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Research article

DEVELOPMENT AND VALIDATION OF AN IMPROVED HPLC-UV METHOD FOR THE DETERMINATION OF TILDIPIROSIN IN HORSE PLASMA

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A simple, rapid, low-cost, and sensitive high-performance liquid chromatographic method was developed to determine tildipirosin in horse plasma. Plasma samples were extracted with diethyl ether, and after evaporation, tildipirosin was determined by reverse-phase chromatography with an ultraviolet detector set at a wavelength of 289 nm. Tildipirosin was separated on a Zorbax Eclipse XDB-C18 column, 150 x 3.0 mm, 5 µm with gradient chromatographic elution. The retention times were 3.0 min and 6.4 min for tildipirosin and tylosin tartrate, respectively. The total run time was 9 minutes in this method.

Calibration curves ranged from 0.1 to 3 µg/mL. The lower limit of detection for plasma was 0.035 µg/mL, and the lower limit of quantitation was 0.1 µg/mL. Both accuracy and precision were always < 12% except for LLOQ < 20%. Mean recovery was 99.5 %. This procedure can be applied to determine tildipirosin concentrations in plasma and be useful to perform pharmacokinetic studies.

Key words: horses, HPLC, plasma, Tildipirosin, validation

INTRODUCTION

Antibiotic resistance has become a serious global problem and is steadily increasing worldwide in almost every bacterial species treated with antibiotics [1]. Abusive prescription of antibiotics, their inappropriate use by patients, and overuse of these substances in the food industry contribute to this [2,3]. However, veterinarians and doctors apply these drugs and provide effective treatments without further spreading of resistance. To combat this global problem the World Health Organization (WHO) established the concept "One Health," where antimicrobial resistance is an ecological

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problem that is characterized by complex interactions involving diverse microbial populations affecting humans and animals' health, as well as the environment [4].

Tildipirosin (TD) is a semi-synthetic tylosin analog with a unique chemical structure characterized by two piperidine substituents on C20 and C23 and a basic mycaminose sugar moiety at C5 of the macrocyclic lactone ring. Owing to three nitrogen atoms accessible to protonation, TD is a tribasic molecule (Figure 1). This antibiotic is exclusively used in veterinary practice, and it has been approved for parenteral treatment of respiratory disease in cattle and swine [1,5]. Moreover, it shows favorable pharmacokinetic properties such as high apparent volume of distribution, high bioavailabilities after extravascular administration, and a long half-life [6-10]. The macrolide analysis in biological fluids is a pre-requisite step for dose optimization of TD therapy based on specific pharmacokinetic information in veterinary medicine is needed before use in other species. For these analyses, tylosin tartrate, which is a mixture of four compounds tylosin A, tylosin B, tylosin C and tylosin D [11], can be used as internal standard (IS) due to similar TD structure (Figure 1).

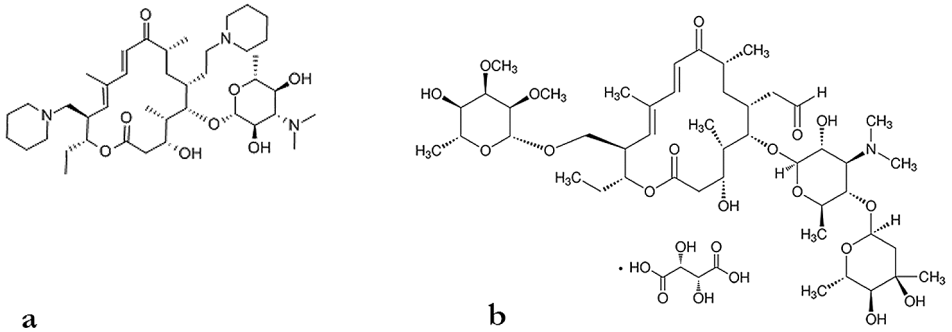

Figure 1. Chemical structure of tildipirosin **(a)** and internal standard tylosin A **(b)**

