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Simultaneous Quantitative Estimation for the Method Development, Validation and Stability Studies of Lesinurad and Allopurinol in Bulk and Pharmaceutical Dosage Form by RP-HPLC

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Abstract
Objective: The objective of the present research work was to develop an innovative, simple, and economic method for estimation of Lesinurad and Allopurinol in bulk and dosage form by RP-HPLC. **Methods:** The chromatographic conditions were performed on Phenomenex Luna C18, 100A, 5µm, 250mmx4.6mm i.d.as stationary phase and mobile phase was prepared with a mixture of Phosphate buffer (pH – 3.9): Acetonitrile flow 1.0 ml/min, with Injection Volume 20µl, at detection wavelength 252 nm and run time at 6.0 min. **Results:** The analytical method is valid for estimation of Lesinurad and Allopurinol over a range of 0-150 µg/ml, 0-15 µg/ml. The results of system suitability test, linearity, precision and accuracy, robustness, specificity, LOD and LOQ and stabilities presented in this report are within the acceptance range, Retention time of Lesinurad and Allopurinol was found to be2.246 and 3.132. **Conclusion:** A specific, sensitive, economic method estimation of Lesinurad and Allopurinol has been developed based on ICH Guidelines with bulk and dosage forms.

Keywords
Lesinurad and Allopurinol, HPLC, Method Development, ICH, Validation, Accuracy, Precision.

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1. INTRODUCTION
Lesinurad inhibits the activity of uric acid transporter 1 (URAT1) and organic anion transporter 4 (OAT4). URAT1 is a major transporter enzyme responsible for reuptake of uric acid from the renal tubules; inhibition of URAT1 function thereby increases excretion of uric acid.[1-5] Allopurinol and its active metabolite, oxypurinol, inhibits the enzyme xanthine oxidase, blocking the conversion of the oxypurines hypoxanthine and xanthine to uric acid.[6-8] Elevated concentrations of oxypurine and oxypurine inhibition of xanthine oxidase through negative feedback results in a decrease in the concentrations of uric acid in the serum and urine.[7-10].

The IUPAC Name of Lesinurad is 2-{[5-bromo-4-(4-cyclopropylnaphthalen-1-yl)-4H-1,2,4-triazol-3-yl] sulfanyl} acetic acid.

Allopurinol, sold under the brand name Zyloprim among others, is a medication used to decrease high blood uric acid levels. It is specifically used to prevent gout, prevent specific types of kidney stones and for the high uric acid levels that can occur with chemotherapy. It is taken by mouth or injected into a vein. Common side effects when used by mouth include itchiness and rash. [11-14] Common side effects when used by injection include vomiting and kidney problems. While not recommended historically, starting allopurinol during an attack of gout appears to be safe. In those already on the medication, it should be continued even during an acute gout attack. While use during pregnancy does not appear to result in harm, this use has not been well studied.

Allopurinol is in the xanthine oxidase inhibitor family of medications. [15]

The IUPAC Name of Allopurinol is 1H,2H,4H-pyrazolo[3,4-d] pyrimidin-4-one.

A detailed literature survey reveals that RP-HPLC methods have been reported for the quantitative estimation of allopurinol and alpha lipoic acid individually in various pharmaceutical dosage forms and in plasma. RP-HPLC methods are reported for the analysis of alpha lipoic acid with other substances and similarly allopurinol with other drugs in combination.[18] As per our detailed literature survey as on date, there are no RP-HPLC methods reported for the simultaneous quantitative estimation of allopurinol and alpha lipoic acid in any matrix either of pharmaceutical dosage forms, plasma, etc.[19] In addition there exist no pharamacoepial RP-HPLC methods available for analysis of these two drugs in combination.[20]

