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Research Article

Development and Validation of a New RP-UFLC Method for the Quantification of Tilorone in Presence of Internal Standard

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Abstract

Tilorone is an anti-viral and immune modulatory drug. It is an inducer of endogenous interferon with low molecular weight. A new RP-UFLC method has been developed and validated for the estimation of Tilorone in bulk and its tablet dosage forms in presence of and internal standard, Eplerenone. Tilorone is used for the treatment of urogenital tract, respiratory infections and viral hepatitis A, B, C. Tilorone stimulates the formation of α, β and γ interferons and produces interferon intestinal epithelial cells, neutrophils, T-lymphocytes and hepatocytes in the body. A mixture of tetra butyl ammonium hydrogen sulphate, acetonitrile and methanol (60: 35:5) was used as mobile phase for the chromatographic study (Isocratic mode) using Agilent C18 column (Detection wavelength 245 nm) with flow rate 0.5 mL/min. Tilorone has shown linearity over the concentration range 0.05-50 µg/mL and the regression equation was y = 0.1075x + 0.0058 with correlation coefficient 0.9999. The LOD and LOQ are found to be 0.0157 and 0.0487 µg/mL respectively. The method was validated as per ICH guidelines. The proposed RP-UFLC method was found to be precise, accurate, and robust for the quantification of Tilorone in tablet dosage forms.

Keywords: Tilorone; RP-UFLC; Eplerenone; Internal Standard; Acetonitrile; ICH Guidelines

Introduction

Tilorone ($C_{25}H_{34}N_2O_3$) is a broad spectrum anti-viral agent. It acts by reducing the reproduction of influenza viruses, herpes viruses, hepato virus infections by the translation of virus-specific proteins in infected cells. It stimulates the synthesis of alpha, beta, gamma interferons [1]. Interferons are produced by the cells of the intestinal epithelium, hepatocytes, T-lymphocytes, neutrophils. It has a molecular formula $C_{24}H_{34}Cl_2N_2O_3$. 2HCl and molecular weight 483.47. Tilorone (Figure 1A) is an orally active interferon inducer. Tilorone is a fluoren-9-ones derivative and it is an alpha 7-nicotinic aceetyl choline receptor agonist. Ekins et al., studied [2] a series of *in vitro* ADMET assays and demonstrated that Tilorone has excellent solubility, high Caco-2 permeability and had no inhibitory activity against five human CYP450 enzymes (3A4, 2D6, 2C19, 2C9 and 1A2) and also has shown that Tilorone has 52% human plasma protein binding with excellent plasma stability.

Eplerenone ($C_{24}H_{30}O_6$) chemically 9,11α-Epoxy-7α-methoxy carbonyl-3-oxo-17α-pregn-4-ene-21,17-carbolactone (Figure 1B) is an anti-hypertensive agent used for the treatment of heart failures either alone or in combination with other drugs. It is an aldosterone antagonist [3]. Eplerenone has been used as an internal standard in the present study.

Zhang et al., developed a HPLC/MS/MS method [4] for the simultaneous quantification of Tiloronoxim and its major active

metabolite, Tilorone in human urine on gradient mode in presence of an internal standard, Metoprolol using a solvent mixture of chloroform and ethyl ether (50: 50, v/v) for the extraction procedure. A mobile phase consisting of methanol and 15 mM ammonium bicarbonate (pH 10.5) prepared in water was used for the chromatographic separation.

Xianhua Zhanga et al., developed a HPLC-MS/MS method [5] for the quantitative determination of Tiloronoxim and its metabolite Tilorone in human blood for which 200 µl human blood was extracted with a mixture of chloroform and ethyl ether (50: 50, v/v) using metoprolol as the internal standard. Chromatographic separation was achieved using Xterra MS C18 column on gradient mode using a mobile phase mixture consisting of methanol and 15 mM ammonium bicarbonate (pH 10.5) prepared in water with positive MRM mode detection on a turbo ion spray source and both the compounds have shown linearity over the concentration range 1-100 ng/ml.

Mathrusri Annapurna et al., developed a derivative spectrophotometric method [6] for the determination of Tilorone using sodium acetate buffer (pH 4.0), borate buffer (pH 9.0), phosphate buffer (pH 2.0) and phosphate buffer (pH 5.0) solutions and the linearity was observed over the concentration range 0.4-14 µg/mL in all the methods.