One analytical method has been reported for measuring TD in biological bovine fluids (plasma, bronchial fluid, and lung tissue). Actually, this method involves sample preparation using solid-phase extraction (SPE) and separation and detection of TD using high-performance liquid chromatography (HPLC) coupled with mass spectrometry (LC/MS/MS) [6]. Several authors have described the pharmacokinetics of this macrolide in different animal species using similar analytical methods [7,12-13]. Although these methods provide high sensitivity with a low limit of quantification values, they require sample preparation by SPE, which is relatively expensive due to the high number of samples to be analyzed in pharmacokinetic studies. Besides, sample preparation and detection require sophisticated equipment LC/MS/MS, which is not commonly available in most laboratories. Up to date, some specific HPLC methods with ultraviolet detection for TD determination in plasma samples have been reported [14-16], but none have been completely validated and detailed according to FDA guidelines [17]. Validated analytical methods for the quantitative evaluation of drugs

in each biological matrix (e.g., blood, plasma, serum, or urine) are essential to conduct pharmacokinetic studies successfully. These validated methods provide critical data to enable the safety and effectiveness of drugs.

In the near future, the pharmacokinetics of this drug will probably be widely studied in different species. Thus, it is of interest to propose a reference method of determination and quantification of this macrolide in different tissue/fluid samples. Therefore, the present study aimed to establish a simple, sensitive, and rapid HPLC method with UV detection to quantify TD in horse plasma samples.

MATERIAL AND METHODS

Chemicals, solvents, and reagents

Tildipirosin was obtained from Cymit Química (Barcelona, Spain), and tylosin tartrate (92.5% of Tylosin A, which was used as internal standard) (IS) was purchased by Merck Life Science (Madrid, Spain). All solvents were of HPLC analytical grade. Acetonitrile (ACN), ethanol (96%), formic acid (98-100%) and water were purchased by Merck Life Science (Madrid, Spain). Potassium di-hydrogen phosphate 99%, di-potassium hydrogen phosphate 99%, diethyl ether 99.7% and sodium hydroxide pearls pure 98 % was supplied by PanReac AppliChem (Barcelona, Spain).

Instrumentation

The LC system consisted of an Agilent series 1220 Infinity (Agilent Technologies Spain, Madrid, Spain) with a dual gradient pump, a manual injector, a thermostatic column compartment, and a variable wavelength detector, all modules belonging to the 1220 series. The system mentioned above was connected to a Gilson 234 Autoinjector for HPLC systems (Gilson Incorporated, Middleton WI, USA). The chromatograms were recorded using Open Lab ChemStation software for the LC system (version A.01.05, Agilent, Spain).

Chromatographic conditions

Chromatographic separation was achieved using a Zorbax Eclipse XDB-C18 column (150 x 3.0 mm, 5 μm) from Agilent (Madrid, Spain) with an Eclipse XDB C18 (4.6 x 12.5 mm x 5 μm) Agilent (Madrid, Spain) guard column. The column temperature was fixed at 30ºC. The mobile phase consisted of 0.3% formic acid in water (solvent A) and acetonitrile (ACN) (solvent B). The gradient program was (minute/A%B%): 0/92:8, 7/50:50, 8-10/92:8, with a constant flow of 1.0 mL/min. The ultraviolet detection was set at 289 nm and 50 μl were injected to HPLC system. The total run time in this method was 9 minutes.

Standard solutions

Stock solutions of TD and IS were prepared at the concentration of 100 μg/mL. For TD, 10.14 mg of this macrolide were dissolved in 2 mL of ethanol (96%), and a solution of phosphate buffer 0.2 M (pH=7.4) was added into a 100 mL volumetric flask. The IS solution was prepared by the dilution of 14.31 mg of tylosin tartrate in 100 mL water for HPLC. The stock solutions were further diluted with water for HPLC to prepare working solutions. Working solutions of TD and IS in water were freshly prepared every week and refrigerated at 4°C. The concentrations of working solutions of TD in water were: 2, 3, 6, 9, 14, 26, 32, 40, 50, and 60 μg/mL.

Preparation of calibration curve and quality controls

Calibration curve (CC) and quality control (QC) samples were prepared with blank plasma spiked with an appropriate amount of TD working solution. After mixing, 40 μL of IS were added. For CC, concentrations 0.1, 0.15, 0.3, 0.45, 0.7, 0.9, 1.3, 1.6, 2, 2.5 and 3 μg/mL were used to compose the linear range. Plasma QC levels used for this validation were 0.1, 0.3, 1.3, and 3 μg/mL.

