

Development and Validation of a Simple and Reliable Method for Determination of Impurities D in Levamisole Oral Solution for Veterinary Use

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Abstract:

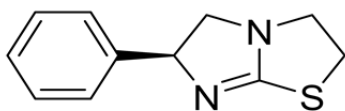
As per British Pharmacopeia (BP), the levamisole containing oral solution should not contain more than 0.5% of tetramisole. In previous studies, the determination of tetramisole has been performed by using LC-MS/MS and CD. In the present study, a simple and reliable ultra-fast liquid chromatography (UFLC) method was developed and validated for the determination of impurities D in Levamisole oral solution (Laying Hen WO, Vetafarm Manufacturing Pty Limited). The calibration curve was linear over a concentration range from 0.15625 to 5.0 µg/ml with a correlation coefficient of 1 and the method was also validated in this concentration range (0.15625–5.0) µg/ml. The developed method was validated with respect to specificity, linearity, and accuracy and there was no peak detected for metabolite D in the Laying Hen WO solution.

Key words: Levamisole, tetramisole, UFLC, method development, Laying Hen WO

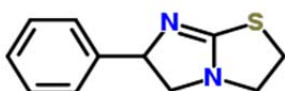
1. INTRODUCTION:

Levamisole ((S)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-b][1,3] thiazole), a phenylimidazothiazole, was originally developed as an anthelmintic but is now used clinically as an immunostimulant and antineoplastic agent (Becq *et al.*, 1996, Krensky *et al.*, 2001).

It is the levorotatory and biologically active isomer of the racemic tetramisole (±)-2,3,5,6-Tetrahydro-6-phenylimidazo[2,1-b] thiazole hydrochloride (**Figure 1**), was discovered by Janssen Pharmaceutical company in 1966 and developed as a broad-spectrum anthelmintic for use in a variety of mammalian and avian species (Stephen W Page, 2008). Levamisole has a similar mode of action to pyrantel and causes spastic paralysis followed by passive elimination of parasites and is rapidly absorbed from the gastrointestinal tract, achieving peak plasma levels within 2 hours, and is eliminated within 3 days (Simon J *et al.*, 2014)



Levamisole



Metabolite D (Tetramisole)

As per British Pharmacopeia (B.P), the levamisole containing oral solution should not contain more than 0.5% of tetramisole. In previous studies, the determination of tetramisole has been performed by using LC-MS/MS and CD (Marc C *et al.*, 2000, Tyrpenou AE *et al.*, 2006, Amin AS *et al.*, 2002). But as per our knowledge, there is no simple and reliable HPLC method has been reported. In the present study, a simple and reliable HPLC method for the determination of tetramisole in Levamisole oral solution (Laying Hen WO, a research & development product of Vetafarm Manufacturing Pty Ltd.) has been developed and validated.

2. MATERIALS AND METHODS:

2.1 Chemicals and reagents:

The certified Levamisole was purchased from Sigma Aldrich, Australia and tetramisole was purchased from ChemBridge Corporation, San Diego, CA 92121, USA. The HPLC grade acetonitrile and methanol was purchased from Merck Millipore, Australia. The orthophosphoric acid was purchased from Chem-Supply, Gillman, SA, Australia. High purity water was prepared by using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

2.2 Apparatus:

Shimadzu UFLC systems consisting of Auto injector and UV detector was used for analysis. In the sample preparation, an ultrasonic instrument (Soniclean, Australia) was used for sonication.

2.3 Chromatographic conditions:

The method was developed by using a Shimadzu UFLC and GEMINI C18 reverse-phase column (250x4.6mm) with a mobile phase consisting of Acetonitrile: Methanol: water (50:30:20) and the pH was adjusted to 4.6 by using orthophosphoric acid. The mobile phase was filtered through nylon 0.45 µm membrane filters and degassed. The flow rate of the mobile phase was 1 mL/min. The column temperature was maintained at 30°C and the eluted

compounds were monitored at the wavelength of 225 nm. The sample injection volume was 20 μ L.

