Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 11 [11] October 2022 : 159-168
©2022 Academy for Environment and Life Sciences, India
Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

Development and Validation of Stability Indicating HPTLC for Determination of Allopurinol in Bulk and Pharmaceutical Dosage Forms and Structural Characterization of Alkaline Degradation Products by MS, IR and NMR

Pallavi M. Patil *1, Mohamad Taleuzzaman2Mayur Tekade3Pranitee V. Godkar4
*1,3,4Department Of Pharmaceutical Chemistry, Modern College of Pharmacy, Nigdi, Pune 411044 (India)
2Maulana Azad University,Village Bujhawar,TehsilLuni Jodhpur, Rajasthan, 342802
pallavipatil_2007@yahoo.com

ABSTRACT
A simple, specific, sensitive, accurate and precise stability indicating methods were described for quantitative determination of the Allopurinol in bulk and in tablets. HPTLC separation were achieved on aluminium plates precoated with silica gel 60 F_{254} as the stationary phase using toluene: methanol: glacial acetic acid (8:2:0.5) as mobile phase. Quantification was done by densitometric scanning at 254 nm. The method was found to give compact and well resolved band for Allopurinol at retention factor 0.43 ± 0.02. The linear regression analysis data for calibration graph showed good linear relationship with r^2 = 0.9963. The method was validated for recovery, precision, robustness, ruggedness and sensitivity as per International conference on Harmonisation (ICH) guidelines. The minimum detectable amount and limit of quantitation were found to be 0.0724 ng/b and and 0.2194 ng/band respectively. Allopurinol was subjected to hydrolysis in acid, alkali, oxidation, photo-degradation and thermal condition. The developed methods were successfully validated as per International Conference on Harmonization guideline (ICH). Allopurinol was subjected to different stress conditions. Stress samples were successfully assay by developed high performance thin layer liquid chromatographic. The proposed developed HPTLC method can be applied for identification and quantitative determination of Allopurinol in bulk drug and pharmaceutical dosage forms.
Keywords *HPTLC; Allopurinol; stability indicating; Validation*

Received 18.09.2022 Revised 06.10.2022 Accepted 19.10.2022

INTRODUCTION

Allopurinol is a tautomeric mixture of 1H-pyrazolo[3,4-d]pyrimidin-4-ol and 1,5dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one [1]. (Fig. 1) It is an official drug in British (BP) and United States (USP) Pharmacopoeias [2, 3] which is used for treatment of gout and hyperuricaemia [4].
It is a structural isomer of hypoxanthine (a naturally occurring purine in the body) and is an inhibitor of the enzyme xanthine oxidase [5]. Allopurinol, decreases uric acid formation and also inhibit purine synthesis [6]. 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 [7].
Allopurinol can be administered either orally or intravenously. The oral bioavailability is about 67 to 90% with a peak plasma concentration occurring within one hour; the volume of distribution is approximately 1.6 L/kg [8]. Allopurinol is principally metabolized by aldehyde oxidase to the active compound oxipurinol [9].
The advantage of HPTLC is that a large number of samples can be simultaneously analysed in a shorter time period. HPTLC facilitates repeated detection of the chromatogram with the same or different parameters.
Stability testing studies give evidence on how the quality of drug differs with moment under the influence of a multiplicity of ecological factor such as humidity, temperature and shelf life to be recognized. In the present study, developed method was validated as per ICH guidelines and used for estimation of Allopurinol under stressed conditions.
There are no earlier reports in the literature involving stability study on ALO by HPTLC. In the present work an attempt has been made to investigate of the alkaline degradation process of the cited drug using

 ©2022 AELS, INDIA

the proposed HPTLC method. Hence, the aim of present work was to develop simple and sensitive stability indicating HPTLC method for quantitative determination of ALO in Pharmaceutical formulations in the presence of its alkaline degradation product.

MATERIAL AND METHODS

The method was developed using Camag - HPTLC instrument using UV detector. The sample was spotted in the form of bands(6mm) with Camag microliter syringe on precoated silica gel aluminium plate gel F 254. The space between two bands was 15 mm and the slit dimension was kept 5 mm x 0.45 mm. The linear ascending development of plates was performed to a distance 8 cm in twin -trough chamber previously saturated for 15 min with the mobile phase toluene : methanol : acetic acid (8:2:0.5 v/v/v)

Materials and Reagents

Allopurinol was supplied from Unicorn Pharma chem Pvt Ltd, Mumbai as gift sample. All chemical and reagents such as Methanol, Hydrochloric acid, hydrogen peroxide, sodium hydroxide, toluene and glacial acetic acid were of Analytical grade and were purchased from Merck Chemicals, India. Unicorn pharma chem Pvt Ltd, Mumbai, India, kindly supplies allopurinol API.

Preparation of standard solution

10 mg of standard Allopurinol was accurately weighed and transferred in 10 mL volumetric flask and dissolved in 10 mL of Methanol.1 mL of solution from the above stock solution was taken and transferred to 10 mL volumetric flask and volume was made up to the mark with methanol. A typical standard chromatogram of Allopurinol (Fig. 2)

Method Validation

Linearity

Calibration curve was constructed by plotting the peak area v/s concentration of Allopurinol and regression equations were calculated for both the methods. The linearity of the method was evaluated by analysis of the six standard solutions at six different concentrations from 100 to 600 ng/band. Peak area was recorded for Allopurinol. (Fig. 3).

