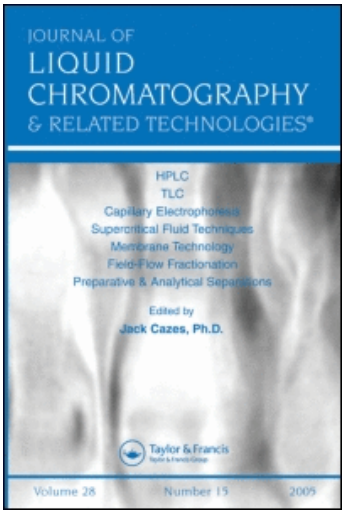

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Determination of Tiamulin in Chickens' Plasma by HPLC with UV-VIS Detection

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Abstract: A rapid, accurate, simple and reproducible high performance liquid chromatographic (HPLC) method for determination of tiamulin in chicken's plasma has been developed and validated. The drug and the standard were eluted from 5 μm X-Terra® RP_{18} (Waters) column (150 × 4.6 mm) at room temperature. The mobile phase was composed of 4 M KH_2PO_4 – acetonitrile (65:35 v/v) (pH adjusted to 2.8). A flow rate was 1.5 mL/min. The effluent was monitored using a UV-VIS detector set at 208 nm. The retention time of tiamulin was about 4.5 min. The suggested technique was characterized by superior performance parameters: linearity $R^2 = 0.9999$, recovery = 83.50%, repeatability RSD ≤ 2.7%. These results demonstrate the validity of the HPLC method for the analysis of tiamulin. This could be a useful tool in the pharmacokinetics studies of tiamulin in animals.

Keywords: HPLC determination, Plasma, Tiamulin, UV-VIS detection

INTRODUCTION

Tiamulin (TIA) is a bacteriostatic agent which belongs to the pleuromutilin group of antibiotics.[1,2] Pleuromutilin was first isolated in 1951 from two

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basidiomycetes species, *Pleurotus mutilus* and *P. passeckerianus*.[2] Tiamulin acts by inhibiting protein synthesis at the ribosomal level. It is active mainly against Gram-positive microorganisms, as well as leptospiras and mycoplasmas.[1–3] Tiamulin is only used in veterinary medicine mainly for treatment of dysentery, pneumonia, and some other pulmonary and gastrointestinal infections in pigs and poultry. There is very little information about the pharmacokinetics of tiamulin in target animals, especially in chickens.[4,5] All investigations were performed many years ago, using microbiological assays. Bioassays, commonly lack sensitivity and specificity, while chromatographic methods are generally preferred for their greater selectivity and simplicity. In literature there is no data about using chromatographic techniques for investigation of pharmacokinetics properties of tiamulin in animals. Several HPLC methods for the determination of tiamulin in various matrices have been described,[6–13] but none of them were adequate for the pharmacokinetics studies. Therefore, the aim of this paper was to work out an HPLC assay for determination of tiamulin in chickens' plasma after administration of therapeutic doses.

EXPERIMENTAL

Animals

The study was conducted on 74 healthy broiler chickens of both sexes, 8–10 weeks of age, and with the initial weight of 1680–2300 g. All chickens were obtained from a poultry breeding farm. The birds were housed in pens. Before the study chickens were marked with numbers. Feed (antibiotic and coccidiostatic free commercial diet) and water were given *ad libitum* throughout the period of study. TIA was administrated individually at the same dose, recommended by producers of veterinary formulations, 40 mg/kg body weight through a stomach tube.

Local Ethical Commission of the University of Life Sciences approved all procedures involved in the study. The birds were randomly allotted to three groups. Group A and B were used to investigate the pharmacokinetics parameters after oral administration of tiamulin. The chickens from group A ($n = 35$) received veterinary formulation "Tiamulina 10%". Chickens from group B ($n = 35$) received veterinary formulation "Tiamutin 12.5%". The last group, group C ($n = 4$), was used to obtained control plasma. TIA was administrated at the same dose, 40 mg/kg body weight, individually by gavage. Blood samples were taken from the brachial veins of each chicken into heparinised tubes at 1, 1.5, 2, 3, 4, 6, and 8 hours after administration of the drugs. The plasma was separated and stored at $-80°C$ until the day of analysis by HPLC.

