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DETECTION AND QUANTIFICATION OF THE EU MARKER RESIDUE OF TIAMULIN IN ANIMAL TISSUES

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Introduction and Aim

Tiamulin is a diterpene antimicrobial agent with a pleuromutilin chemical structure, which is mainly active against Gram-positive micro-organisms and *Mycoplasma*. In rabbits, tiamulin is used for the treatment of Epizootic Rabbit Enteropathy. Maximum Residue Levels (MRLs) for rabbit tissues have been established by the European Union for the marker residue, i.e. the sum of metabolites that may be hydrolysed to 8-α-hydroxymutilin, at 100 µg kg^{-1} for muscle and 500 µg kg^{-1} for liver.[1]

In the literature, only one method has been reported for the determination of the marker residue of tiamulin in animal tissues. This method, based on gas chromatography (GC) with electron capture detection (ECD), was very time-consuming, resulting in a sample throughput of 3 to 5 samples per person per day.[2]

The **aim of this study** was to develop and validate an LC-MS/MS method for the quantitative determination of 8-α-hydroxymutilin in rabbit tissues.

Sample preparation

- ***Method A*, with alkaline hydrolysis** ⇒ to quantitate the sum of tiamulin and metabolites that may be hydrolysed to 8-α-hydroxymutilin in rabbit tissues
- ***Method B*, without alkaline hydrolysis** ⇒ to investigate the stability of 8-α-hydroxymutilin (in matrix, freeze-thaw stability)

1 g of minced tissue (muscle or liver)

+ 5 mL of 0.01 N HCl in water/aceton (50/50, v/v)
extraction, 20 min
centrifugation, 10 min, 4000 rpm

upper liquid phase

defatting using 5 mL of *n*-hexane

evaporation of 4 mL of lower phase (50 °C, N_2) to 2 mL

+ 3 mL of 7 N NaOH
vortex mixing

for calibrator and QC samples: spiking with 8-α-hydroxymutilin

alkaline hydrolysis, 45 °C, 20 min

+ 2 mL concentrated HCl
vortex mixing, cooling
+ 10 mL ethylacetate
extraction, 20 min
centrifugation, 10 min, 4000 rpm

evaporation of 9 mL of organic phase until dryness (45 °C, N_2)

+ 250 µL 0.1 % formic acid in water
vortex mixing
filtration using Millex® Nylon 0.22 µm syringe filter

in vial → LC-MS/MS analysis

HPLC conditions

- **HPLC**: Surveyor autosampler Plus and MS Pump Plus, autosampler t°: 5 °C (Thermo Scientific)
- **Column**: Hypersil Gold, 50 mm x 2.1 mm, dp: 1.9 µm, 50 °C + pre-column of the same type (Thermo Scientific)
- **Gradient elution:** see Table 1

Table 1. Gradient programme

Time (min)	MF A (%)	MF B (%)	Flow rate (µl/min)
0.0	75	25	300
4.3	75	25	300
5.0	10	90	300
7.3	10	90	300
8.0	75	25	300
12.0	75	25	300

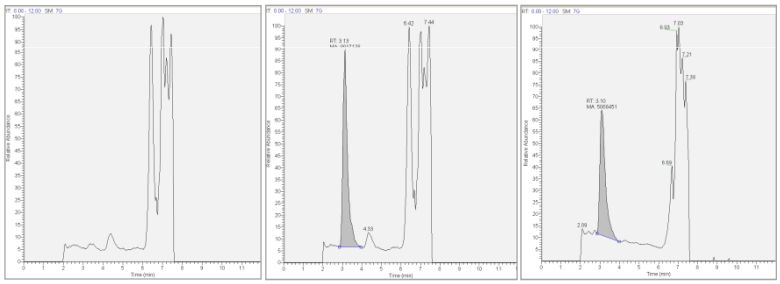
⇒ 8-α-hydroxymutilin eluted at retention time (Tr) of 3.10 min (see Figure 1B)

MS/MS conditions

- **Instrument:** TSQ Quantum Ultra® (Thermo Scientific)
- **Ionization mode**: positive electrospray ionization
- **Pseudo MS/MS**: 8-α-hydroxymutilin could not be fragmented and therefore the mass spectrometer was operated in the pseudo MS/MS mode (see Table 2)