Hima Bindu et al., developed a stability indicating RP-UFLC method [7] for the determination of Tilorone in bulk and tablet dosage forms using C18 Agilent column on isocratic mode using a mixture of Acetonitrile: 0.1% Triethylamine (pH adjusted to 3.2 with ortho phosphoric acid) as mobile phase with 0.5 mL/min as flow rate (Detection wavelength 270 nm) and the linearity was shown over the concentration range 0.1-20 µg/mL.

Manishankar and Mathrusri Annapurna developed a RP-HPLC method [8] for the assay of Tilorone using Shimadzu Model HPLC system with C8 Agilent column and PDA detector on isocratic mode using a mixture of Tetra butyl ammonium hydrogen sulphate: Acetonitrile (61: 39) as mobile phase with 0.5 mL/min as flow rate (Detection wavelength 264 nm) and the linearity was shown over the concentration range 0.05-40 µg/mL.

The authors have proposed a new RP-UFLC for the quantification of Tilorone in tablet dosage forms in presence of and internal standard, Eplerenone by employing an ion pairing agent as an aqueous phase and the method was validated as per ICH guidelines [9].

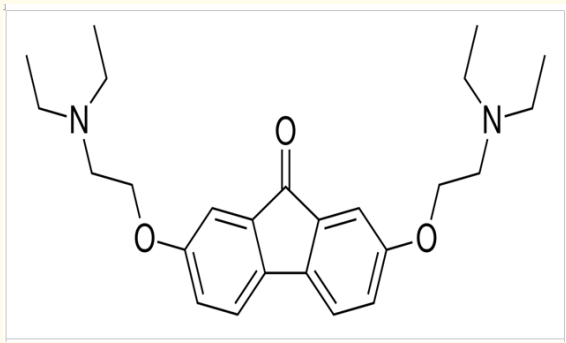


Figure 1A: Chemical structure of Tilorone (TL).

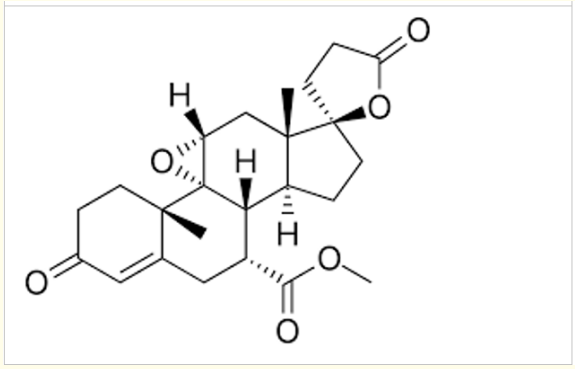


Figure 1B: Chemical structure of Eplerenone (EP) (Internal standard).

Materials and Methods

Instrumentation and chemicals

Acetonitrile (HPLC grade) and tetra butyl ammonium hydrogen sulphate (TBHS) were procured from Merck (India). Shimadzu Model UFLC system with C8 Agilent column and PDA detector were used for the chromatographic analysis. Tilorone is available with brand names AMIXIN (Label claim: 125 mg) and LAVOMAX (Label claim: 125 mg) as film coated tablets. Tilorone was supplied by HONOUR Labs Limited (Hyderabad, India) as gift sample.

Preparation of 10 mM tetra butyl ammonium hydrogen sulphate solution (pH 3.5)

Tetra butyl ammonium hydrogen sulphate (Mol wt 339.5 grams/mole) is an ion pairing reagent. etra butyl ammonium hydrogen sulphate (10 mM) solution was prepared by dissolving accurately 3.395 grams in HPLC grade water in a 1000 ml volumetric flask and the solution was sonicated for 30 mins, filtered through a membrane filter and then used as mobile phase.

Preparation of tilorone stock (1000 µg/mL) solution

25 mg of Tilorone was accurately weighed and dissolved in a 25 mL volumetric flask and made up to the volume with HPLC grade acetonitrile (1000 µg/mL). This stock solution was diluted as per the requirement with the mobile phase and filtered through membrane filter before use.

Method validation

Linearity study

A series of solutions (0.05 - 50 µg/mL) were prepared from the stock solution along with 10 µg/mL of IS and diluted with mobile phase. 20 µL of each of these solutions were injected in to the UFLC system and the peak areas of Tilorone as well as the IS were noted from the chromatogram. A calibration curve was drawn by plotting the concentration of Tilorone on the x-axis and the corresponding mean peak area ratio values on the y-axis.

Precision study

Precision study was performed for Tilorone solutions on the same day with three different concentrations (10, 20 and 40 µg/mL) (Intraday) and on three successive days (Inter-day) using three different concentrations (10, 20 and 40 µg/mL) in presence of IS. The peak areas of Tilorone with respect to IS were noted from the corresponding chromatograms and the peak area ratio was calculated and finally the % RSD was calculated.

