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Development and Validation Rp-Hplc Method for the Estimation of Topiroxostat in Its Pharmacetical Dosage Form

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Abstract
An optimized isocratic method using RP- HPLC was performed and validated for Topiroxostat tablet dosage form. The new developed RP-HPLC method was highly precise, specific, reliable and accurate for the analysis of Topiroxostat tablet dosage form. Also the reduced retention time of drug peak shows the method was time saving Topiroxostat tablet dosage form. Also the reduced retention time of drug peak shows the method was time saving and inexpensive. Mobile phase: Mixed phosphate buffer: Acetonitrile (45:55). at 276 nm. Higher percentage recovery shows that the methods are free from interference of the excipients used in the commercial formulation.

Keyword: RP-HPLC, Topiroxostat, FTIR. UV

1. Introduction

Xanthine oxidase inhibitors are primarily used in the clinical prevention and treatment of gout associated with hyperuricemia. The archetypal xanthine oxidase inhibitor, Allopurinol has been shown to have other beneficial effects such as a reduction in vascular reactive oxygen species and mechano-energetic uncoupling. This chapter discusses these properties and their relevance to human pathophysiology with a focus on Allopurinol as well as newer xanthine oxidase inhibitors such as Febuxostat and Topiroxostat.

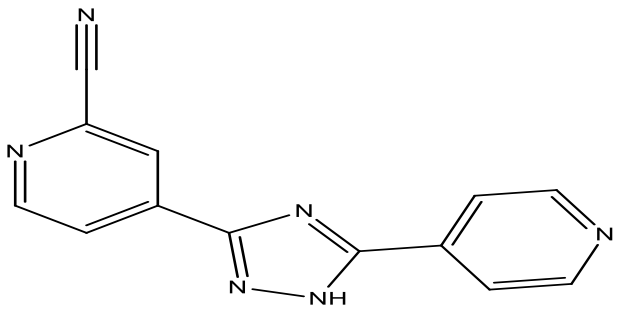


Figure No. 1.1 Molecular structure of topiroxostat

Topiroxostat is a [4-[5-(4-Pyridinyl)-1H-1,2,4-triazol-3-yl]-2- pyridine carbonitrile] non-purine XOR inhibitor, approved in Japan in 2013 for the treatment of patients with hyperuricaemia. There is limited

experience internationally with this agent. Topiroxostat behaves initially as a competitive type inhibitor to xanthine oxidoreductase before forming a strong covalent linkage to molybdenum via oxygen in the hydroxylation reaction intermediate [Chen et al. 2016]. It also displays a potent non-covalent competitive type inhibition of XOR with a Ki value of 5.7 10 9 M [Matsumoto et al. 2011]. Topiroxostat has good oral bioavailability with a half-life of up to 7.5 h after oral administration. It is predominantly eliminated in the urine. It is a strong inhibitor of Cyp 2C9 and has no inducing effect on CYP enzymes. Topiroxostat has a greater inhibitory effect on plasma XOR compared to tissue XOR (the opposite is observed with febuxostat) (Nakamura et al. 2016). Mouse models of minimal change nephrotic syndrome demonstrated that nitrotyrosine and 8-hydroxy-2-deoxyguanosine (8-OHdG) were significantly ameliorated by topiroxostat (Kawamorita et al. 2017).

The recently reported TROFEO trial (Sezai et al. 2017) in hyperuricaemic patients with cardiovascular disease comparing the effects of febuxostat and topiroxostat showed similar urate, antioxidant, anti-inflammatory and reno-protective effects for both drugs. The renoprotective effects of topiroxostat for hyperuricemic patients with overt diabetic nephropathy (ETUDE) study concluded that high-dose topiroxostat (160 mg/day) significantly reduced L-Fatty Acid Binding Protein (FABP), a validated biomarker of tubulointestitial damage and oxidative stress (Mizukoshi et al. 2018). There has not been any direct head-to-head antioxidant effect comparison between allopurinol and topiroxostat.

1.1 HPLC Introduction

High Performance Liquid Chromatography (HPLC) was derived from the classical column chromatography and, is one of the most important tools of analytical chemistry today. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. High Performance Liquid Chromatography (HPLC) is more versatile than gas chromatography (GC) since, it is not limited to volatile and thermally stable samples, and the choice of mobile and stationary phases is wider [5].