Precision

Repeatability, intra-day and inter-day precision studies were carried out by estimating corresponding responses three times on the same day and three times on different days for one concentration of Allopurinol and results are reported in terms of % relative standard deviation.(Table 1)

Limit of detection and limit of quantification

The limit of detection and limit of quantification were calculated using following as per ICH guidelines

$$LOD = 3.3 \times S.D./S$$

$$LOQ = 10 \times S.D./S$$

Where = SD the standard deviation of the precision and S is the slope of the calibration curve. (Table 2)

Robustness

Robustness is the measure of capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters. The robustness was studied by changing mobile phase composition, saturation time. The plates were prewashed with methanol and activated at 100^0 C ± 5^0 C. Time from spotting to chromatography and from chromatography to scanning was varied from 20, 30 and 40 min. (Table 3)

Accuracy

The analysed samples were spotted with extra 80, 100 and 120% of the standard Allopurinol and the mixture was analysed by the proposed method. The experiment was conducted in triplicate and % recovery was calculated. (Table 4)

Specificity

Specificity of the method was ascertained by analyzing standard drug and samples of equivalent concentration. The specificity of the method was establishing sample together with the reference standard.[10]

Forced degradation studies

Acid degradation studies

10 mg of Allopurinol was separately dissolved in 10 mL of 0.1 N HCL and kept for 3 hrs at 80^0 C. From this solution, 1 mL was withdrawn and neutralized with 0.1 N NaOH and diluted to 10 mL methanol (Fig. 4).

Alkali degradation studies

10 mg of Allopurinol was separately dissolved in 10 mL of 0.5 N NaOH and kept for 3 hrs at 80 ^{0} C. From this solution, 1 mL was withdrawn and neutralized with 0.1 N HCl and diluted to 10mL methanol (Fig.5).

Oxidative degradation studies

10 mg of Allopurinol was dissolved in 10 ml of 6% H_2O_2 in 10 ml volumetric flask. This solution heated for 3 hrs. and kept for 24 hrs. at room temperature in dark. From the above solution 1 ml was withdrawn and make up with methanol up to 10 ml. (Fig. 6).

Thermal degradation studies

For thermal decomposition drug powder was kept at 80^0 C for 12 hrs. From powder solution (100µg/ml) was prepared and analysed for thermal degradation study (Fig. 7).

Photo degradation studies

A sample of the drug was exposed to a near ultraviolet lamp in a UV chamber in petri dish for 24 hrs and solution of 100µg/ml was prepared and analysed for photolytic degradation (Fig. 8). [11]

Extensive alkaline stress studies

In order to evaluate the stability indicating property of the developed HPTLC method stress studies were carried out under ICH recommended conditions. Intentional degradation was tried by exposing the ALO to following stress conditions: alkaline (1 N NaOH at 80^0c) ability of the proposed method to measure the analyte response in presence of its degradation product was studied.

Structure prediction of alkaline degradation of Allopurinol

Structure prediction of alkaline degradation of allopurinol in to a series of 10 test tubes, 2.5 mL of allopurinol working solution 100 µg/mL was transferred and mixed with 2 mL of 1.0 M sodium hydroxide. The test tubes were allowed to stand in a thermostatically controlled oven at 100^0C and then were removed from the oven, one by one at 15 min time interval up to 180 min. The test tubes were immediately inserted into an ice-bath to terminate the degradation reaction and then were put in another water bath set at room temperature. The contents of the test tubes were transferred into a 10.0 mL volumetric flask and diluted to required volume with methanol. The proposed HPTLC method was applied for the determination of the remaining intact allopurinol at each time interval from its corresponding regression equation. A plot of log of the remaining concentration versus time in minutes was then obtained to determine the alkaline degradation process as depicted in. Structural elucidation of the obtained degradation product was achieved by IR, NMR and Mass spectrophotometry. (Figure 9,10,11,12).

Table 1 Statistical evaluation of precision of developed method

Sr. No.	Intra-day precision			Inter day precision		
	%Label claim*	S.D. (±)	R.S.D.	%Label claim*	S.D. (±)	R.S.D.
1	99.25	0.2501	0.2519	98.96	0.7477	0.7556

Table 2 Results of LOD and LOQ

Parameters	Allopurinol
LOD (ng/band)	0.0724
LOQ (ng/band)	0.2194

Table 3 Result of robustness study

Chromatographic Changes			
Mobile phase composition (± 0.1 ml)	Level	Peak Area	R_fValue
7.9: 2.1: 0.5	– 0.1	2311.4	0.43
8: 2: 0.5	0	2315.6	0.42
8.1: 1.9: 0.5	+ 0.1	2309.5	0.44
	R.S.D.	0.135	
Duration for chamber saturation (± 2 min)		Peak Area	R_fValue
13 min	- 2	2278.4	0.46
15 min	0	2259.7	0.44
17 min	+ 2	2254.5	0.45
	R.S.D.	0.554	
Spotting to development		Peak Area	R_fValue
15 min	-	2321.8	0.39
20 min	-	2349.6	0.41
25 min	-	2354.3	0.41