Accuracy study

The accuracy study was performed using the standard addition method. In the standard addition method, a known amount of the standard drug solution (Analyte/API) is added to the sample or formulation solution. It means the extracted solution from the dosage forms is spiked with a known amount of the standard or API solution of the analyte and the peak area obtained from the chromatogram was substituted in the calibration curve and the concentration was calculated. In this study the recovery values and the % RSD values were also calculated. In this method 50%, 100% and 150% of the pure Tilorone (API) solutions were added to a fixed concentration of the extracted Tilorone formulation solution along with 10 µg/mL of IS and the solutions were made up to volume with the mobile phase. 20 µL of each of these resultant solutions were injected (n = 3) in to the UFLC system and the percentage recovery was calculated along with % RSD.

Robustness study

Robustness is one of the important validated parameters and in this study small changes such as flow rate (± 0.1mL/min), mobile phase ratio (± 5%), pH and detection wave length (243 nm and 247 nm) were incorporated in the optimized method and 20 µL of 20 µg/ml of solution consisting of 10 µg/mL of IS was injected (n = 3) in to the UFLC system and the % RSD was calculated.

Assay of tilorone tablets

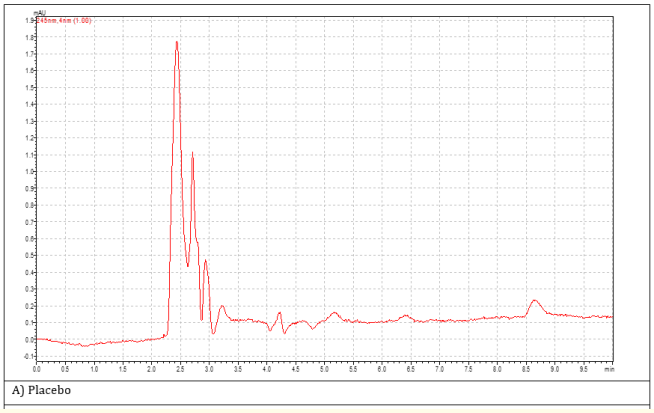
20 tablets of Tilorone were procured, weighed and powdered. Powder equivalent to 25 mg Tilorone was weighed accurately and extracted with HPLC grade acetonitrile. The extracted solution was further diluted with the mobile phase, sonicated for half an hour and filtered through 0.45 mm membrane filter and then 20 μL of these solutions were injected (n = 3) in to the UFLC system and the mean peak area ratio was noted from the peak areas of Tilorone and the IS from their respective chromatogram. The percentage of purity was calculated from the mean peak area ratio value from the calibration curve.

Results and Discussion

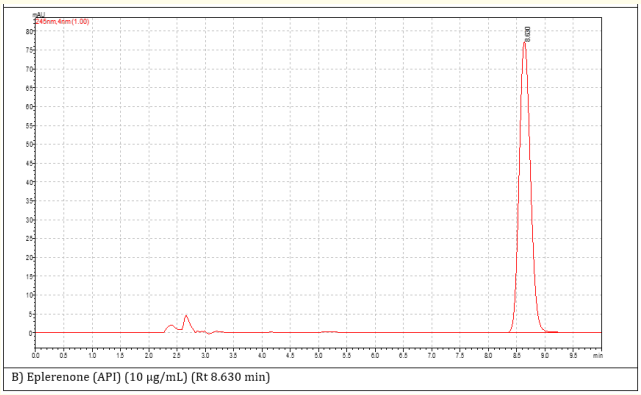
A new reverse phase liquid chromatographic method has been developed for the determination of Tilorone in tablet dosage forms in presence of an internal standard, Eplerenone using Shimadzu Model UFLC system. An internal standard is added to the analyte samples to find more accurate results. During the construction of calibration curve the ratio of the peak area of the analyte (Tilorone) to that of the internal standard is plotted against the concentration of the analyte. A detailed review of the literature was given in table 1. A mixture of tetra butyl ammonium hydrogen sulphate, acetonitrile and methanol (60: 35:5) was used as mobile phase for the chromatographic study (Isocratic mode) using Agilent C18 column and PDA detector (Detection wavelength 245 nm) with flow rate 0.5 mL/min.

Table 1: Literature survey.

