

Development and Validation of Reverse Phase HPLC Method for Simultaneous Estimation of Allopurinol and Lesinurad in its API and Pharmaceutical Dosage Form

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ABSTRACT

A new, reliable, and validated reverse phase-high-performance liquid chromatography (HPLC) method was developed to quantify the amount of allopurinol and lesinurad simultaneously in solid (tablet) dosage form. A clear chromatographic division was attained on inertsil ODS (4.6 x 250 mm, 5 mm) column, and a mixture of 0.1% trifluoroacetic acid and methanol in the ratio of 40:60 v/v was used as mobile phase. The rate of flow was set at 1 mL/min, and UV detection was achieved at λ_{max} of 255 nm. Injection volume was set to 20 μ L. The correlation coefficient of 0.999 was established, and the accurateness was found to be 100.69 and 100.49 for both the drugs, respectively. Therefore, the developed method was simple, specific, precise, and stable. Hence, the method can be employed to estimate the said drugs in other pharmaceutical formulations.

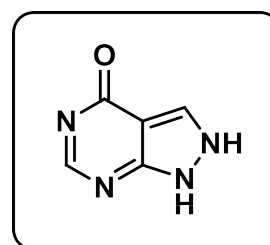
Keywords: Allopurinol, Degradation studies, HPLC, Lesinurad, Vacuum freeze, Validation.

1. INTRODUCTION

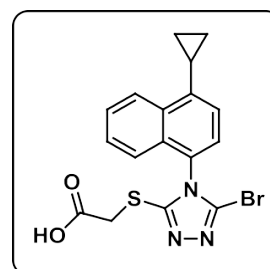
Allopurinol ($C_5H_4N_4O$) is a xanthine oxidase inhibitor that inhibits the synthesis of uric acid in certain conditions like gout and kidney diseases. It is considered as a standard treatment for hyperuricemia. It possesses a purine skeleton, which is structurally an isomer of a natural purine base, hypoxanthine and inhibits xanthine oxidase enzyme, which oxidizes hypoxanthine to xanthine (Arellano et al., 1993; Goicoechea et al., 2010). As a result, uric acid production is prevented, which is the desired approach for the treatment of gout. Hence, it is used as an anti-gout agent. It is also used in the case of patients with inflammatory bowel syndrome (Ermer et al., 2001; Stamp, 2014).

Lesinurad ($C_{17}H_{14}BrN_3O_2S$) is a selective uric acid reabsorption inhibitor (SURI) that inhibits URAT1 transporter. URAT1 is responsible for the reabsorption of filtered uric acid from the renal tubular lumen during excretion in kidneys (Hoy, 2016) and is employed for the treatment of gout in combination with allopurinol, a hypoxanthine isomer that inhibits xanthine oxidase enzyme. It also inhibits OAT4, which is URAT protein that leads

to diuretic-induced hyperuricemia (Borstad et al., 2014; Perez-Ruiz et al., 2016; Bardin et al., 2017). Duzallo was the first drug consists of the mixture of allopurinol and lesinurad, for the treatment of gout as well as other allied



Allopurinol (1, 2 - dihydro - 4H - pyrazolo [3, 4 - d] pyrimidin - 4 - one)



Lesinurad (2 - {[5 - bromo - 4 - (4 - cyclopropyl)naphthalen - 1 - yl]-4H-1, 2, 4 - triazol -3- yl] sulfanyl} acetic acid)

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diseases approved by the Food and Drug Administration (FDA) in 2017.

Literature survey reveals that numerous analytical methods have been accounted for the quantitative and qualitative determination of allopurinol individually in the pharmaceutical formulations and a few methods, in combination with lesinurad after the approval by FDA in 2017 for using HPLC method development (Revathi et al., 2016; Dastiagiramma et al., 2018). The developed methods were mostly available for allopurinol alone. Few methods were available for quantification of allopurinol in combination with lesinurad (Attia et al., 2018) but, to best our knowledge, a validate RP-HPLC method has not been established yet. Hence the present study reported a simple, reliable, accurate, rapid, and validated RP-HPLC method for the quantification of allopurinol and lesinurad in a combined dosage form.

