



ISSN (E): 2277- 7695  
ISSN (P): 2349-8242  
NAAS Rating: 5.23  
TPI 2021; SP-10(7): 548-553  
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[www.thepharmajournal.com](http://www.thepharmajournal.com)  
Received: 11-04-2021  
Accepted: 02-06-2021

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## Validation of simple isocratic HPLC assay method for determination of tilmicosin concentration in chicken plasma and meat

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### Abstract

The development and validation of a simple accurate method based on HPLC with ultra-violet detection for the quantification of tilmicosin in chicken plasma and meat is described. Tilmicosin is a popular macrolide antibacterial used in poultry for the control and therapy of Mycoplasmosis in poultry. There is a need to assay measure Tilmicosin concentrations in plasma and meat of chicken for pharmacokinetics and residue studies. This paper attempts to describe a simple sensitive HPLC assay. Tilmicosin was extracted from the plasma using a single-step deproteinization by perchloric acid. Chromatographic separation of tilmicosin from interfering components was achieved with a C<sub>18</sub> column, mobile phase consisting of 0.1M ammonium formate + acetonitrile + methanol in the ratio of 60:30:10 v/v (pH adjusted with trifluoroacetic acid) was used. The chromatogram was monitored at a wavelength of 287 nm. The column temperature was maintained at 30 °C. The tilmicosin standard peaks in the plasma observed at the retention time of 7-7.5 min. The method was linear from 0.05 to 5µg.ml<sup>-1</sup> ( $r^2 \geq 0.998$ ), and tilmicosin had a mean recovery from plasma of 100.66%. The coefficient of variation of inter-day and intra-day quality control samples was less than 15%. The method described is suitable for chicken meat and plasma and is easily adoptable in analytical laboratories.

**Keywords:** Tilmicosin, HPLC, chicken, plasma, meat

### 1. Introduction

Tilmicosin, a relatively new semisynthetic macrolide antibiotic derived from tylosin has been found to have good activity against many Gram positive, as well as some Gram-negative organisms. It is classified as medium spectrum antibiotic as it is highly active against mycoplasma, anaerobic bacteria and Gram-positive bacteria. It is extensively used in veterinary medicine for the treatment of pneumonia, CRD, arthritis and other infections caused by susceptible organisms [1]. Determination of drug concentration in plasma and tissues is very important in pharmacokinetic studies and drug residue studies. There are different assay methods available to detect tilmicosin concentration like microbiological assay and immunological assay (ELISA). But these methods lack sensitivity and specificity, as they are unable to identify the exact nature of the antibiotic residue. Some of the advanced techniques like LC-MS, GC-MS, LC-MS-MS also available. All these studies used detection systems (mass spectrometry) more sophisticated than UV detection, which are sometimes not available in the majority of the analytical laboratories or with no significant differences in sensitivity with UV detection methods. Thus the most prominent place among them is occupied by high performance liquid chromatography. The HPLC method is very effective in monitoring veterinary drugs and that technique has been reported for the determination of tilmicosin concentrations in various biological matrices. Laboratories involved in analytical research are constantly evolving newer assay techniques to get a robust, sensitive, simple method that will suit the assay across matrices.

### 2. Materials and Methods

#### 2.1 Drugs and chemicals

Pure Tilmicosin standard 100% (Sigma Aldrich private limited), Acetonitrile HPLC grade (Merck Specialities Private Limited, Mumbai), Methanol HPLC grade (Merck Specialities Private Limited, Mumbai), Ammonium Formate (Himedia Laboratories Private Limited), Other chemicals used were of analytical grade.

## 2.2 Equipment

Analytical Balance (KERN 870), Cooling centrifuge (Eppendorf, Germany), Deep freezer -20 °C (Voltas Ltd., India), Digital pH meter (ph 510, Eutech Instruments, Singapore), Ultra Performance Liquid Chromatography system (Shimadzu, Japan) with PDA detector (Shimadzu, Japan), Water purification system (Milli Q, Millipore, USA), Ultrasonicator (Vibracell, Sonics and materials, New Town), Vortex (Cyclomixer, REMI, India).

