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Determination of tildipirosin residues in rabbit's serum and tissues by High Performance Liquid Chromatography

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Abstract

An accurate precise, reliable and reproducible high-performance liquid chromatographic (HPLC) method for the quantization of tildipirosin in rabbit's serum and different tissues has been developed and validated. The tildipirosin was separated using C18 column (4.6 mm i.d., 250 mm, 5 µm particle size) at 30^0C with a mobile phase consisting of (0.02 M ammonium acetate: methanol) (40:60), adjust pH to 3.5 by phosphoric acid. A UV detector set at 289 nm, retention time of tildipirosin was approximately at 2.7 min and the flow rate was 1.2 ml/min. Tildipirosin was extracted from serum and tissues by Acetonitrile followed by centrifugation and evaporation. Tissue extraction required cleaned-up by solid-phase extraction (SPE) on C18 cartridges using 50 mm ammonium acetate elution. The method was validated with respect to specificity, linearity, accuracy, precision, robustness as per ICH Q2 (R1) guidelines. The developed method was accurate, precise and rapid for simultaneous estimation of tildipirosin in rabbit's serum and different tissues.

Keywords: *Tildipirosin, Serum, Tissues, Rabbit, HPLC*

Introduction

Veterinary drugs are mainly used in livestock production for prevention of diseases (prophylactic), treatment of diseases (therapeutic), modification of physiological functions as well as improvement of growth and productivity (growth promoters) **(WHO, 2018).** They are used throughout the world and comprise a broad variety of classes of chemical compounds including vaccines, antimicrobials, antiparasitic, etc. **(Moreno and Lanusse, 2017).** Many antimicrobials that are used in livestock are identical or closely related to antimicrobials used in humans. It has also been showed that about 80% of all antibiotics administered in veterinary field are used as growth promoters and in most cases; this exceeds the total antibiotics use for human medical care **(Vishnuraj *et al.,* 2016).** However, recent reports have revealed that the use of antimicrobial drugs in large amounts could result in deposition of antimicrobial residues in muscle and organs of animal **(Sanz *et al.,* 2015).** Consumption of these residues in animal products (especially through meat and meat products) may cause health risk to consumers including development of antibiotic resistance and hypersensitivity reaction. It is therefore imperative that animal products (particularly meat and milk) should be analyzed to ensure that residues do not exceed maximum residue limits **(Vragović *et al.,* 2011).**

Among the antimicrobials that are commonly used in livestock production are macrolides.

Macrolides are characterized by extensive partitioning into tissues where they achieve multifold higher concentrations relative to that observed in the blood plasma **(Anado´n, and Reeve-Johnson 1999)**.]

Tildipirosin (TD) is a semi-synthetic derivative of Tylosin. It is a tribasic molecule and has 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 **(Rose *et al.,* 2012).** Its antimicrobial activity is attributed to inhibition of bacterial protein synthesis through binding to the 23S ribosomal ribonucleic acid (rRNA) of the 50S ribosomal subunit of bacterial cells, **(Schluenzen *et al.,* 2001).** Compared with other macrolides, Tildipirosin has a long half-life and maintains a high concentration in lung tissue **(Rose *et al.,* 2012**; **Menge *et al.,* 2012**). So, it is extremely used in veterinary practice for parenteral treatment of respiratory diseases in cattle and pigs **(EMEA, 2011).**

Several studies on the pharmacokinetics, pharmacodynamics, particularly, distribution, and metabolism have been conducted in various species, including pigs **(Rose *et al.,* 2012**; **Torres *et al.,* 2016)** and cattle. However, no information is available on the pharmacokinetic characteristics of TD through i.v and i.m. administration in rabbits which could be accessed so, the aim of this study was to focus in developing method that can be utilized to investigate Tildipirosin residues in rabbit serum and tissues. This method includes sample clean up with solid phase Extraction (SPE) and analytical determination with high performance liquid chromatography (HPLC) coupled with ultraviolet detector (UV). **(Menge *et al.,* (2012)**; **Rose *et al.,* (2012**).