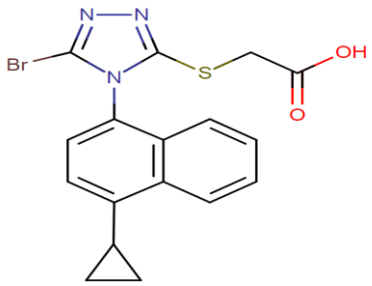


Fig no.1-Structure of Lesinurad

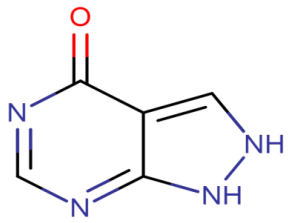


Fig no.2- Structure of Allopurinol

2. EXPERIMENTAL

2.1 Materials and Methods:

Pharmaceutical grade working standard Lesinurad and Allopurinol were obtained from Syncorp Pvt. Laboratories, Hyderabad, India. All chemicals and reagents were HPLC grade and were purchased from S D Fine-Chem Limited & Loba Chemie Pvt Ltd, Mumbai, India.

2.2 Instrumentation:

The analysis was performed using HPLC (Waters-717 series) with PDA detector and data handling system EMPOWER2 software, UV-Visible double beam spectrophotometer (ELICO SL-159), analytical balance 0.1mg Sensitivity (SHIMADZU), pH meter (Labindia), ultra sonicator. The column used is Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d. (as Stationary phase) with the flow rate 1.0ml/min (isocratic).

2.3 Sample & Standard Preparation for the Analysis

Accurately weighed 10mg of Lesinurad and 10mg of Allopurinol working standard were transferred intoa10mL and 10ml of clean dry volumetric flasks. About 7mL and 70ml of Diluents are added and sonicated to dissolve it completely and made volume

up to the mark with the same solvent. (Stock solution) Further 1ml of allopurinol and 1ml of Allopurinol the above stock solution was pipette into a 10mlvolumetricflaskand diluted up to the mark with diluents.

2.4 Selection of wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It is scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Lesinurad and allopurinol, so that the same wave number can be utilized in HPLC UV detector for estimating the Lesinurad and allopurinol. While scanning the Lesinurad and allopurinol solution we observed the maxima at 252 nm.

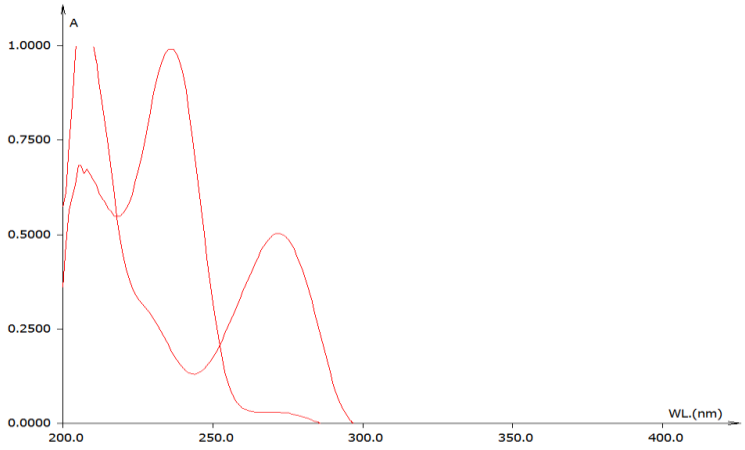


Fig-2: UV Spectrum for Lesinurad and Allopurinol

2.5 Method Development

2.5.1. Preparation of Phosphate buffer:(PH:3.9):

Weighed 0.50grams of Disodium hydrogen phosphate and 0.301grams of potassium dihydrogen phosphate was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to3.9 with orthophosphoric acid.

2.5.2 Preparation of Mobile Phase:

The mobile phase was prepared with the combination of Phosphate Buffer (pH- 3.9) and Acetonitrile at the volume of 1000ml. 600ml of Phosphate Buffer and 400ml of Acetonitrile were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

2.5.3 Summary of Optimized Chromatographic Conditions:

The Optimum Chromatographic conditions obtained from experiments can be summarized as below:

Table-1: Summary of Optimised Chromatographic Conditions

Mobile phase	Phosphate Buffer (3.9): Acetonitrile
Column	Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.
Flow rate	1.0 ml/ min.
Wavelength	252nm
Sampling System	Automatic
Temp. of Auto sampler	Ambient
Volume of injection	20µl
Run time	06 min.
Mode of Separation	Isocratic