## 2.3 Separation of plasma and meat and preservation

Blood samples from drug free birds were centrifuged at 3500 rpm for 10 min to separate the plasma using microcentrifuge. The plasma samples were separated and stored at -20 °C until assayed. Chicken tissues were collected from slaughter houses and preserved at -20 °C until assayed.

## 2.4 Assay of tilmicosin

The concentration of tilmicosin in plasma was determined by using Ultra Performance Liquid Chromatography (UPLC) (Shimadzu, Japan) in Translational Research Platform for Veterinary Biologicals (TRPVB), Madhavaram, Chennai.

## 2.5 UPLC conditions

The UPLC system consisted of a pump (Prominence, LC 20 AD), Photo Diode Array detector (Prominence, SPD-M20A), autosampler (Prominence, SIL-20 AC HT), column oven (Shimadzu, CTO-10AS VP) and LC Solution software for data analysis. A reverse phase column C<sub>18</sub> column (Syncronis, particle size 5µm, 4.6 x 250mm, (Thermo scientific., USA) served as a stationary phase.

## 2.6 Preparation of standard curves

Drug-free poultry plasma was used to prepare standards by spiking the aliquots of plasma with tilmicosin of known concentrations ranging from 0.01 to 5µg.ml<sup>-1</sup>. The plasma was subjected to liquid-liquid extraction and analyzed as described. The calibration curve was constructed in the range of 0.1 to 5 µg.ml<sup>-1</sup> of tilmicosin. The standard curve was prepared by plotting peak area against concentration of the drug. The standard curves were linear in the range of 0.01 to 5 µg.ml<sup>-1</sup> with r<sup>2</sup> value is 0.998. Similarly chicken meat was also used to prepare the standards. Meat was obtained from slaughter. The meat was homogenized with water at the rate 10gm in 10 ml water (Purified water). One gm of the homogenate was centrifuged at 8000 rpm for 10 min. The supernatant was liquid-liquid extraction as described.

## 2.7 Extraction procedure

For extraction of tilmicosin from plasma, a simple liquid - liquid extraction method was used. The plasma was deproteinized using the perchloric acid. To 475µl of plasma, 25µl of perchloric acid was added. For meat sample to 475 µl, 25 µl of perchloric acid was added. After immediate vortexing using the vortex mixer for 1 minute, the samples were subjected to centrifugation at 3500 rpm for 5 min. The supernatant was collected and filtered using nylon membrane filters (0.2 µm pore size) and 10 µl of this filtrate was injected into HPLC for analysis. In addition to perchloric acid, acetonitrile and methanol were also tried as deproteinizing agents for comparison of performance.

## 2.8 Mobile phase

Different mobile phase compositions were used to optimize elution of tilmicosin using a HPLC assay [2-4]. Optimal

chromatographic separation was achieved using isocratic mobile phase which consisted of 0.1M ammonium formate (pH adjusted to 5 using trifluoroacetic acid) and acetonitrile and methanol in the ratio of 60:30:10.

## 2.9 Chromatography conditions

In the HPLC separation, the pump was set at a flow rate of 1.2 ml/min. The detector setting was kept at 287 nm using a UV detector. A reverse phase C<sub>18</sub> column was used which was maintained at 30 °C. The retention time of the peak elution was 7.0-7.5 min.

## 2.10 Quantification

The regression formula obtained from the calibration curve was used to quantify the concentrations of tilmicosin in plasma by substituting respective peak area.

$Y = a + Bx$  where,

Y - Peak area; a - y intercept; B - slope of the calibration curve; x - concentration (µg.ml<sup>-1</sup>)

## 2.11 Analytical recovery

The analytical recovery of the method was studied by external standard technique. It was determined by adding tilmicosin to drug-free chicken plasma and to mobile phase to yield concentrations of 0.1, 0.5 and 2.0 µg.ml<sup>-1</sup>. Plasma standards were subjected to liquid-liquid extraction. Plasma and mobile phase standards were analyzed as described above. Recovery was calculated for plasma standards as a ratio of the peak areas obtained for plasma-based standards and those obtained for mobile phase based standards. For each concentration, three determinants were made. Percent recovery was calculated according to the regression formula.

$$\text{Percent recovery} = \frac{n \sum XY - (\sum X)(\sum Y)}{n \sum X^2 - (\sum X)^2}$$

Where, X = concentration of drug spiked, Y = concentration found by assay method (recovery x concentration spiked, n = number of observations.