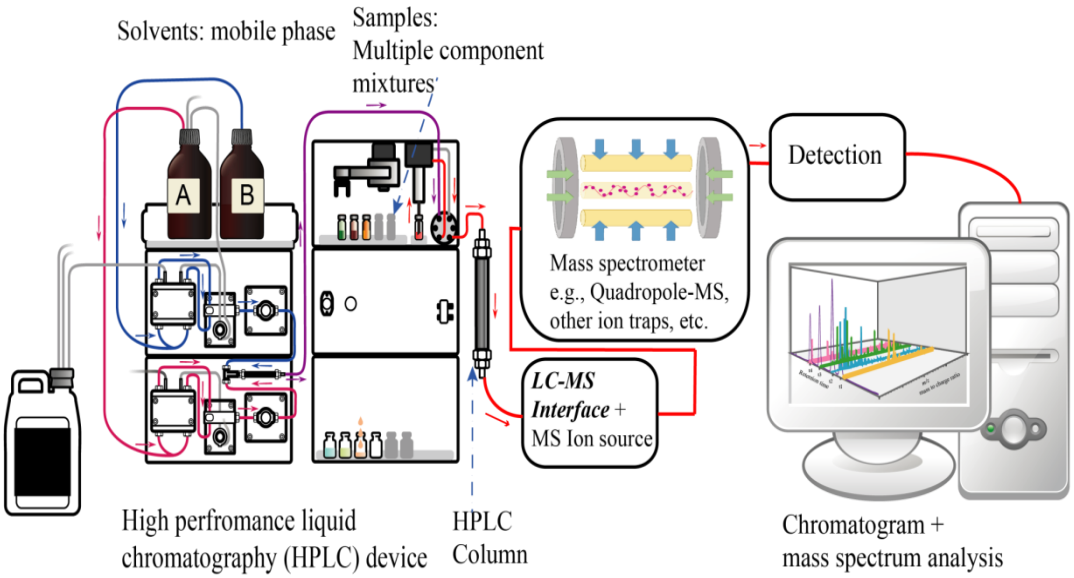


Figure 1.2; Schematic representation of HPLC-MS instrument

1.2 Method development

Analytical method development and validation studies play an important role in discovery, development and manufacture of pharmaceuticals [6-9]. These methods used to ensure the identity, purity, potency, & performance of the pharmaceutical drug products. There are many factors to consider when developing methods. The initially collect the information about the analyte's physicochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis (i.e., suitable wavelength in case of UV detection) . The majority of the analytical development effort goes into validating a stability indicating HPLC–method. The goal of the HPLC-method is to try & separate quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degradants. Steps involve in method development are:

1. Understand the physicochemical properties of drug molecule.
2. Set up HPLC conditions.
3. Preparation of sample solution for method development.
4. Method optimization.
5. Validation of method.

2.Experimental

2.1 Instrumentation

The high-performance liquid chromatography (HPLC) of Shimadzu SCL-10A$_{VP}$ inbuilt with binary pump (LC-10AT$_{VP}$), UV detector (SPD-10A$_{VP}$), Rheodyne 20µl loop capacity manual injector (P/N 77251) was used throughout the analysis. The LC-Solution software was used to interpret the HPLC reports. Zodiac-100 C8 (5µm; 150 x 4.6 mm ID.) column was purchased from zodiac life sciences. (Hyderabad, India) was used throughout the analysis. Digital weighing balance (ME-204) purchased from Mettler-Toledo (USA), ultra-sonicator Labman® purchased from UltraChrom Ltd, India. Digital pH meter from Mettler-Toledo was purchased from (Mumbai-India). 50 µ micro-syringe was purchased from Hamilton USA. 0.20µ and 0.45µ nylon membrane filters were purchased from Phenomenex® Mumbai, India.

2.2 Reagents and reference samples

The reference standard; topiroxostat was obtained as a gift sample from Yarrow chem Ltd. Ammonium formate was purchased from Merck Ltd. (Mumbai-India) HPLC grade acetonitrile, methanol and HPLC grade water were purchased from Merck (Mumbai, India). 0.20µ and 0.45µ nylon membrane filters were used and purchased from UltraChrom Innovatives Pvt. Ltd. (India). All other chemicals and reagents were used of HPLC grade.

2.3 Selection of solvent and wavelength

Topiroxostat is soluble in water and methanol and partially soluble in acetonitrile. Hence, standard stock solution of topiroxostat was prepared in acetonitrile-methanol-water (20:60:20% v/v). Topiroxostat shows maximum UV absorbance (λ_{max}) at 340 and 290 nm wavelength; but it is more sensitive at 340 nm wavelength so that this 340 nm was selected throughout the HPLC Analysis.

2.4 Preparation of standard solution

Exactly, 7 mg of topiroxostat standard was weighed and dissolved in 7 ml of acetonitrile-methanol-water (2:4:1, v/v) to get 1000 ppm (1000 µg/ml) solution. It was sonicated for 4 minutes and then as per the need, serial dilutions were made to get the final concentration of 100 ppm for the determination of repeatability, precision (intraday and interday/intermediate) and robustness.

2.5 Chromatographic conditions

20 µl of freshly prepared stock solution of topiroxostat was injected into the Zodiac-100 C8 (5µm; 150 x 4.6 mm ID.) column and eluted using the mobile phase as solvent A; 15mM ammonium formate (AF) and solvent B; acetonitrile-methanol (70:30, v/v) at 1.0 ml/mins flow rate for 10 mins. Separation was carried out at room temperature and monitored at 240 nm wavelength.

2.6 System suitability studies

Freshly prepared stock solution of topiroxostat (100 ppm) was injected 6 times to determine the closeness of results achieved for relative standard deviation (RSD) in percentage; The calculated values should always less than 2%. Moreover, other system suitability parameters including, retention time, capacity factor (k'), theoretical plates (N), tailing factor/peak asymmetry (As) and separation factor (α) were tested and evaluated.

2.7 Sample preparation for drug accuracy studies

Exactly 5 tablets of topiroxo-20 mg manufactured by Alkem pharmaceuticals Ltd, consisting 20 mg of topiroxostat was weighed and the average weight was calculated. The average weight of tablet was 108 mg. They were mixed and crushed to fine powder into the mortar and pestle. An accurately weighed amount of the finely powdered equivalent to 2 mg was dissolved in 4 ml of acetonitrile-methanol-water (1:2:1, v/v) to get 500 ppm. It was then ultrasonicated for 5-10 mins and then filtered through 0.45µ nylon filter. Furthermore, serial dilutions were made in accordance to get the final concentration 100 ppm of topiroxostat. The solution was then sonicated and analysed as per the chromatographic condition mentioned in section 5.x.