Chemicals and Reagents

The drugs used in the study: "Tiamulina 10%" (Chemifarma, Italy) and "Tiamutin 12.5%" (Novartis, Switzerland) were purchased from pharmaceutical companies. The HPLC grade acetonitrile and hexane were purchased from POCH Chemical Company (Gliwice, Poland). The HPLC grade ethyl acetate was purchased from Merck (Germany). Other chemicals were analytical grade (Na_2HPO_4 – disodium hydrogen phosphate, H_3PO_4 – phosphate acid, Na_2CO_3 – sodium carbonate, $C_4H_6O_6$ – tartaric acid) and were obtained from POCH. An analytical standard Vetranal® (Tiamulin fumarate, 250 mg) was purchased from Sigma-Aldrich (Germany). Water was purified by the reverse osmosis method with Milli-Q-Plus 185 system (Millipore, Molsheim, France).

Apparatus

The chromatographic system used was a Varian liquid chromatograph (Varian, Palo Alto, USA). It consisted of a solvent delivery pump (STAR 9002), a 10 μL volume manual injector and a variable wavelength UV-VIS detector (all Varian Analytical Instruments, USA). Chromatographic separations were performed using a Waters X-Terra® RP_{18}, 5 μm, (4.6 × 150 mm) column (Waters, USA). The chromatographic data were collected and processed with the Varian Star Chromatography Workstation Version 4.51 (Varian, Palo Alto, USA) software installed on an IBM-PC Pentium computer.

Chromatographic Conditions

For HPLC analysis of TIA, the solution (10 μL) was injected into the column and eluted at room temperature under isocratic conditions with a 65:35 (v/v) mixture of 4 M KH_2PO_4 and acetonitrile (pH = 2,8) as a mobile phase. The mobile phase at a flow rate was constant at 1.5 mL/min, and a column effluent was monitored at a wavelength of 208 nm. All analyses were performed in an ambient temperature. The retention time of tiamulin under these conditions was approximately 4.5 min.

Extraction Procedure

Frozen plasma samples were thawed to room temperature prior extraction. In the liquid-liquid extraction (LLE) procedure, the tiamulin was extracted into an organic solvent mixture and then concentrated by back

extraction into an aqueous tartaric solution. A sample of 0.5 mL of test plasma was transferred into a vial and mixed with 2.5 mL 1% aqueous solution sodium carbonate and then extracted with 2,5 hexane-ethyl acetate (3/1 v/v). Extraction was performed by shaking for 10 minutes with an up and down shaker and phases were separated by centrifugation for 20 min at 4000 rpm. The upper supernatant layer (1 mL) was centrifuged once more for 20 min at 4000 rpm; 1 mL of upper supernatant (organic) was carefully transferred into the second vial and the extract was evaporated almost to dry under a stream of nitrogen. The residue was redissolved into 0.1% (w/v) buffered aqueous solution tartaric acid (0.3 mL) by ultrasound and acetonitrile. The solution of 10 μL was injected into the HPLC system. For the recovery study, a standard solution of tiamulin was added to blank pig's plasma.

Preparation of Standard Solutions

A stock solution (3600 ng/mL) of tiamulin was prepared by dissolving 36 mg of the compound in 1000 mL of distilled water. Working standard solutions ranging from 360 to 2880 ng/mL were prepared by appropriate dilution of the stock solution with distilled water.

RESULTS AND DISCUSSION

Literature contains little information about tiamulin per se, and its determination in biological matrices. According to our best knowledge, there is no publishing data about using HPLC for determination of tiamulin in plasma after administration in therapeutic doses. Until this time, pharmacological investigation of the kinetics of tiamulin in animals with the use of microbiological techniques was conducted.[5,10,14–16] Tiamulin in medicated formulations was determined by HPLC-UV and HPLC with diode array detection,[6,9–11,13] and TLC.[8] In premixes, tiamulin was determined by HPLC-DAD.[6] To determine tiamulin in eggs and honey, the same investigators used LC/MS.[7,12] In all these cases, tiamulin was found in much higher concentrations than that which consisted in the plasma of medicated animals.