Table 2. MS/MS conditions

Parent ion (m/z)	Product ion (m/z)	Scan width (sec)	Scan time (sec)	Collision energy (%)	Tube lens (V)
337.25	337.25	0.01	0.30	5	85



(A) (B) (C)

Figure 1. LC-MS/MS chromatogram of (A) a blank rabbit liver sample, (B) a blank rabbit liver sample spiked with 500 µg kg^{-1} 8-α-hydroxymutilin (= MRL level) and (C) a liver sample of a rabbit that received 16 mg tiamulin base/kg BW/day via the drinking water for 14 consecutive days and that was slaughtered at 1 h after withdrawal of the medicated drinking water (concentration of the marker residue 8-α-hydroxymutilin: 454 µg kg^{-1}).

Validation

Validation was performed in rabbit muscle and liver according to European Guidelines[3,4].

–**Linearity:** 50 – 2000 µg kg^{-1}, r ≥ 0.99, g ≤ 10 %

–**Accuracy:** muscle (50 – 100 – 200 µg kg^{-1}), liver (250 – 500 – 1000 µg kg^{-1}) → within -20 % to +10 % of theorethical concentration

–**Within-run precision:** muscle (50 – 100 – 200 µg kg^{-1}), liver (250 – 500 – 1000 µg kg^{-1}); relative standard deviation (RSD) < RSD_{max} = 15 % for conc. ≥ 10 µg kg^{-1} < 100 µg kg^{-1}; RSD_{max} = 10 % for conc. ≥ 100 µg kg^{-1}

–**Between-run precision:** muscle (50 – 100 – 200 µg kg^{-1}), liver (250 – 500 – 1000 µg kg^{-1}); relative standard deviation (RSD) < $RSD_{max} = 2^{(1-0.5\log C)}$

–**LOQ:** 50 µg kg^{-1} for both muscle and liver tissue

–**LOD:** S/N ≥ 3, ***method A***: muscle, 11.9 µg kg^{-1}; liver, 20.6 µg kg^{-1}; ***method B***: muscle, 27.8 µg kg^{-1}; liver, 21.9 µg kg^{-1}

–**Specificity:** no interferences from endogenous compounds (see Figure 1A)

–**Stability**:

- in acetonitrile during storage at ≤ - 15 °C (stock solution, at least 189 days) or at 2 – 8 °C (working solutions, at least 20 days)
- in matrix during storage at ≤ - 15 °C: at least 9 months
- in extract during storage at 2 – 8 °C: at least 1 day
- during three freeze-thaw cycles at ≤ - 15 °C

References. [1]Commission Regulation (EU) No 37/2010; [2]Markus J.R. and Sherma J., *J. AOAC Int.*, 1993, 76(2) 451 – 458; [3]EMEA/CVMP/573/00; [4]EMA/CVMP/VICH/463199/2009

Results and discussion

- **Sample preparation**:
  - extraction recovery: 66.2 % (muscle) – 75.5 % (liver); signal enhancement/suppression: 51.7 % (muscle) – 43.3 % (liver); apparent recovery: 34.2 % (muscle) – 32.5 % (liver)
  - up to 50 samples can be analysed per person and per day (= advantage compared to reported GC-ECD mehtod[2])
- **Alkaline hydrolysis**: conversion of tiamulin and related metabolites to 8-α-hydroxymutilin was optimal at a temperature of 45 °C and a hydrolysis time of 20 min
- **Quantitation**:
  - no suitable internal standard could be found and therefore the method of external standardization was applied
  - ⇒ importance of working with exact volumes during the sample preparation procedure
- **Method validation**: results fell within the ranges specified
- **Applicability of the method**: was demonstrated by the analysis of 8-α-hydroxymutilin in rabbit liver and muscle tissues that were taken during a residue study with tiamuline in rabbits after the administration via the drinking water (dose: 16 mg tiamulin base/kg BW/day, 14 consecutive days)

Conclusions

Developed LC-MS/MS method:

➢straightforward → up to 50 samples can be analysed per person per day

➢in-house validated

➢can be used for the analysis of 8-α-hydroxymutilin, the EU marker residue for tiamulin, in animal tissues