2.8 Sample preparation for Linearity/Calibration studies

1000 ppm (1000 µg/ml) of standard stock solution of topiroxostat was made. Subsequently, serial dilutions of five different concentrations ranging between 3.12–50 ppm were made, ultrasonicated and then analysed as per the chromatographic condition in section 5.x. Furthermore, the calibration curve (linearity graph) was plotted by calculating the peak area against known concentration to determine regression equation, regression coefficient (R^2), limit of quantification (LOQ) and limit of detection (LOD).

2.9 Precision studies of the proposed method

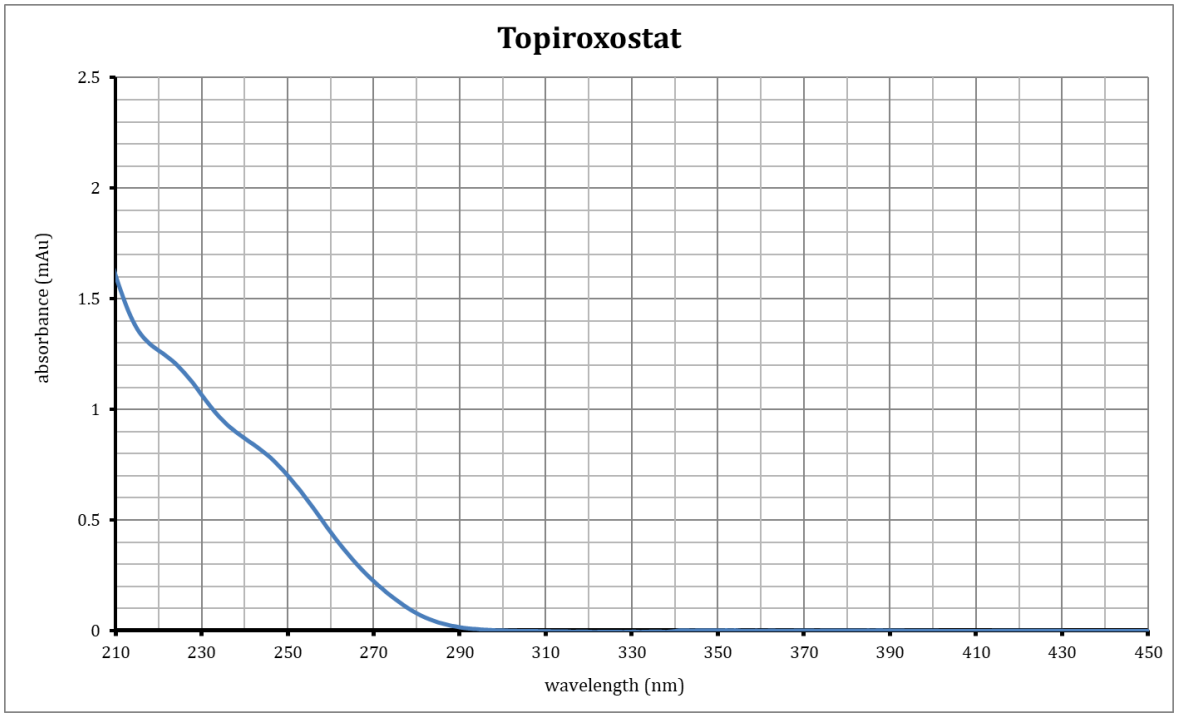
Freshly prepared stock solution of topiroxostat (100 ppm) was analyzed thrice within the same day (intraday precision) and three successive days (intermediate precision) were tested and evaluated. Furthermore, their mean, standard deviation and relative standard deviation (RSD) were calculated which should be less than 2% as per the ICH guidelines.

2.10 Robustness for the chromatographic method

The flow rate of the mobile phase was changed by 1.00 ±1 decimal from 1 mL/min to 1.1 mL/min and to 0.9 mL/min to evaluate the effect of the flow rate on separation pattern of topiroxostat. Similarly, small but deliberate variation of organic modifier as solvent B (70%) was changed by ±2% in its isocratic elution mode to investigate the effects on retention time (t_R), capacity factor (k'), tailing factor (t_f) and theoretical plates (N). Finally, the effect of wavelength was monitored by making deliberate variation from 240±2 nm to 242 and 238 nm and the differences in retention time (t_R), capacity factor (k'), tailing factor (t_f) and theoretical plates (N) were tested and evaluated. Robustness study was performed as per the procedure mentioned under the chromatographic condition under the section 5.5.

3. Results and Discussion

1. UV spectroscopy of topiroxostat



UV spectroscopy of topiroxostat

3.1 HPLC analysis of Topiroxostat

Discussion; RP-HPLC was carried out at two different wavelength, 240 and 290 nm wavelength since UV spectra of topiroxostat HCl represents that it has λmax values at 240 and 290 nm. However, as observed the peak sensitivity of topiroxostat was higher in 240 than 290 nm wavelength. Moreover, as seen in trial (Fig. 2) the elution of topiroxostat was quite late and hence, it is not affordable such elongated retention value for single drug analysis. Therefore, further studies were performed in 240 nm wavelength.

