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Pre-study and in-study validation of an ultra-high pressure LC method coupled to tandem mass spectrometry for off-line determination of oxytetracycline in nasal secretions of healthy pigs☆

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ABSTRACT

In order to quantify oxytetracycline (OTC) in nasal secretions of healthy pigs after intramuscular injection of OTC at doses of 10, 20 and 40 mg/kg bodyweight, an original method based on ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) was developed and fully validated. Sample preparation consisted in protein precipitation preceded by the addition of a releasing protein reagent. Metacycline (MTC) was used as internal standard. Separation was carried out at 65 °C in the gradient elution mode on a short analytical column filled with Acquity BEH C_{18} stationary phase. The mobile phase consisted in a mixture of water and acetonitrile containing 1 mM of oxalic acid and 0.1% (v/v) of formic acid. The triple quadrupole mass spectrometer operated in the positive electrospray ionization mode; OTC and MTC were detected using multiple reaction monitoring. The pre-study and in-study validation of this bioanalytical method was performed by applying a novel strategy based on total measurement error and accuracy profiles. The maximum risk of observing future measurements falling outside the acceptance limits during routine as well as the measurements uncertainty were also estimated.

1. Introduction

Oxytetracycline (4S, 4aR, 5S, 5aR, 6S, 12aS)-4-dimethylamino-1, 4, 4a, 5, 5a, 6, 11, 12a-octahydro-3, 5, 6, 10, 12, 12a-hexahydroxy-6-methyl-1, 11-dioxonaphthacene-2-carboxamid (Fig. 1) is a broad-spectrum antibacteriostatic widely used in the veterinary treatment of respiratory and gastro-intestinal infectious diseases in pigs and several other species [1–5]. The formulations used are injectable, oral solution and feed medication. There is no active metabolite described for oxytetracycline (OTC).

Several pharmacokinetic studies of OTC in pig plasma have been published [1–5]. However, the successful of an antimicrobial therapy requires the knowledge of local concentrations at the site of infection. The nasal cavity is the first respiratory compartment where the microbial contamination may occur and the nasal secretions are the site where bacteria establish and multiply. Consequently, the concentrations of OTC in nasal secretions may provide information upon which prediction of efficacy can be made that is more realistic than the information provided by concentrations in plasma. However, the determination of OTC in nasal secretions of healthy pigs requires the development of a sensitive and reliable analytical procedure. As far as we know, no quantitative data on the normal or pathological concentrations of OTC in the nasal secretions of pigs have been published. The major problem is related to the very small quantities of nasal secretions collected, particularly in healthy pigs [6]. Moreover, the nasal secretions of pigs can be considered as protein rich samples. The development of a sample preparation method adapted to these low volumes and the use of a sensitive detection mode coupled to liquid chromatography (LC) are needed.

Several methods have been published for the analysis of OTC in biological media. They are based mainly on LC coupled to a technique of sample preparation, essentially protein precipitation, liquid–liquid extraction and solid-phase extraction (SPE) [7–9].

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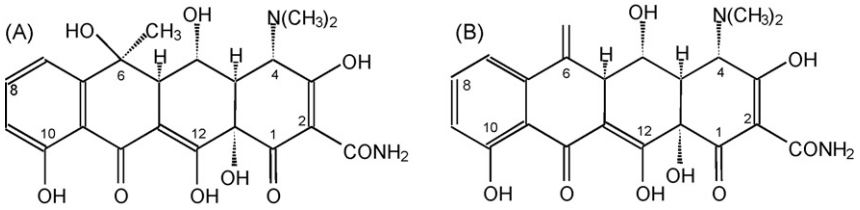

Fig. 1. Chemical structures of oxytetracycline (A) and metacycline (B).

A suitable method for the determination of OTC in nasal secretions of healthy pigs was selected by taking into account the simplicity of the sample preparation procedure, the concentration range and the analysis time of the entire analytical procedure. The latter consisted in a simple sample clean-up by protein precipitation preceded by the addition of a releasing protein reagent and coupled to an ultra-high pressure LC method followed by tandem mass spectrometry (UPLC–MS/MS). UPLC is a relatively recent chromatographic technique based on the use of columns packed with sub-2 μm particles for applications up to 15,000 psi. Significant gains in efficiency, resolution, detectability and analysis time can be obtained. Its hyphenation to mass spectrometers capable of high speed acquisitions is particularly interesting for high throughput quantitative bioanalysis.

The aim of this study was to valid the developed UPLC–MS/MS method in order to quantify OTC in nasal secretions of healthy pigs during a pilot study on the fluctuations of the sensitivity of nasal commensal bacteria to OTC administered intramuscularly at doses of 10, 20 and 40 mg/kg bodyweight [10]. A novel strategy based on total measurement error and accuracy profiles was applied successfully for the pre-study and in-study validation of this bioanalytical method. The maximum risk of observing future measurements falling outside the acceptance limits during routine as well as the measurements uncertainty were also estimated.

2. Experimental

2.1. Chemicals and reagents

Oxytetracycline hydrochloride (OTC, CAS No. 2058-46-0, purity ≥95%) and metacycline (MTC, CAS No. 3963-95-9, grade Vetranal®, analytical standard) used as internal standard (Fig. 1) were supplied by Sigma Chemical Company (Saint-Louis, MO, USA). They were used without further purification. The other chemicals were supplied as follows: oxalic acid, formic acid and perchloric acid ($HClO_4$), all of analytical grade, were obtained from VWR international (Darmstadt, Germany); methanol and acetonitrile of UPLC grade were purchased from Biosolve (Valkenswaard, The Netherlands) and the water used in all experiments was purified on a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

2.2. Instrumentation

The Acquity™ UPLC system from Waters (Milford, MA, USA) was equipped with a binary solvent delivery manager, a column heater/cooler module and a sample manager. It was coupled to a Quattro Premier™ XE tandem mass spectrometer. The separation was performed on a short analytical column (50 mm × 2.1 mm, i.d.) filled with Acquity BEH C_{18} stationary phase (particle size: 1.7 μm) from Waters. The control system and the data acquisition were performed through the Masslynx ™ software (version 4.1). The e-noval® 2.0 software (Arlenda, Liege, Belgium) devoted to the validation of physico-chemical methods was applied to obtain the accuracy profiles as well as the validation results.