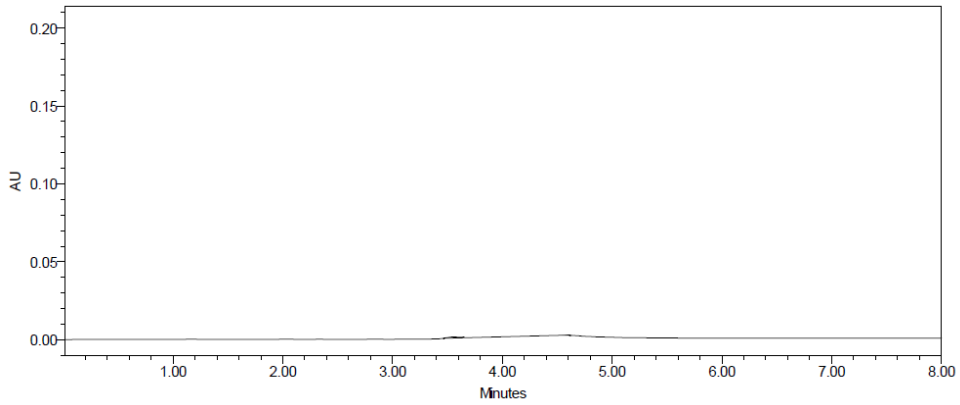
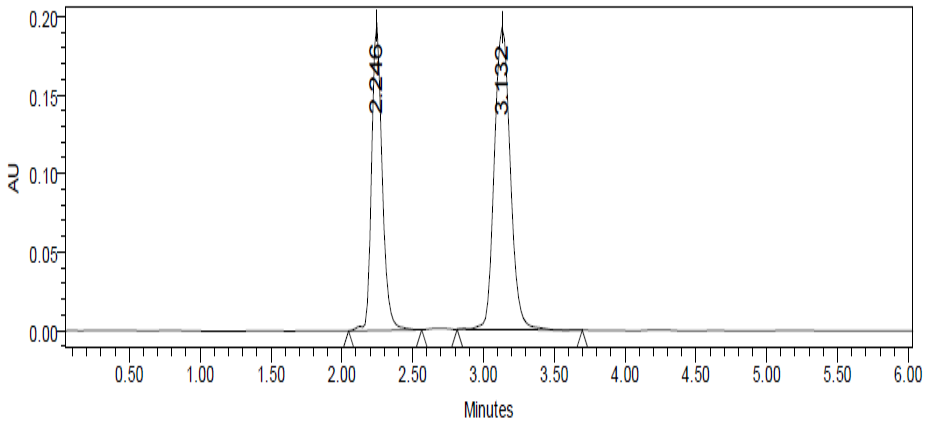


Fig-3: Chromatogram for Blank Preparation



Fig–4: Chromatogram of Lesinurad and Allopurinol in Optimized Condition

2.6 Method validation:

2.6.1 Linearity & Range:

Calibration standards at five levels were prepared by appropriately mixed and further diluted standard stock solutions in the concentration ranges from 0-150 µg/ml for Lesinurad and concentration ranging from 0-15 µg/ml for Allopurinol. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs. Chromatograms of each solution were recorded.