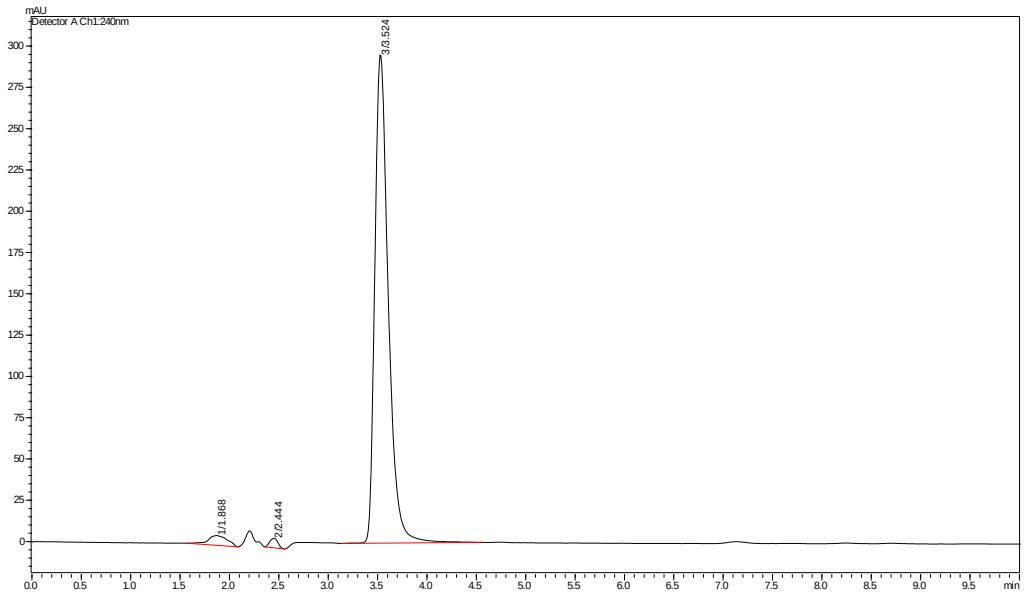


Figure3.1 ; method development of topiroxostat by RP-HPLC method

Table 1; method development of topiroxostat by RP-HPLC method

Peak#	Ret. Time	Area	Height	Area%	T.Plate #	Resolution	k'	Tailing F.	Separation
1	1.868	79936	5924	2.7314	454.122	--	0	0.912	0
2	2.444	32892	5721	1.1239	3548.892	2.235	0.308	1.103	0
topiroxostat	3.524	2813768	295657	96.1447	3160.523	5.21	0.886	1.479	2.878

Analytes: topiroxostat (100 ppm)
Column: Zodiac C8 (5µ, 150 X 4.6mm. ID.)
Mobile Phase: A; 15mM ammonium formate; B; acetonitrile-methanol (70:30 v/v)
Flow rate: 1mL/min
Elution mode: isocratic mode
Elution program: A-B (30:70, v/v)
Wavelength selected: 240nm wavelength
Temperature: 30°C temperature
Run time: 10 minutes

3.2. Method validation

The method was validated according to ICH guidelines.

3.2.1 Repeatability

Implementing the procedure mentioned under experimental section (5.3), the standard reference sample of topiroxostat was tested for six injections within the same day. The % RSD was calculated and found it is less than 2%. It represents the proposed method accepted all basic characteristics of ICH guidelines