2.3. Chromatographic conditions

All chromatographic experiments were carried out in the gradient elution mode, using a mobile phase composed of mixtures of water/acetonitrile (95:5, v/v) (phase A) and water/acetonitrile (5:95, v/v) (phase B), both containing 1 mM of oxalic acid and 0.1% of formic acid. The injection volume was 1 μl. Metacycline was used as internal standard. The mobile phase was delivered at a constant flow-rate of 0.7 ml min^{-1} according to the following program:

- From 0 to 0.5 min: isocratic elution; A: 99.9% – B: 0.1% (v/v).
- From 0.5 to 7.1 min: linear gradient; A: 99.9–0.1% – B: 0.1–99.9% (v/v).
- From 7.1 to 7.6 min: isocratic elution; A: 0.1% – B: 99.9% (v/v).
- From 7.6 to 7.7 min: back to the initial conditions; A: 99.9% – B: 0.1% (v/v).

The analytical column was maintained at 65 °C. The triple quadrupole mass spectrometer operated in the positive electrospray ionization mode (ESI+). OTC and MTC were detected using multiple reaction monitoring (MRM) of the specific transitions m/z 461 > 426 and 443 > 426, respectively. The other mass spectrometry settings were as follows: capillary voltage of 0.25 kV; cone voltage of 23 and 28 V for OTC and MTC, respectively; collision energy of 19 and 16 eV for OTC and MTC, respectively; desolvation gas flow of 900 l/h; cone gas flow of 50 l/h; desolvation temperature of 350 °C and source temperature of 135 °C.

2.4. Sample clean-up procedure

After thawing and vortex-mixing, a volume of 10 μl of nasal secretions was introduced into an eppendorf polypropylene tube. Sample clean-up consisted to add 10 μl of a protein releasing reagent (solution of perchloric acid at 0.6% (v/v)) and to vortex-mix, before to precipitate the proteins by the addition of 10 μl of the same acid at a higher concentration (6%, v/v) and to vortex-mix again. The samples were then centrifuged at 1500 × g for 10 min. Finally, 20 μl of 0.6% perchloric acid containing 1 μg/ml of MTC, 2 mM of oxalic acid and 0.2% of formic acid were added to 20 μl of supernatant. 1 μl of this mixture was injected into the UPLC system.

2.5. Solutions

2.5.1. Solutions used for method development

A stock solution of OTC at a concentration of approximately 1100 μg/ml was daily prepared in 0.6% perchloric acid. Several intermediate solutions (from 0.44 to 220 μg/ml) were then obtained by dilution in the same acid solution. All solutions used during method development were prepared by diluting 10-fold these intermediate solutions in 0.6% perchloric acid or in blank nasal secretions.

2.5.2. Solutions used for method validation

Two different stock solutions of OTC at a concentration of approximately 1100 μg/ml were first prepared in 0.6% perchloric

acid in order to furnish two kinds of independent samples: calibration standards and validation standards.

For calibration, several intermediate solutions were obtained by dilution of the first stock solution in 0.6% perchloric acid. The concentration levels were 0.44, 1.1, 2.2, 11, 44, 110 and 220 μg/ml. A volume of 100 μl of these solutions was then added to 1000 μl of blank nasal secretions in order to constitute pooled samples at the following concentrations: 40, 100, 200, 1000, 4000, 10,000 and 20,000 ng/ml. At each concentration level, two calibration standards were treated and three calibration curves were constructed during the validation step.

As for the validation standards, the same preparation procedure was applied from the second stock solution of OTC. The concentration levels of the different intermediate solutions were 0.44, 1.32, 44 and 209 μg/ml. 100 μl of these solutions were then added to 1000 μl of blank nasal secretions in order to constitute pooled samples used as validation standards and quality control (QC) samples in routine analysis. The final concentrations of OTC were 40, 120, 4000 and 19,000 ng/ml. At each concentration level, four replicates were prepared and three independent series were performed.

2.5.3. Solutions used for routine analysis

Healthy pigs were treated by intramuscular injection of OTC (Terramycin®, Pfizer, Brussels, Belgium) at 10, 20 and 40 mg/kg bodyweight. Prior to sample collection, a sedative mixture of xylazine (Xyl-M®, VMD, Brussels, Belgium) at 2 mg/kg bodyweight and ketamine (Ketamine®, CEVA, Brussels, Belgium) at 10 mg/kg bodyweight was administered to the pigs, which were anaesthetised after 10 min by intravenous injection of thiopental (Pentothal®, Abbott Laboratories, IL, USA). Nasal secretions were then absorbed on a swab fixed at the end of a flexible rod inserted deeply into the nasal cavity. After centrifugation of nasal secretions from the swab at 13,000 × *g*, the supernatant was introduced in an adequate vial and stored at −80 °C. Just before the analysis of OTC, the samples were thawed and vortex-mixed. A sample volume of 10 μl was treated as previously described.