2. MATERIALS AND METHODS

Allopurinol was obtained as a gift sample from Harman Finochem Ltd., Mumbai, and lesinurad was obtained from A. R. Life Sciences Pvt. Ltd., from Hyderabad, India. Potassium dihydrogen phosphate was purchased from Finer Chemical Ltd., Hyderabad, India. HPLC grade acetonitrile and methanol were procured from E-Merck, Hyderabad. All other chemicals and solvents used were of analytical grade.

2.1 Instruments

HPLC system (Waters, USA) consisting of empower-II software, quaternary pump, manual rheodyne injector, and photodiode-array detection (PDA) detector was used for analysis. They were processed by using micropipettes made up of borosil, centrifuge, and ultra-sonicator.

2.2 Optimized Conditions of Chromatography

Instrument	:	Waters HPLC (2695 separations module)
Temperature	:	Ambient
Column	:	Inertsil ODS (4.6 x 250 mm, 5 mm)
Mobile Phase	:	0.1 % Trifluoro Acetic Acid buffer & Methanol (40:60)
Flow Rate	:	1 mL/min
λ_{\max}	:	255 nm
Injected Volume:	:	20 μ L
Run time	:	10 min.

2.2 Buffer and Mobile Phase Preparation

2.3.1 0.1% Trifluoro Acetic Acid (TFA)

Dissolved 1 mL of trifluoro acetic acid in 1000 mL of HPLC water with a pH adjusted up to 3.0. The obtained solution was passed through 0.44 μ m membrane filter and sonicated for ten minutes.

2.3.2 Mobile Phase Preparation

400 mL (40%) of prepared buffer solution was mixed with 600 mL (60%) of Methanol HPLC. By using an ultrasonic water bath, the dissolved gases were removed by degassing for ten minutes and then passed through 0.45 μ m filter membrane under vacuum filtration. The same was used as diluents (Ahuja & Rasmussen, 2011; Snyder et al., 2012; Dastiagiramma et al., 2018).

2.4 Standard and Sample Solution of Allopurinol and Lesinurad

2.4.1 Preparation of Standard Solution

0.06 gm of allopurinol and 0.04 gm of lesinurad were weighed accurately and transferred into a 0.1 L dry volumetric flask. A total of 7 mL of diluents was added to the mixture and sonicated to dissolve the residual particles. Final volume was adjusted by mixing the same solvent (stock solution). 1.5 mL of the above stock solution was pipetted to 10 mL volumetric flask, and the final volume was diluted with the diluent.

2.4.2 Preparation of Sample Solution

Accurately weighed 10 tablets were crushed in mortar and pestle. An equivalent amount of 0.06 g of allopurinol and 0.04 g lesinurad samples were transferred into a 0.1 L dry volumetric flask add to which 7 mL of diluent was added and subjected to sonication for 15 min to dissolve the residue. The final volume was adjusted up to the mark with the same solvent and is passed through 0.45 μ m injection filter. 1.5 mL of allopurinol and lesinurad from the stock solution was taken and pipetted to 10 mL volumetric flask and diluted. The final volume was made with diluent.

2.5 Procedure

The HPLC system was injected with 20 μ L of the standard, sample and peak areas were measured for allopurinol and lesinurad peaks. The percentage assay was

calculated by using the following formula, (Ahuja and Dong, 2005; Connors, 2007).

$$\% \text{ Assay} = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{\text{Avg. wt.}}{\text{Label claim}} * \frac{P}{100} * 100$$

Where, AT = Average area count of sample
AS = Average area count of standard
WS = Weight of working standard (mg)
P = % purity

2.6 Method Validation

The present analytical method was validated as per ICH guidelines with reverence to various parameters like linearity, precision, specificity and accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness (Bakshi & Singh, 2002; Breaux et al., 2003; Swartz and Krull, 2018).

2.6.1 System Suitability

The prepared standard solutions were injected into the HPLC chromatographic system and evaluated for various parameters such as theoretical plates, tailing factor, retention time resolution, and asymmetric factor. The results are given in Table 1.

2.6.2 Assay

20 µl of the sample and standard solutions were injected separately into the HPLC system and the peak areas were measured. The percentage quantity of both the drugs was then calculated and reported in Table 2.

2.6.3 Precision

Equivalent concentration solutions of allopurinol and lesinurad were injected six times, and % RSD was determined for both the drugs.

2.6.4 Ruggedness

The ruggedness was determined by measuring the analyst to analyst variation assay of two different analysts.