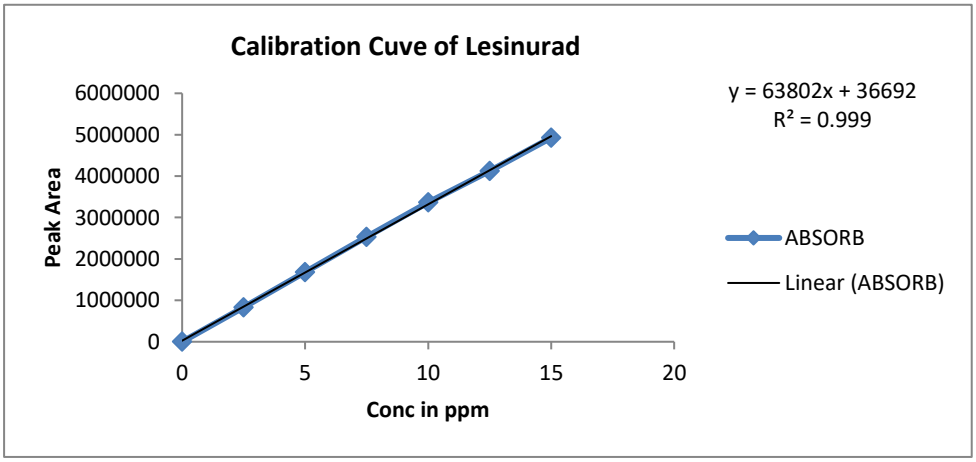


Fig-8: Standard curve for Lesinurad

Table-2: Linearity Results for Lesinurad

CONC. (µg/ml)	AUC (n=6)
0	0
25	1599471
50	3357873
75	4832464
100	6353428
125	7963787
150	9645631

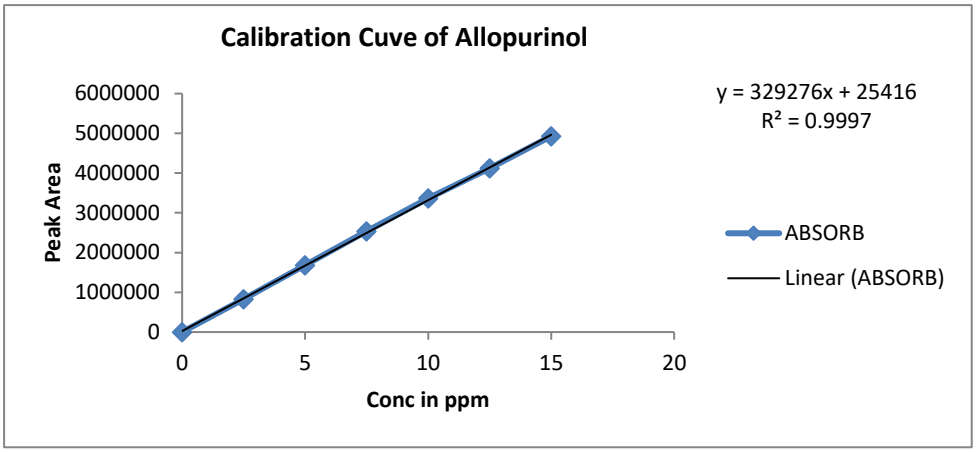


Fig-9: Standard curve for Allopurinol

Table-3: Linearity Results for Allopurinol

CONC.(µg/ml)	MEAN AUC (n=6)
0	0
2.5	831153
5	1684215
7.5	2533451
10	3363412
12.5	4125535
15	4927109

2.6.2. Accuracy:

Recovery study*:* For Lesinurad

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of LESINURAD were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 63802 x + 36692.

Recovery study: Allopurinol

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of ALLOPURINOL were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 32927x + 25416.

Table-4: Accuracy Readings for Lesinurad

Sample ID	Concentration (µg/ml)		Peak Area	%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered			
S_1 : 80 %	8	7.885787	539821	98.57234	Mean= 99.12496%
S_2 : 80 %	8	7.886179	539846	98.57724	S.D. = 0.952926
S_3 : 80 %	8	8.018025	548258	100.2253	% R.S.D.= 0.96133
S_4 : 100 %	10	9.963967	672413	99.63967	Mean= 100.35948%
S_5 : 100 %	10	10.1793	686152	101.793	S.D. = 1.241466 R.S.D.= 1.158668
S_6 : 100 %	10	9.964578	672452	99.64578	
S_7 : 120 %	12	11.80891	790124	98.40757	Mean= 98.40757%
S_8 : 120 %	12	11.91113	796646	99.25943	S.D. = 0.582486
S_9 : 120 %	12	11.9426	798654	99.5217	% R.S.D. = 0.59191