3. Results and discussion

3.1. Selection of suitable UPLC–MS/MS conditions

A pronounced peak tailing could be observed in the chromatographic separation of oxytetracycline due to the formation of chelate complexes with metal ions and the presence of a dimethylamino group bound to C4 (Fig. 1). The addition of oxalic acid as ligand combined to the use of a column packed with a suitable stationary phase (hybrid phase) allowed the improvement of peak symmetry. However, the concentration of oxalic acid was limited to 1 mM, since it is a non-volatile compound which could cause clogging at the interface of the ion source. In addition, the application of a gradient elution is also an effective way to solve peak tailing, as demonstrated by Snyder and Dolan [11]. Indeed, in the gradient mode, the eluting power of mobile phase is increased during the time of peak elution and the tail of the peak is always under the influence of the stronger mobile phase when compared to the elution peak front, reducing the peak tailing and the peak width [11]. Satisfactory gradient conditions were rapidly obtained by optimizing only the gradient steepness. Under the selected gradient conditions, OTC and MTC were separated within 2.5 min, as shown in Fig. 2.

The product ion mass spectra from collision-induced dissociation of the protonated molecules $[MH]^+$ of OTC and MTC are illustrated in Fig. 3. The analytes of interest were detected using MRM of the transitions *m*/*z* 461 > 426 and 443 > 426 for OTC and MTC, respectively. The mass spectrometry parameters were optimized in order to obtain maximum detectability and selectivity.

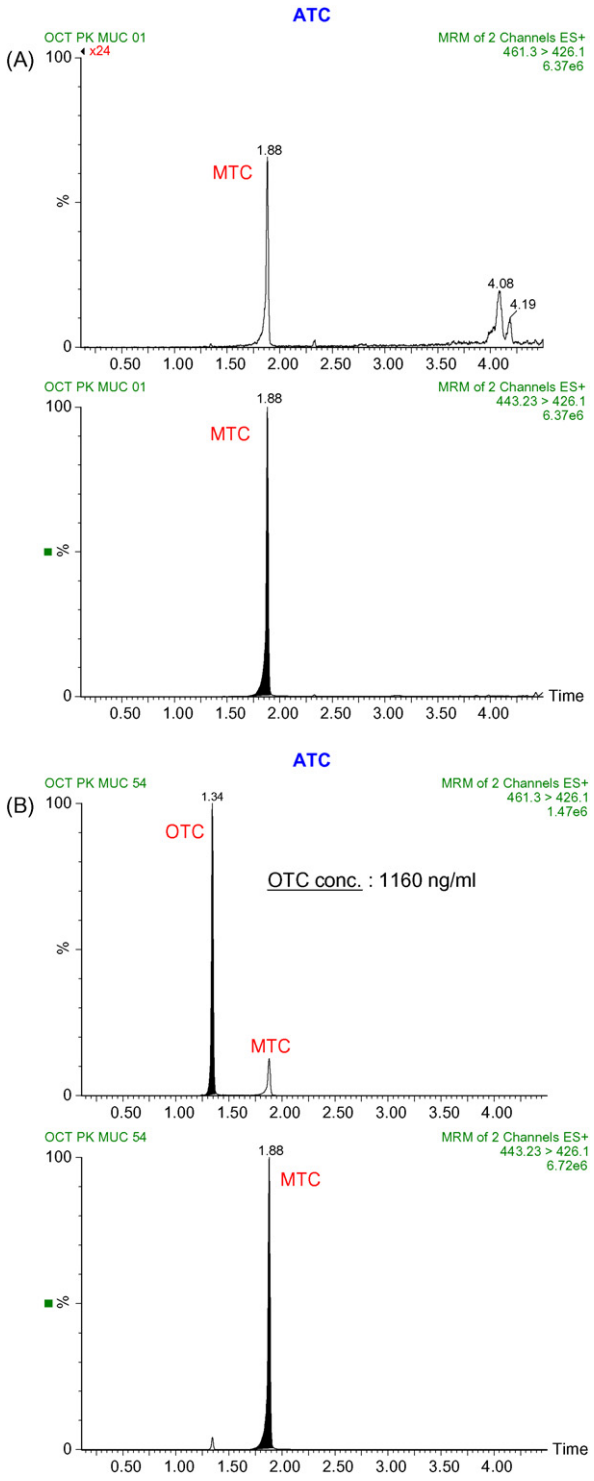

Fig. 2. Selectivity and applicability of the method to routine analysis: chromatograms of blank nasal secretions of healthy pigs (A) and a real sample of nasal secretions from a healthy pig treated by intramuscular injection of OTC at 10 mg/kg bodyweight and collected after 1 h (B).

3.2. Development of the sample clean-up procedure

Since a sensitive and selective detection mode, namely tandem mass spectrometry, was selected, it was decided to test a simple

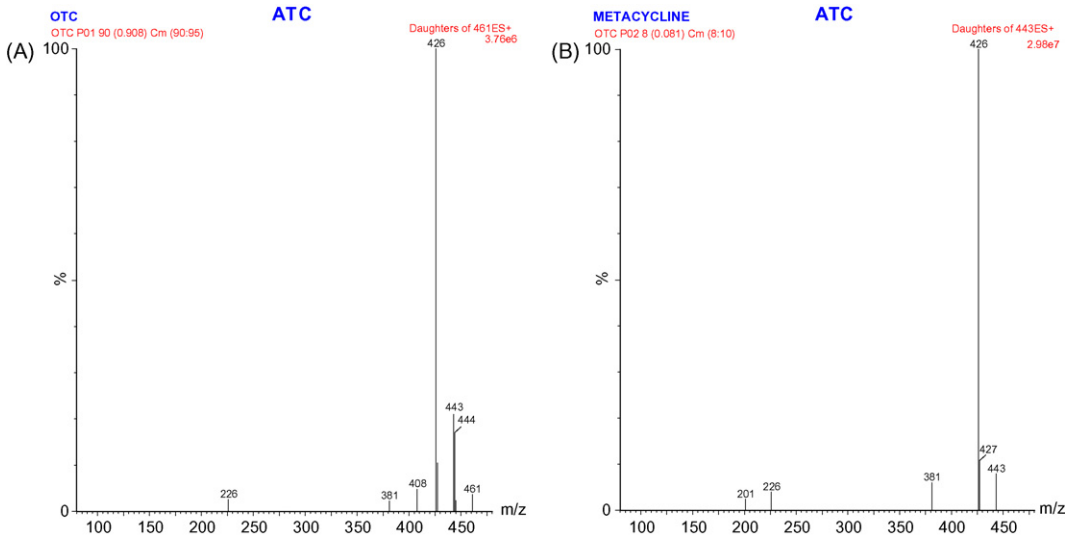

Fig. 3. Product ion mass spectra of OTC (A) and MTC (B).

sample clean-up procedure. Protein precipitation (PP) remains a popular technique widely used in bioanalysis [12]. A manual PP procedure was applied in the present study. The number of samples to be analysed was not high enough to justify its automation.