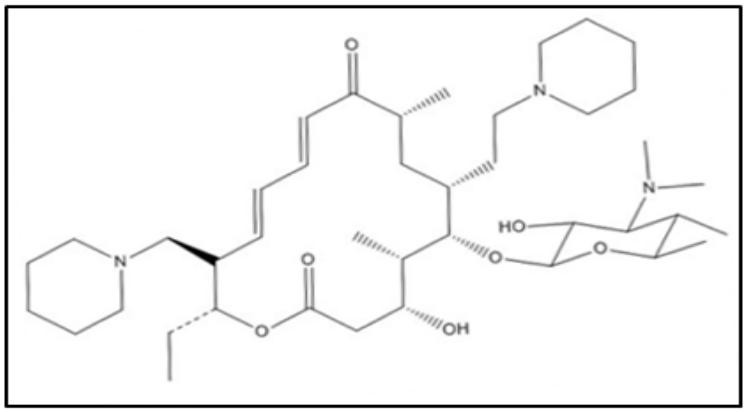

Fig. (1): Chemical structure of Tildipirosin.

Materials and Methods

Materials:

Tildipirosin standard TD (purity of 98%; Cat No. CS-T-62075) was supplied by Clear synth co. Methanol and acetonitrile were of HPLC-grade. Ammonium acetate was of analytical grade. Water was purified before use in a Milli -Q system (Millipore, Bedford, MA, USA). Solid phase extraction (SPE) of Agilent C18-E cartridge (500 mg, 6 ml; Phenomenex, Milford, MA, USA) was used for clean up the extracts.

HPLC apparatus and chromatographic conditions:

Agilent Series 1200 quaternary gradient pump, Series 1200 auto sampler, Series 1200 UV Vis detector, and HPLC 2D Chemstation software. The chromatographic column was a reversed phase C18 column (Zorbax column, 4.6 mm i.d., 250 mm, 5 µm). The UV detector wavelength was set at 289 nm. The mobile phase consisted of 0.02M ammonium acetate: methanol (40:60), adjust pH to 3.5 by phosphoric acid. The mobile phase was degassed daily by

passing it through a 0.45 µm nylon membrane filter (Millipore, Bedford, MA, USA). The mobile phase was pumped isocratically at flow rate of 1.2 ml/min. the separation was achieved at 30°C, with injection volume at 10 ul.

Standard solutions

Stock standard solution of TD compound was prepared by dissolving 10 mg of it in 10 ml of methanol to obtain a final concentration of 1 mg/ml. Stock solution was stored in amber glass at -20^0 C and stable for at least 4 weeks. Stock solutions were diluted with purified water to obtain the fortification solution at a concentration of 10 ppm, which was freshly prepared daily.

Calibration curve of serum was constructed by spiking blank serum with various volumes of fortification solution to yield a concentration range of 0.025, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 µg/ml (calibration samples) and spike blank serum to prepare quality control (QC) samples at 0.30, 0.75 and 2.0 µg/ml.

calibration curve of tissues was constructed by spiking blank rabbit tissues (muscle, liver, kidney) with various volumes of fortification solution to yield a concentration range of 0.1, 0.2, 0.5, 1.0, 2.0 and 4.0 µg/mg (calibration samples) and spike blank tissues to prepare quality control (QC) samples at 0.2, 0.4 and 0.6 µg/gm for muscle, at 1.0, 2.0 and 3.5 µg/gm for liver and at 1.5, 3.0 and 4.5 µg/gm for kidneys.

Determination of Tildiprosin concentration in serum.

The extraction was applied according to **Menge *et al.*, (2012)**. Serum sample (200 µl) was extracted with 1 ml of acetonitrile for protein precipitation, and mixed for 1 min. followed by centrifugation at 3300g at 10°C for 10 min., the supernatant was evaporated with gentle nitrogen pressure stream at 45°c then reconstituted with 200 µl of 50 mM ammonium acetate buffer. 10 µl of sample was injected into HPLC system.

During analysis of calibration serum samples, triplicate QC samples were analyzed in the same day and in consecutive six days.

Determination of TD concentration in tissues.

The extraction was done according to **Rose *et al.*, (2012**) with some modifications. Half gram of homogenized spiked tissue was mixed with 2 ml of Acetonitrile. The mixture was shaken for 30 min. followed by centrifugation at 3300 xg for 10 min at 10°C. The supernatant was collected transferred to glass tubes and extraction was repeated again with 2 ml of Acetonitrile. Vortex was done for 1 minute followed by centrifugation again, supernatant collected and the combined supernatants was evaporated to dryness with nitrogen evaporator water bath at 45°C. The residues were reconstituted with 3 ml of 50 mM ammonium acetate buffer then applied to SPE cartridge, previously conditioned with 1.5mL methanol and 2.5mL of 50 mM ammonium acetate buffer. The cartridge containing the sample eluted with 2 ml of methanol slowly then 1mL of eluent was evaporated with gentle nitrogen pressure stream at 45°C then reconstituted with 0.25 ml of (50 mM ammonium acetate buffer: methanol) (50:50); 10µL of the aliquot was injected into the HPLC system.