## 3. Results

### 3.1 Method validation-precision

The precision of the analytical method was determined by evaluating intra-day and inter-day variation. Three different concentrations of the standards were subjected to assay as described above on the same day at different times (intra-day) or on different dates (inter-day). The coefficients of variation of the results obtained were expressed to ensure the repeatability and reproducibility of the assay. The precision of the assay denoting the reproducibility was expressed as Coefficient of variation over inter-day and intra-day assay were less than 15% at three different concentrations.

### 3.2 Sensitivity of the assay

The sensitivity of assay was expressed in terms of Limit of detection and Limit of Quantification. The LOD and LOQ of the method used were calculated by the formulas as per the reference (ICH guideline, 2005). Using the calibration curves obtained, the formulae as below were applied to arrive at the LOD and LOQ of the assay.  $LOD = (3.3 \times \text{intercept})/\text{slope}$ ,  $LOQ = (10 \times \text{intercept})/\text{slope}$ . The limit of detection obtained in the present study was 0.03 µg.ml<sup>-1</sup>. The limit of quantification obtained in the present study was 0.06 µg.ml<sup>-1</sup>.

### 3.3 Recovery

The ability of the assay procedure to extract the tilmicosin

from the plasma matrix is expressed in terms of recovery. The recovery for tilmicosin from plasma in this study is 100.66%.

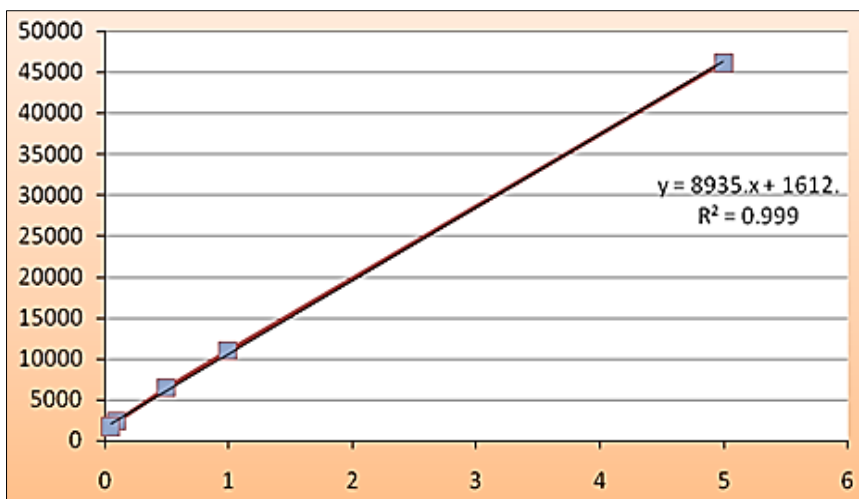
**Table 1:** Analytical recovery of tilmicosin from chicken plasma

Concentration spiked (( $\mu\text{g}\cdot\text{ml}^{-1}$ ))	Mobile phase peak area	Plasma spiked peak area	Percentage range	Mean	Recovery percentage
0.05	1132	793	142.75	128.82	100.66
0.05	1972	2310	85.37		
0.05	730	461	158.35		
0.5	6253	7848	79.68	106.78	
0.5	5846	3317	176.24		
0.5	6401	9937	64.42		
1	6253	15219	41.09	66.39	
1	9868	8866	111.30		
1	10979	23471	46.78		