	R.S.D.	0.75	
Development to scanning		Peak Area	R_fValue
10 min	-	2245.7	0.43
15 min	-	2239.5	0.42
20 min	-	2236.7	0.43
	R.S.D.	0.2055	
Volume of mobile phase (± 1 ml)		Peak Area	R_fValue
9.5	– 1	2289.7	0.45
10.5	0	2278.6	0.46
11.5	+ 1	2292.5	0.43
	R.S.D.	0.321	

Table 4 Results of recovery studies

Sr. No	Level of recovery	Conc. (µg/ml) Std Sample	Mean	Recovered Conc.	% Recovery
1	80%	10+8	4184.3	17.88	99.34
2	100%	10+10	4610.667	19.70	98.51
3	120%	10+12	5123.333	21.89	99.52

Table 5 Result of degradation studies

Sr. No.	Stress Condition	Temperature and Time	% assay of active substance	Rf values of degraded peaks
1.	Acid(0.1 N HCl)	80^0C for 3 hr	95.52%	0.52
2.	Alkali(0.5 M NaOH)	80^0C for 3 hr	92%	0.34
3.	Oxide(6 % H_2O_2)	80^0C for 3 hr	94.36%	0.38
4.	Thermal degradation	80^0C for 12 hr	98.18%	0.56
5.	Photo degradation	24 hr	99.20%	0.52

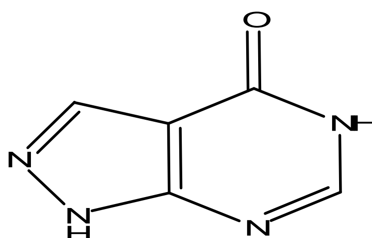

Figure 1 Structure of Allopurinol.

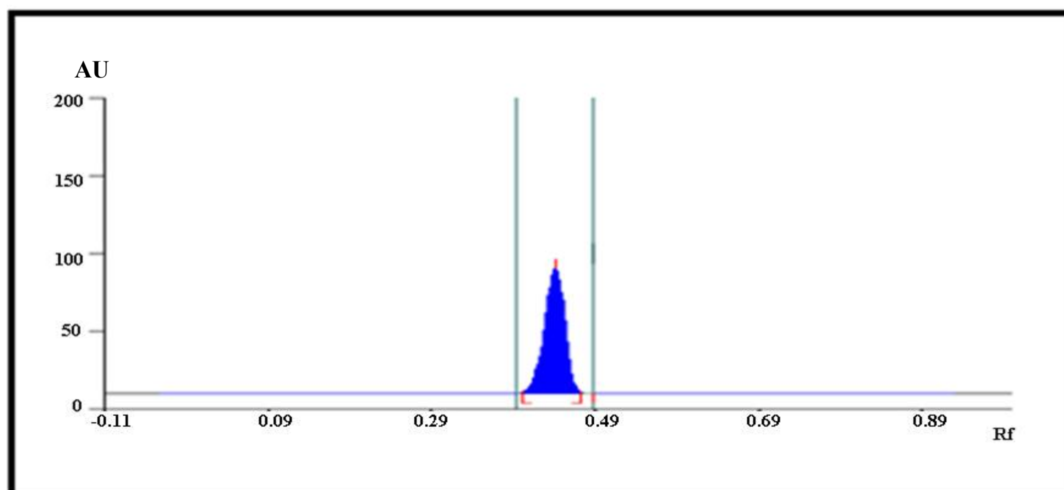

Figure 2A typical standard chromatogram of Allopurinol(Rf=0.48)