The horse plasma was obtained from seven clinical healthy horses without drug administration records in the last 60 days. Blood samples were collected from the jugular vein into heparinized tubes and centrifuged at 600 g for 10 min, the obtained plasma was transferred to polypropylene tubes. Finally, the plasma was stored at -40 ºC until it was used.

Sample preparation

40 μL of IS solution (100 μg/mL) was added to 500 μL of plasma. After mixing, 1000 μL diethyl ether and 100 μL of NaOH 1M were added. Further, this solution was homogenized in a vortex for 1 min, followed by centrifugation for 10 min at 6000 g. The organic phase was extracted (550 μL) and transferred to another polypropylene tube and evaporated for 30 m at room temperature (20°C) in a SpeedVac Vacuum Concentrators (Fisher Scientific, Madrid, Spain). The residue was reconstituted with 75 μL of the mobile phase, and 50 μL was injected into the HPLC system.

METHOD VALIDATION

Method validation for horse plasma was performed according to FDA guidelines for bioanalytical method validation [17]. The following parameters were evaluated: linearity, lower limit of detection (LLOD), lower limit of quantitation (LLOQ), accuracy, precision, recovery, selectivity and carry over.

Linearity, detection, and quantitation limits

The linearity of the proposed chromatographic method was examined by analyzing a series of eleven concentrations of TD plus IS in horse plasma. Calibration curves were obtained by plotting the ratio peak-area compound/IS versus known nominal concentrations of TD. Three replicates of each level were assayed. The lower limit of detection of TD was stablished as the concentration that provides a signal-to-noise ratio ≥ 3. The lower limit of quantitation was accepted as the lowest concentration on the calibration curve that can be determined with acceptable precision (coefficient of variation % ≤ 20).

Intraday, inter-day precision and accuracy

The accuracy and precision of the method were estimated by measuring samples spiked with IS at four different concentrations: QC LLOQ, QC low, QC medium and QC high. Intra-day precision and accuracy were calculated on a single day using five replicates at each concentration level. Inter-day, precision and accuracy were evaluated using five replicates at each concentration level, over three consecutive days. The accuracy was calculated with the following expression: ((measured concentration - nominal concentration)/ nominal concentration) x 100. The accuracy results should be $\pm$ 15% of nominal concentration, except $\pm$ 20 % at LLOQ. Regarding the precision values, results should be $\leq$ 15% CV (variation coefficient) and $\leq$ 20% for LLOQ.

Recovery

Recovery tests were analyzed at three concentrations (low, medium, and high QCs). Five samples were assayed at each concentration level. Recovery (%) was calculated as: (mean peak area extracted plasma samples / mean peak area extracted blank plasma spiked with the analyte post-extraction) $\times$ 100.

Selectivity and carry over

The selectivity of this method was studied by analyzing six samples of drug-free plasma, free of interference at the retention times of TD and the IS. Potential injection carryover effects were excluded by analyzing blank samples (n = 6) of plasma directly after injection of a set of samples containing a high TD concentration. The carryover effects should not exceed 20% of LLOQ.

RESULTS

Linearity, detection, and quantitation limits for plasma

The peaks corresponding to TD and Tylosin A were obtained at 3.0 min and 6.4 min, respectively. Small peaks near Tylosin A are due to Tylosin B, C, D although these peaks were neither identified nor quantified (Figure 2). Calibration curves were plotted

by the area ratio of TD/ IS versus the nominal concentration of TD μg/mL. The concentration range was linear from 0.1 to 3 μg/mL. The linear regression equation of TD in plasma was y=1.1287 (±0.000) x – 0.03568 (±0.012), with a regression coefficient values of r^2 = 0.9986. Finally, the LLOQ and LOD were 0.1 μg/mL and 0.035 μg/mL, respectively. The results are shown in Table 1. These values indicate that the proposed method is suitable and has adequate sensitivity for determining TD concentrations in plasma by HPLC with ultraviolet detection.