The routine analytical method for monitoring levels of tiamulin in poultries' and pigs' tissues was gas chromatography with electrochemical detection. Metabolites of tiamulin in animals' tissues were hydrolyzed to a α-hydroxymutilin and measured as 8-α-hydroxymutilin equivalents. The sum of residues that can be hydrolyzed to form 8-α-hydroxymutilin were identified as the marker of tiamulin residue in tissues. For this

reason this method couldn't be used in pharmacokinetics studies when we have to determine the parent compound.

We were looking for a simple, effective, timesaving, and precise HPLC method to determine tiamulin in chickens' plasma after its administration in therapeutic doses. The development method was validated by the determination of the following parameters: linearity, precision and accuracy, limit of detection (LOD), limit of quantification (LOQ), stability, and recovery.

Linearity

The linearity of the method was evaluated by constructing the calibration curve. Six standard solutions of tiamulin in the range of 360–3600 ng/mL were prepared and then injected (10 μL, in triplicate) in order to construct a 6 point calibration curve (peak area vs. concentration). Before injection, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. The equation for the calibration curve was $y = (1.608 \pm 0.009) \times -(14 \pm 20)$ and the correlation coefficient (R^2) was 0.9999.

The plot is shown in Figure 1.

Precision/Accuracy

The precision of the method was determined by calculating the relative standard deviation (RSD %) for the repeated measurements and the accuracy as the standard deviation (SD) between nominal and measured concentrations. Five drug free plasma samples (1.0 mL) were spiked with

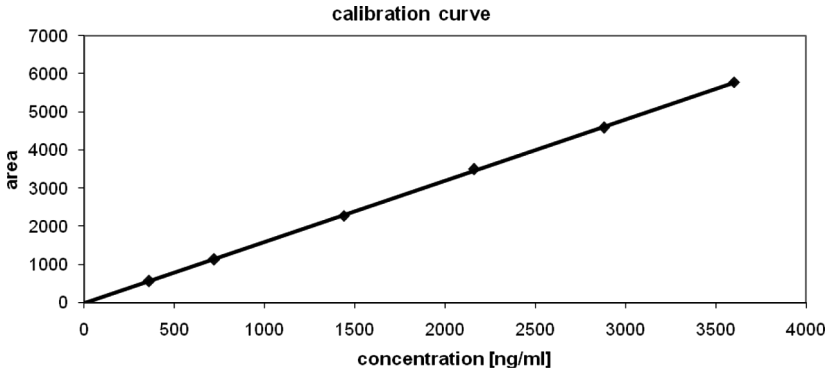

Figure 1. A calibration curve of tiamulin.

the tiamulin standard to 1000 ng/mL. All samples were processed according to the procedure described in the section Extraction Procedure. The area of a peak corresponding to an initial amount of TIA (S_p) in the spiked sample was calculated according to the equation:

$$S_p = \frac{2.5 \cdot S}{0.835}$$

where S is a peak of TIA obtained after LLE, 0.835 is the recovery, and 2.5 is a stoichiometric factor. Sp was next used in the determination of the TIA amount from the calibration curve. The equation of the curve was $y = (1.608 \pm 0.009) \times -(14 \pm 20)$. The calculated SD was 26.6 ng/mL and relative RSD was 2.7%.

Recovery

The recovery of the LLE procedure was assessed by analyzing the extract of pigs' plasma samples containing tiamulin (spiked at 1000 ng/mL). Six replicate extractions were assayed. Three additional unfortified control blank plasma samples were analyzed to determine the potential for chromatographic interferences. The recovery from the tested plasma was calculated according to the equation:

$$\text{Recovery}[\%] = \frac{2.5S}{S_{\text{std}}} \cdot 100\%$$

where S is a peak area of OTC obtained from the analysis of a spiked plasma samples (1000 ng/mL), $S_{std} = 1575$ is a peak area for the standard (1000 ng/mL), and 2.5 is a stoichiometric factor. The calculated TIA recovery was 83.5%.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The lowest absolute concentration of analyte in the sample that can be detected, but not necessarily quantified (LOD), and the lowest concentration of analyte in the sample that can be determined with acceptable precision and accuracy (LOQ) were calculated.