Table no.5: Accuracy Results for Allopurinol

Sample ID	Concentration (µg/ml)		Peak Area	%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered			
S_1 : 80 %	8	7.991557	288554	99.89446	Mean= 100.365066%
S_2 : 80 %	8	8.073526	291253	100.9191	S.D. = 0.517385
S_3 : 80 %	8	8.022535	289574	100.2817	% R.S.D.= 1.08
S_4 : 100 %	10	10.05582	356524	100.5582	Mean= 100.821506%
S_5 : 100 %	10	9.995202	354528	99.95202	S.D. = 1.026781
S_6 : 100 %	10	10.19543	361121	101.9543	% R.S.D.= 1.165758
S_7 : 120 %	12	12.14641	425361	101.2201	Mean= 100.8581233%
S_8 : 120 %	12	12.18204	426534	101.517	S.D. = 0.896462
S_9 : 120 %	12	11.98047	419897	99.83727	% R.S.D. = 0.88883

2.6.3. Precision:

2.6.3.1. Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Lesinurad and Allopurinol (API). The percent relative standard deviation was calculated for Lesinurad and Allopurinol are presented in the Table-4.

Table-6: Data showing repeatability analysis for Lesinurad

HPLC Injection Replicates of Lesinurad	Peak Area (AUC)
Replicate – 1	6152684
Replicate – 2	6212311
Replicate – 3	6135241
Replicate – 4	6087958
Replicate – 5	6125685
Average	6142776
Standard Deviation	45516.96
% RSD	0.740984

Table-7: Data showing repeatability analysis for Allopurinol

HPLC Injection Replicates of Allopurinol	Peak Area (AUC)
Replicate – 1	3378546
Replicate – 2	3368541
Replicate – 3	3298786
Replicate – 4	3352468
Replicate – 5	3412131
Average	3362094
Standard Deviation	41582.74
% RSD	1.236811

2.6.3.2. Intermediate precision:

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Lesinurad and Allopurinol revealed that the proposed method is precise.

Table-8: Data for Lesinurad Analysis

Conc. Of Lesinurad (API) (µg/ml)	Observed Conc. Of Lesinurad (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
80	79.98	1.07	80.05	0.93
100	100.10	0.87	99.95	0.79
120	119.91	0.75	120.08	0.18

Table-9: Data for Allopurinol analysis

Conc. Of Allopurinol (API) (µg/ml)	Observed Conc. Of Allopurinol (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.04	0.84	7.94	0.99
10	10.07	0.36	10.05	0.88
12	11.96	0.48	12.06	0.74

2.6.4. Method Robustness:

Influence of little changes in optimized chromatographic conditions like changes in flow rate (± 0.1ml/min), mobile phase ratio (±2%), Wavelength of detection (±2nm) and Acetonitrile content in mobile phase (±2%) studied to measure the robustness of the method are also in favour of (Table-36, % RSD < 2%) the developed RP-HPLC method for the analysis of Lesinurad and Allopurinol (API).

Table-10: Result of Method Robustness Test for Lesinurad

Change in parameter	% RSD
Flow (1.2 ml/min)	0.45
Flow (0.8 ml/min)	0.90
Less organic	0.21
More organic	0.11
Wavelength of Detection (272 nm)	0.74
Wavelength of detection (268 nm)	0.96

Table no-11: Result of Method Robustness Test for Allopurinol

Change in parameter	% RSD
Flow (1.2 ml/min)	0.88
Flow (0.8 ml/min)	0.97
Less organic	0.14
More organic	0.15
Wavelength of Detection (242 nm)	0.46
Wavelength of detection (238 nm)	0.87

2.6.5. LOD & LOQ:

The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

2.6.6. System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-12.

Table-12: Data of System Suitability Parameter

S. No.	Parameter	Limit	Result
1	Resolution	Rs> 2	3.15
2	Asymmetry	T ≤ 2	Lesinurad =0.28 Allopurinol =0.37
3	Theoretical plate	N > 2000	Lesinurad =4257 Allopurinol= 4365

2.6.7 Estimation of Lesinurad and Allopurinol in Tablet Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally, the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicated to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase). The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded. A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in the chapter results and discussion.

Table-13: Assay of marketed formulation

Brand name of Tablets	Labeled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Duzallo (Ironwood Pharmaceuticals)	200/300	199.95 (±0.08)/ 299.96 (±0.02)	99.97 (±0.854), 99.98(±0.635)

2.6.8 Stability studies:

The API (Lesinurad and Allopurinol) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation, photolytic degradation and oxidative degradation.

