FACULTEIT DIERGENEESKUNDE
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This project is funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through contract RF 14/6287 DOSERESIST.

Quantitative determination of florfenicol in pig manure by LC-MS/MS to assess the exposure of the gut microbiota to florfenicol

Joren De Smet, Siska Croubels, Patrick De Backer, Mathias Devreese
Department of Pharmacology, Toxicology and Biochemistry
Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; joren.desmet@ugent.be

Introduction

The current posology of veterinary antimicrobial drugs is mainly established solely on clinical efficacy, without taking resistance selection of pathogenic or commensal bacteria into account. Moreover, dosage regimens of antimicrobials in animal husbandry commonly show considerable variability, even between manufacturers. In order to assess the effect of different dosage regimens (administration route and dosage) on resistance selection in the intestinal commensal microbiota, data on the exposure are mandatory.

Florfenicol is a broad-spectrum antimicrobial, increasingly used in veterinary medicine. When administered in pigs, florfenicol has a complete oral and intramuscular bioavailability and it is mainly renally excreted, even though a significant part (up to 24%) is excreted via the faeces. This is interesting with regards to **exposure of the gut microbiota to the antimicrobial active molecule both after oral and intramuscular administration**.

Hence, the objective of this study was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to **quantify florfenicol in intestinal content of pigs**.

Materials & Methods

Sample pre-treatment

A rapid and effective sample pre-treatment (figure 1) was developed based on liquid-liquid extraction (LLE). One gram of faeces was homogenized and diluted tenfold in PBS. A 1.0 g subsample of this dilution was spiked with 25.0 µL internal standard solution (10.00 µg/mL florfenicol-d_3). For optimal extraction, 20.0 µL of 1M NaOH was also added (pH 10). After vortex mixing and equilibrating, 7.5 mL of EtOAc was added, followed by roller mixing and centrifugation. A clear supernatant was subjected to a nitrogen stream until dryness. The extract was reconstituted in a vial and an aliquot of 10.0 µL was injected onto the LC-MS/MS instrument.

Instrumentation and analysis

Liquid chromatography was performed using a Hypersil Gold™ column (Reversed Phase, 50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column (10mm x 2.1mm i.d., dp: 5µm). Mobile phases for chromatographic separation consisted of 0.1% acetic acid in H_2O (A) and ACN (B) following a gradient elution. Flow rate was set at 300 µL/min. This LC system was connected to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, with electrospray ionization in negative mode (Figure 2). Acquisition was performed in the selected reaction monitoring mode. For florfenicol and its internal standard, following transitions were followed (*quantification ion): m/z 356.00 > 185.00/336.00*, florfenicol-d_3: m/z 359.00 > 188.00, 339.00*.

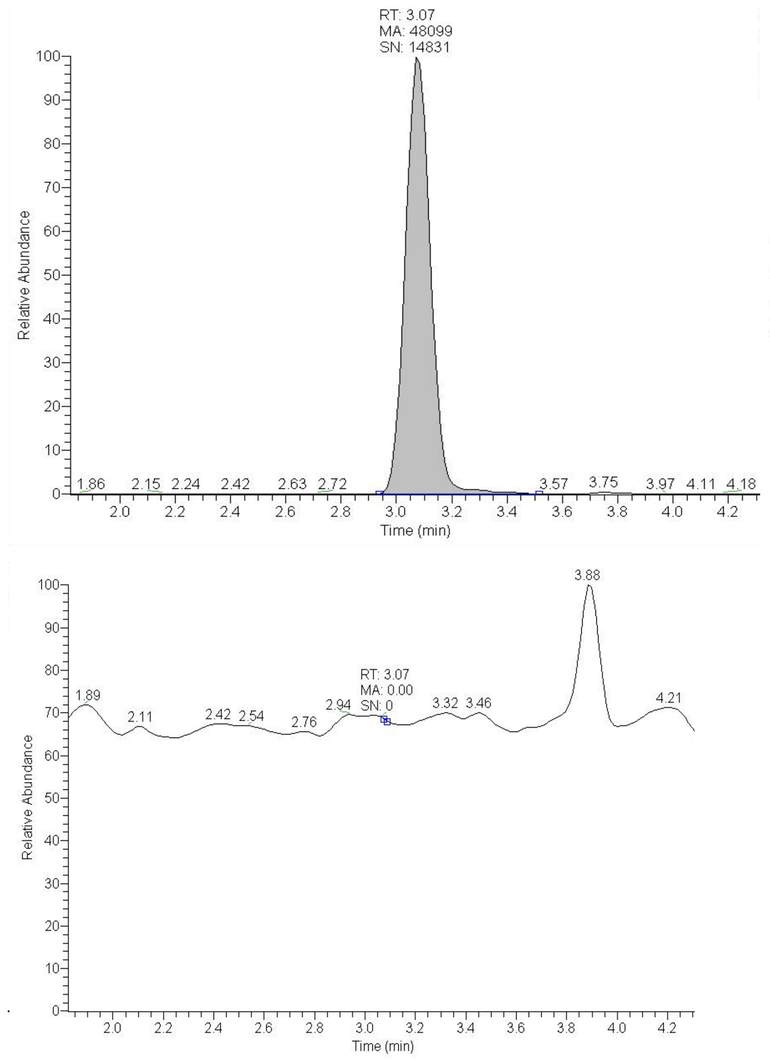

Figure 2. Chromatogram of a manure sample spiked at LOQ level ($1.00 \cdot 10^{-2}$ µg/g, top) versus a blank sample (bottom).