 ©2022 AELS, INDIA

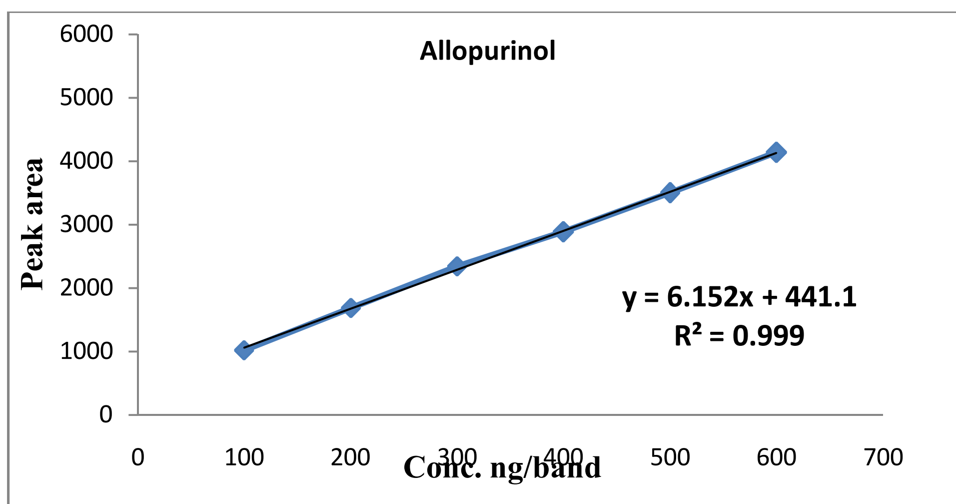

Fig. 3: Calibration curve of Allopurinol at 254 nm

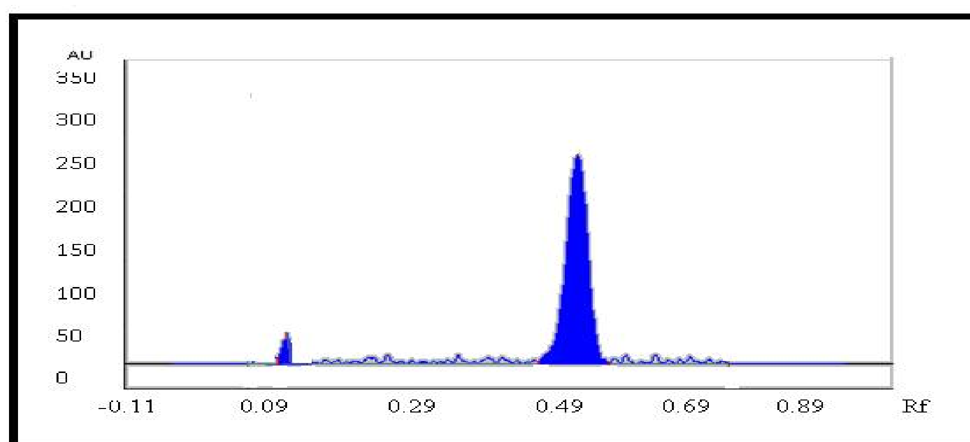

Figure 4 Chromatogram of acid (0.1N HCl) treated sample

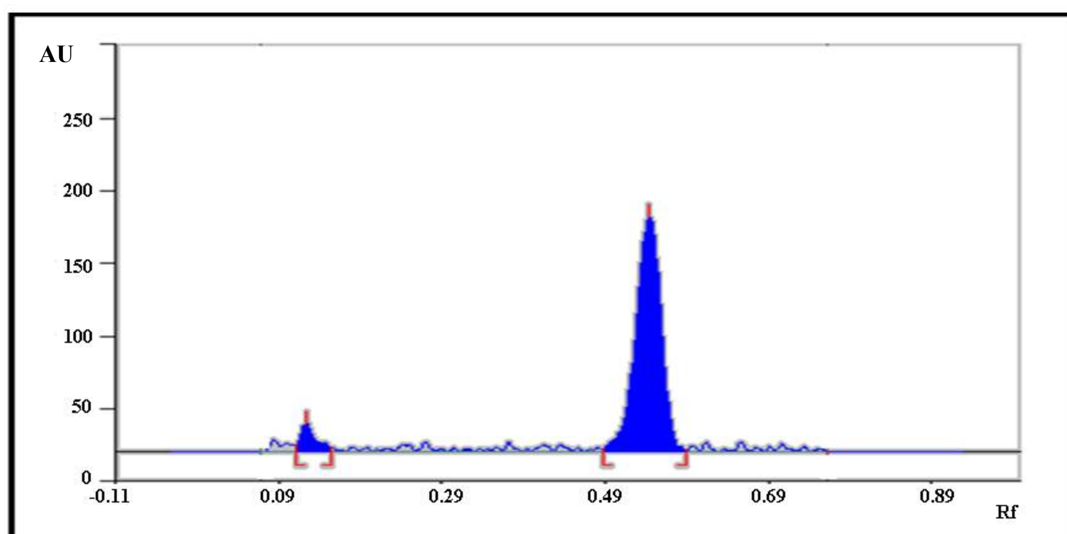

Figure 5 Densitogram of alkali (0.5 N NaOH) treated sample