Repeatability studies of Topiroxostat

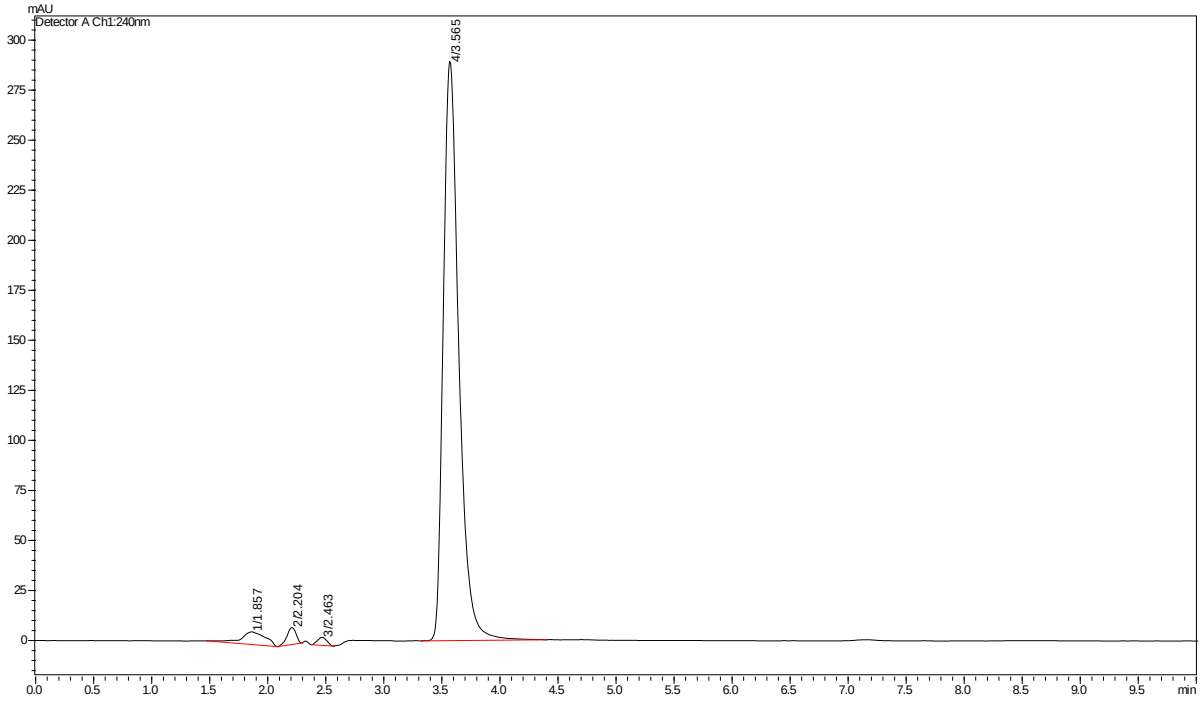


Figure 3.2; repeatability studies of topiroxostat

Table 2; repeatability of Topiroxostat

Peak#	Ret. Time	Area	Height	Area%	T.Plate #	Resolution	k'	Tailing F.	Separation
1	1.857	93198	6320	3.2544	306.043	--	0	0.831	0
2	2.204	46559	8533	1.6258	3116.546	1.191	0.187	0.89	0
3	2.463	22408	4086	0.7825	3517.128	1.603	0.327	1.056	1.749
topiroxostat	3.565	2701589	28950	94.3373	3380.447	5.357	0.92	1.443	2.817

Table 3; Repeatability data of Topiroxostat

S. No.	Drug Name; topiroxostat Peak Area; Conc. 100 ppm
1	2813768
2	2701589
3	2771991
4	2807891
5	2771191
6	2829629
Mean	**2782677**
STD. DEV.	**46104.81301**
RSD (%)	**1.66**

3.2.2. System suitability studies of topiroxostat

The proposed RP-HPLC method for the quantification of topiroxostat was validated as per the ICH guidelines and therefore, system suitability studies specifically, including the variables such as repeatability, linearity, precision (interday/intermediate), robustness studies were tested, evaluated, and displayed in in short Table 4. The tailing factor (T) values <2 represented that the peak width is under the acceptance criteria as per the ICH guideline since both symmetric and asymmetric factors were found of equal magnitude. The separation factor (α) and resolution (Rs) for topiroxostat was significantly higher than the minimum requirement as per the ICH guidelines. As demonstrated, the proposed HPLC method signifies a high degree of reproducibility for the quantification of topiroxostat. System suitability test reveals the factors such as, theoretical plate (N), capacity factor (k'), resolution (R), and separation factor (α), tailing factor (T), Mean±SD and RSD% which should in acceptable range for at least 6 successive injections of same analytes.

Table No. 4; System suitability of topiroxostat

System suitability parameters	Topiroxostat	Acceptable Values
Theoretical plates (*N*)	3160	≤ 2000
Capacity Factor (*K'*)	0.89	≤ 0.5
Resolution (*R*)	---	≤ 1.5

Selectivity/Separation factor (α)	2.87	> k’
Asymmetry/Tailing factor (*T*)	1.47	< 1.5
Retention time (t_R)	3.52 min.	> k’
Wavelength of Detection (nm)	240 nm	> 200 nm
Repeatability (% RSD)	1.67	< 2%
Intra-Day Precision (% RSD)	0.12-1.56	< 2%
Inter-Day Precision (% RSD)	0.12 – 1.94	< 2%
Linearity range	3.9 – 62.5 µg.ml^{-1}	NA
Regression equation	y = 3061x + 27326	NA
SE of intercept (S_e)	0.9993	NA
SD of intercept (S_a)	10102.02409	NA
Correlation Coefficient (r^2)	24744.80439	NA
LOQ[a] (µg.mL^{-1})	8.08 µg.mL^{-1}	NA
LOD[a] (µg.mL^{-1})	2.42 µg.mL^{-1}	NA

Precision studies of Topiroxostat

The precision of HPLC method reflects its closeness to the agreement among the series of repetitive results, derived after multiple sampling of the same homogenous mixture of selected drugs under the given conditions. As displayed in Table 5; for intermediate variability for precision studies, this method is significantly precise over selected tested range of topiroxostat. Moreover, the peak area of the studied samples was also correlated with selected concentration; where the % RSDs were <2%. The RSDs were observed well below 2% that reflects an acceptable precision with minimum variations of the proposed method.

Implementing the procedure mentioned under experimental section (5.3), the sample solution of topiroxostat of three replicates of selected similar concentrations; were tested and evaluated in three successive days (interday/intermediate precision). The % RSD was calculated and it is found less than 2%; for all analytes.

