TIAMULIN HYDROGEN FUMARATE – VETERINARY USES AND HPLC METHOD OF DETERMINATION IN PREMIXES AND MEDICATED FEEDING STUFFS

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Approved antimicrobial substances were treated as feed additives and used also as growth promoters until 2006. Regulation No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition changed the situation and at the moment antibiotics in feeds, except coccidiostats and histomonostats, can be used only as medicated feedingstuff.

Medicated feedingstuffs are produced only on prescription from approved premixes in authorized manufacturing place. In agreement with EU and Polish legislation, both production process and usage of medicated feedingstuff has to be strictly monitored and controlled. The main aims of the control process are determination of active ingredient concentration, homogeneity of the product and accordance with GMP. Among antimicrobials mainly used in veterinary medicine are tetracyclines, amoxicillin, tylosin, tiamulin and sulfonamides.

Tiamulin is mainly used for swine and poultry but has also some application in cattle, goats and sheep treatment. Its antibacterial activity against Mycoplasma spp., Gram positive bacteria such as streptococci and staphylococci and obligate anaerobes is well known. Tiamulin is a drug of choice in treatment of swine dysentery caused by Brachyspira hyodeysentreiae and bacterial pneumonia caused by Pasteurella multica. It is also used in porcine intestinal adenomatosis (Lawsonia intracellularis), arthritis (Mycoplasma hyosynoviae, M. bovis) and chronic sinusitis (2–5).

The mechanism of tiamulin bacteriostatic activity, as well as other pleuromutilins, is based on blockage of protein synthesis in bacterial cell. Thanks to its strong affinity to 23 rRNA in 50S ribosomal subunit tiamulin joins to peptidyl transferase centre during initiation of translation, inhibiting peptide bond formation at this point of the process (6, 7). This antibiotic does not affect peptide binding when elongation has started.

Tiamulin resistance is rare but emerging and cross-resistance to tylosin and erythromycin occurs (2). It is probably due to mutations in ribosomal L3 protein as well as in 23sRNA itself, which leads to higher flexibility of peptidyl transferase centre region and ability to overcome tiamulin inhibition (6, 7).

Tiamulin is a base sparingly soluble in water more often used as hydrogen fumarate salt, what increases its solubility dramatically. Diterpenic core of the molecule consists of three rings: cyclo-pen-
tanone, cyclo-hexyl and cyclo-octane with side-chain attached to C14 (Fig. 1). Changes in tiamulin solubility in water, depending if it is a free base or salt, lay on the basis of extraction process for premixes and medicated feeds samples.

There are published methods for determination of tiamulin and its metabolites in various matrixes such as soil (8), liquid manure (9), tissues samples (10–13), honey (14), medicated feeds (5, 15) and premixes (16–18). Quantitative analysis use different techniques like thin layer (19) or liquid chromatography (5, 14, 16) as well as gas chromatography (20, 21), well established is also microbiological assay. The aim of this study was to optimize extraction procedure and HPLC determination method of tiamulin hydrogen fumarate in medicated feeding stuffs and premixes available on Polish market.

EXPERIMENTAL

Chemicals and reagents
Tiamulin hydrogen fumarate stock solution 1.0 mg/mL – accurately weighed 10.0 mg of tiamulin hydrogen fumarate analytical standard (Sigma) was dissolved and diluted to 10 mL with 0.1% L-tartaric acid (Sigma) water solution.

Working standard solution – all needed concentration of tiamulin standards were prepared by dilution of stock solution with 0.1% tartaric acid water solution.

Reagents: Acetonitrile (HPLC grade, POCH), methanol (HPLC grade, POCH), ethyl acetate (POCH), hexane (J.T. Baker), water (HPLC grade), ammonium carbonate (Sigma), sodium carbonate anhydrous (Fluka), nitrogen 5.0 (Messer) were used in the course of the study.

Samples of commercially available premix with 10% tiamulin content were used for all experiments. Commercial medicated feedingstuff and spiked samples prepared in Department of Veterinary Pharmacy served for method verification purposes.

Equipment
Agilent 1200 series chromatographic LC system equipped with binary pump (G1322A), degasser (D1379A), autosampler (G1329A), thermostated column compartment (G1316A) and VWD detector (G1314D) controlled by Chemstation software was used. LC system was equipped with C18 Gemini NX 5 µm, 4.6 × 150 mm column and precolumn with the same stationary phase (Phenomenex). Column temperature was maintained at 25°C.

Analytical balance (Competence CP323P-OCE, Sartorius), grinding mill (ZM200, Retsch), orbital shaker (KS501digital, IKA), vortex (TTS2, IKA), centrifuge (MPW-6K15, MPW Med Instruments) and block heater (Reacti-Therm III, Pierce) were used for extraction procedure.