 ©2022 AELS, INDIA

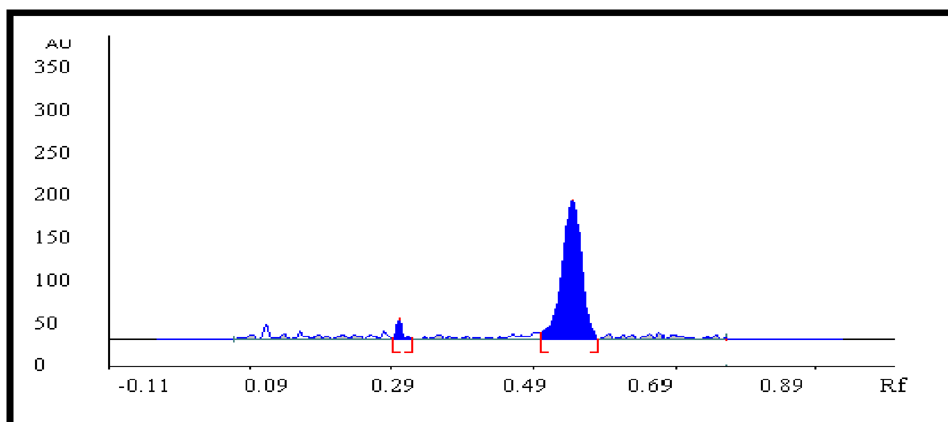

Figure 6 Densitogram of oxide (6% H_2O_2) treated sample

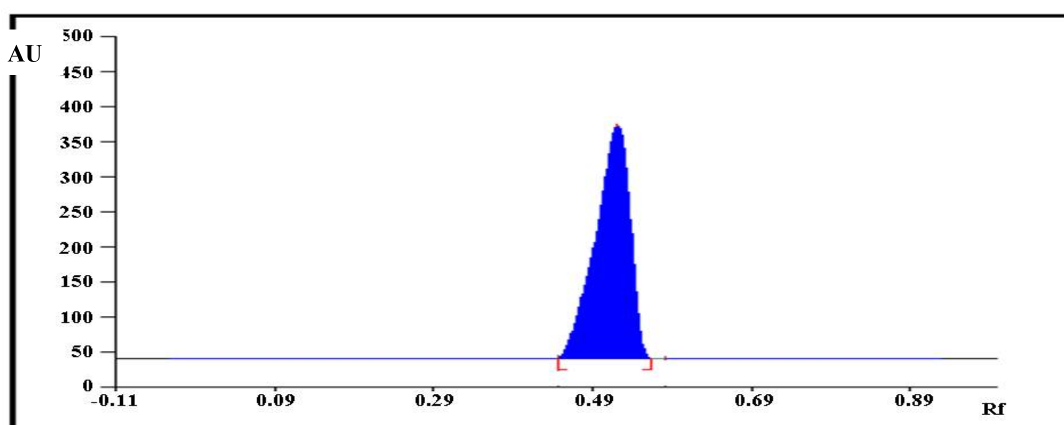

Figure 7 Densitogram of heat treated sample(12 hrs)

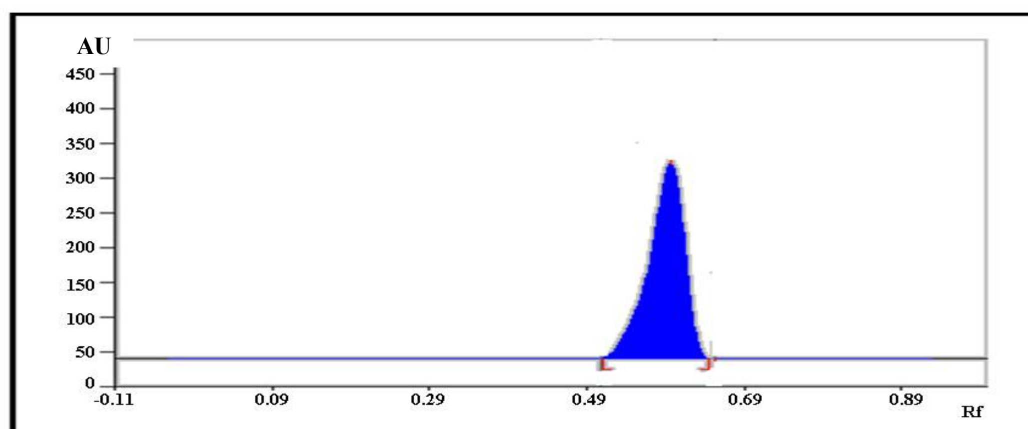

Figure 8 Densitogram of sample exposed to UV radiations (24 hrs)