2.6.5 Accuracy

The accuracy of the developed method was done by recovery studies by the addition of standard drug solution to pre-analyzed sample solution at three different levels 50, 100, and 150%. The percentage recovery and percentage mean recovery were determined for the drugs and shown in Table 7.

2.6.6 Limit of Detection (LOD)

$$LOD = \frac{3 \sigma}{S}$$

Where, σ = standard deviation of the response
S = slope of the calibration curve

2.6.7 Limit of Quantification (LOQ)

$$LOQ = \frac{10 \sigma}{S}$$

Where, σ = standard deviation of the response
S = slope of the calibration curve

2.6.8 Robustness

Robustness is done by changing the flow rate and the organic phase of the mobile phase.

2.7 Degradation Studies

2.7.1 Hydrolytic Degradation (Acidic condition)

Stock solution measuring 1.5 mL was pipetted to 10 mL volumetric flask, and 3 mL of 0.1 N HCl was added. Then, the volumetric flask was maintained at 60°C for 24 hours followed by neutralizing using 0.1 N NaOH. The final volume was adjusted to 10 mL with diluents, which was then filtered and stored in vials.

2.7.2 Hydrolytic Degradation (Alkaline condition)

Stock solution measuring 1.5 mL was pipetted to 10 mL volumetric flask, and 3 mL of 0.1 N NaOH was added. Then, the volumetric was maintained at 60°C for 24 hours, followed by neutralizing using 0.1 N HCl. The final volume was adjusted to 10 mL with diluent. The solution was filtered and place in vials (Blessy et al., 2014).

2.7.3 Thermal Induced Degradation

Allopurinol and lesinurad sample was kept in a Petri dish and placed in a hot air oven maintained at 110 °C for 3 hours. The samples were then diluted with the diluents, injected into HPLC and analyzed.

2.7.4 Degradation due to Oxidation

Stock solution measuring 1.5 mL was pipetted to 10 mL volumetric flask and 1 mL of 12.5% w/v of H₂O₂ was added. The final volume was adjusted to the mark with diluent. The volumetric flask was kept at room temperature for 15 minutes, and the solution was subjected to filtration followed by placing in vials.

2.7.5 Photo Degradation

Stock solution measuring 1.5 mL was pipetted to 10 mL volumetric flask and exposed to sunlight for 24 hrs. The final volume was made up with the diluents till the mark. The solution was filtered with 0.45 microns syringe filters and transferred in vials (Reynolds et al., 2002; Ngwa, 2010; Baertschi et al., 2016).

3. RESULTS

3.1 System Suitability

It was found from the obtained data that all the system suitability parameters such as retention time, resolution, tailing, and plate count have shown uniformity and was within the limit. The results are tabulated, and from the observed data, it can be concluded that the system was suitable for analysis.

3.2 Assay

Standard and sample solution injected as described under experimental work. The percentage of the assay was found to be 100.87 and 100.18. The corresponding results are shown in Table 2.

3.3 Validation Parameters

3.3.1 Linearity

Linearity was found in the range of 30 - 150 µg/mL for allopurinol and 20-100 µg/mL for lesinurad with a linearity correlation coefficient of 0.999 and 0.999 which shows