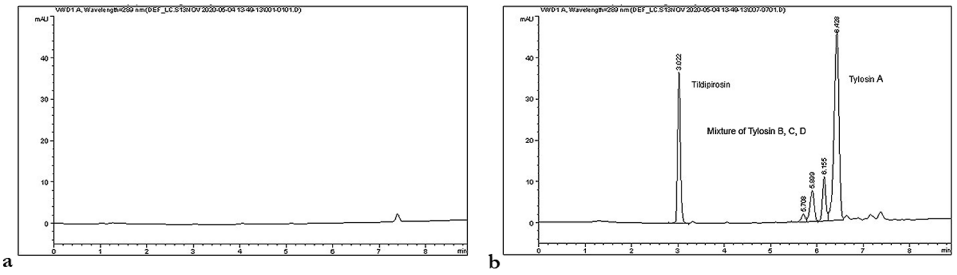

Figure 2. Chromatograms of tildipirosin and tylosin A in horse plasma by HPLC. **(a)** Blank plasma; **(b)** Blank plasma spiked with tildipirosin 0.45 μg/mL and 40 μl of tylosin A (IS) 100 μg/mL.

Table 1. Validation parameters of tildipirosin in horse plasma samples

Validation parameters	Tildipirosin
Linearity range	0.1-3.0 μg/mL
Slope	1.1287
Intercept	-0.03568 (± 0.012)
Correlation coefficient, r^2	0.9986
LLOD	0.035 μg/mL
LLOQ	0.1 μg/mL

Intraday and inter-day precision and accuracy

Precision and accuracy results are shown in Table 2. Intra-day and inter-day precision were evaluated at four QCs concentration levels: for plasma QCs 0.1, 0.3, 1.3, and 3 μg/mL, and five replicates were recorded. The CV precision values in plasma samples were < 10.5 % for intra-day and < 12.0 % for inter-day. The same procedure was performed for intra-day and inter-day accuracy for plasma samples. The accuracy ranged from –10.1 to 18.1 %. For LLOQ the accuracy results were < 20 %. Relevant results were obtained, indicating that the method is reliable for the quantitative determination of horse plasma.

Table 2. Intraday and inter-day precision and accuracy for tildipirosin in horse plasma samples.

Nominal concentration (µg/mL)	Mean concentration ± SD (standard deviation) (µg/mL)	CV (%)	Accuracy (%)
Intra-day runs			
0.100	0.118 ± 0.006	7.6	18.1
0.300	0.306 ± 0.009	3.6	2.1
1.300	1.319 ± 0.075	5.9	1.5
3.000	3.152 ± 0.223	7.1	5.1
Inter-day runs			
0.100	0.110 ± 0.012	5.8	10.5
0.300	0.290 ± 0.018	5.3	-3.3
1.300	1.169 ± 0.131	11.9	-10.1
3.000	2.828 ± 0.208	7.3	-5.7

Recovery

The recoveries of TD from plasma were measured at low, mid, and high QC levels 0.3, 1.3 and, 3.0 µg/mL, by comparing extracted samples (n = 5) with blanks spiked with the analyte post-extraction. Mean recoveries at these three levels were from 99.50 ± 7.86 The results are reported in Table 3. Excellent recoveries were obtained, indicating that this method was efficient.

Table 3. Mean ± SD recovery of tildipirosin in horse plasma

Nominal Concentration (µg/mL)	Recovery (%) (Mean ± SD)
0.3	102.4 ± 5.8
1.3	95.2 ± 10.0
3.0	100.8 ± 7.8

Selectivity and carry over

Six blank plasmas were analyzed and there were no endogenous interferences with the same retention times of TD and IS (Figure 2). These chromatograms were compared with spiked plasma samples. Moreover, well-resolved peaks for TD and IS were observed. Adequate results were obtained, indicating the high selectivity of the method for plasma. Finally, the carryover effects have not been shown due to in six blank plasmas, there were no peaks at the same TD retention time after running a set of plasma samples with high concentrations of TD (n = 6).

DISCUSSION

Method development

This work aimed to achieve a satisfactory method for the determination of TD in horse plasma with HPLC/UV detection. In the literature, four methods have been reported using HPLC-UV for TD detection in plasma [10,14-16] with running time over 20 minutes and using diethyl ether as an extraction reagent [14-16]. Other authors have analyzed TD in the plasma using HPLC/MS/MS with solid-phase extraction to concentrate and purify TD plasma samples [13]. One disadvantage of this method is this extraction process is time-consuming, and the use of cartridges and reagents significantly increases the price of each analysis.

On the other hand, some researchers have developed techniques by ultra-high-performance liquid chromatography-tandem mass spectrometry for the determination of macrolides in different matrices [18-20]. It should be noted, mass spectrometry (MS) has become the technique of choice for the analysis of all macrolides in food, biological and environmental samples due to its sensitivity and specificity. However, LC/MS suffers from a high matrix effect that affects deeply the accuracy, precision, and sensitivity [21]. Moreover, LC/MS/MS is not commonly available in most laboratories.