Intraday precision studies of topiroxostat

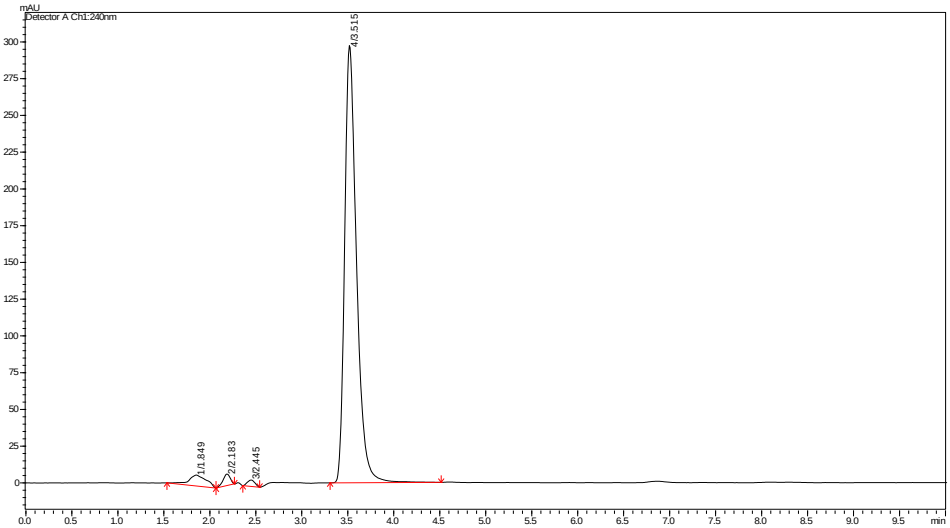


Figure 3.3; intraday precision studies of topiroxostat

Table 5; intraday precision studies of Topiroxostat

Peak#	Ret. Time	Area	Height	Area%	T. Plate#	Resolution	k'	Tailing F.	Separation
1	1.849	98281	7394	3.4675	332.815	--	0	0.878	0
2	2.183	42768	7836	1.5089	3049.89	1.185	0.181	0.898	0
3	2.445	24443	4338	0.8624	3382.789	1.609	0.322	1.061	1.786
topiroxostat	3.515	2668834	29734 9	94.1612	3399.835	5.228	0.901	1.432	2.795

Table 6; Intraday precision data of Topiroxostat

Drug Name: Topiroxostat					
S. No.	Concentration (ppm)	Area	Average	Std. Deviation	%RSD
1	100 ppm	2813768	2809850	3393.09	0.12
	100 ppm	2807891			
	100 ppm	2807891			
2	100 ppm	2701589	2748257	40417.65	1.47
	100 ppm	2771991			
	100 ppm	2771191			
3	100 ppm	2701589	2724263	42550.21	1.56
	100 ppm	2697851			
	100 ppm	2773348			
	Range of % RSD				**0.12-1.56**

3.2.4. Interday precision studies of topiroxostat

Implementing the procedure mentioned under experimental section (5.3), the sample solution of topiroxostat of three replicates of selected similar concentrations; were tested and evaluated in three successive days (interday/intermediate precision). The % RSD was calculated and it is found less than 2%; for all analytes; shown in Table 6.

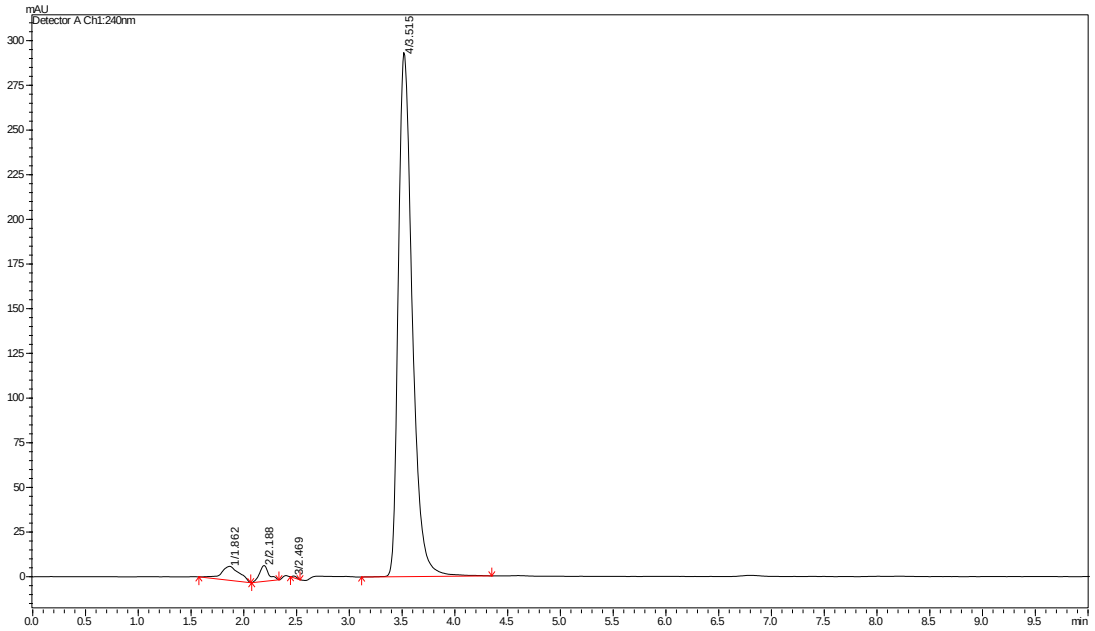


Figure 3.5; Interday precision studies of topiroxostat

Table 7; Interday precision studies of topiroxostat

Peak#	Ret. Time	Area	Height	Area %	T.Plate #	Resolution	k'	Tailing F.	Separation
1	1.862	92626	7801	3.2479	544.068	--	0	0.903	0
2	2.188	54403	8912	1.9077	2850.203	1.348	0.175	1.2	0
3	2.469	2492	856	0.0874	13678.15	2.266	0.326	1.629	1.864
Topiroxostat	3.515	2702316	293331	94.757	3361.465	6.396	0.887	1.476	2.722

Table 8; Interday precision data of topiroxostat

Drug Name: Topiroxostat					
S. No.	Concentration (ppm)	Area	Average	Std. Deviation	%RSD
Day 1	100 ppm	2813768	2809850	3393.09	0.12
	100 ppm	2807891			
	100 ppm	2807891			
Day 2	100 ppm	2702316	2730453	28758.95	1.05
	100 ppm	2759796			
	100 ppm	2729248			

Day 3	100 ppm	2796921	2745124	53186.08	1.94
	100 ppm	2690650			
	100 ppm	2747802			
	Range of % RSD				**0.12-1.94**

3.2.5. Robustness studies of Topiroxostat

Robustness of HPLC Method represents its ability to remain unaffected by small but deliberate variations in separation parameters to ascertain its reliability during routine analysis. In this method, robustness was established by making deliberate changes in flow rate (1.0 ± 0.1 ml/minutes), organic modifier (70% ± 2% ml), and temperature (28°C ± 2°C).