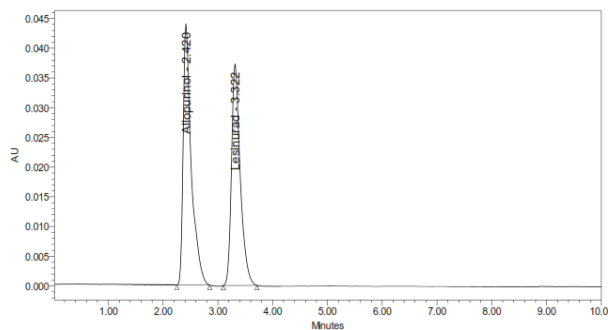


Fig. 1: Chromatogram of system suitability for allopurinol and lesinurad

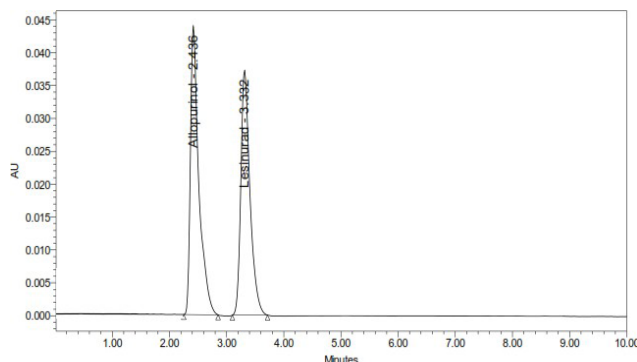


Fig. 2: Chromatogram for standard allopurinol and lesinurad

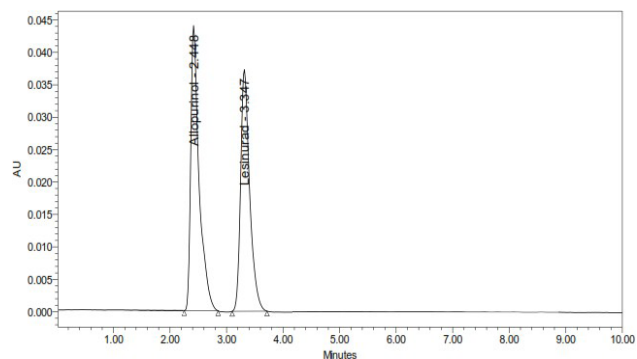


Fig. 3: Chromatogram for sample allopurinol and lesinurad

Table 1: System suitability parameters for allopurinol and lesinurad

S. No.	Name	RT(min)	Area (µV sec)	Height (µV)	USP resolution	USP tailing	USP plate count
1	Allopurinol	2.420	466482	43853	3.16	1.53	3467.19
2	Lesinurad	3.322	371997	37483	3.16	1.40	5115.36

Table 2: Assay results of allopurinol and lesinurad

Name	Claim of label (mg)	% Assay
Allopurinol	300	100.87
Lesinurad	200	100.18

Table 3: Linearity results of allopurinol and lesinurad

S. No.	Allopurinol		Lesinurad	
	Concentration of solution (µg/mL)	Peak area	Concentration of solution (µg/mL)	Peak area
1	30	158922	20	125433
2	60	319278	40	254978
3	90	467255	60	369727
4	120	642988	80	503762
5	150	783655	100	623994

that the method is capable of producing good sensitivity and useful for routine analysis (Table 3 and Table 4).

3.3.2 Precision

For the present developed RP-HPLC method, the precision was determined by using quality control samples. The method show precision of 0.5 and 0.8, indicating the method was precise when performed on different days also. The percentage of relative standard deviation (RSD) was reported in Table 5.

3.3.3 Ruggedness

There was no significant change in assay content and system suitability parameters at different conditions of ruggedness like day to day and system to system variation. The method show ruggedness of 0.5 and 0.5, which reveals that the method is precise. The percentage of relative standard deviation (RSD) was reported in Table 6.

Table 4: Analytical performance parameters of allopurinol and lesinurad

Parameters	Allopurinol	Lesinurad
Slope (m)	5255.7	6242.4
Intercept (c)	1174.7	860.48
Correlation coefficient (R ²)	0.999	0.999

Table 5: Precision results of allopurinol and lesinurad

Injection	Peak area (Allopurinol)	Peak area (Lesinurad)
1	468873	362588
2	465388	370267
3	468873	370468
4	463548	368962
5	467126	370882
6	469978	369764
Average	467297.7	368821.8
Standard deviation	2444.7	3124.4
% RSD	0.5	0.8

Table 6: Ruggedness Results of Allopurinol and Lesinurad

Injection	Peak area (Allopurinol)	Peak area (Lesinurad)
1	462866	370757
2	469272	368674
3	463875	368562
4	465887	365298
5	463867	367744
6	465387	369977
Average	465192.3	368502.0
Standard deviation	2283.3	1904.4
%RSD	0.5	0.5

3.3.4 Accuracy

The total recovery was found to be 100.69 and 100.49% for allopurinol and lesinurad. The validation of the developed method shows that the accuracy is well within limits (Table 7).

3.3.5 Limit of Detection (LOD)

The LOD for allopurinol and lesinurad were found to be 3.03 and 2.98, which is in acceptance criteria given in Table 8.