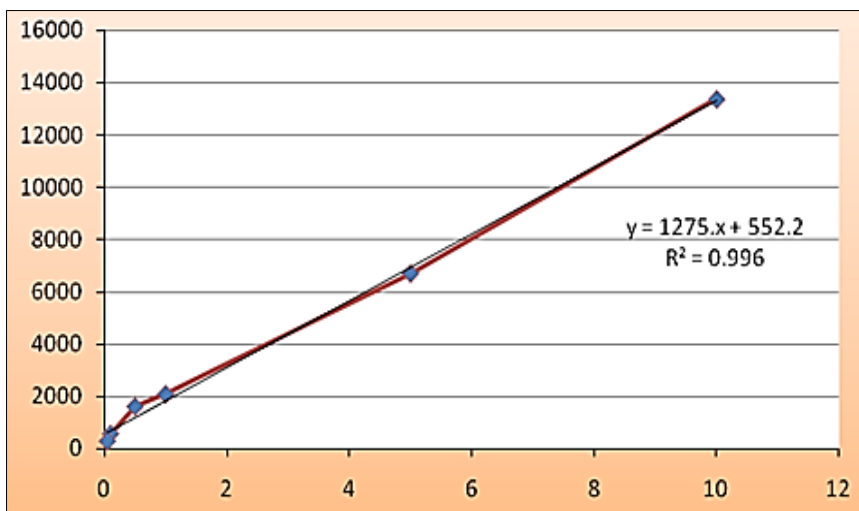
### 3.4 Calibration curve

Calibration curves were constructed based on values for tilmicosin standard solutions prepared in mobile phase or plasma. Graphs using concentration ( $\mu\text{g}/\text{ml}$ ) in X axis and the

corresponding peak areas in Y axis were plotted. The calibration curves for mobile phase and plasma returned with  $R^2$  value of 0.999, which is indicative of very high reliability over a broad range from 0.05  $\mu\text{g}$  to 5  $\mu\text{g}/\text{ml}$ .



**Fig 1:** Standard curve of tilmicosin spiked in chicken plasma



**Fig 2:** Standard curve of tilmicosin spiked in chicken tissue (Muscle)

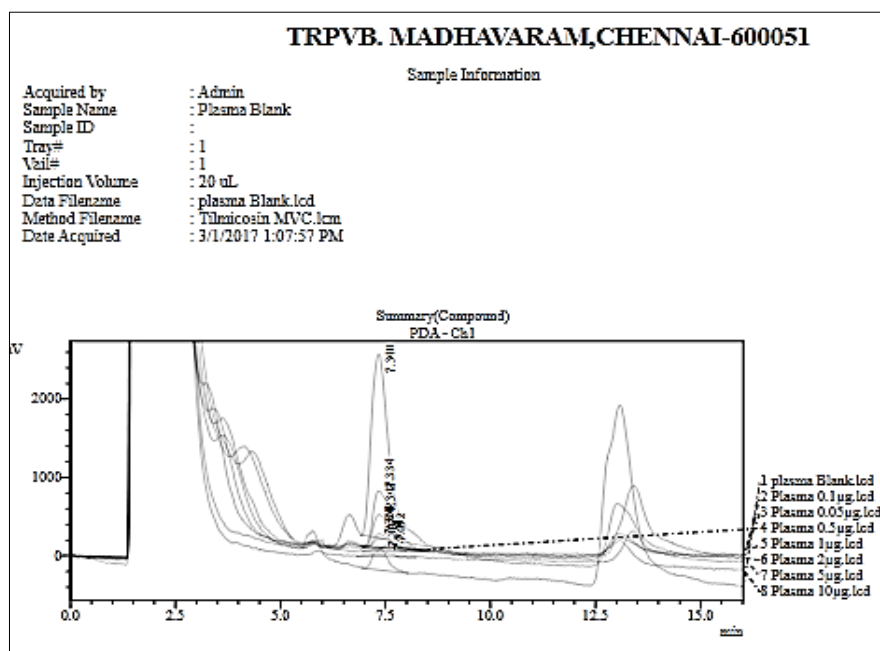


Fig 3: Overlay report of tilmicosin for different concentrations in chicken plasma

## 4. Discussion

### 4.1 Methods to detect tilmicosin concentration in chicken plasma

#### 4.1.1 Microbiological assay method

Microbiological assay has been used to screen samples for tilmicosin in plasma [5, 6]. But these microbiological assay methods have lack of sensitivity and specificity, as they are unable to identify the exact nature of the antibiotic residue.

#### 4.1.2 Immunological methods

Other analytical methods (screening or confirmatory) e.g., Enzyme Linked Immunosorbent Assay (ELISA) [7, 8], Immuno chromatographic method [9], Quantum dot-based immunoassay [10], Indirect Competitive Enzyme-Linked Immunosorbent Assay [11] have also been described for assay of tilmicosin.

