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A Validated and Stability Indicating Ultra Performance Liquid Chromatographic Method for Allopurinol in Pharmaceutical Preparation

* V. Sreeram ** M. V. Basaveswara Rao
*** Srinivasa Rao Karumuri

* Department of chemistry,P.G.Centre,P.B.Siddhartha College of Arts & Science, Vijayawada-520 010.

** Department of Chemistry and Pharmacy, Krishna University, Machilipatnam-521 001, Andhra Pradesh, India

*** Department of Electronics & Instrumentation, Lakireddy Bali Reddy College of Engineering, Mylavaram, A.P, India

ABSTRACT

A simple, selective, linear, precise, and accurate ultra performance liquid chromatography method was developed, optimised and validated for the quantification of synthetic Allopurinol in pharmaceutical dosage form. Isocratic elution at a flow rate of 0.55 mL/min was employed on C18 1.7 µm(2.1 x 50 mm) or equivalent column at ambient temperature. The mobile phase consists of 0.05m monobasic Ammonium Sulphate (Ammonium Di-hydrogen Phosphate) filter through 0.2µm. The UV detection wavelength was 254 nm, and 0.5µL sample was injected. The Flow rate was found to be 0.55 ml/min The retention time for Allopurinol was ± 0.9 min. The percent RSD for accuracy of the method was found to be 0.9%. The correlation coefficient (R2) for Allopurinol is 1.000. The average percent Recovery is varying from 98.0 -101.0. The method for the Dissolution of Allopurinol 5 mg Tablets complies with the requirements for Specificity, System suitability, Linearity, Accuracy and Method precision across the range of 50 % to 150 % at working concentrations of 0.020 mg/ml. The theoretical plates were found to be 3864 and reproducibility at 1.3 %. The method was validated as per the guidelines. The method can be successfully applied for Allopurinol in the rapid and reliable determination of Allopurinol in pharmaceutical dosage form. The method is therefore acceptable as valid and stability indicating.

Keywords : Allopurinol, UPLC, UV-Dection, % Recovery , Accuracy

Introduction:

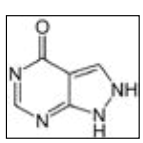


Fig :1

The Systamatic IUPAC Name for allopurinol is 1H-pyrazolo[3,4-d]pyrimidin-4(2H)-one and the Molecular formulae is $C_5H_4N_4O$. Allopurinol is a purine analog; it is a structural isomer of hypoxanthine (a naturally occurring purine in the body) and is an inhibitor of the enzyme xanthine oxidase. [1] Allopurinol, decreases uric acid formation and may also inhibit purine synthesis.[2] Subsequently the uric acid lowering capacity of allopurinol was noted, and the drug went on to be developed for its more famous use: to treat hyperuricemia (excess uric acid inblood plasma) and its complications. [3] Allopurinol was also commonly used in chemotherapeutic treatments to treat 'tumor lysis syndrome' (as these regimes can rapidly produce severe acute hyperuricemia) although it has gradually been replaced by urate oxidase therapy. [4] Allopurinol can cause severe pancytopenia if given with full-dose mercaptopurine or its pro-drug azathioprine, due to the inhibition of xanthine oxidase that metabolizes mercaptopurine.[5] The use of allopurinol in combination with azathioprine or mercaptopurine has been revived.[6] More recently, it was discovered that this co-therapy greatly improves the outcome for patients that do not respond to thiopurine monotherapy when treatinginflammatory bowel disease (IBD), specifically Crohn's disease.[7] Co-therapy has also been shown to greatly improve hepatoxicity side effects in treatment of IBD.[8] Co-therapy invariably requires dose reduction of the thiopurine, usually to 1/3 of the standard dose depending upon the patient's genetic status for thiopurine methyltransferase (TPMT).[9] Allopurinol can be used in patients with poor kidney function. A study of allopurinol use in patients withchronic kidney disease decreases C-reactive protein, in patients with chronic kidney disease. In addition, allopurinol reduces cardiovascular and hospitalization risk in these subjects.[10] A mechanistic study in patients with chronic heart failure has shown that the actions of allopurinol may be due to its inhibition ofxanthine oxidase rather than a urate-lowering effect.[11] Allopurinol is used as an add-on drug for refractory epilepsy, which inhibits glutamine release from excitatory neurons.[12] Allopurinol can lower blood pressure in mild hypertension.[13] Allopurinol is almost completely metabolized to oxypurinol within two hours of oral administration for this reason, oxypurinol is believed responsible for the majority of allopurinol's effect.[14] First, its dosing is complex.[15] Some patients are hypersensitive to the drug,[16] Studies have found HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions.[17] More rarely, allopurinol can also result in the depression of bone marrow elements, leading to cytopenias, as well as aplastic anemia. Moreover, allopurinol can also cause peripheral neuritis in some patients, although this is a rare side effect. Another side effect of allopurinol is interstitial nephritis.[18] It is suspected to cause congenital malformations when used during pregnancy, and should be avoided whenever possible by women trying to conceive.[1]According to