During analysis of calibration spiked samples, triplicate QC samples were analyzed in the same day and in consecutive six days.

Method validation:

Application of analytical method validation through specific criteria according to **ICH, (2005)** (International Conference on Harmonization), which are specificity, Linearity and Range, precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ), robustness and system suitability test (SST).

Linearity and range:

The relationship between TD peak area and the known concentrations of the analyze (standards), which is used as the calibration curve can be explained by a similar regression model. To have a robust calibration line (or curve), a series of replicates of each standard (at least three replicates of 6–8 expected range of concentration values) are recommended. The calibration curves were evaluated by correlation coefficient, slope, and intercept.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

The parameters LOD and LOQ were deter-

mined using the slope of the calibration curve (a) and the standard deviation value of the response (S) according to Eq.(LOD= 3.3*S/a) and LOQ= 10*S/a), as the LOD. The LOQ was identified as the lowest plasma and tissue concentrations of the standard curves that could be quantified with acceptable accuracy, precision and variability

Selectivity and specificity:
Selectivity is the ability of a method to determine a particular analyte in a complex matrix without interference from other ingredients of the matrix. It can be calculated by comparing the chromatograms obtained after injection of a blank sample with and without the analyte or analytical solutions and with and without the matrix components.

Accuracy and recovery:
Accuracy is the most important aspect of validation. It is a measurement of the systematic errors affecting the method. To estimate the accuracy of a method, by spiking known amount of analyte in the blank matrix (QC samples) and calculating the percentage of recovery from the matrix.

Precision:
The term precision is defined as the closeness of repeated individual measurements of an analyte under specified conditions. This term is demonstrating the repeatability and reproducibility of the method and expressed as the coefficient of variation (CV%) (RSD%).

Robustness:
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage (ex, mobile phase composition and PH, column temperature, wave length…. etc.)

System Suitability Test (SST):
It is generally performed to evaluate the suitability and effectiveness of the entire chromatographic system not only prior to use but also during the time of analysis. The most important SST parameters which are investigated are Resolution (R), repeatability (RSD— relative standard deviations of peak response and retention time), column efficiency (N), and Tailing factor (T).

Results:
Linearity and range, LOD, LOQ, accuracy, recovery and precision results were illustrated in table (1).

Table (1). Validation sheet of analytical method

Parameter	Serum	Muscle	Liver	Kidney
Range (ppm)	0.025-2ppm	0.1-4 ppm		
Retention time (min.)	2.79			
Regression equation	y = 4071.3 x - 25.362	y = 3828.2 x - 75.81	y = 2885.8 x + 88.995	y = 3408.1 x - 100.58
Correlation coefficient (R^2)	0.9999	0.9981	0.9989	0.9984
Slope (a)	4071.3	3828.2	2885.8	3408.1
Intercept (b)	25.362	-75.81	88.995	-100.58
LOD (ppm)	0.007	0.014	0.015	0.014
LOQ (ppm)	0.021	0.041	0.045	0.043
Accuracy	99.9 ± 0.611	91.8 ± 0.6	88.8 ± 0.47	96.4 ± 0.17
Recovery %	98.7- 101.1	89.2- 93.2	85.9- 94.3	92.7- 101.82
Intra-day precision (RSD%)	0.16	0.45	0.63	1.81
Inter-day precision (RSD%)	0.94	1.63	1.73	2.63

Specificity:
The equilibrated chromatograms of tildipirosin either in serum, muscle, liver and kidneys samples were demonstrated specific at retention time 2.701 min. There was no impurities or excipient interference between the different extracted spiked matrixes and pure standard **Figure (2, 3, 4, 5, 6).**