 ©2022 AELS, INDIA

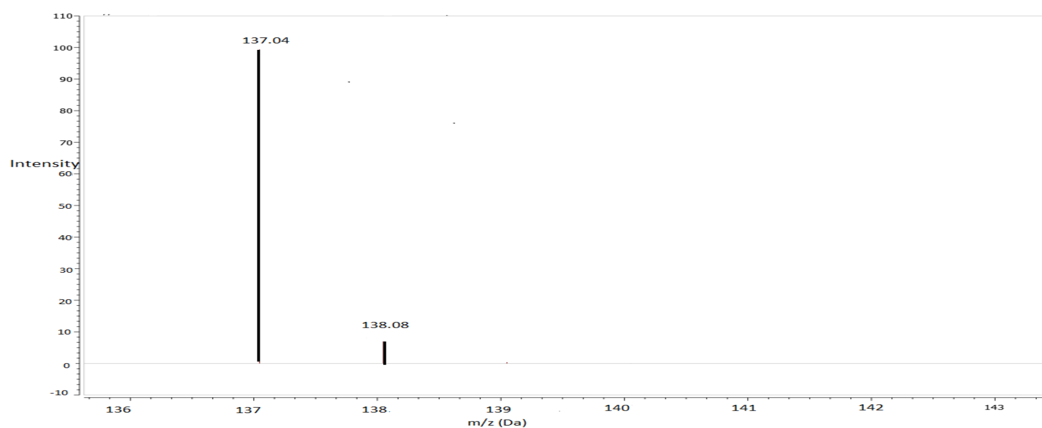

Figure 9 MS of Allopurinol

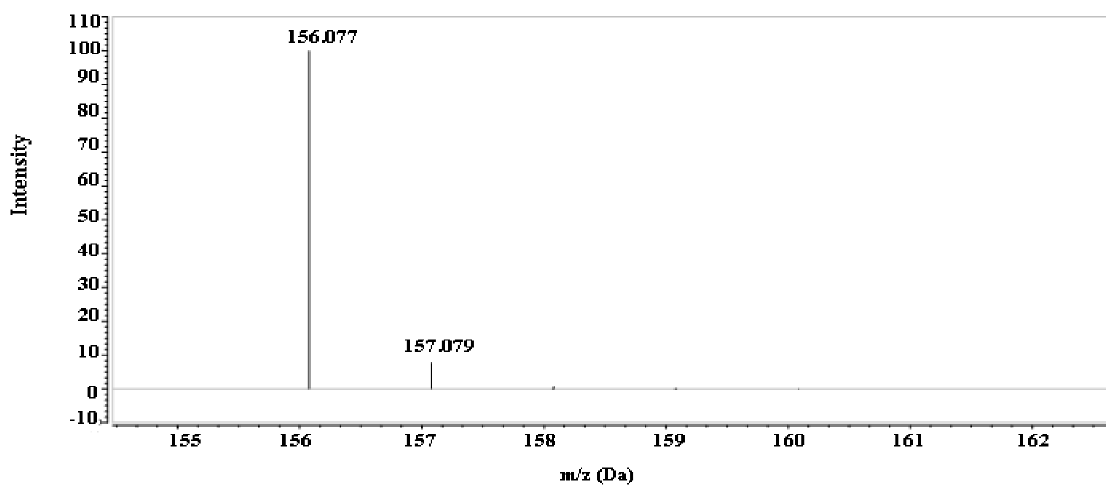

Figure 10 MS of Allopurinolalkaline degradation

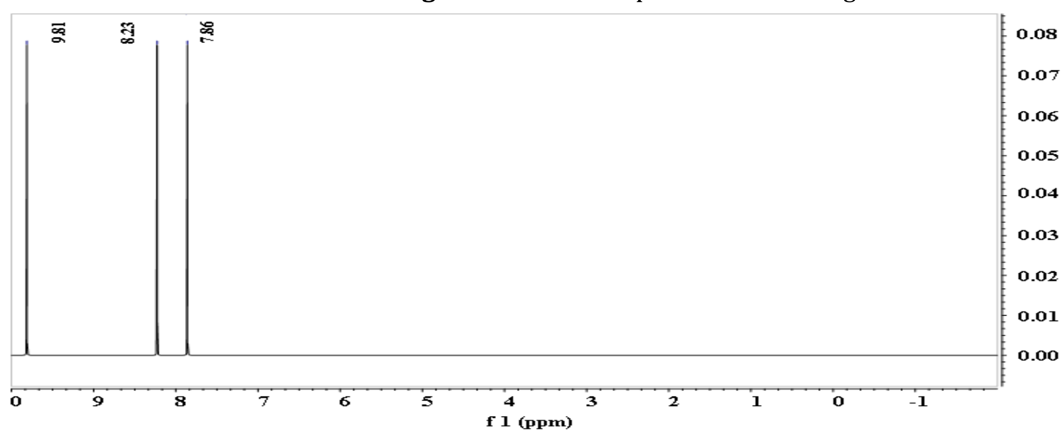

Figure 11 NMR of Allopurinol

 ©2022 AELS, INDIA

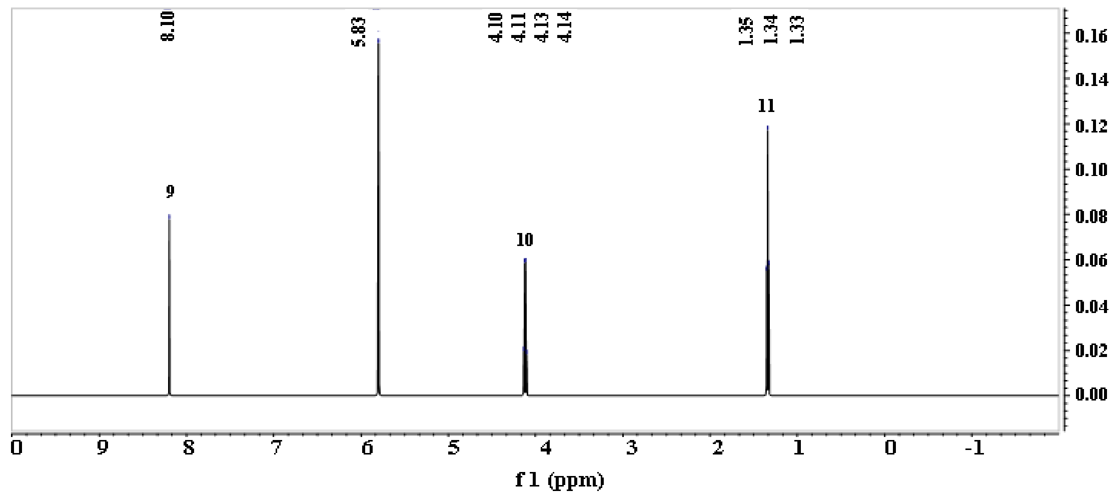

Figure 12 NMR of Allopurinolalkaline degradation

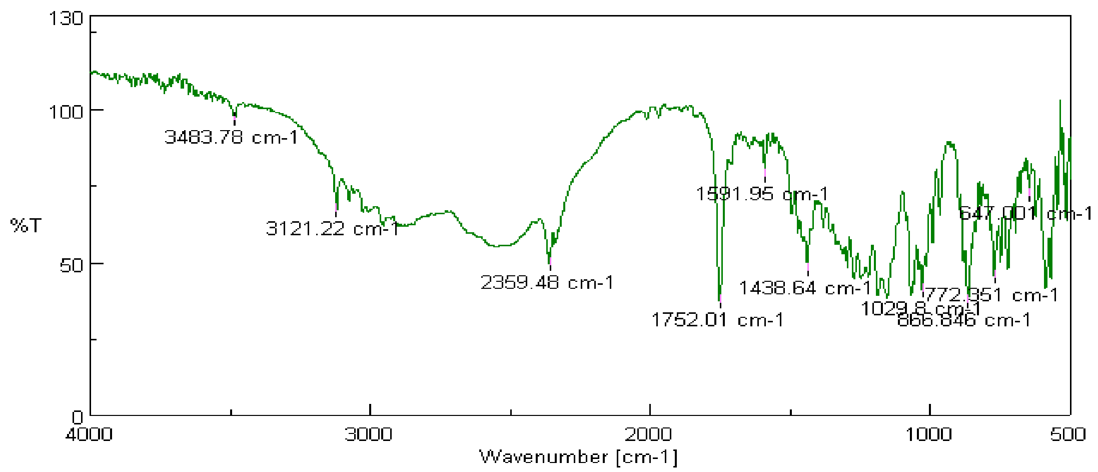

Figure 13IR spectra of Allopurinol

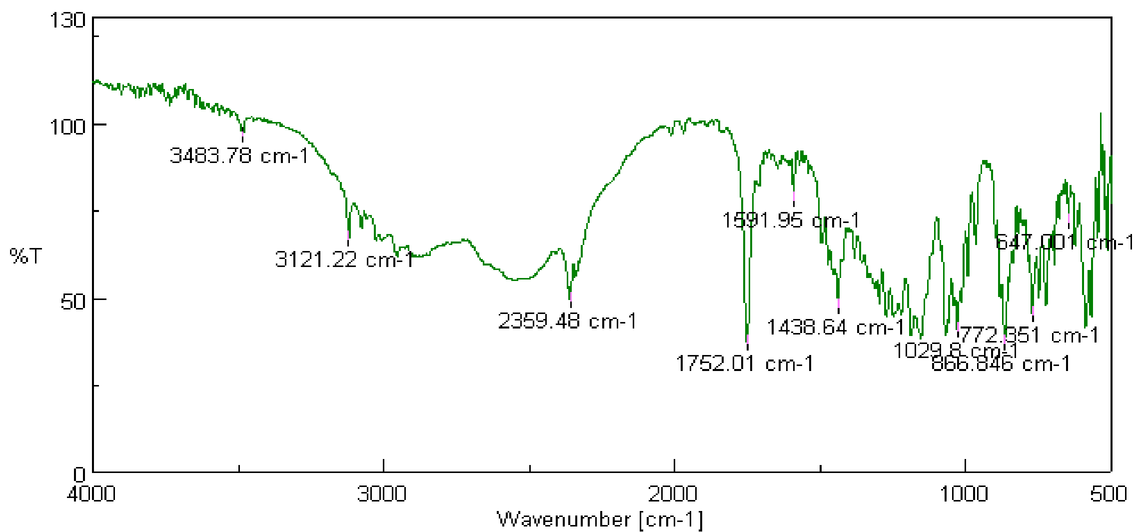

Figure 14 IR spectra of degradant product

 ©2022 AELS, INDIA

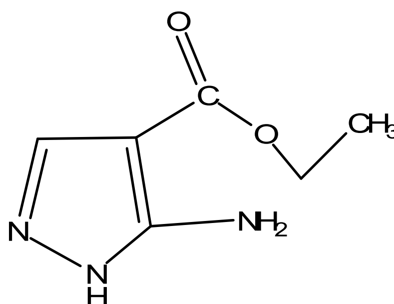

Figure 15 Structure of Allopurinol alkaline degradation

RESULT AND DISCUSSION

Prime objective of development and validation of HPTLC method for determination of Allopurinol was run and should be accurate, precise, reproducible, robust and stability indicating.

Development of optimum mobile phase

Different composition of the mobile phase for HPTLC analysis were tested in order to obtain high resolution and reproducible peaks. The desired objective was achieved using toluene: methanol: glacial acetic acid (8:2:0.5) as the mobile phase. The wavelength of 254 nm was found to be optimal for the highest sensitivity. Sharp and well-defined peaks for the Allopurinol were obtained at 0.43 ± 0.02 when the chamber was saturated with the mobile phase for 15 min at room temperature.
HPTLC studies on Allopurinol under different stress conditions suggested that Allopurinol is susceptible to hydrolytic, oxidative and photolytic stress conditions.

Calibration curve

The linear regression data for the calibration curves showed good linear relationship over the concentration range of 100-600 ng/band. Linear regression equation was found to be y = 6.153x + 441.2. The regression coefficient ($r^2 = 0.999$) is generally considered as evidence of acceptable fit.