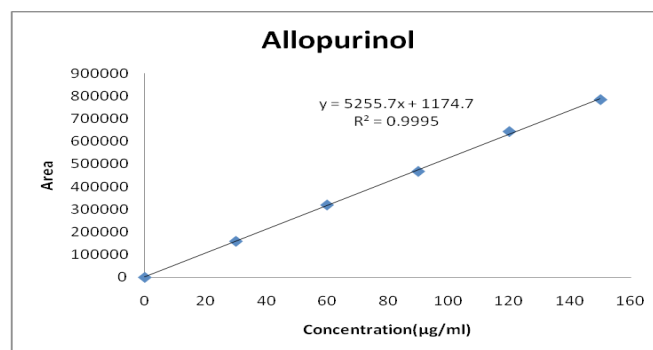


Fig. 4: Calibration graph for allopurinol

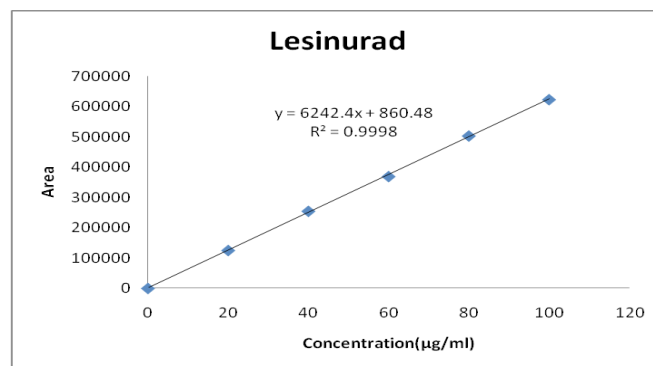


Fig. 5: Calibration graph for lesinurad

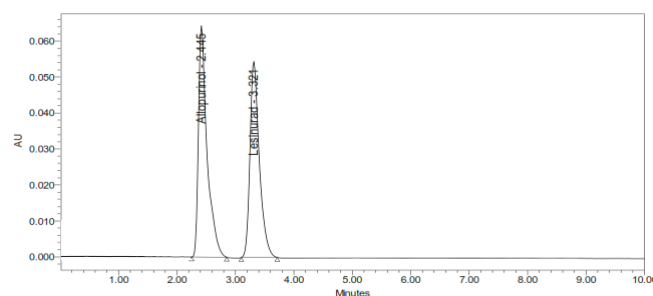


Fig. 6: 50% Accuracy chromatogram

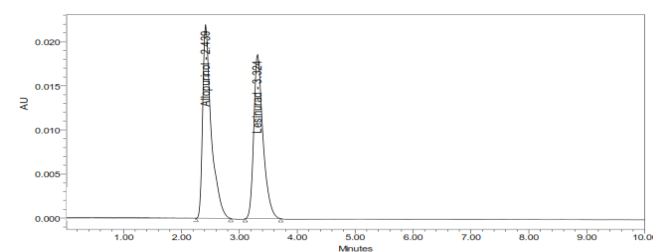


Fig. 7: 100% Accuracy chromatogram

Table 7: Accuracy (Recovery) data for allopurinol

	% Concentration (at specification level)	Peak area	Amount added (mg)	Amount found (mg)	Recovery (%)	Mean recovery (%)
Allopurinol	50 %	233682	30	30.19	100.64	100.69
	100 %	466589	60	60.29	100.48	
	150 %	703223	90	90.86	100.96	
Lesinurad	50%	186179	20	20.10	100.50	100.49
	100%	373282.3	40	40.30	100.75	
	150%	556834.7	60	60.12	100.20	

*Average of three determinations

Table 8: Results of LOD

Name of drug	Baseline noise (µV)	Obtained signal (µV)	S/N ratio
Allopurinol	63	191	3.03
Lesinurad	63	188	2.98

Table 9: Results of LOQ

Name of drug	Baseline noise (µV)	Obtained signal (µV)	S/N ratio
Allopurinol	63	631	10.02
Lesinurad	63	629	9.98

Table 10: Variations in flow for allopurinol and lesinurad

S. No.	Drug	Rate of flow (mL/min)	Results of system suitability		
			USP resolution	USP tailing	USP plate count
1	Allopurinol	0.9	3.16	1.53	3587.65
2		1.0	3.16	1.53	3494.63
3		1.1	3.16	1.53	3427.86
1	Lesinurad	0.9	3.31	1.41	5348.76
2		1.0	3.16	1.40	5231.87
3		1.1	2.97	1.39	5207.38