In order to select the most suitable protein releasing and/or deproteinization reagents, different additives were tested for the elimination of proteins and other macromolecules from biological samples. Only 10 μl of biological matrix were treated. According to the results presented in Table 1, the following procedure was selected for sample clean-up: to add a protein releasing reagent (0.6% (v/v) of $HClO_4$) to the biological matrix before to precipitate the proteins by the addition of the same acid at a higher concentration (6%, v/v). After centrifugation and addition of the internal standard, a low volume of supernatant was injected into the UPLC system. Under these conditions, the analyte recoveries were practically 100%.

Even if this kind of sample preparation is more laborious than on-line SPE, the analysis time was reduced two times in comparison to a method previously developed for the determination of OTC in nasal secretions by on-line SPE coupled to LC followed by fluorescence detection [13]. In addition, a significant phenomenon of carry-over was observed by applying this method (data not shown).

3.3. Method pre-study validation

The pre-study validation of an analytical method is mandatory before its routine application in order to ensure its fitness for purpose [14–17]. The purpose of any quantitative analytical method is to quantify the target analytes with a known accuracy [18–20]. To demonstrate this, a novel validation strategy was introduced which uses accuracy profiles based on tolerance intervals for the total or measurement error, including both bias and standard deviation for intermediate precision [18–23]. Such an approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current strategies that compare point estimates of observed bias and precision. The originality of this strategy is that the decision about the method validity is based on the prediction to obtain accurate results in the future conditionally to the amount and quality of results obtained during the validation phase [22,24]. The concept of accuracy profile was also used to select the most appropriate regression model for calibration, to determine the limit of quantitations (LOQ) and the range over which the method can be considered as valid. Furthermore, this strategy allows computing the risk of having future results falling outside the acceptance limits or specifications. This is also an increasing request of regulatory requirements [25,26]. For this accuracy profile approach, two parameters must be set by the analyst, the acceptance limits on one hand and the risk of having future measurements falling outside these acceptance limits on the other hand (1-β). The acceptance limits were set at ±30% in order to meet the requirements of AAPS guidelines [27]. Such limits are generally suggested for the determination of macromolecules using ligand-binding assays. Nevertheless, according to these guidelines, it is proposed that the acceptable total error for both methodologies (chromatographic method and ligand-binding methodology) should be less than 30%. Therefore, these acceptance limits were considered. The risk of having future results falling outside the acceptance limits was set a priori at 10% (1-β), however it is important to notice that as demonstrated by Boulanger et al. [28], the minimum value of this risk must be set at 20% in order to conciliate the pre-study validation requirements of the US Food and Drug Administration (FDA) [15] with those of in-study validation.

Table 1
OTC recovery from spiked nasal secretions.

Sample (10 μl)	Protein releasing reagent (10 μl)	Deproteinization reagent (10 μl)	Absolute recovery (%, $n = 2$)
Aqueous	–	–	97
Nasal secretions	–	MeCN/MeOH (3/1)	78
Nasal secretions	–	$HClO_4$ (6%)	90
Nasal secretions	$HClO_4$ (0.6%)	$HClO_4$ (6%)	99

MeCN: acetonitrile; MeOH: methanol; OTC concentration: 2.5 μg/ml; other operating conditions: as given in Section 2.

3.4. Method selectivity and matrix effect

The absence of matrix interferences at the retention time of OTC was demonstrated in Fig. 2, which illustrates chromatograms obtained after analysis of a sample of blank nasal secretions from six different pigs spiked with MTC, the IS, and a real sample from a healthy pig treated by intramuscular injection of OTC at 10 mg/kg bodyweight and collected after 1 h. Moreover, according to the FDA guidelines [15], for MS-based bioanalytical methods which use nonisotopically labelled IS, the absence of unmonitored co-eluting compounds should be checked by the determination of matrix