#### 4.1.3 Chromatographic methods

Chromatographic methods are generally preferred for their greater selectivity and simplicity. Liquid chromatography [12] Gas chromatography coupled with mass spectrometry; liquid chromatography electrospray mass spectrometry [13, 14], Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry [15], liquid chromatography-tandem mass spectrometry [16] are also described for assay of tilmicosin. All these studies used detection systems (mass spectrometry) more sophisticated than UV detection, which are sometimes not available in the majority of the analytical laboratories or with no significant differences in sensitivity with UV detection methods. HPLC occupies a very important role in analytical laboratories. Laboratories tend to prefer methods that are feasible and do not involve lot of sophistication. At the same use of HPLC assures required sensitivity. Thus HPLC method can be very effective in monitoring veterinary drugs and that technique has been reported for the determination of tilmicosin concentrations in various biological matrices.

### 4.2 Standardization parameters of HPLC assay method for tilmicosin

In this study standardized HPLC method to quantify the levels

of tilmicosin in plasma was reported. Many papers have been published on the assay method for tilmicosin [3, 4, 17-21]. After going through the methods described, the authors felt the need for developing a simple sensitive technique which will be uniform for chicken plasma and meat.

### 4.3 Extraction procedure

A suitable extraction procedure is required for any matrix to bring the analyte molecule out of the matrix. The procedure should be simple, quick and less cumbersome. In this study the extraction procedure followed as in [3, 17] was adopted with a few modifications. But still, the method described allows tilmicosin to be extracted, in a simple and reliable way from the plasma. The method is also highly sensitive and specific to tilmicosin. In our method, the volume of plasma needed is also very less (0.5 ml) which was enough to obtain a good sensitivity, recovery and specificity. This point is pertinent in smaller species including poultry.

Other reported methods used different solvents such as acetonitrile [16] methanol [20, 21] phosphoric buffers etc. for tilmicosin extraction [17]. Even though, use of perchloric acid needs almost safety measures as they are highly corrosive in nature, the extraction percentage was better than solvents like acetonitrile and methanol as described in other methods. All these methods use large quantities of sample to increase the sensitivity, this therefore implies increasing the number of extraction steps, which involves loss of precision and accuracy of the method and the volume of solvents employed in order to achieve a good resolution and specificity of the method.

Extraction with SPE cartridges [21] has the disadvantages that it needs a high accuracy and repeatability and a lot of attention at many points of extraction. Furthermore, it is more expensive than liquid-liquid extraction. Moreover the isocratic method overrides the repeatability problem observed by the use of gradient elution, which also needs extra time to achieve column conditioning.

### 4.4 Problems in tilmicosin assay

The major factor hampering the determination of tilmicosin is that it consists of at least three structurally similar metabolites

viz., T-1, T-2, and T-3 along with parent compound. Metabolite T-1(N-desmethyl tilmicosin) is the only major tilmicosin metabolite. In most of the assay procedures described the peak obtained corresponds to T-1, T-2 and T-3 were impurity and minor degradation products respectively [1]. The appearance of two to three peaks may also be observed in some chromatograms whereas there may be a single peak with two split cones. Another factor contributing the difficulty is mainly the pH. Tilmicosin is unstable at  $\text{pH} < 4$  and  $\text{pH} > 9$ . Both blood and serum samples were protected from direct light due to photosensitivity of tilmicosin and highly photo degraded [21]. Moreover tilmicosin has a relatively strong ultraviolet (UV) absorption and falls between 282-289 nm, the spectrum range in which most of the compounds absorption spectrum coincides. This spectrum within the visible range is a major hampering factor in getting the compound standardized.

One of the mobile phase used for standardizing the tilmicosin included 0.2M ammonium acetate + water + acetonitrile + methanol in 20:32:24:24 ratio (pH adjusted with 5 with 50% acetic acid) [3]. It responded well with mobile phase but lacked proper eluting capacity with plasma based standards and showed multiple peaks. The mobile phase with 0.05 M of  $\text{NaH}_2\text{PO}_4$  with acetonitrile in ratio of 67:33 v/v (pH adjusted to 2.5 with 50% orthophosphoric acid) [2] was also inadequate since no peaks were observed in plasma. Extraction with acetonitrile showed no peaks at same time with methanol showed peaks but not much as like in perchloric acid extraction method.