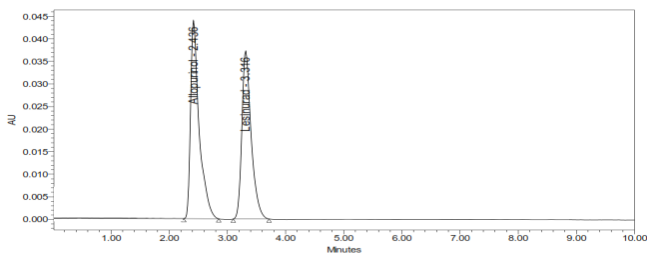


Fig. 8: 150 % Accuracy Chromatogram

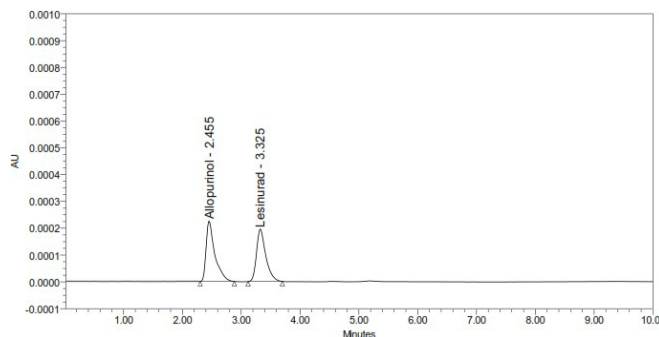


Fig. 9: Chromatogram of Allopurinol, Lesinurad showing LOD

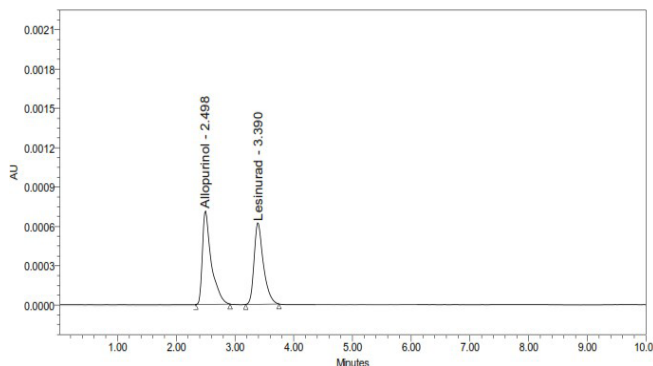


Fig. 10: Chromatogram of allopurinol, lesinurad showing LoQ

3.3.6 Limit of Quantification (LOQ)

The LOQ for allopurinol and lesinurad were found to be 10.02 and 9.98 which is in accepted criteria given in Table 9. This shows that the method is having good system suitability and precision under given set of conditions.

3.3.7 Robustness

The standard and samples of allopurinol and lesinurad were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count and are in limits (Table 10).

Table 11: Results for the stability of allopurinol and lesinurad

Sample Name	Allopurinol		Lesinurad	
	Area	% Degraded	Area	% Degraded
Standard	463442.7	-	369747	-
Acid	447882	3.36	338774	8.38
Base	444972	3.99	334928	9.42
Peroxide	436676	5.78	347222	6.09
Thermal	429847	7.25	349852	5.38
Photo	441856	4.66	358733	2.98