[20] chromatographic separation was achieved on Zorbax SB C8 (1.8µm, 4.6mm X 50mm) column using gradient elution of potassium dihydrogen phosphate buffer (pH 2.50, 0.025M) and methanol at flow rate of 1.0 ml/min. UV detection was 230 nm. Run time was 10 min. By the Studies of [21] the validation showed for allopurinol lower and upper limits of quantification of 0.5 and 10 mg/L. The assay was linear over the concentration range of 0.5–10 mg/L. Intra- and inter-day precision showed coefficients of variation <15%; accuracy was within 5% for allopurinol with a resolution factor >1.5. Serum levels of 66 patients prescribed allopurinol 300 mg/day were determined using this HPLC–UV method. Measured serum allopurinol and oxipurinol concentrations in clinical practice showed large variability with a range of <0.5–4.3 mg/L for allopurinol. According to[22] reveals that UPLC is a better technique than HPLC in terms of performance and speed, so it was selected. The method was developed using Acetonitrile and Ammonium Acetate buffer (pH 6.7) and Kromacil column C18 (50×2.1mm, 3.5µ) as a stationary phase at a flow rate of 0.25ml/min. The precision was found to be within the limits. The range of linearity for drug was between 80µg/ml and 120µg/ml. Solution stability study showed the drug was not stable for more than 2 h at 25˚C but stable at 5˚C.. According to [23] a Development and Validation of Stability-Indicating Assay Method by UPLC for a Fixed Dose Combination of Atorvastatin and Ezetimib has been developed. Studies about the [24] Solid Oral Dosage concludes that the chromatographic separation was achieved on Zorbax SB C8 (1.8µm, 4.6mm X 50mm) column using potassium dihydrogen phosphate buffer (pH 2.50, 0.025M) and methanol at flow rate of 1.0 ml/min. UV detection was performed on 230 nm. Run time was 10 min within limits. Therefore the method was validated for accuracy, repeatability, reproducibility and robustness. Linearity, LOQ and LOD were established for Allopurinol and its known impurities

EXPERIMENTAL

Instrumentation:

Peak UPLC containing variable wavelength programmable UV-Visible detector and Rheodyne injector was employed for investigation. The chromatographic analysis was performed on a C18 1.7 µm (2.1 mm x 50 mm). Degassing of the mobile phase was done using a Loba ultrasonic bath sonicator. A Denwar Analytical balance was used for weighing the materials.

Chemicals and Solvents:

The reference sample of Allopurinol 300.00 mg/tablet (277.50 – 322.50 mg/tablet) was obtained from Jollc. Ammonium Dihydrogen phosphate, Methanol and Water used was of HPLC grade and purchased from Merck Specialties Private Limited, Mumbai, India.