Since the results with other mobile phase were not satisfactory owing to inconsistent results and failure of elution, the following mobile phase consisting of 0.1M ammonium formate + acetonitrile + methanol in the ratio of 60:30:10 v/v (pH adjusted with trifluoroacetic acid) was used [4]. The flow rate was kept at 1.2 mL/min. The chromatogram was monitored at a wavelength of 287 nm. The column temperature was maintained at 30 °C. The linearity, recovery, precision and sensitivity of the method were excellent thus suggesting the above method can be used for conducting pharmacokinetic studies of tilmicosin.

#### 4.5 Characterization of the method of standardization

The important features that govern the utility of the analytical method include linearity, sensitivity, specificity, precision and recovery.

##### 4.5.1 Linearity

Linearity was observed for curve range of 0.01-10  $\mu\text{g}\cdot\text{ml}^{-1}$ . The correlation coefficient ( $r^2$ ) for the calibration curve was 0.998 and 0.9977 [16] and 0.99 [23] and  $r^2$  greater than 0.99 [22].

##### 4.5.2 Sensitivity

The sensitivity of the assay method was given by LOD and LOQ. The LOD was 0.03  $\mu\text{g}\cdot\text{ml}^{-1}$  whereas the LOQ was found to be 0.06  $\mu\text{g}\cdot\text{ml}^{-1}$ . In comparison the sensitivities quoted by other authors were LOD-0.025  $\mu\text{g}\cdot\text{ml}^{-1}$  and LOQ-0.05  $\mu\text{g}\cdot\text{ml}^{-1}$  [17], LOD-0.005  $\mu\text{g}\cdot\text{ml}^{-1}$  and LOQ-0.015  $\mu\text{g}\cdot\text{ml}^{-1}$  [3] in chicken plasma and LOD-0.048  $\mu\text{g}\cdot\text{ml}^{-1}$ , LOQ-0.070  $\mu\text{g}\cdot\text{ml}^{-1}$  in equine plasma. The sensitivity was found to be adequate since it was lower than the Minimum Inhibitory Concentrations (MIC) quoted for tilmicosin against sensitive infections. The MICs of tilmicosin against mycoplasma are in the range of 0.0125 to 0.1. For residues in chicken meat the method will be able to detect lesser than the quoted MRLs for

chicken (0.075  $\mu\text{g}/\text{gram}$  [22]).

##### 4.5.3 Specificity

The specificity of the method (i.e., the ability of the test method to detect truly negative samples as negative) was demonstrated by analyzing control (drug free) samples. The term specificity indicates exclusivity of the peaks without overriding or overlapping peaks due to other compounds or matrix effects. In this though the retention time was slightly longer (7.0-7.5 min.), there were no overlapping peaks at the retention time observed.

##### 4.5.4 Precision

Analytical precision indicates reproducibility of the method over different laboratories or different time points. This is crucial especially when studies are to be planned over a long period of time esp. in pharmacokinetic studies involving large number of samples. The precision of a method is given by coefficient of variation. Accordingly the method used in the present study can be considered to be precise with the coefficients of variation less than or close to 15%, whereas [17] the CV ranged from 1.5% to 9%, 3.06% to 4.08% [3] and 2.7% to 10.1% [20], 4.4 to 8.1% in equine plasma [22].

##### 4.5.5 Recovery

In any matrix it is important to ensure that the analyte of interest is maximally extracted from the matrix without impurities. An indication of the same is given by the recovery expressed in percent. In this study the absolute recovery and the analytical recovery are in the range of 100% or slightly more which indicated complete recovery. Whereas, 96.7% to 100.8% in chicken plasma [17].

Thus in this study, a simple HPLC assay method for the assay of tilmicosin based on easy extraction steps, isocratic mobile phase is described, which is suitable for chicken plasma and meat. The method will be very useful in analytical laboratories handling poultry samples for pharmacokinetic and residue studies.

#### 5. Acknowledgement

The author wish to thank the Dean, Madras Veterinary College, TANUVAS, Chennai for providing infrastructure to complete this work. They are also grateful to Huvepharma (SEA), Pune for donating tilmicosin standard drug for analysis.

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