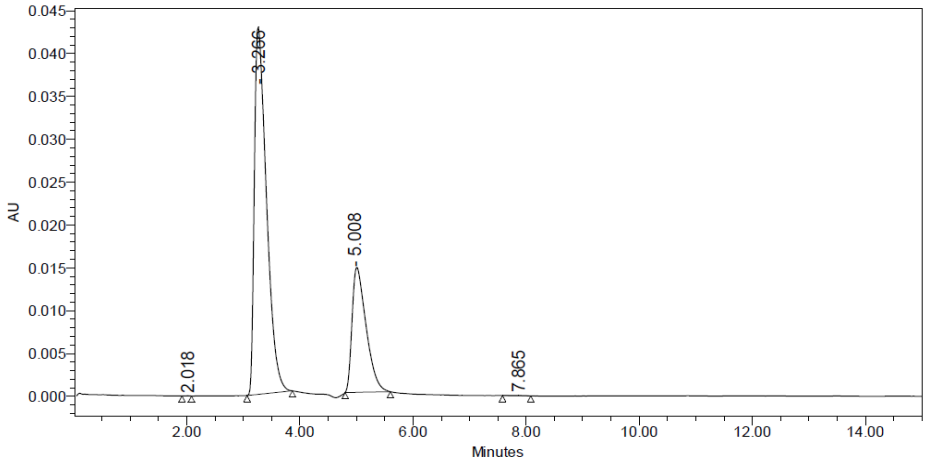


Fig-5: Chromatogram for Acid Degradation

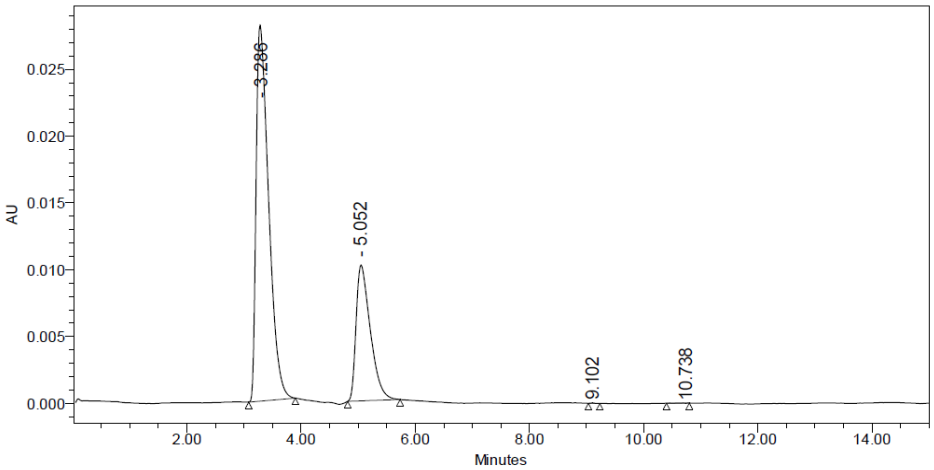


Fig-6: Chromatogram for Basic Degradation

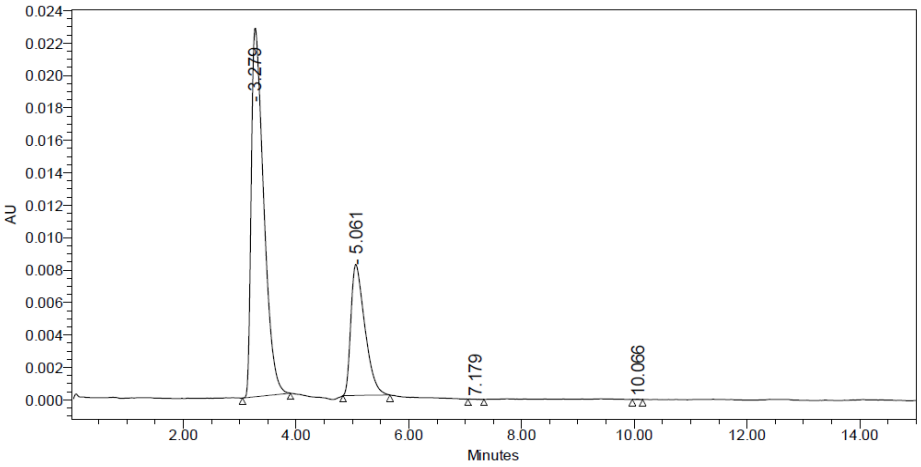


Fig-8: Chromatogram for Thermal Degradation

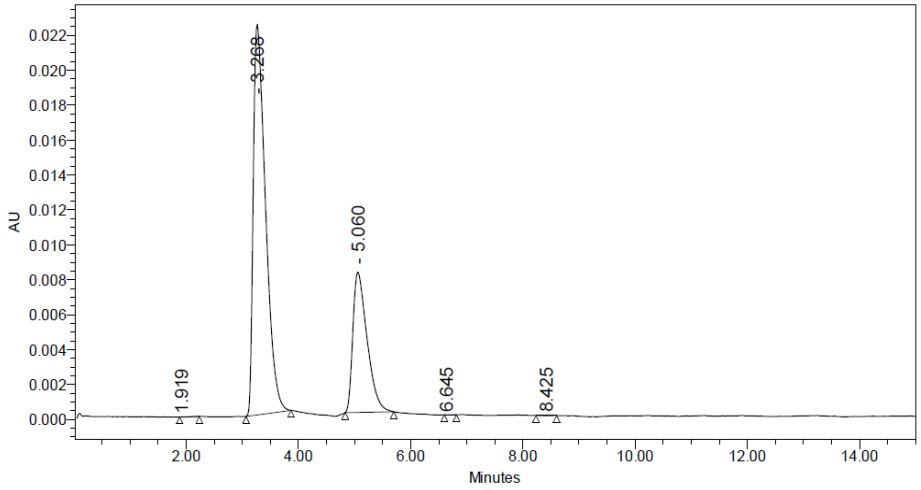


Fig-9: Chromatogram for Photolytic Degradation

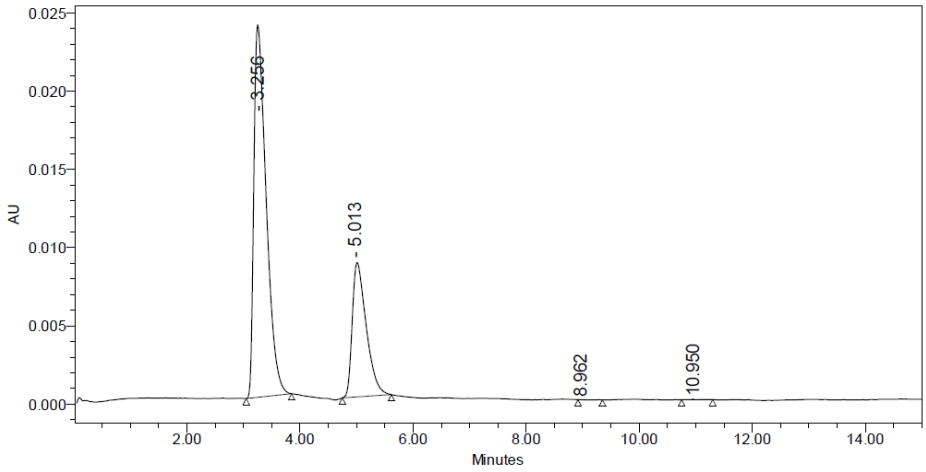


Fig-10: Chromatogram for Oxidation with 3% H_2O_2 Degradation

Table-14: Results of Stress studies of Lesinurad and Allopurinol API.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.95	4.07	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	59.41	40.51	100.00
Thermal Degradation (50 ^{0}C)	24Hrs.	79.23	20.42	100.00
UV (254nm)	24Hrs.	97.21	2.63	100.00
3% Hydrogen peroxide	24Hrs.	96.36	3.24	100.00

3. RESULTS

The optimized chromatographic conditions were Phenomenex Luna C18, 100A, 5µm, 250mmx4.6mm i.d.as stationary phase and mobile phase was prepared with a mixture of Phosphate Buffer: Acetonitrile = 60:40 (pH-3.9) flow 1.0 ml/min, with Injection Volume 20µl, at detection wavelength 252 nm and run time at 6.0 min. In these chromatographic conditions the peak was pure,

sharp, symmetric and found a greater number of theoretical plates.