Regarding the mobile phase conditions, the use of acidic mobile phases was the best option instead of preparing buffer solutions in HPLC/UV detection and is considerate greener and eco-friendly method due to it is buffer-free. The advantages of this mobile phase are a faster and simple preparation and a reduced tendency to precipitate and clog inside the chromatographic system. Moreover, gradient elution and isocratic elution were also investigated. Finally, gradient elution was chosen to obtain short running times.

Some usual C18 columns were checked in our laboratory: a Brisa LC^2, C18 column (150 mm x 4.6mm i.d x 5 μm) (Teknokroma, Barcelona, Spain), an ODS C18 column (250 mm x 4.6mm i.d x 5 μm) (Análisis Vínicos, Tomelloso, Spain), a Kromasil C18 column (250 mm x 4.6mm i.d x 5 μm) (Teknokroma, Barcelona, Spain) and a Zorbax Eclipse XDB-C18 column (150 x 3.0 mm i.d x 5 μm) from Agilent (Madrid, Spain). Finally, the Zorbax Eclipse column was chosen as it showed the best resolution.

Validation

The LLOQ value for plasma was 0.1 μg/mL. This value was similar to the values published in the literature [10,14]. Therefore, the proposed method is suitable and has adequate sensitivity for determining TD concentrations in horse plasma.

Excellent recoveries for horse plasma (from 95.2 ± 10.0 to 102.4 ± 5.8 %) were obtained. Other authors have reported similar results for pig plasma (from 84 ± 1.02 % to 102 ± 0.53 %) [14]. However, lower recoveries (from 79 % to 82 %) have been

described in horse [16], and in ewe plasma samples (88.9 %) determinations [10] than in the present study. Moreover, the obtained intra-day and inter-day assays coefficients in plasma were < 20 % for LLOQ and < 15 % for low, medium, and high QCs. Consequently, our results indicate that this method was efficient and reproducible.

CONCLUSION

A rapid (9 min), cost-saving, sensitive and reproducible method for the determination of TD in horse plasma samples was developed by HPLC with ultraviolet detection, according to FDA guidelines. Furthermore, this validated method could be applied to clinical studies, routine analyses and pharmacokinetic studies using a short running time.

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Ethical approval

The conducted research is not related to animals use. No ethical approval was obtained because this study did not involve laboratory animals and only involved non-invasive procedures.

Authors' contributions

JS, VH, PM and EE participated in conceptualization, methodology, validation, investigation, original draft, writing and review of the manuscript. JS participated in the acquisition of data and analysis. JS, VH and PM participated in the interpretation of data. PM and EE made supervision, reviewed and editing of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy of the integrity of any part of the work are appropriately investigated and resolved.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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RAZVOJ I VALIDACIJA POBOLJŠANE HPLC-UV METODE ZA ODREĐIVANJE TILDIPIROZINA U PLAZMI KONJA

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Razvijena je jednostavna, brza, jeftina i osetljiva tečna hromatografska metoda visokih performansi za određivanje tildipirozina u plazmi konja. Uzorci plazme su ekstrahovani dietil etrom, a nakon isparavanja tildipirozin je određen hromatografijom sa obrnutom fazom sa ultraljubičastim detektorom postavljenim na talasnu dužinu od 289 nm. Tildipirozin je odvojen na Zorbak Eclipse KSDB-C18 koloni, 150 cm, 3,0 mm, 5 mm sa gradijentnom hromatografskom elucijom. Retenciona vremena su bila 3,0 min, odnosno 6,4 min za tildipirozin i tilozin tartrat. Ukupno vreme izvršenja ove metode bilo je 9 minuta. Kalibracione krive su bile u rasponu od 0,1 do 3 μg/mL. Donja granica detekcije za plazmu bila je 0,035 μg/mL, a donja granica kvantifikacije bila je 0,1 μg / mL. Tačnost i preciznost su uvek bili < 12% osim za LLOQ < 20%. Prosečan oporavak bio je 99,5%. Ovaj postupak se može primeniti za određivanje koncentracija tildipirozina u plazmi i biti koristan za izvođenje farmakokinetičkih studija.