2.4 Standard solution preparation:

Methanol was used as a diluent. A stock solution containing 1mg/mL of tetramisole was prepared by dissolving an appropriate amount of drug in diluent. Appropriate dilutions were made with diluents to obtain solution containing 0.15625, 0.3125, 0.625, 1.25, 2.5 and 5 μ g/mL.

3.RESULTS AND DISCUSSION:

3.1 Method development and optimization:

An UFLC method using isocratic conditions for the analysis of levamisole and metabolite D was developed at 225 nm wavelength. At this wavelength, the drug has sufficient absorption and low quantities of metabolite D may be detected correctly. Furthermore, the calibration curves obtained at 220 nm was of good linearity. The mobile phase which was composed of Acetonitrile: Methanol: water (50:30:20) gave best resolution and sensitivity with a reasonable short run time. A GEMINI C18 reverse- phase column (250x4.6mm) column was selected over a Luna C8 reverse- phase column (250x4.6mm), to achieve good peak shape and symmetry. The injection volume was varied between 5 and 50 μ L, finally 20 μ L was chosen, because bigger volumes imply wider peaks without much enhancement of the signal-to-noise ratio. The flow rate of the mobile phase was kept 1 mL/min and the column temperature was maintained at 30°C.

3.2 Validation of the method:

The developed method was validated as per ICH guidelines. The following validation characteristics were addressed: system suitability, specificity, accuracy, and linearity.

Table 1: System suitability tests result

Injection	Retention Time	Area
1	3.688	324965
2	3.689	324836
3	3.689	324357
4	3.688	324311
5	3.688	325446
6	3.689	325507
7	3.690	324545
Mean	3.690	324852
Standard Deviation	0.00076	487.654
%RSD	0.020	0.150

Table 2: Linearity data

Concentration (μ g/mL)	Area	Slope	Intercept	Correlation Coefficient
0.15625	11888	64542	2490.4	1.00
0.3125	22287			
0.625	43007			
1.25	83860			
2.5	164524			
5	324708			

Table 3: Results of recovery study

Sample	Spiked level (μ g/mL)	% Recovery(n=7)	% RSD(n=7)
1	4	98.168	0.236
2	5	103.004	0.310
3	6	101.547	0.774

3.3 System suitability:

The system suitability test was used to ensure that the UFLC system and procedures are adequate for the analysis performed. Parameters of this test were column efficiency (number of theoretical plates), and reproducibility as relative standard deviation (RSD) of peak area and retention time of seven injections of standard solution. During performing the system suitability test, in all cases RSD of the peak areas was $\leq 2.0\%$, the number of theoretical plates per column was 39810, and the USP tailing factor was ≤ 2.0 . The results are summarized in **Table 1**.

3.1 Specificity:

The ability of this method to separate and accurately measure the peak of interest indicates the specificity of the method. The specificity of the method was checked by injecting metabolite D standard and the diluting solvent methanol. There is no interference from the diluting solvent at the retention time of analyte peak (**Figure 2**).

3.5 Linearity:

Linearity of the method was studied by analyzing standard solutions at six different concentrations levels ranging from 0.15625 to 5 μ g/mL. The calibration curve was constructed by plotting the peak area against the corresponding concentration injected, using the least square method. The calibration curve values of slope, intercept, and correlation coefficient for metabolite D are 64542, 2490.4 and 1, respectively. The high value of the correlation coefficient indicated a good linearity (**Table 2 & Figure 1**).