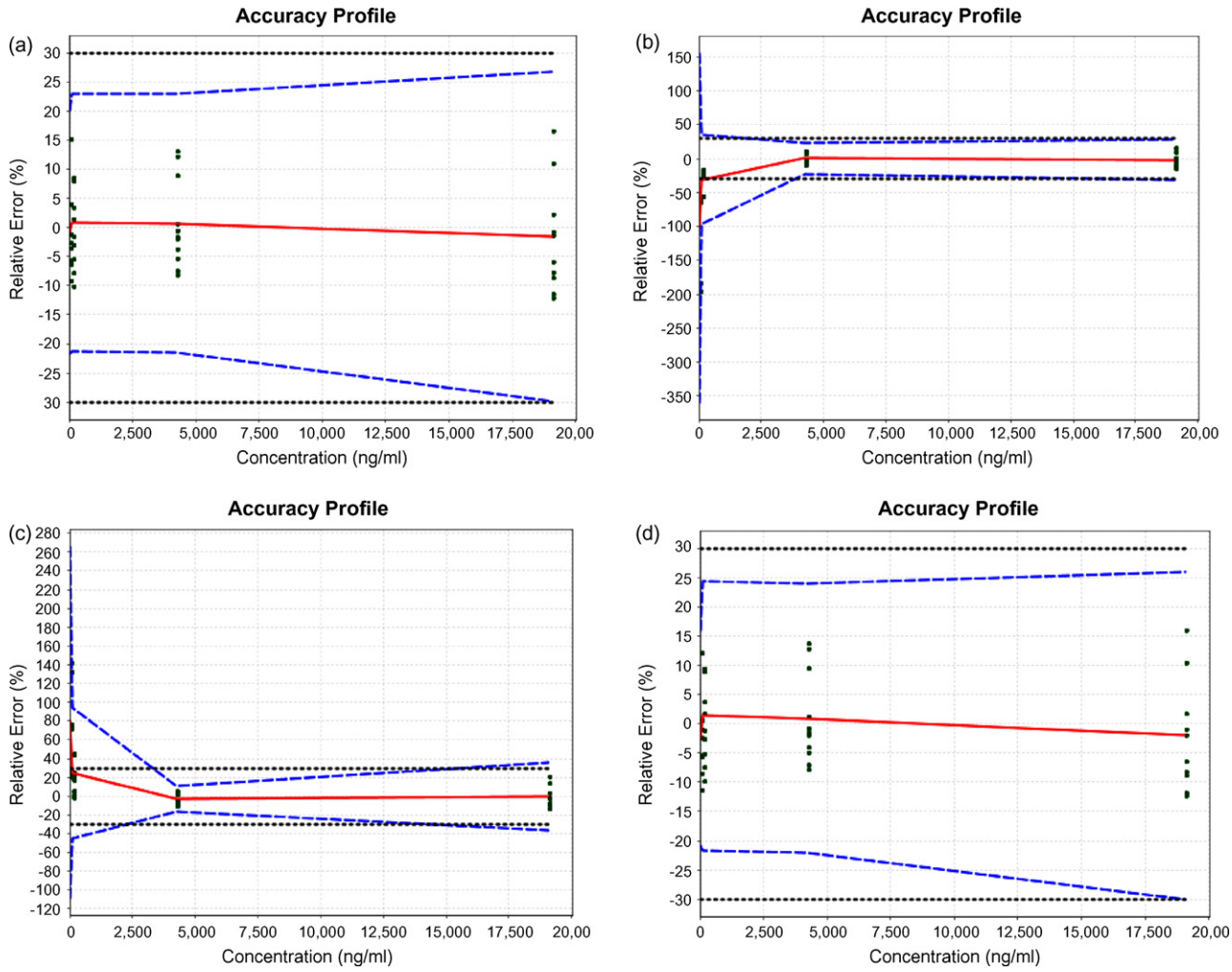

Fig. 4. Accuracy profiles for the quantification of OTC in nasal secretions using (A) a linear regression model after logarithmic transformation, (B) a simple linear regression model, (C) a quadratic model and (D) a weighted $1/X$ linear regression model. Relative bias (—), acceptance limits (····), beta expectation tolerance limits (– – –), and relative back-calculated concentrations (■).

effect. The quantitative measure of matrix effect can be termed as Matrix Factor (MF), which is defined as follows:

$$\mathrm{MF} = \frac{A}{B}$$

where A and B are the analyte peak responses in the presence of matrix ions and in the absence of matrix ions, respectively.

Post-extraction blank samples spiked with OTC and MTC and neat solutions at the same concentration level were analysed. The peak response was the ratio of the peak area for OTC versus that of MTC.

In the present method, the MF was close to 1, which signifies that there was no matrix effect. In addition, in order to predict the variability of matrix effects in samples from individual subjects, it is recommended to determine the MF for six independent sources of the same matrix. The variability in matrix factors, as measured by the coefficient of variation (CV), should be less than 15%. The CV calculated was 6.3%, which also demonstrates the absence of matrix effect.

Moreover, with respect to analyte recovery, the extraction efficiency was consistent and reproducible over the dosing range. Irrespective of the concentration level, the analyte recovery was practically 100%. In the presence of matrix effect, a recovery of about 100% would be not very probable.

3.5. *Fit for the purpose of selecting the calibration curve*

Several response functions were fitted to the calibration standards. The analytical response was the peak area ratio of OTC versus MTC. From every response function obtained for OTC, the concentrations of the validation standards were back-calculated allowing to compute, by concentration level, the upper and lower β-expectation tolerance limits at 90% (β%) level by introducing the estimations of the standard deviation for intermediate precision and the relative bias.

From these data, different accuracy profiles were plotted to select the best fit for regression model according to the intended use of the analytical method [18–23]. As shown in Fig. 4, four response functions, namely the simple linear regression model, the linear regression model after logarithmic transformation, the quadratic regression model and the weighted $1/X$ linear regression model (X, being the concentration level), were tested for the quantification of OTC in nasal secretions. The acceptance limits were set at ±30%.

As can be seen in Fig. 4, the accuracy profiles obtained from the linear regression model after logarithmic transformation and from the weighted linear regression model are comparable. Nevertheless, when using the weighted regression, the lower tolerance limit for the highest concentration level was −30.1% and was slightly outside the lower acceptance limit. By applying the linear regression after logarithmic transformation, the tolerance intervals were

totally included inside the acceptance limits, even at the highest concentration level (−29.9%, as lower tolerance limit). Therefore, this regression model was finally selected as response function. It allowed demonstrating the ability of the method to quantify the studied analyte in nasal secretions over the whole concentration range considered.

The responses functions obtained for each series by applying the selected regression model are presented in Table 2.

3.6. Precision and trueness

Intermediate precision variance and bias are not the validation criteria to decide on but their estimates are necessary to assess the quality of the analytical method. Results of repeatability (intra-day precision), time-different intermediate precision, and trueness, as resulted from validation standards, are summarized in Table 2. At the studied concentration levels, the relative standard deviations (R.S.D.s) for intermediate precision were maximum 10%. The overall relative bias never exceeded 2%. All values were within the limits of the FDA guiding principles recommended for bioanalytical method validation [15].