The mobile phase:

0.05 M Monobasic Ammonium Phosphate (Ammonium dihydrogen phosphate) 90:10 v/v was prepared and used as mobile phase.

Sample (Capsule) solution:

Weigh 20 tablets determine the average mass and grind to a fine powder. Accurately weigh 100 mg of sample into a 50 ml volumetric flask. Add 10 ml of 0.1 N sodium hydroxide and shake mechanically for 10 minutes. Make up to volume with water and mix well. Transfer 4 ml of this solution and 2 ml of internal standard to a 200 ml volumetric flask. Dilute to volume with mobile phase and mix well. Filter both sample and standard through 0.2 **µ**m filter, discarding the first 5 ml of filtrate.

Standard solution of the drug:

Dissolve about 50 mg of Hypoxanthine in 20 ml of 0.1 N sodium hydroxide, shake mechanical until dissolved (about 10 minutes), dilute with water to 50 ml volumetric flask and mix well. This is prepared on the day of use.

Method Development

Detection wavelength:

The spectrum of 10ppm solution of the Allopurinol was recorded separately on UV spectrophotometer. The peak of maximum absorbance wavelength was observed. The spectra of Allopurinol were showed maximum absorbance at 254nm.

Choice of stationary phase:

Preliminary trials have performed with different types, configurations and from different manufacturers. Finally the expected separation and peak shapes were obtained on chromosil C18 1.7 µm (2.1 mm x 50 mm).

Selection of the mobile phase:

In order to get sharp peak, low tailing factor and base line separation of the separation of the components, a number of experiments were carried out by varying the composition of various solvents and flow rate. Indifferent combinations were tested as mobile phases on a C18 1.7 µm (2.1 mm x 50 mm). 0.05 M Monobasic Ammonium Phosphate (Ammonium dihydrogen phosphate) was proved to be the most suitable of all the combinations since the chromatographic peak obtained was better defined and resolved and almost free from tailing.

Flow rate:

Flow rates of the mobile phase were changed from 0.5 – 1.5 mL/min for optimum separation. It was found from the experiments that 0.55 ml/min flow rate was ideal for the successful elution of the analyte.

Optimization of chromatographic conditions:

Chromatographic conditions are required to be optimized. These optimized conditions were

Followed for the determination of Allopurinol in bulk samples and in its formulations.

Validation of Proposed Method:

The proposed method was validated as per ICH guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, Repeatability, Range, Robustness, system suitability, limit of detection, limit of quantification and solution stability.

Specificity

The specificity of method was performed by comparing chromatograms of blank, standard and sample (prepared from formulation). The solvent and placebo solutions must contain no components, which co-elute with the Allopurinol. The peak purity results from the photo diode-array analysis must show that the Allopurinol peak is pure – i.e. the purity angle (PA) must be less than the threshold angle (TH). The solutions were injected using the conditions specified in the method of analysis.

Linearity

Linearity was performed by preparing mixed standard solutions of Allopurinol at different

Concentration levels including working concentration mentioned in experimental condition i.e. 10 ppm. The response was read and the corresponding chromatograms were recorded. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The correlation coefficient of the regression line for Allopurinol should be greater than or equal to 0.999. The Y-intercept of the line should not be significantly different from zero, i.e. the assessment value (z) falls within the specified limits only when +2 > z > -2. Five solutions containing 50, 75, 100, 125, and 150 % of Allopurinol, relative to the working concentrations of 0.020 mg/ml, were prepared and injected. A linear regression curve was constructed, and the correlation coefficients (R^2) and assessment values calculated.

Assay of Allopurinol tablets

Chromatographic conditions as optimized were followed for

the determination of Allopurinol in bulk samples and in its Formulations. The chromatogram of standard Solution was shown in Figure 2.A - 2.C. For Chromatogram – 1 no significant peak was detected, Chromatogram - 2 Peak due to Allopurinol eluted at about 0.9 minutes Chromatogram -3 No significant peak was detected. And the peak purity results were shown in the Figure 2.D – 2.F and the results are tabulated in the Table: 1.