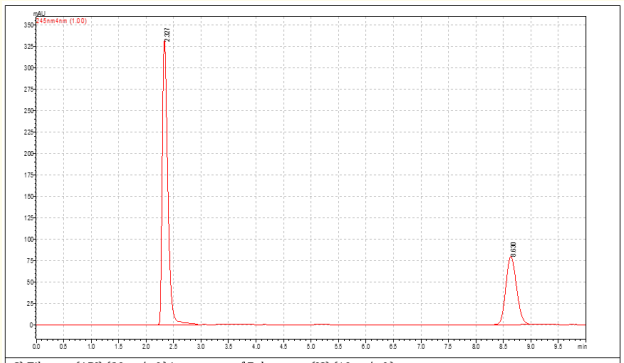
Method	Mobile phase (% v/v)	Linearity (µg/mL)	Comments	Ref
HPLC-MS/MS Metoprolol (Internal standard)	Methanol: 15 mM Ammonium bicarbonate (pH 10.5)	0.001-0.1	Human urine (Gradient mode)	[4]
HPLC-MS/MS	Methanol: 15 mM Ammonium bicarbonate (pH 10.5)	0.001-0.1	Human Blood Gradient mode	[5]
Spectrophotometry	Sodium acetate buffer (pH 4) Borate buffer (pH 9.0) Phosphate buffer (pH 2.0) Phosphate buffer (pH 5.0)	0.4-14	Good linearity	[6]
RP-UFLC	0.1% TEA: Acetonitrile (pH 3.2 adjusted with OPA)/(40:60)	0.1-20	Stability indicating	[7]
RP-HPLC	Tetra butyl ammonium hydrogen sulphate: Acetonitrile (61: 39)	0.05-40	Ion pairing agent	[8]
RP-UFLC Eplerenone (Internal standard)	Tetra butyl ammonium hydrogen sulphate: Acetonitrile: Methanol (60: 35:5)	0.05-50	Ion pairing agent	Present method



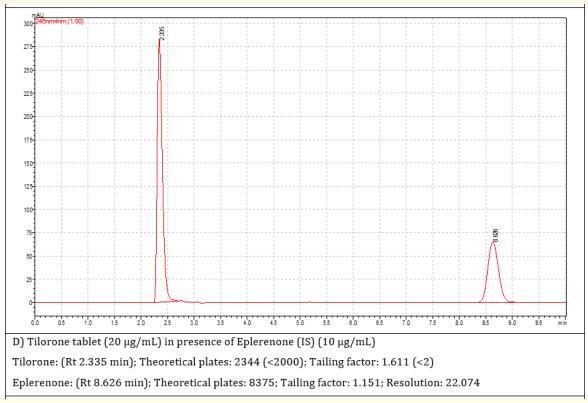
A) Placebo



B) Eplerenone (API) (10 µg/mL) (Rt 8.630 min)



C) Tilorone (API) (20 µg/mL) in presence of Eplerenone (IS) (10 µg/mL)
Tilorone: (Rt 2.327 min); Theoretical plates: 2272 (<2000); Tailing factor: 1.631 (<2)
Eplerenone: (Rt 8.630 min); Theoretical plates: 8868; Tailing factor: 1.172; Resolution: 22.436



D) Tilorone tablet (20 µg/mL) in presence of Eplerenone (IS) (10 µg/mL)
Tilorone: (Rt 2.335 min); Theoretical plates: 2344 (<2000); Tailing factor: 1.611 (<2)
Eplerenone: (Rt 8.626 min); Theoretical plates: 8375; Tailing factor: 1.151; Resolution: 22.074

Figure 2: Representative chromatograms of Tilorone in presence of Eplerenone (IS).

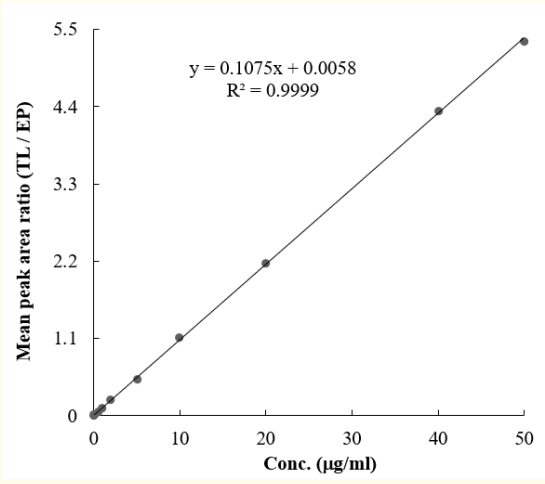


Figure 3: Calibration curve of Tilorone in presence of IS.