The LOD ($LOD = 3 \cdot S_{xy}/\text{slope}$, where S_{xy} is a standard deviation) was 47.5 ng/mL and the LOQ ($LOQ = 10 \cdot S_{xy}/\text{slope}$) was 158 ng/mL.

For tiamulin in plasma the standard curves were linear in the investigated areas from 360 to 3600 ng/mL. The correlation coefficient (R^2) was 0.9999. The high value of the coefficient indicated good linearity

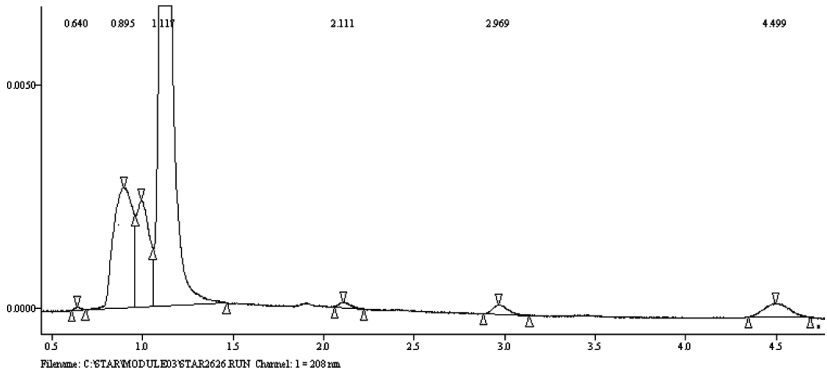

Figure 2. Chromatogram of TIA standard (water solution); retention time – 4.499.

of the calibration curve for the method in the considered concentration range. The chromatogram of TIA standard is shown in Figure 2. The chromatogram obtained from the plasma sample of the bird, which received tiamulin orally is shown in Figure 3. The method presented in this paper was fully validated. It is selective and sensitive enough for the determination of TIA in plasma samples after administration in therapeutic doses and showed good linearity. This makes it valuable and adequate in many applications, particularly in veterinary medicine studies. The described assay offers a number of significant advantages compared to previously published chromatography methods for detection and quantification of TIA in various matrices. In all previously described techniques,[6–13] the sample sizes used for the extraction procedure (from

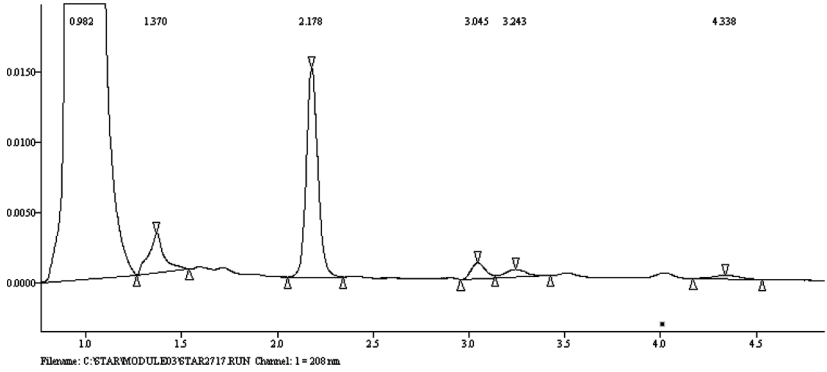

Figure 3. Chromatogram of plasma sample taken from a chicken one hour after administration of "Tiamulina 10%"; retention time –4.338.

10 g to 5000 g) was too big for pharmacokinetics studies in birds. We greatly reduced the sample size (to about 0.5 mg) and the extraction volume makes our method adequate for these investigations and also more cost effective. It also reduces waste generated by the method. The extraction procedure is quite simple and no derivatization is required.

CONCLUSIONS

Results demonstrate that the suggested technique is characterized by satisfactory performance parameters: linearity $R^2 = 0.9999$, recovery = 83.5%, repeatability RSD $\leq$ 2.7%. In conclusion, the HPLC method described in this paper is simple, sensitive, reproducible, and able to treat numerous samples in a relatively short period of time. Furthermore, the assay is applicable to pharmacokinetics studies of TIA in chickens.

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