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A Validated Stability Indicating RP-UPLC Method for the Quantitative Determination of Potential Impurities of Allopurinol

Sajan PG[*1], Rohith T[1], Santosh Patil[2], Mantelingu K[3], Rangappa K S[3], Kumara M N[4]
1. Deepta Laboratories, No.77-78/1, Vishweshwaranagar, 2nd stage, Industrial Suburb, Mysore – 570008
2. Biocon Limited, Plot No. 2,3,4,5 & 6, Bommasandra Jigani Link Road, Bangalore, 560099
3. Department of Chemistry, Manasagangothri, University of Mysore, Mysore-570006
4. Chemistry department, Yuvaraja's college, University of Mysore, Mysore-570005

ABSTRACT

A simple, sensitive, selective and stability indicating UPLC method has been developed for the quantitative determination of potential impurities of allopurinol active pharmaceutical ingredient. Allopurinol, its five impurities and degradation products were separated efficiently by using the mobile phase consisted of sodium perchlorate (10 mM, pH 3.0)and acetonitrile on a HSS T_3P stationary phase in gradient elution profile. Forced degradation study confirmed that the newly developed method was specific and selective to the degradation products. The newly developed UPLC method was validated according to ICH guidelines considering five impurities to demonstrate specificity, precision, linearity, accuracy and stability indicating nature of the method. Regression analysis showed correlation coefficient value greater than 0.99 for allopurinol and its five impurities. Detection limit of impurities was in the range of 0.002–0.006% indicating the high sensitivity of the newly developed method. Accuracy of the method was established based on the recovery obtained between 91.7% and 106.6% for all impurities.

Keywords: Allopurinol; Impurities; RP-UPLC; Validation; Forced Degradation.

*Corresponding Author Email sajanpg@gmail.com
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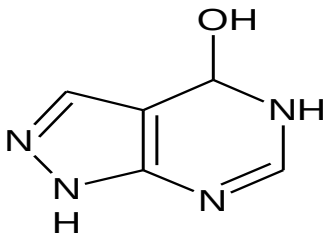
INTRODUCTION

Allopurinol is chemically tautomeric mixture of 1H-pyrazolo[3,4-d]pyrimidin-4-ol and 1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one[1]. It is a structural isomer of hypoxanthine and is an inhibitor of the enzyme xanthine oxidase. Xanthine oxidase is responsible for the successive oxidation of hypoxanthine and xanthine, resulting in the production of uric acid, the product of human purine metabolism[2]. In addition to blocking uric acid production, inhibition of xanthine oxidase causes an increase in hypoxanthine and xanthine. While xanthine cannot be converted to purine ribotides, hypoxanthine can be salvaged to the purine ribotides adenosine and guanosine monophosphates. Increased levels of these ribotides may cause feedback inhibition of amidophosphoribosyl transferase, the first and rate-limiting enzyme of purine biosynthesis. Allopurinol, therefore, decreases uric acid formation and may also inhibit purine synthesis[3]. Chemical structure of allopurinol is given in Figure 1. Allopurinol is an official drug in USP, EP, BP, and IP. Several analytical methods such as spectrophotometric[4-8], capillary zone electrophoresis [9], polarography[10], second derivative oscillopolarography [11] and room temperature phosphorescence methods[12] are reported for estimation of allopurinol in bulk drug, formulations and in biological matrices. Some HPLC methods are reported for estimation of allopurinol in its biological samples [13-15]. Estimation of allopurinol in pharmaceutical dosage form by HPLC methods is also reported[16]. Extensive literature survey reveals that very few LC methods and no UPLC methods have been reported for the analysis allopurinol drug substance. Hence it was felt necessary to develop an accurate, rapid, selective and sensitive stability indicating UPLC method for the determination of allopurinol impurities.



Molecular weight = 138.13, Molecular formula = $C_5H_6N_4O$

Figure 1: Structure of allopurinol

Objective of the current study was to develop a stability indicating UPLC–UV method for the quantitative determination of impurities in allopurinol and check the suitability of the method as per ICH guidelines. EP reported five impurities and use two isocratic LC methods for the quantification of allopurinol impurities. USP use gradient LC method however the separation between impurity-2 and impurity-3 is very poor. The newly developed UPLC method

separates all impurities with short run time.

MATERIALS AND METHODS

Reagents and Chemicals

Samples of allopurinol and standards of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 (Table 1) were received from Deepta laboratories, Mysore, India. HPLC grade methanol and acetonitrile was purchased from Rankem, Mumbai, India. Deionized water was prepared using a Milli-Q plus water purification system from Millipore (Bedford, MA, USA).Analytical reagent grade of sodium perchlorate anhydrous, dipotassium hydrogen orthophosphate, potasium hydrogen phosphate, diammonium hydrogen orthophosphate, ammonium hydrogen phosphate, ammonium acetate, sodium hydroxide, hydrogen peroxide and hydrochloric acid were purchased from Merck India Limited (Mumbai, India).

Instruments

The LC method development, validation and forced degradation (stress) studies were done using Agilent 1290 Infinity UPLC equipped with PDA detector (Agilent technologies, CA, USA). The data were collected and the peak purity of the allopurinol peak was checked using Chemstation software. The photolytic degradation was carried out using TP 0000090G (Thermo Lab equipment's Pvt. Ltd., Mumbai, India) photolytic chamber.