Table 1. Validation results for within- and between-run accuracy and precision.

Theoretical concentration (µg/g)	Mean concentration ± SD (µg/g)	Precision (RSD %)	Accuracy (%)
[a]$1.00 \cdot 10^{-2}$	$1.03 \cdot 10^{-2} \pm 1.23 \cdot 10^{-3}$	10.9	3.0
[a]$1.00 \cdot 10^{-1}$	$1.10 \cdot 10^{-1} \pm 4.88 \cdot 10^{-3}$	4.4	9.9
[a]1.00	$0.94 \pm 0.51 \cdot 10^{-1}$	5.4	-5.8
[b]$1.00 \cdot 10^{-2}$	$0.99 \cdot 10^{-2} \pm 1.01 \cdot 10^{-3}$	10.1	-1.3
[b]1.00	$0.90 \pm 0.58 \cdot 10^{-1}$	6.5	-9.9

[a] Between-run accuracy and precision (n=6) on three different days of analysis.
[b] Within-run accuracy and precision (n=6)
SD: standard deviation; RSD: relative standard deviation Acceptance criteria: accuracy: -20% to +10%, within-run precision (RSD_{max}): ≥ 10 ng/g < 100 ng/g: 15%; ≥ 100ng/g: 10%, between-run precision: $RSD_{max} = 2^{(1-0.5\log \text{concentration})}$

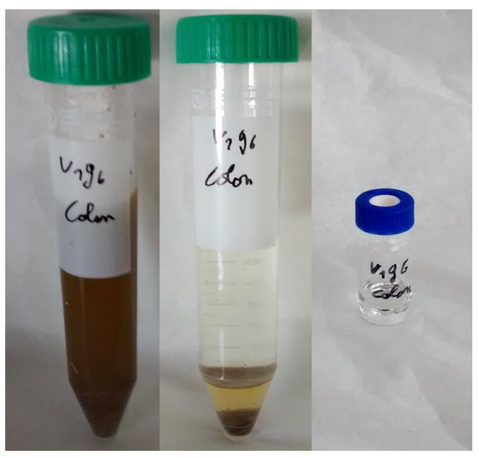

Figure 1. Sample pre-treatment from manure (left) to clear reconstitution (right).

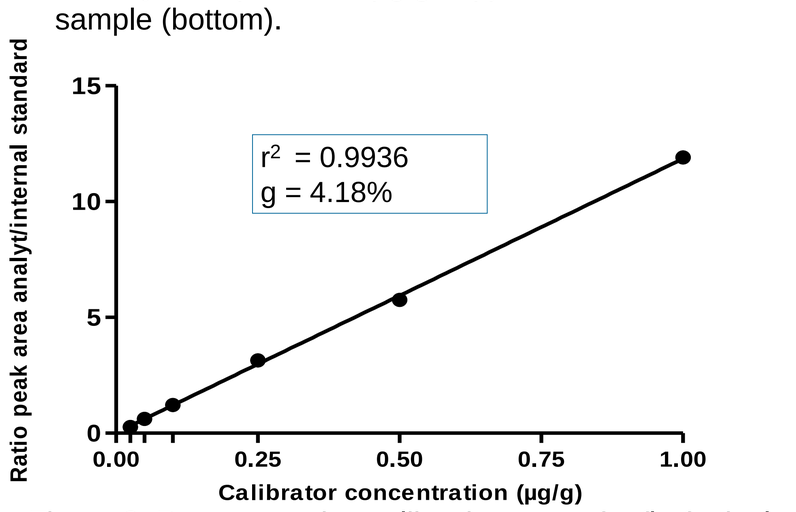

Figure 3. Representative calibration curve for florfenicol. Validation criteria for correlation coefficient (r^2) is > 0.99 and for goodness-of-fit (g) < 10%.

Results & Conclusion

All validation procedures were carried out compliant with European and international guidelines[1,2]. All crucial validation parameters were evaluated, namely linearity, accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), extraction recovery (R_E) and signal suppression and enhancement (SSE). A matrix-matched approach was used for calibration of the method in manure. The calibration curve for florfenicol ($1/x^2$ weighing) was linear over the working concentration range ($1.00 \cdot 10^{-2}$ – 1.00 µg/g). Linearity was evaluated based on correlation coefficient (r) and goodness-of-fit (g) as seen in figure 3. Next the LOQ level was determined at $1.00 \cdot 10^{-2}$ µg/g and the LOD at $2.9 \cdot 10^{-3}$ µg/g. Accuracy and precision were determined at different concentration levels (each n=6); namely the LOQ-level, an intermediate level and a high level; an overview is given in table 1.

In conclusion, a fast, accurate and precise method was developed for the quantitative determination of florfenicol in pig manure. This method will be further used to assess intestinal florfenicol concentrations in duodenum, jejunum, ileum, cecum, colon and rectum, after antimicrobial therapy in pigs. Determining the concentrations of the drug in intestinal samples is necessary with regards to assessing the exposure of the gut microbiota to florfenicol and evaluating the magnitude of possible antimicrobial resistance selection.

[1] *VICH GL49: Guidance for Industry: Studies to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs in Food-Producing Animals: Validation of Analytical Methods used in Residue Depletion Studies (Februari 2011)*
[2.] *2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044)*