3.7. Accuracy

The accuracy of the results should be estimated by considering the overall measurement error, i.e. the simultaneous combination of the systematic and random error components, arising from the experimental results [18–23,29,30]. This is achieved by computing the β-expectation tolerance interval or accuracy profile which guarantees that routine results will be with minimum risk of eccentricity from the conventional true values. Indeed, as long as the upper and lower bounds of the tolerance intervals of the future individual results at the studied concentration levels entirely fit the ±30% acceptance boundaries, the bioanalytical method is defined as accurate. The upper and lower β-expectation tolerance limits in relative value (%) are presented in Table 2 as a function of the concentration of the validation standards. Since the tolerance intervals are included in the ±30% acceptance limits irrespective of the concentration level, the analytical method can be declared as providing accurate results.

3.8. Linearity

In order to demonstrate linearity of the results, a regression line was fitted on the back-calculated concentrations of the validation standards as a function of the introduced concentrations by applying the linear regression model based on the least squares method [18–23,30]. The results attesting the linearity are presented in Table 2.

3.9. Limits of quantitation and detection

The lower and upper limits of quantitation (LLOQ and ULOQ) are considered as the lowest and highest analyte concentrations, which can be reliably determined with acceptable accuracy under the given experimental conditions. The lowest and highest concentration levels studied in the nasal secretions were considered as the LLOQ and the ULOQ, respectively, since the accuracy profile, which characterizes the overall measurement error, is confined within the acceptance limits, as shown in Table 2. The limit of detection (LOD), estimated on the basis of the mean intercept of the calibration line build in the matrix and the residual variance of the regression [31], was 13 ng/ml.

3.9.1. Evaluation of carry-over

According to the FDA guidelines [15], blank samples (1 or more) should be injected after a standard or a sample containing a high concentration of the compound of interest. In the present study, a blank sample was analysed after each set of calibration standards and each series of QC samples. The calibration standards and the QC samples were injected from the lowest concentration level to the highest concentration. The peak area obtained with the blank samples should be less than 20% of the peak area observed after analysis of a biological sample at a concentration equivalent to the LLOQ.

During method validation and routine analysis, the results obtained by injecting blank samples were comprised between 0 and 5% of the response obtained at the LLOQ. These results attest the absence of carry-over, which could be also explained by the low volume injected (1 μl).

3.10. Determination of stability

The conditions used in stability studies should reflect situations likely to be encountered during the handling of the samples and their analysis. The stability of the stock solutions, the post-preparative and bench-top stabilities and the long-term stability were tested. The freeze-thaw stability was not considered. Due to low volumes collected for the incurred samples, the latter were thawed and discarded after analysis. In routine, the pooled samples used to prepare the calibration standards and QC samples were divided into small portions before storage at −80 °C. Only fresh portions were thawed at each analysis day and discarded after use.

3.10.1. Stability of the stock solutions (OTC and MTC)

The stability of the stock solutions was evaluated at room temperature for 6 h. In this study, both stock solutions were not refrigerated or frozen, but were discarded just after the preparation of pooled samples. The time needed to prepare these pooled samples was below 4 h. The analysis of the solutions was performed in triplicate at T_0 and $T_{6\,h}$. The peak responses were comparable, attesting the stability of the stock solutions of OTC and MTC.

3.10.2. Post-preparative and bench-top stabilities

According to the FDA guidelines [15], these experiments can be combined, in order to demonstrate the overall process stability. As recommended, three aliquots of spiked nasal secretions at LLOQ and ULOQ were thawed at room temperature and kept at this temperature for 4 h in order to cover at least the time needed to treat the samples. Afterwards, the samples were extracted according to the described sample clean-up procedure and stored at room temperature for 48 h. They were analysed at T_0 and after 6, 24 and 48 h. In comparison to initial time, the mean calculated ratios (±S.D.) for the LLOQ samples were 0.97 (±0.06), 0.94 (±0.07) and 0.89 (±0.08) at $T_{6\,h}$, $T_{24\,h}$ and $T_{48\,h}$, respectively. The values obtained for the ULOQ samples were 0.98 (±0.03), 0.95 (±0.09) and 0.91 (±0.06), respectively. These results attest the post-preparative and bench-top stabilities of OTC and MTC during a period of at least 48 h.

3.10.3. Long-term stability

In order to meet the recommendations of the FDA guidelines for bioanalytical method validation, the long-term stability was estimated by analysing QC samples and two incurred samples stored at −80 °C, 6 months after validation. As recommended, three aliquots of QC samples at LLOQ and ULOQ and two incurred samples from the first analytical batch were reanalysed. The mean calculated ratios (±S.D.) were 0.98 (±0.10), 0.99 (±0.09) for the LLOQ and ULOQ samples, respectively, and 0.95 (±0.11) and 0.98 (±0.07) for the two incurred samples. These results confirm the stability of OTC in nasal

Table 2
Results of the validation of the analytical method dedicated to the quantification of OTC in nasal secretions.