3.4 Degradation Studies

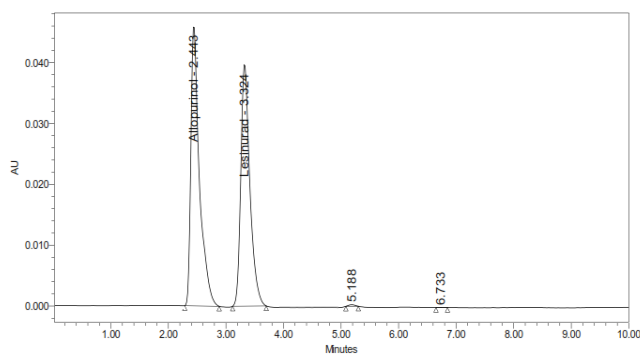


Fig. 11: Acid Degradation Chromatogram

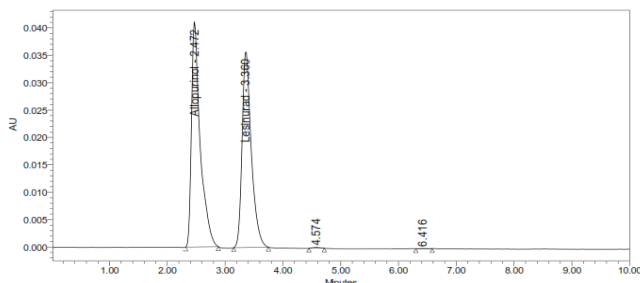


Fig. 12: Base degradation chromatogram

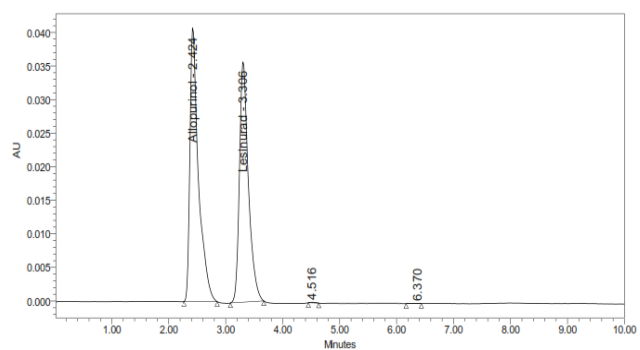


Fig. 13: Peroxide degradation chromatogram

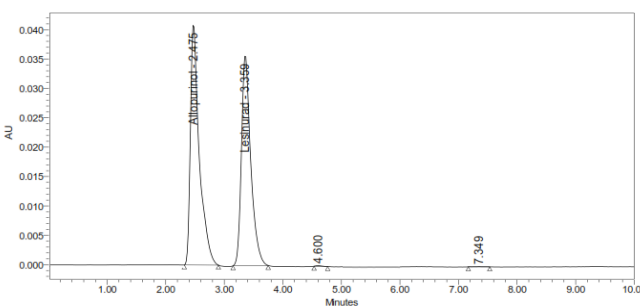


Fig. 14: Photo-degradation chromatogram

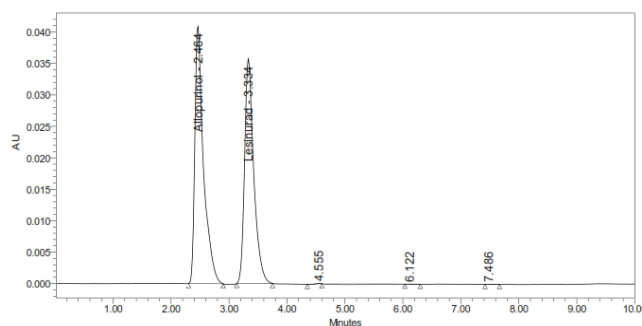


Fig. 15: Thermal degradation chromatogram

4. DISCUSSION AND CONCLUSION

In the present developed reverse phase-HPLC method, a satisfactory separation with good peak symmetry was obtained with inertsil ODS (4.6 × 250 mm, 5 mm) column using mixture of mobile phase containing 0.1% trifluoro acetic acid buffer and methanol (40:60) at a flow rate of 1 mL/min. Quantification was achieved at UV detection at 255 nm based on peak area. The present optimized method was validated according to the guidelines of ICH (Bakshi and Singh, 2002; Breaux et al., 2003; Swartz and Krull, 2018).

For system suitability, the method was validated for linearity, precision, specificity and accuracy, LOD, LOQ, and robustness. The system suitability parameters such as retention time, resolution, tailing, and plate count have shown uniformity and % RSD was less than 1 and found to be within limits. Hence it was concluded that the system was suitable to perform the assay.

The method was observed to be simple and accurate as the recovery of both the drugs was found to be >100%. The chromatographic isolation was clear and there was no interference indicating that the method was specific. The LOD and LOQ values of both the drugs are within the desired criteria. The results of degradation studies were <10% as the acceptance criteria was NMT 15%. Therefore, the method was also found to be stable. There is no interference of any excipients peaks with standard and analytic peak and hence it proves that the method is selective.

From the results obtained, it is evident that the developed method can be employed to estimate allopurinol

and lesinurad in other pharmaceutical formulations as well.

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