The results obtained in method validation were

Linearity and Range: Linearity range was found to be 0-150 µg/ml for Lesinurad. The correlation coefficient was found to be 0.999, the slope was found to be 63802 and intercept was found to be 36692 for Lesinurad.

Linearity range was found to be 0-15 µg/ml for Allopurinol. The correlation coefficient was found to be 0.999, the slope was found to be 32927 and intercept was found to be 25416 for Allopurinol.

Accuracy:

Lesinurad: From the Accuracy Method, we observed that the mean % Recovery of the drug are 99.12496%, 100.35948% and 98.40757% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.96133%, 1.158668% and 0.59191% respectively.

Allopurinol: From the Accuracy Method, we observed that the mean % Recovery of the drug are 100.3650867%, 100.82150% and 100.858123% which is within the range of 98-102% and %RSD is within the range <2 i.e. 0.5717%, 1.018414% and 0.8888% respectively.

Repeatability: The repeatability study which was conducted on the solution having the concentration of about 100 µg/ml for Lesinurad and 10 µg/ml for Allopurinol (n =5) showed a RSD of 0.740984% for Lesinurad and 1.236811% for Allopurinol. It was concluded that the analytical technique showed good repeatability.

LOD and LOQ: The LOD was found to be 0.19 µg/ml and 0.65 µg/ml and LOQ was found to be 0.5 µg/ml and 0.8 µg/ml for Lesinurad and Allopurinol respectively which represents that sensitivity of the method is high.

Assay: The assay of Duzallo Tablets containing 200 mg of Allopurinol & 300 mg of Lesinurad was found to be 99.97 % and 99.98% respectively.

Degradation studies: The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Lesinurad and Allopurinol were stable only in oxidative stress conditions and photolytic stress conditions. The results of stability studies are given in the following Table-14.

4. DISCUSSION

To develop a precise, linear, specific RP-HPLC method for analysis of Lesinurad and Allopurinol, different chromatographic conditions were applied, and the results observed were compared with the methods available in literatures.

H.M. Sowjanya, et al. Chromatography was carried out on a Zorbax C18 (4.6 x 150mm, 5µm) column using a mixture of Methanol: Phosphate Buffer pH 3.9 (55:45v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 255nm. The retention time of the Lesinurad and Allopurinol was 2.061, 2.462 ±0.02 min respectively. The method produces linear responses in the concentration range of 1-5µg/ml of Lesinurad and 100-500µg/ml of Allopurinol. The method precision for the determination of assay was below 2.0%RSD. [21]

A.Ashok Kumar*et al The optimized method uses reverse phase column, Enable C18G (250 X 4.6 mm; 5µ), a mobile phase of acetonitrile: 0.02M ammonium acetate buffer adjusted to pH 4.6 in the proportion of 50:50 v/v, flow rate of 0.8 ml/min and a detection wavelength of 210 nm using a UV detector. Results: The developed method resulted in allopurinol eluting at 3.01 min and alpha lipoic acid at 8.42 min. Both the drugs exhibited linearity in the range 50-175 µg/ml. The precision is exemplified by relative standard deviations of 0.83 % for allopurinol and 1% for alpha lipoic acid. Percentage Mean recoveries were found to be in the range of 98-102, during accuracy studies. The limit of detection was obtained as 3 ng/ml for allopurinol and 0.5 µg/ml for alpha lipoic acid, while the limit of quantitation was obtained as 10 ng/ml for allopurinol and 1µg/ml for alpha lipoic acid. [22]

The result shows the developed method is yet another suitable method for assay which can help in the analysis of in formulations.

5. CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed and validated for the analysis of Lesinurad and Allopurinol API. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Lesinurad and Allopurinol indicated that the developed method is specific for the estimation of Lesinurad and Allopurinol. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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