Validation criterion	Results (linear regression after logarithmic transformation, calibration range (m = 7): 40–20,000 ng/ml)		
	Series 1	Series 2	Series 3
Response function (k = 3; n = 2)			
Slope	0.998	0.9957	0.9928
Intercept	−3.793	−3.767	−3.814
r^2	0.9998	0.9998	0.9994

Validation criterion	Results
Trueness (k = 3; n = 4), relative bias (%)	
42 ng/ml	−0.7
127 ng/ml	0.8
4200 ng/ml	0.7
19,000 ng/ml	−1.6
Precision (k = 3; n = 4), repeatability/intermediate precision (R.S.D.%)	
42 ng/ml	3.8/7.8
127 ng/ml	3.0/7.7
4200 ng/ml	3.8/8.1
19,000 ng/ml	5.7/10.7
Accuracy (k = 3; n = 4), relative β-expectation lower and upper tolerance limits (%)	
42 ng/ml	[−21.6; 20.2]
127 ng/ml	[−21.3; 22.9]
4200 ng/ml	[−21.5; 22.9]
19,000 ng/ml	[−29.9; 26.8]
Linearity (k = 3; n = 4)	
Range (ng/ml)	[42; 19,000]
Slope	0.9838
Intercept	28.77
r^2	0.9878
LOD (ng/ml)	13
LLOQ (ng/ml)	42
ULOQ (ng/ml)	19,000

k: number of series; n: number of replicates per series; m: number of concentration levels.

secretions when storing at −80 °C during a period exceeded the time comprised between the first incurred sample collection and the end of routine analysis, which was of approximately 4 months.

3.11. Risk and uncertainty assessments

As stated previously, the maximum risk (1-β) to have future measurements outside the acceptance limits was set at 10%. However the effective probability to obtain such measurements was estimated using the accuracy profiles. As shown in Table 3, the maximum risk of observing future measurements falling outside the acceptance limits during routine analysis is maximum 15% for the quantification of OTC in nasal secretions at the highest concentration level of the dosing range and around 8% for all other levels. It can be noticed that according to the 4-6-15 rule of the FDA guidance [15], a risk of 33% is tolerated for routine analysis.

The estimation of measurements uncertainty is an important parameter to provide for quantitative analytical methods if one wishes to thoroughly interpret and compare results against legal thresholds, acceptance limits or other laboratories results. One major advantage of the applied validation methodology is that it can, without any additional experiments, give an estimation of measurement uncertainty [32]. On this basis, several estimations of uncertainty were computed for the quantification of OTC in nasal secretions and are presented in Table 4. The expanded uncertainty was computed using a coverage factor of k = 2 [33–35] representing an interval around the results where the unknown true value can be observed with a confidence level of 95%. As shown in Table 4, the relative expanded uncertainty of OTC in nasal secretions did not exceed 24%, irrespective of the concentration level. In other words, this means that with a confidence level of 95% the unknown true value is situated at maximum ±24% around the measured result.

Table 3
Risk in % of having future measurements falling outside the ±30% acceptance limits in routine analysis for the quantification of OTC in nasal secretions using the selected response function.

Concentration level (ng/ml)	Risk (%)
42	6.4
127	8.1
4,200	7.8
19,000	15.3

3.12. Method in-study validation

The analytical method dedicated to the quantification of OTC in nasal secretions was then used in routine. One run of 92 samples was performed whose in-study validation was made using 12 QC samples at four concentration levels ranging from 42 to 19,000 ng/ml. This complies with the FDA requirements for assessing in-study validation which requires at least QCs at three concentration levels, namely a low QC at 3 times the LLOQ, a mid one and a high one [15]. However, a QC sample at the LLOQ was added to increase the reliability of the decision about the run acceptance and about the results of the unknown samples that may be obtained near this concentration level. As stated in the previous section, the linear regression with logarithmic transformation model was selected as response function since it was predicted to provide the most accurate results.

With the back-calculated results of the QC samples, it is then possible on one hand to evaluate whether the prediction, and so the response function selection, was adequate or not and, on the other hand to decide about the acceptability of the run according to the 4-6-15 rule of the FDA [15]. Fig. 5 represents the

Table 4
Uncertainty estimates for each concentration level of the validation standards related to the quantification of OTC in nasal secretions. The coverage factor of the expanded uncertainty was set to $k = 2$.

Concentration (ng/ml)	Uncertainty of the bias (ng/ml)	Uncertainty (ng/ml)	Expanded uncertainty (ng/ml)	Relative expanded uncertainty (%)
42	1.7	3.7	7.4	17.6
127	5.3	11.1	22.2	17.5
4200	181.8	388.9	777.7	18.4
19,000	1061.0	2305.0	4611.0	24.2

observed (dots) relative errors of the three QC samples at each concentration level (1 = 42 ng/ml, 2 = 127 ng/ml, 3 = 4200 ng/ml and 4 = 19,000 ng/ml) obtained during the routine use together with the in-study validation acceptance limit of ±15% (continuous lines) and their respective upper and lower β-expectation tolerance limits obtained during the pre-study validation (horizontal dashes). As shown in this figure, it can be concluded that the response function selected seems to be adequate, since all results of the QC samples are included in their respective β-expectation tolerance intervals. Furthermore, since all those results were also fully included in the ±15% acceptance limits, the run could be accepted.

In order to focus the attention of the analyst about the role and importance of measurement uncertainty, their estimates obtained during the pre-study validation were used to put in perspective the decision about the acceptability of the run. As stated in the previous section to thoroughly compare results to regulatory acceptance limits, the use of the results value together with their measurement uncertainty is needed. Fig. 6A–D shows the results (dots) of each QC sample complemented with their relative expanded uncertainty (coverage factor $k = 2$; horizontal dashes) for the levels 1–4, respectively. Additionally the ±15% acceptance limits are represented on each figure. As can be seen, the decision about the acceptability of the run can be mitigated. Indeed, none of the relative expanded uncertainty intervals of the QC samples results are fully included in the ±15% acceptance limits. Therefore, it cannot be guaranteed that the true values of these results are included in the ±15% acceptance limits since these intervals represent a region where it is expected to find the true value of the result 95 times out of 100. For three concentration levels (levels 1–3; cf. Fig. 6A–C), the relative expanded uncertainty intervals step only a little bit outside the acceptance limits and the question about the acceptability of this situation remains. On the other hand, for the highest concentration level (level 4; cf. Fig. 6D) the situation is more explicit, since approximately half of the relative expanded uncertainty intervals of each QC sample steps outside the ±15% acceptance limits. Measurement uncertainty can play an important role, since the reliability of the decisions made using the results obtained can be different when taking into account or not this parameter.