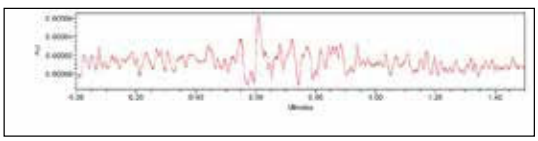


Figure 2.A - Chromatogram - 1

Figure 2.B - Chromatogram - 2

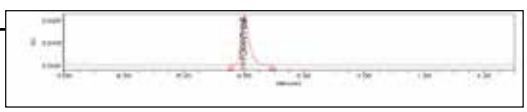
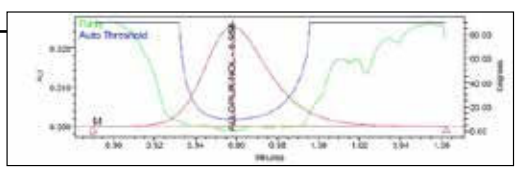
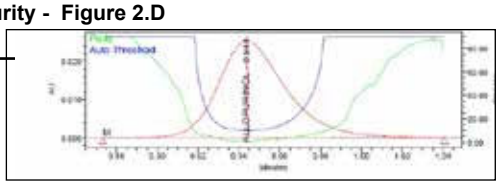


Figure 2.C - Chromatogram -3

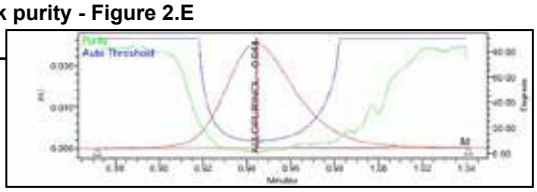
Chromatographitc results



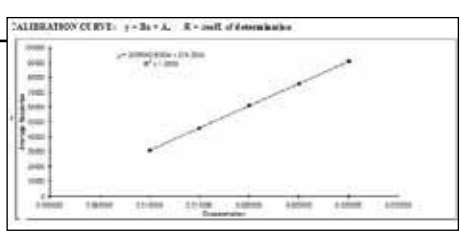
Peak purity - Figure 2.D



Peak purity - Figure 2.E



Peak purity - Figure 2.F



Sample No	Concentration	Response - 1	Response - 1	Average Response
50%	0.010002	31071	30587	30829
75%	0.015003	46030	46055	46043
100%	0.020004	61338	61154	61246
125%	0.025005	76162	75719	75941
150%	0.030006	90968	91170	91069

Table - 1

Intermediate Precision

Intermediate Precision of an analytical procedure expresses intra-laboratory variations of the repeatability test performed by a different analyst, on a different day, and using different reagents, mobile phases and solvents. The % RSD due to Allopurinol concentration for the six samples must be less than or equal to 2.0 %. The mean results obtained in the repeatability, and the intermediate precision must not differ by more than 3.0 %. Six separate sample preparations were assayed according to the method of analysis. The % RSD for intermediate precision is 0.9 %. The intermediate precision and repeatability comply as they differ by 0.7 %. Results are tabulated in the Table: 2.

Sample	Allopurinol - Results (mg/tab)
1	292.35
2	295.61
3	293.77
4	293.33
5	297.68
6	299.55
Mean	295.38
% RSD	0.9

Sample	Allopurinol - Mean Results (mg/tab)
Repeatability	298.23
Intermediate Precision	295.38
Mean	296.89
% RSD	0.7

Table - 2

Accuracy

The accuracy of an analytical method expresses the closeness of test results obtained by that method to the true value. The percentage recovery of the active compounds, for each solution prepared, must be within 98.0 – 102.0 % of the actual amount. Sample solutions were spiked with known concentrations of Allopurinol to result in concentrations of 0.01 mg/ml, 0.015 mg/ml, 0.020 mg/ml, 0.025 mg/ml, and 0.030 mg/ml representing respectively 50, 75, 100,125, and 150 % of Allopurinol relative to the working concentration of 0.020 mg/ml. The above samples were injected in duplicate according to the method of analysis. From the accuracy results, the percentage recovery values for Allopurinol satisfy the acceptance criteria for accuracy across the range of 50 % - 150 %. Results are tabulated in the Table: 3.