Method validation

Linearity, precision, accuracy and robustness

Tilorone has shown linearity over the concentration range 0.05-50 µg/mL and the regression equation was y = 0.1075x + 0.0058 with correlation coefficient 0.9999. The LOD and LOQ are found to be 0.0157 and 0.0487 µg/mL respectively. The typical chromatogram of placebo and Eplerenone (IS) were shown in figure 2A and 2B. The chromatogram of and Tilorone standard in presence of IS was shown in figure 2C.

The % RSD in intraday and inter-day precision was found to be 0.27-0.79 (Table 3) and 0.52-0.94 (Table 4) respectively (<2.0%) indicating that the method is precise. The % RSD in accuracy was found to be 0.58-1.11 (<2.0%) with a recovery of 98.83-99.70% showing that the method is accurate (Table 5). In robustness study the % RSD was found to be 0.23-0.72 which is less than 2.0 indicating that the method is robust (Table 6). Form the method validation parameters it is concluded that the method is precise, accurate and robust.

Table 2: Linearity study.

Conc. (µg/mL)		*Mean peak area		Peak area ratio (TL/EP)	% RSD
TL	EP	TL	EP		
0	0	0	0	0	0
0.05	10	5607	1074987	0.01	0.21
0.1	10	11672	1074825	0.01	0.62
0.5	10	55409	1075022	0.05	0.54
1	10	114154	1074927	0.11	0.71
2	10	234169	1075099	0.22	0.44
5	10	553854	1074837	0.52	0.34
10	10	1200154	1074961	1.12	0.46
20	10	2334810	1075048	2.17	0.54
40	10	4659872	1075037	4.34	0.27
50	10	5740463	1074993	5.34	0.35

*Mean of three replicates.

Table 3: Intraday precision study.

Conc. (µg/mL)	*Mean peak area		*Mean peak area ratio ± SD (% RSD)
	TL	EP	
10	1197924	1074897	1.12 ± 0.0030 (0.27)
20	2298793	1074936	2.14 ± 0.0109 (0.51)
40	4661574	1075007	4.34 ± 0.0343 (0.79)

*Mean of three replicates.

Table 4: Interday precision study.

Conc. (µg/mL)	*Mean peak area		*Mean peak area ratio ± SD (% RSD)
	TL	EP	
Day 1	2298793	1074936	2.14 ± 0.0111 (0.52)
Day 2	2299014	1074894	2.14 ± 0.0178 (0.83)
Day 3	2298957	1074798	2.14 ± 0.0201 (0.94)

*Mean of three replicates.

Table 5: Accuracy study.

Spiked Conc. (µg/mL)	Formulation (µg/mL)	Total Conc. (µg/mL)	*Mean Conc. (µg/mL) ± SD (% RSD)	% Recovery
10 (50%)	20 20 20	30 30 30	29.91 ± 0.1735 (0.58)	99.70
20 (100%)	20 20 20	40 40 40	39.53 ± 0.4111 (1.04)	98.83
30 (150%)	20 20 20	50 50 50	49.81 ± 0.5529 (1.11)	99.62

*Mean of three replicates.

Table 6: Robustness study.

Parameter	Condition	*Mean peak area ratio (TL/EP) ± SD (% RSD)
Flow rate (± 0.1 ml/min)	0.4 0.5 0.6	2.13 ± 0.0143 (0.67)
Detection wavelength (± 2 nm)	243 245 247	2.14 ± 0.0049 (0.23)
Mobile phase composition Tetra butyl ammonium hydrogen sulphate: Acetonitrile: Methanol (± 5 %, v/v)	65: 30: 5 60: 35: 5 55: 40: 5	2.14 ± 0.0088 (0.41)
pH (± 0.01 unit)	3.4 3.5 3.6	2.15 ± 0.0155 (0.72)

*Mean of three replicates.

Assay of tilorone tablets in presence of eplerenone (IS)

The proposed validated RP-UFLC method was applied for the assay of Tilorone tablet dosage forms. Figure 2D represents the typical chromatogram of Tilorone tablets in presence of Eplerenone (IS) was shown in which Tilorone was eluted at Rt 2.335 min with theoretical plates 2344 (<2000) and tailing factor: 1.611 (<2) whereas the IS was eluted at Rt 8.626 min with theoretical plates 8375 (<2000) and tailing factor: 1.151 (<2) with very good resolution of about 22.074 (>2) indicating that the method is selective and all the system suitability parameters were within the acceptable criteria. The percentage of recovery was found to be 99.93.

Conclusion

The proposed RP-UFLC method for the determination of Tilorone in in presence of tablet dosage forms was validated as per ICH guidelines. This method is very much useful for the quantitative determination of Tilorone in biological fluids and also for the pharmacokinetic studies.

Acknowledgement

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