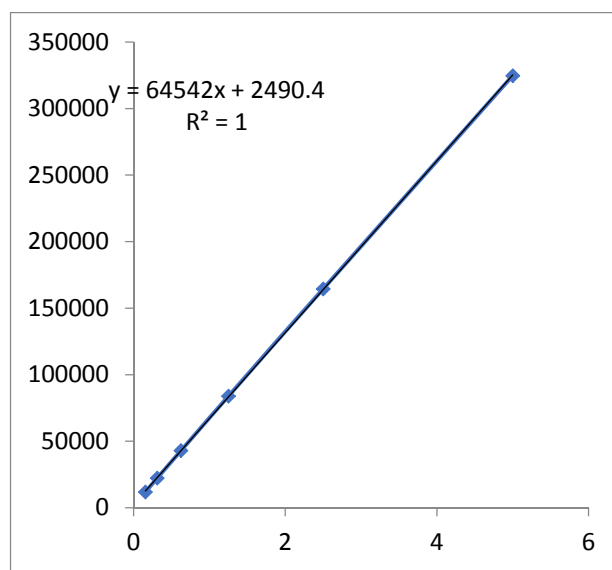


Figure 1: Linearity of Metabolite D

3.6 Accuracy:

The method accuracy was evaluated by spiking the placebo with concentration approximately equivalent to 80,100 and 120 percent of maximum allowable concentration (5 µg/mL). Each of the concentrations was performed 7 times. The averages of these recoveries are detailed in **Table 3**. The average of the recoveries of these spikes with concentrations ranging from 3.94 µg/mL to 6.06 µg/mL.

3.7 Determination of metabolite D in Laying Hen WO solution:

By using the validated method, the detection of metabolite D in Laying Hen WO solution, Vetafarm Pty Limited was attempted. But there was no peak found for metabolite D in Laying Hen WO solution of both ambient and accelerated conditions **Figure 3-7**.

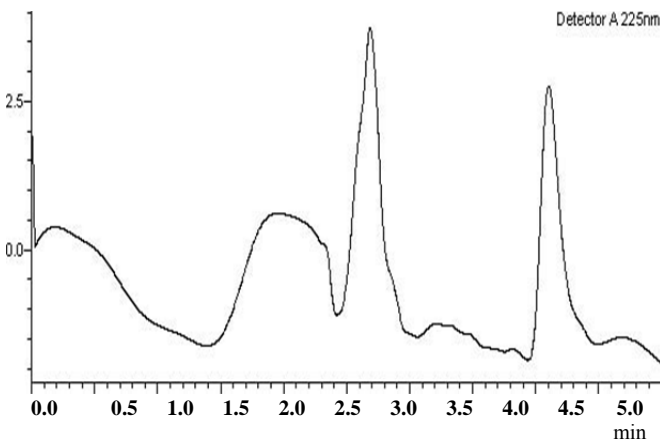


Figure 2: Chromatogram of Diluting solvent (MeOH) showing no interference with analyte retention time

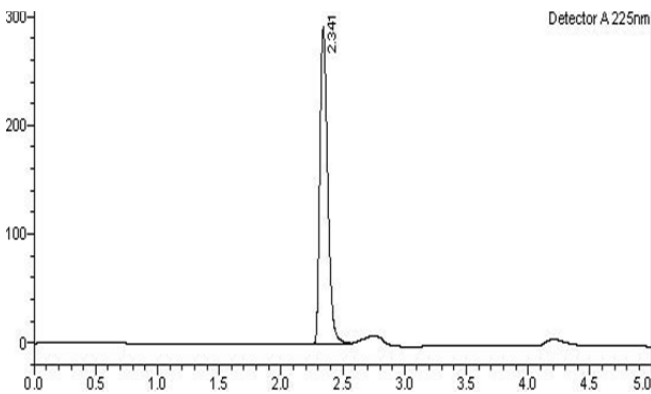


Figure 3: Chromatogram of Levamisole in Laying Hen WO solution at ambient condition

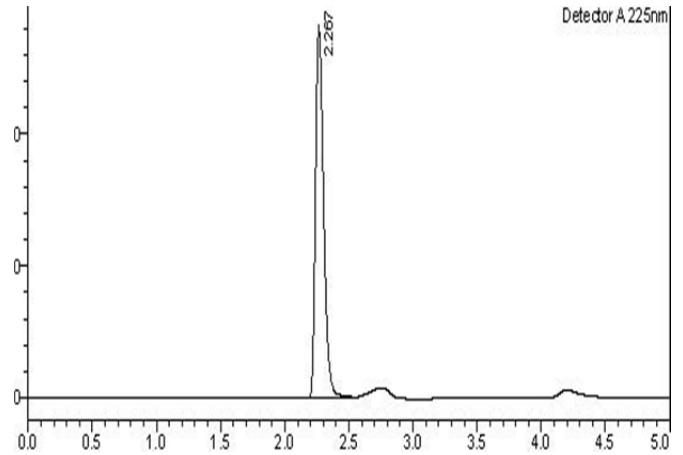


Figure 4: Chromatogram of Levamisole in Laying Hen WO solution at accelerated condition (40°C)