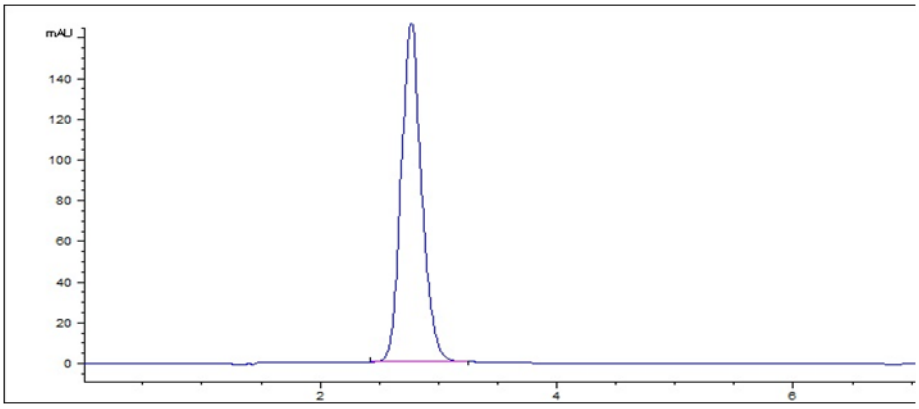

Fig. (2): Chromatogram of pure standard at a conc. of 1000ppb

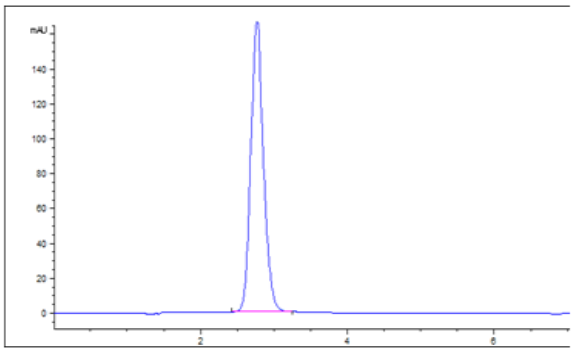

Fig. (3): Chromatogram of standard in blank serum at a conc. of 1000ppb

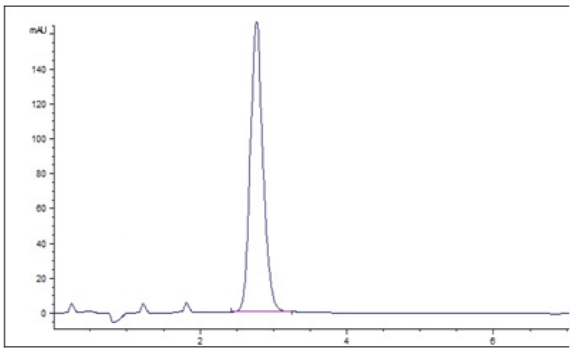

Fig. (4): Chromatogram of standard in blank muscle at a conc. of 1000ppb

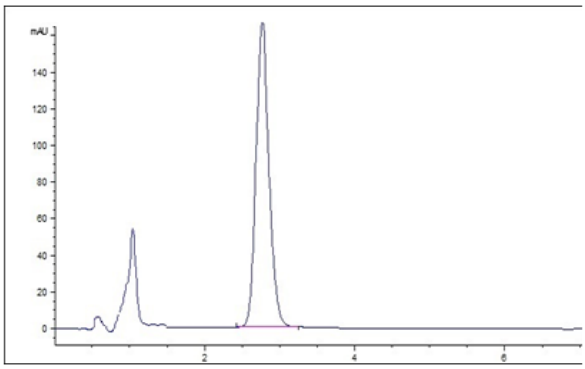

Fig. (5): Chromatogram of standard in blank liver at a conc. of 1000ppb

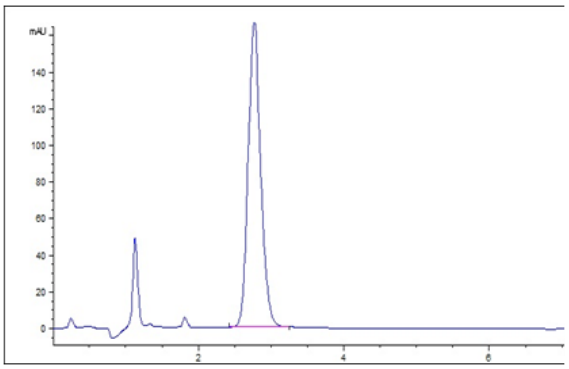

Fig. (6): Chromatogram of standard in blank kidneys at a conc. of 1000ppb

Robustness:

The results of the robustness of the assay method are listed in Table (3). Method robustness that was checked after deliberate alterations of the mobile phase composition, flow, pH, and temperature showed that the changes of the operational parameters did not lead to any essential changes in the performance of the chromatographic system. Considering the result of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust

Table (3). Influence of moderate changes in chromatographic conditions:

parameter	Modification	Peak area	Mean	SD	RSD%
Mobile phase composition	41.8: 58.2	92.6	93.7	1.056	1.1
	40: 60	94.7			
	38.2: 61.8	93.85			
Wavelength of UV	286	89.3	93.6	1.99	2.13
	289	94.7			
	292	94.8			
Column Temperature	27	95.1	94.3	1.001	1.06
	30	94.7			
	33	93.2			
Pooled RSD	1.96%				

System suitability test (SST):
The limits which are considered for the SST parameters and results of the present study are listed in table **(4).**

Table (4). System suitability test parameters FOR TD at a concentration of 1 ppm (n=6):

		Serum	Muscle	Liver	Kidneys	Acceptance criteria
Retention time (RT)	**mean**	2.79	2.781	2.78	2.8	RSD <1.0%
	RSD	0.06	0.18	0.09	0.089	
Tailing factor (Tf)	**mean**	1.0368	1.12	1.035	1.14	≤2.0
	RSD	0.066	0.03	0.03	0.028	
Theoretical plates (N)	**mean**	10773.3	9876	9865	9767.5	>2000
	RSD	1.9	1.9	1.36	1.6	
Peak Response	**mean**	4010	3901	3885	3701	RSD <1.0%
	RSD	1.9	0.8	0.72	1.1	
Symmetry factor	**mean**	0.56	0.6	0.62	0.61	≤1.0
	RSD	0.001	0.001	0.01	0.001	

Discussion
The method is selective for tildipirosin because interference between both peaks or between them and any detectable inactive or degradative material peaks were not found. Peak purity for tildipirosin peaks was checked indicating that they are pure from any other excipients or impurities or derivative materials. Thus, method of analysis was qualified and reliable to demonstrate and detect any expected change in the drug extract from serum and muscle tissues during stability studies. The obtained data were analyzed statistically regarding *y,* intercept and correlation coefficient measurements and the resulted data declared best linearity.

In our study, the developed validated method was simple and rapid as establishment of many changes like isocratic mobile phase instead of gradient one according to **Rose *et al.,* (2012)**. Isocratic system is simpler to use especially when the pump system does not have the facility to do gradient system. On the other hand, in the present study the run time reduced from 15 minutes in most other methods to 10 min. Also, the retention time reduced from 12.5 min. according to **Lei *et al.,* (2018)** to 2.7 min in this case**.**

In this study we used UV detection, the selection of an optimal wavelength was critical. Preliminary spectrophotometric scans of proteins and DNA absorb within this range (220-300 nm) according to **Brescia, (2012),** using this wavelength to analyze samples extracted from biological matrices was not feasible, so the most prominent absorbance at 289 nm as **Lei *et al.,* (2018).**

The best acceptable sample preparation protocol which be efficient, rapid, selective and easy. Herein, the extraction procedures achieved this purpose with high efficiency and selectivity.

In consideration of the structural similarities between tildipirosin and tilmicosin, and the known cardiotoxic potential of the latter substance, there were many studies to know the cardiovascular effect of tildipirosin which revealed lower cardiotoxicity than tilmicosin and the same pharmacological effect **EMEA, CVMP., (2011).** So that, presence of good and efficient method for its detection and quantification in biological samples was very important purpose and this was our target. High performance liquid chromatography (HPLC) is a widely-used technique for the analysis of compounds, and has evolved into a primary technique for the evaluation of nonvolatile small molecules **(Bansal, S. and DeStefano, A., 2007; Lee, 2003).** As tildipirosin is a nonvolatile compound, and since HPLC methods are often used for the detection and quantitation of macrolides compounds **(Bhuwapathanapun and Gray, 1977),** we decided to develop a HPLC method, with an emphasis on its practical applications for future pre-clinical and clinical studies.

Conclusion

In summary, a sensitive and reliable HPLC method for the determination of tildipirosin in rabbit serum and different tissues was developed and validated according to **ICH (2005)**. The extraction and quantitation provide accurate and reproducible results, with acceptable intra- and inter-day precision. The developed method was simple and accurate.

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