Sample	Theoretical	Actual	% Recovery	Average % Recovery
50 %	24.52	24.18	98.6	98.6
	24.52	24.18	98.6	
75 %	36.77	36.78	100.0	99.9
	36.77	36.70	99.8	
100 %	49.03	49.59	101.1	100.6
	49.03	49.03	100.0	
125 %	61.29	61.85	100.9	101.0
	61.29	61.97	101.1	
150 %	73.55	74.15	100.8	100.7
	73.55	74.01	100.6	

Table - 3

Method Precision

The precision of an analytical procedure expresses the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogenous sample.

Repeatability

This parameter determines the repeatability of assay results under the same operating conditions over a short period of time. The % RSD due to Allopurinol concentration for the six samples must be less than or equal to 2.0 %. Six separate sample preparations were analyzed according to the method of analysis. Results are tabulated in the Table: 4.

Sample number	Allopurinol Results (mg/tab)
1	303.43
2	300.08
3	298.52
4	294.33
5	299.61
6	293.38
Mean	298.23
% RSD	1.3

Table - 4

Range
Range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Based on the accuracy results, the range for the assay of Lonol PT 300 mg Tablets is 150.00 – 450.00 mg/tab of Allopurinol, which represents 50 % to 150 % of the working concentration.

Robustness
The robustness study was performed by slight modification in flow rate of mobile phase, pH of

the buffer and composition of the mobile phase. Allopurinol at 7 ppm concentration was analyzed under these changed experimental conditions.

System Suitability
System suitability is a measure of the performance and chromatographic quality of the total analytical system – i.e. instrument and procedure. The % RSD of the peak responses due to Allopurinol for the six replicate injections must be less than or equal to 2.0 %. The tailing factor of the peak due to Allopurinol must not be more than 2.0. The theoretical plate count must not be less than 2000. Six replicate injections of working standard solution were injected according to the method of analysis. The percentage relative standard deviation (% RSD) for the peak responses was determined. Results are tabulated in the Table: 5.

Sample	Allopurinol Area	Allopurinol Tailing	Allopurinol Tangent
1	59413	1.4	3866
2	59566	1.3	3875
3	59604	1.3	3858
4	59542	1.3	3853
5	59633	1.3	3880
6	59564	1.3	3853
Mean	59554	1.3	3864
% RSD	0.1		

Table - 5

Limit of detection and Limit of quantification
Limit of detection (LOD) is defined as the lowest concentration of analyte that gives a detectable response. Limit of quantification (LOQ) is defined as the lowest Concentration that can be quantified reliably with a specified level of accuracy and Precision. For this sample was dissolved by using Mobile Phase and injected until peak was disappeared. After 0.4ppm dilution, Peak was not clearly observed. So it confirms that 0.4ppm is limit of Detection and 0.89ppm dilution is Limit of Quantification. For this study six replicates of the analyte at lowest concentration were Measured and quantified. The LOD and LOQ of Allopurinol are given in Table-6.

Parameter	Measured volume
Limit of Detection	0.4ppm
Limit of Quantification	0.89ppm

Table - 6

Results and Discussion
Allopurinol is stable under UV light exposure. No components are seen to co-elute with Allopurinol peak, and the peak purity results indicate that Allopurinol peak can therefore be considered spectrally pure. The analytical system complies with the requirements specified by the system suitability. The correlation coefficient (R^2) for Allopurinol is 1.000.The plot is a straight line, and the assessment value (z) is 1 for Allopurinol. The % RSD due to Allopurinol concentration for the assay meets the requirements for reproducibility at 1.3 % respectively. The method for the assay of Lonol PT 300 mg Tablets complies with the requirements for Specificity, System suitability, Linearity, Accuracy and Method precision across the range of 50 % to 150 %. The method is therefore acceptable as valid and stability indicating.

Conclusion
The statistical evaluation of the proposed method revealed its good linearity, reproducibility and validation for different parameters and can be used for the rapid and reliable determination of Allopurinol in tablet formulation. All these factors lead to the conclusion that proposed method is accurate, precise, sensitive and rapid and can be applied successfully for estimation of Allopurinol in bulk and pharmaceutical formulations without interference and with good sensitivity.

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