Robustness studies was determined by small variation in separation parameters like effect of temperature, flow rate, eluent composition, temperature, pH, wavelength, injection volume on selected separation variables including capacity factor (k'), resolution (Rs), tailing factor (*Tf*), separation factor, theoretical plates (N)and peak area.

Therefore, increased the flow rate by +0.1 ml/minutes, marginally reduced the t_R values of all selected drugs and impurities whereas reducing it, extended slightly the t_R values of same drugs. Similarly, slight variation in organic solvent concentration, considered here as CAN-MeOH (90:10 v/v) 20%±2% for topiroxistat have not made any significant changes. Furthermore, small variation in temperature by 28°C ± 2°C has not made any significant changes in the retention pattern of all selected drugs .

As resulted, the robustness studies for all drugs and impurities were almost unchanged by small variations which clearly signified that the proposed HPLC method obliged all minimum requirements led by the ICH guidelines.

Robustness data of Topiroxostat

Variables	**Topiroxostat**			
	t_R (min)	k'	T_f	N
Flow rate (+0.2 mL.min^{-1})	3.21	0.89	1.43	3271
Flow rate (-0.2 mL.min^{-1})	3.9	0.89	1.46	3548
CH3OH (+2%)	3.36	0.77	1.47	3295
CH3OH (-2%)	3.56	0.93	1.41	3787
Temperature (+2˚C)	3.51	0.89	1.46	3403
Temperature (-2˚C)	3.51	0.89	1.46	3388
Mean ± S.D.	3.51±0.23	0.88±0.05	1.45±0.02	

3.2.7. Caliberation/linearity studies of topiroxostat

The linearity of HPLC method represents its ability to explicit the results that should proportional to the concentration of studied analytes within a selected range. Therefore, over the selected concentrations of topiroxostat, its corresponding area was highly proportional since in all studies their regression coefficients (R2) were nearby 0.999 which almost close to 1 which itself represented a high degree of linearity. (Table No. . Furthermore, the LOD and LOQ were calculated based on the standard deviation of

the response and the slope of the regression equation. As observed, the LOD and LOQ for all selected compounds were well below the 5 µg/ml which signifies the selected wavelength is more sensitive to detect the lowest quantity of drugs either from pharmaceutical drugs or biological fluids.

3.2.7 Linearity data of Topiroxostat

Name of Drug: Topiroxostat		
S. No.	Concentration (µg.mL^{-1})	Area
1	50	1545708
2	25	811745
3	12.5	429776
4	6.25	205497
5	3.125	123307
6	1.5625	61653.5
Regression Equation		y=30616x + 27326
Correlation coefficient (R^2)		0.9992
Std. error of intercept		10102.02409
Std. Dev. Of intercept		24744.80439
LOQ		8.08 µg/ml
LOD		2.42 µg/ml

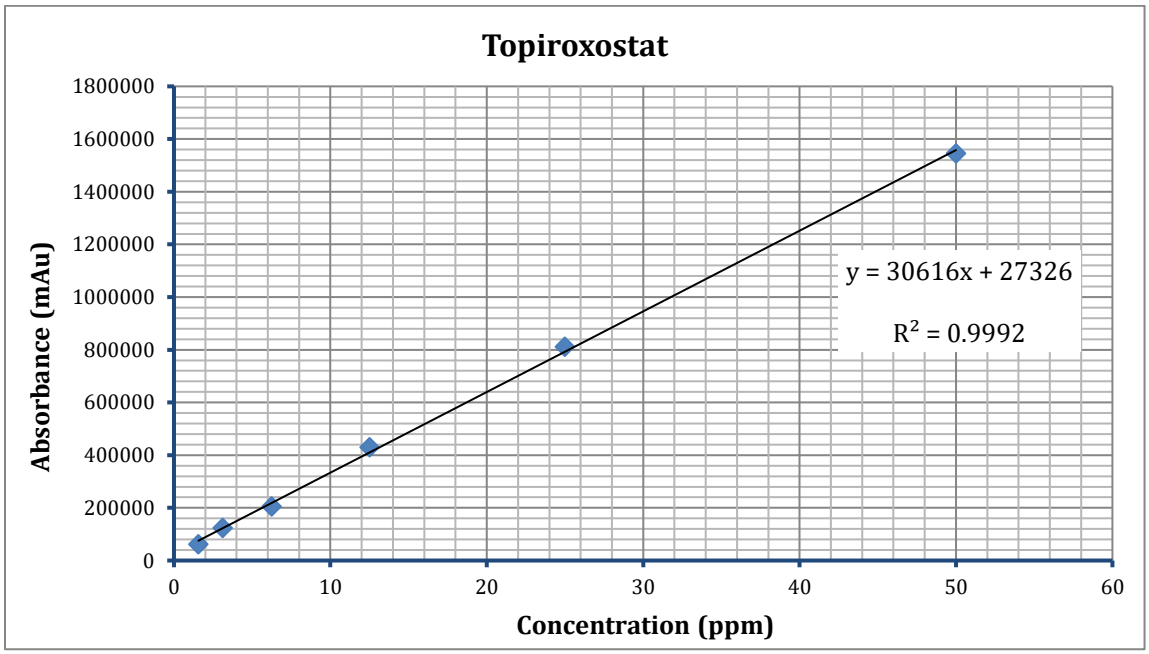


Figure 3.6 linearity graph of topiroxostat

3.2.8 . Accuracy studies of topiroxostat

Percentage drug accuracy of three different concentrations; 80%, 100% and 120% (injected thrice) to estimate the topiroxostat from marketed formulation and results obtained have been reported in Table 7.34. Accuracy can be studied by applying the calibration curve; the Y-intercept and the slope of the graph were used to determine the % drug recovery, attributed to the developed method for the simultaneous quantification of selected drugs or by comparing with similar concentration of reference standard.

