed. A simple sample preparation and 13-min single chromatographic run allow to perform multiple analyses within one working day. The method validation parameters demonstrate its good accuracy, precision, specificity and sensitivity. Furthermore, the preliminary results of the experiment with incurred samples proved that the method presented in the study can be suitable for the analysis of tulathromycin distribution and its depletion in pig tissues.

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Notes and references

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1 N. A. Evans, *Vet Ther.*, 2005, **6(2)**, 83.
2 D. Gàler, S. Hessong, B. Beato, J. Risk, P. Inskeep, C. Weerasinghe, R. P. Schneider, C. Langer, J. LaPerle, D. Renouf, A. Bessire, E. Español, R. Rafka, C. Ragan, W. Boettner, T. Murphy, D. Keller, H. Benchaoui and M. N. Nowakowski, *J. Agric. Food Chem.*, 2004, **52**, 2179.
3 P. L. Boner, D. W. Gottschall and H. Kim-Kang, *J. AOAC Int.*, 2011, **94(2)**, 436.
4 H. A. Benchaoui, M. Nowakowski, J. Sherington, T. G. Rowan and S. J. Sunderland, *J Vet Pharmacol. Ther.*, 2004, **27(4)**, 203.
5 M. A. Nowakowski, P. B. Inskeep, J. E. Risk, T. L. Skogerboe, H. A. Benchaoui, T. R. Meinert, J. Sherington and S. J. Sunderland, *Vet Ther.*, 2004, **5(1)**, 60.
6 European Medicines Agency: Committee for veterinary medicinal products – Tulathromycin – Summary Report (2), EMEA/MRL/894/04-Final
7 Commission Regulation 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, *Off. J Eur. Union*, **L15**, 1-71.
8 J. A. Tarbin, D. Chan, J. F. Kay and M. Sharman, *Proceedings of the EuroResidue VII Conference*, Egmond ann Zee, 2012, 65.
9 P. A. Martos, S. J. Lehotay and B. Shurmer, *J. Agric. Food Chem.*, 2008, **56**, 8844.
10 C. Juan, J. C. Molto, J. Manes and G. Font, *Food Control*, 2010, **21**, 1703.
11 P. A. Martos, F. Jayasundara, J. Dolber, W. Jin, L. Spilsbury, M. Mitchell, C. Varilla and B. Shurmer, *J. Agric. Food Chem,*. 2010, **58**, 5932.
12 E. Scheuch, J. Spieker, M. Venner and W. Siegmund, *J. Chromatogr. B*, 2007, **850**, 464.
13 X. Wang, Y. F. Tao, L. L. Huang, D. M. Chen, S. Z. Yin, A. Ihsan, W. Zhou, S. J. Su, Z. L. Liu, Y. H. Pan and Z. H. Yuan, *J. Vet Pharmacol. Ther.*, 2012, **35(3)**, 282.
14 A. Gajda, A. Posyniak and T. Błądek, *Bull Vet Inst Pulawy*, 2013, **57**, 191.
15 Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *Off. J Eur. Commun.*, **L 221**, 8-36.