Validation of method

Specificity of the method is its ability to measure accurately and specifically the analyte of interest in the presence of sample matrix. Value of the peak purity index was found to be higher than 0.999 indicated that the proposed methods are specific.
Intraday precision and inter day precision were measured to evaluate the precision of the method. (Table1).The repeatability of sample application and measurements of peak area were expressed in terms of coefficient of variations and were found to be very low.
The LOD and LOQ were determined from slopes of linear regression curves. LOD and LOQ were found to be 0.0724 ng/band & 0.2194 ng/band. (Table 2).
Robustness was studied by determining effects of small variation of mobile phase composition (±2%), chamber saturation period, development distance and scanning time. No significant changes were observed, indicating the method was robust. System suitability test results were within the acceptable range (Table 3), indicated that the system is suitable for the intended analysis.
Average recovery of Allopurinol from the formulation was 99.123%, which shows the method is accurate and free from interference of excipients present in the formulation(Table 4).
Peak of stress degradants led to peak of Allopurinol was not significantly shifted in the presence of degradation peaks indicating property of the method. The chromatogram obtained from Allopurinol contained additional peaks at 0.12 in acid – induced degradation and 0.53 in the base – induced degradation. The concentration of the drug was different from the initial concentration, indicating that Allopurinol undergoes degradation under acidic and basic condition. The chromatogram of Allopurinol degraded with hydrogen peroxide, photochemical and neutral condition showed well separated spots of pure Allopurinol. However, there was no additional peak of degraded sample. (Table 5)
In contrast to chemical degradation photo degradation shows the formation of alkaline degradant (Figure 18). From the MS, IR and NMR data the alkaline degradation product was predicted. (Figure 15) The mass spectrum of DG was characterized by the appearance of the molecular ion peaks at 157.07 m/z which confirm the molecular weight of the suggested degradation product. (Figure 9,10)
From NMR spectra, Chemical shift at 8.10 shows that presence of aromatic ring. Chemical shift at 5.83 and 4.13 shows that presence of alkene group (C=C) bond in aromatic group in structure. Also, chemical shift at 1.34 show presence of alkyl group. (Figure 11,12)
The IR spectrum (KBr) of DG was characterized by the absorption frequency of NH2- band as a doublet at 3483.7 cm^{-1} and C=O band as 1752.01 cm^{-1}, C-H at 1438 cm^{-1}, C-O stretch at 1029 cm^{-1}.(Figure 13,14)

CONCLUSION
This study presents simple and validated stability indicating HPTLC methods for estimation of allopurinol in the presence of degradation products. This method can be used to determine the purity of commercially available drug by detecting and relating impurities. The method could effectively separate and quantify the drugs from their degradation products so it can be regarded as specific and stability indicating. This method can be used as a quality control tool for routine quantitative analysis of allopurinol.

ACKNOWLEDGEMENT
I very much thank my guide, PE's Modern college of Pharmacy, Pune for her guidance, kind help and constant encouragement at every step during the progress of my work. The authors gratefully acknowledge D. Y. Patil College of pharmacy, Pune, India.

REFERENCES
1. Sweetman, S.C.,(2009). In Martindale The Complete Drug Reference, Pharmaceutical Press, London.552.
2. The British Pharmacopoeia, Her Majesty's. The stationary office, London 2009.
3. The United States Pharmacopeia, National Formulary. The United States Pharmacopoeia convention Inc. 2007, 25-30.
4. Breithaupt, H., Goebel G., (1981).Determination of Allopurinol and oxipurinol in biological fluids by High-Performance Liquid Chromatography. J .Chromatogr.B. 226, 237-242.
5. Pacher, P., Nivorozhkin, A., Szabó C., (2006). Therapeutic Effects of Xanthine Oxidase Inhibitors: Renaissance Half a Century after the Discovery of Allopurinol. Pharmacological Reviews, **58**(1): 87–114.
6. Cameron, J.S., Moro, F., Simmonds, H.A.,(1993). Gout, uric acid and purine metabolism in paediatric nephrology."PediatrNephrol. **7**(1):105–118.
7. Elion, G.B., (1989). The purine path to chemotherapy (Nobel lecture in physiology or medicine) Science. 244(4900)41–47.
8. Pea, F., (2005).Contrib. Nephrol.147:35–46.
9. Reiter, S., Simmonds, H.A., ollner, N.Z et al., (1990).Clin.Chim.Acta.187, 221–234.
10. ICH, Q2A. Text on Validation of Analytical Procedures, International Conference On Harmonization*,* Geneva, 1994, 1-5.
11. ICH. Stability Q1AR2 Testing of New Drug Substances and Products. International Conference on Harmonization*.* IFPMA, Geneva, 2003.

CITATION OF THIS ARTICLE
P M. Patil, M Taleuzzaman, M Tekade, P V. Godkar. Development and Validation of Stability Indicating HPTLC for Determination of Allopurinol in Bulk and Pharmaceutical Dosage Forms and Structural Characterization of Alkaline Degradation Products by MS, IR and NMR. Bull. Env.Pharmacol. Life Sci., Vol 11 [11] October 2022 : 159-168

 ©2022 AELS, INDIA