Premix sample extraction
Twenty five mL of HPLC grade water was added to 500 mg premix sample which was then extracted by vortexing for 1 min followed by 15 min/250 rpm on orbital shaker. Centrifugation was then applied and 20 mL of supernatant transferred to 100 mL volumetric flask. Extraction step was repeated in the same conditions and both extracts were pooled together and diluted to volume with HPLC grade water. Further dilution of obtained solution was performed if necessary.

Medicated feeding stuff sample extraction
Sample of 2.0 g of tested medicated feedingstuff was transferred to Erlenmeyer flask and 30 mL of 1% sodium carbonate water solution was added followed by 30 mL of hexane: ethyl acetate mixture. Sample was extracted for 15 min/250 rpm on orbital shaker and then left for separation of phases. Around 40 mL of liquid was poured to test tube and centrifuged for 10 min/2000 × g. 20 mL of upper organic phase was then brought to dryness and residue dissolved in 0.1% tartaric acid solution. The same procedure was used for blank and spiked samples preparation.

RESULTS AND DISCUSSION

LC conditions
Optimization of chromatographic conditions included mobile phase composition and pH, sample solvent and injection volume as well as wavelength of detection. Methanol : acetonitrile : 1% ammonium carbonate was chosen as mobile phase and different ratios of acetonitrile to ammonium carbonate solution were tested. Also the effect of buffers pH was checked in range of 8.6 – 9.9. The best peak shape
and retention time was obtained while using 50:25:25 mixture of solvents mentioned above and pH of ammonium carbonate around 9.1. This allows shortening the time of mobile phase preparation, as there is no need of pH adjustment, and time of analysis as the retention time of tiamulin is around 8 minutes.

Sample solvents such as methanol, 0.1% tartaric acid solution, water, 50:50 acetonitrile: ammonium carbonate buffer mixture and mobile phase were analyzed. No significant differences were noted for most of mentioned solvents except of methanol which caused broadening of the peak. Water for premix samples and 0.1% tartaric acid for standard and feeding stuffs samples were finally chosen due to extraction procedures.

Also different detection wavelengths were tested. The highest response and best peak shape gave analysis with detection at $\lambda = 208$ nm, where tiamulin shows maximum of absorption (Fig. 2). Twenty µL injection volume was firstly used but it was concluded that 50 µL injection increase sensitivity of the method.

**Sample preparation**

Extraction procedures for both medicated premixes and feedingstuffs samples were optimized as well. As it was already mentioned, tiamulin hydrogen fumarate is freely soluble in water while in the form of premix so it was decided to use method published by Cancho Grande et al. (16) with water as an extraction solvent. Different sample size and single or duplicate extraction cycle procedures were tested. It was found that double extraction gives better recoveries on the level of 93–99% and variation coefficient below 2%. Sample size does not have big influence on recoveries or repeatability of results so it was decided to use 500 mg sample to be able to determine tiamulin content in premixes with different label concentration in range of 2–45%.

Feedingstuff is more complex matrix and extraction approach is also different than one presented for premixes. Various parameters were tested. No significant changes in recovery or repeatability in correlation to time of extraction were noted and 15 min procedure was selected to shorten sample preparation step. Sample size to solvents volume ratio was basic for efficiency of extraction process. Extraction of 2.0 g sample with 30 mL of 1% sodium carbonate solution and hexane: ethyl acetate mixture gave satisfying results. Furthermore liquid-liquid extraction of organic phase versus drying under nitrogen flow was tested. Both methods gave similar result but the latter one is simpler and quicker so it was used in the proceedings.

**Method characterization**

Identity of tiamulin was confirmed by comparison of chromatograms of tiamulin hydrogen fumarate standard solution and those of sample extracts. Analysis of blank and reagent samples as well as solvents used allows to conclude that proposed method is specific as no interfering peaks were observed. Also precision in the means of repeatability and reproducibility was tested. Results obtained for 5 separate preparations of the same premix sample returned value of 93% recovery with coefficient of variation CV% = 1.5%. Series to series reproducibility for 3 series of samples (9 samples altogether) was calculated as CV% = 1.5%.

Figure 3 depicts peak area in correlation to theoretical concentration of premix extract which was calculated on basis of extraction procedure, sample volume and repeatability.

![Figure 2. Effect of wavelength changes on detector response](image-url)
weight and label concentration of premix. Correlation coefficient of $R^2 = 0.9999$ shows that method is linear in range of concentrations 0.02 – 0.9 mg/mL.

**CONCLUSIONS**

In the course of the study sample extraction process and analytical method of determination of tiamulin hydrogen fumarate concentration in premixes and medicated feedingstuff was optimized. It can be be concluded that presented method is specific, precise and linear at the tested range of concentrations as well as quick and relatively inexpensive. It can be also suggested that after further verification, with representative number of commercial samples, and validation it can be used as routine method for tiamulin determination in the above drug formulations.

**REFERENCES**