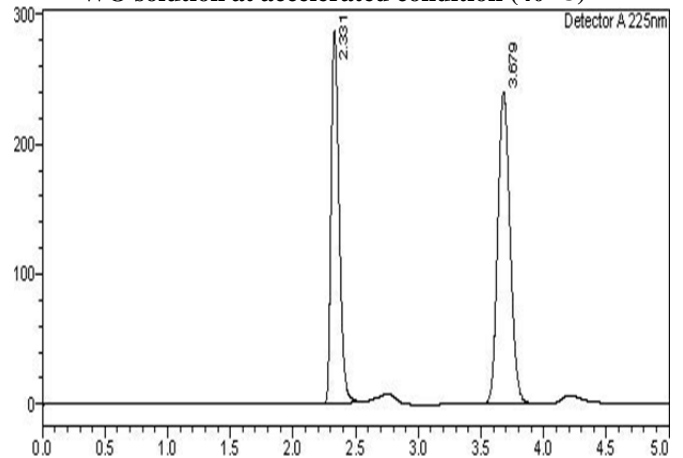


Figure 5: Chromatogram of Laying Hen WO solution and Metabolite D

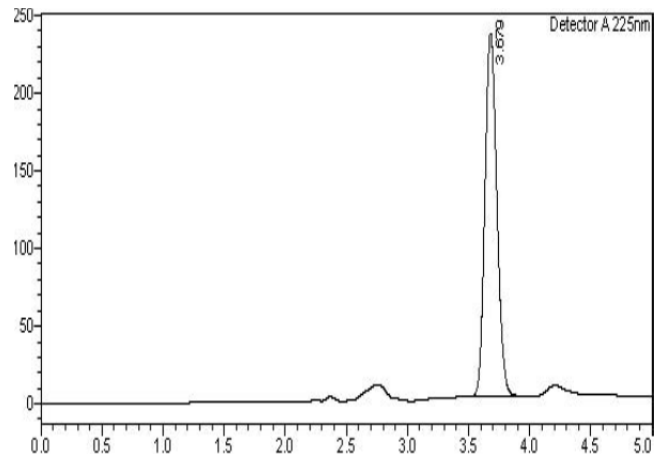


Figure 6: Chromatogram of metabolite D reference standard

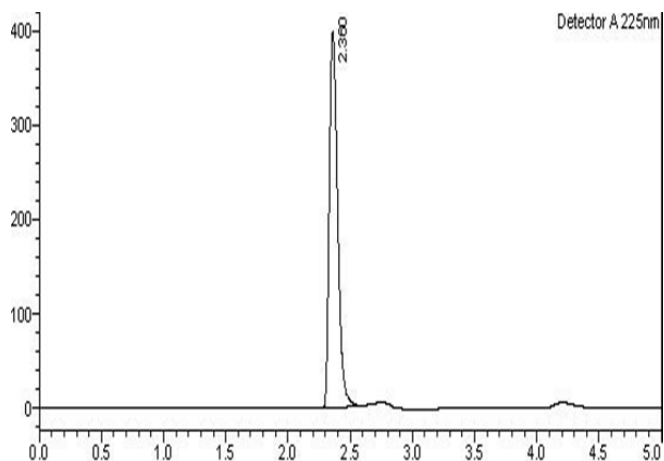


Figure 7: Chromatogram of Levamisole reference standard

CONCLUSION:

In the present study, a simple and reliable method was developed, and validated for the determination of metabolite D in Laying Hen WO solution. There was no peak detected for metabolite D in the analysed formulation.

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