As resulted, the achieved drug recovery of topiroxostat was in the range of 100.4-100.7 and 100-105, respectively. As recommended by International conferences of Harmonization (ICH) guidelines the drug recovery should be within the range of 90-110% (100±10 %) and the RSD in percentage should be less than 2%. Hence, the calculated drug recoveries for simultaneous estimation of topiroxostat represents the drug recovery were in the acceptance limit given by ICH guidelines.

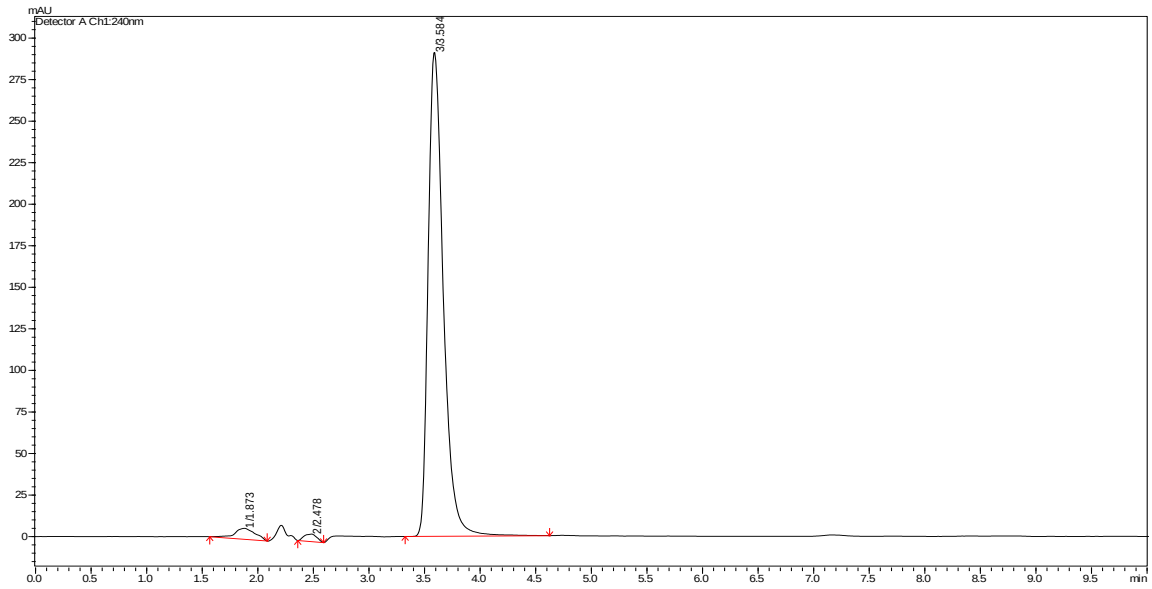


Figure 3.7; accuracy studies; topiroxostat API analysis of 100 µg/ml concentration

Table 9; topiroxostat API analysis of 100 µg/ml concentration

Peak#	Ret. Time	Area	Height	Area%	T.Plate #	Resolution	k'	Tailing F.	Separation
1	1.873	86037	6629	2.9745	460.88	--	0	0.877	0
2	2.478	35295	4488	1.2202	2100.153	2.14	0.323	0.959	0
topiroxostat	3.584	2771191	290960	95.8053	3337.282	4.759	0.913	1.494	2.826

Table 10. Accuracy data of topiroxostat

Drug Name: topiroxostat			Drug content: 25 mg		Marketed formulation: Topiroxo-25 Tablet		
Std. conc. (%)	Std. (ppm)	Peak area	Drug (%)	Drug (ppm)	Peak area	Avg. peak area	Drug Rec. (%)

100%	100 ppm	2771191	80	80	2204090	2203210	99.38%
				80	2202330		
			100	100	2697851	2735599.5	98.72%
				100	2773348		
			120	120	3608002	3611376	108.60%
				120	3614750		
			Drug recovery Range (%) as per ICH = 100±10%				98.72 % - 108.60%

4. Conclusion

From collective results and discussion, it has been concluded that the proposed RPHPLC-UV analytical method for the quantification of topiroxostat in both bulk and tablet formulation has obliged the ICH guidelines. Moreover, as per the ICH guidelines, the developed method has complied the linearity range (calibration data), accuracy/drug recovery studies (%), repeatability, precision studies (intraday and interday/intermediate), and robustness parameters. In addition, as per the ICH guidelines, the system suitability test performed for topiroxostat has achieved all guidelines; including, tailing factor (*T*), separation factors (α), theoretical plates (*N*), capacity factor (*k'*), resolution (*R*) and RSD (%). The validated stress degradation studies under thermal, oxidative, alkali and acid ascertained few degradation products for topiroxostat.

Hence, this developed and validated method for investigation by C18 based RP-HPLC can be used for the routine analysis of estimation of topiroxostat from marketed formulation.

5. References

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