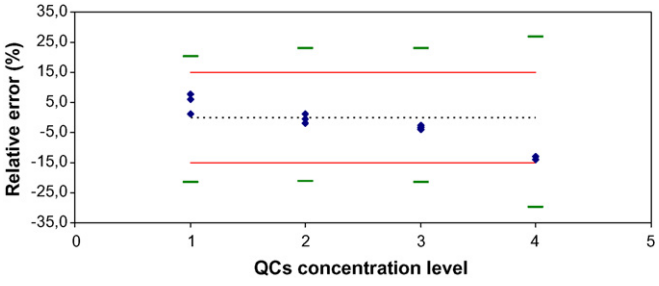

Fig. 5. Results of the in-study validation. QC samples by concentration level (level 1 = 42 ng/ml, level 2 = 127 ng/ml, level 3 = 4200 ng/ml, level 4 = 19,000 ng/ml) of OTC in nasal secretions expressed in relative values (%). The dots are the relative back-calculated concentrations, the continuous lines are the ±15% acceptance limits for the run; the upper and lower horizontal dashes represent the upper and lower β-expectation tolerance limits obtained during the pre-study validation of the method at each concentration level. The dotted line is the 0% relative error line.

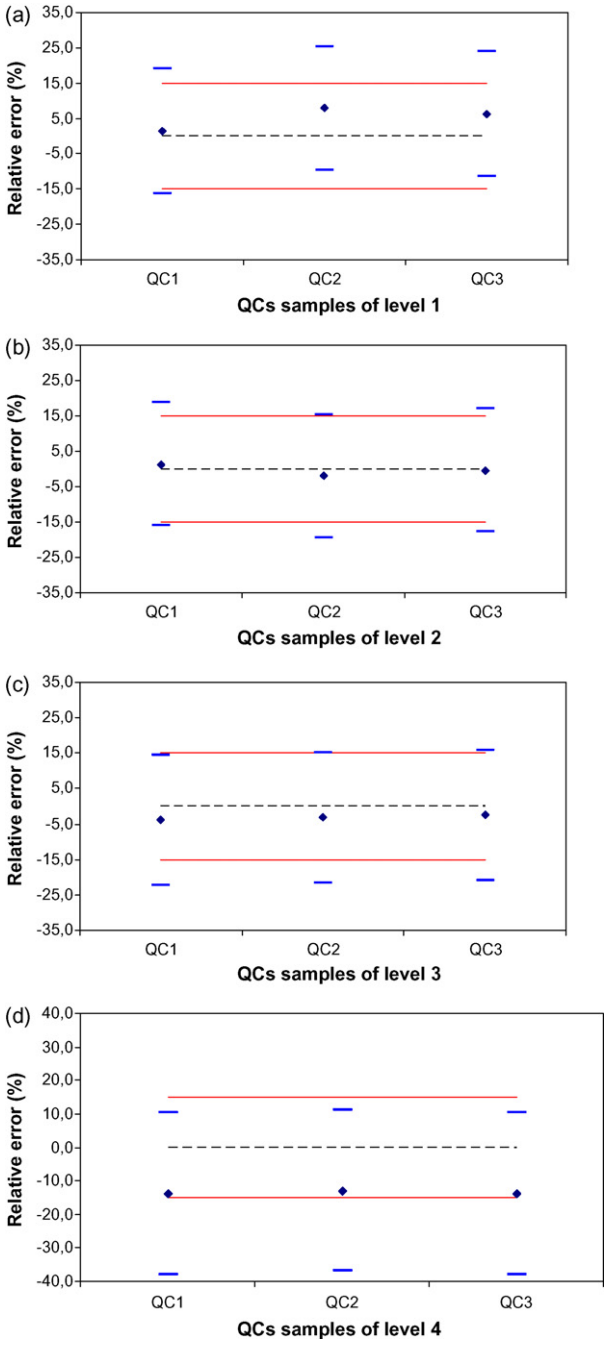

Fig. 6. Results of each in-study validation QC sample together with their respective relative expanded uncertainty (coverage factor $k = 2$) by concentration level: (A) level 1 = 42 ng/ml, (B) level 2 = 127 ng/ml, (C) level 3 = 4200 ng/ml, (D) level 4 = 19,000 ng/ml. The dots are the relative back-calculated concentrations, the continuous lines are the ±15% acceptance limits for the run; the upper and lower horizontal dashes represent the upper and lower relative expanded uncertainty limits. The dotted line is the 0% relative error line.

4. Conclusions

This paper describes the development as well as the pre-study and in-study validation of an UPLC–MS/MS method for the determination of oxytetracycline in nasal secretions of healthy pigs. Owing to the use of a sensitive and selective detection mode, sample preparation was simple and consisted in manual protein precipitation prior to the chromatographic separation.

An original strategy, based on total measurement error and accuracy profiles as a decision tool, allowed to demonstrate that the method was reliable for its intended use over a dosing range comprised between 42 and 19,000 ng/ml. The maximum risk of observing future measurements falling outside the acceptance limits during routine analysis was maximum 15% for the quantification of OTC in nasal secretions at the highest concentration level of the dosing range and around 8% for all other levels. All the validation results complied with the FDA recommendations. The measurements uncertainty was also estimated.

The method was then applied successfully for a pilot study on the fluctuations of the sensitivity of nasal bacteria to OTC administered by intramuscular injection to pigs at different doses (10, 20 and 40 mg/kg bodyweight). From the results obtained during the in-study validation, the method applicability was demonstrated, since all results of the QC samples were included in their respective β-expectation tolerance intervals.

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