Chromatographic conditions

The chromatographic separations were achieved on Waters Acquity UPLC HSS T_3P column (100 mm length × 2.1 mm ID with 1.7µm particle size, Waters corporation, MA, USA). Mobile phase A consisted, 10 mM sodium perchlorate, pH adjusted to 3.0 with dilute sodium hydroxide and mobile phase B consisted acetonitrile with a gradient programme (T_{min}A:B) $T_0$100:00, $T_4$100:00, T_{14}30:70 with a post-run time of 2.5 min. The column temperature was maintained at 20 $^{\circ}$C and the detection was carried out at 220 nm. The flow rate was set to 0.5 mL/min. The test concentration was about 200 µg/mL and the injection volume was 3µL. A degassed mixture of solution A (0.2g sodium hydroxide dissolved in 10mL water and made up to 100 mL with methanol) and mobile phase A was used as diluent for standard and sample preparations.

Sample preparation for forced degradation studies

Stress study is a complementary part of stability testing wherein influence of environmental factors like pH, temperature, humidity, oxygen and light are evaluated on a drug substances and products. Stress testing of the drug substance was performed as per ICH guidelines Q1 (R2) and it can help to identify the likely degradation products, which can in turn help to establish the

degradation pathways, the intrinsic stability of the molecule and specificity of the proposed method. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. About 10 mg each of allopurinol sample was transferred into four separate 50 mL volumetric flasks and subjected to forced degradation study under acidic (1N HCl, at 70 °C, 2 h), basic (1 N NaOH, at 70 °C, 2h) and in water (70 °C for 48 h). The stressed samples of acid and base degradation were neutralized with 1N NaOH and 1N HCl respectively and made up to volume with diluent. Oxidative degradation was carried out using 5% hydrogen peroxide (70 °C for 1h). Solid state stability of the drug substance was carried out by (a) thermal degradation at 105 °C for 48 h and (b) photolytic degradation was performed by keeping 500 mg of each solid sample in two separate loss on drying bottles (LOD, dark control and photolytic exposure) in photo stability chamber model TP 0000090G (Thermo Lab equipments Pvt. Ltd., Mumbai, India). Samples were exposed to get a minimum exposure of 1.2 million lux hours for light and 200 Wh/m2 for ultraviolet region. Samples were withdrawn at appropriate times and subjected to LC analysis using 200 µg/mL sample concentration.

Preparation of stock solutions for method validation

A test preparation of 200 µg/mL of allopurinol API sample was prepared by dissolving in 4mL solution-A and made up to volume with mobile phase A. A stock solution of impurities was prepared by dissolving 5 mg each of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5and 5 mg of allopurinol in 4ml solution-A and made up to 50 mL with mobile phase A. Transferred 5 mL of each individual stock solution into a 100 mL volumetric flask and made up to volume with diluent. From this stock solution, standard solution of 0.40 µg/mL of each impurity and 0.40 µg/mL of allopurinol was prepared.

RESULTS AND DISCUSSION

Method development

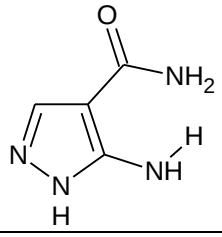
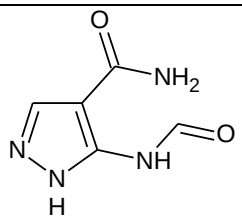
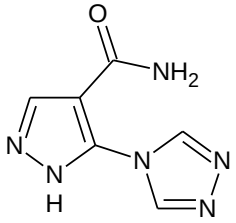
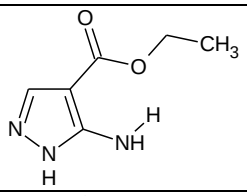
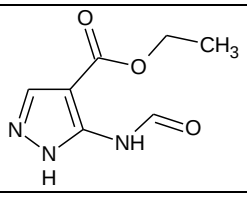
The main objective of the method development was to achieve efficient separation of impurities, degradation products generated during stress studies, increase the solution stability of the sample and a short run time method. All impurities are listed in Table-1 and named based on their elution pattern. Allopurinol is a polar compound with strong intermolecular hydrogen bonding and limited solubility in both polar and non polar media. It dissolves in dilute solutions of alkali hydroxides however in alkaline conditions, allopurinol degrades with time. Mixture of 0.05N sodium hydroxide solution and methanol is used as diluent for sample preparation which significantly improved the solution stability of the sample. Allopurinol has UV-absorption

maxima at about 220nm wavelength. Hence detection at 220nm was selected for method development purpose. Allopurinol is a very weak acid with a dissociation constant (pka) of 9.4 and is therefore essentially unionized at all physiological pH values[17]. Because of high polarity, the retention of allopurinol and its impurities in the column was quite challenging during method development. Parameters, such as choice of analytical column, pH of buffer, mobile phase composition and proportion, detection wavelength and other factors were exhaustively studied. The resolution between Imp-2 and Imp-3 were very poor when different stationary phase viz; C_{18}, RP_{18}, phenyl and CSH were used in different mobile phases containing buffers like di-potassium hydrogen orthophosphate, potassium hydrogen phosphate, di-ammonium hydrogen orthophosphate, ammonium hydrogen phosphate, ammonium acetate and sodium perchlorate using organic modifiers such as methanol, acetonitrile and isopropyl alcohol in the mobile phases. Use of HSS T_3P column, 10mM sodium perchlorate as mobile phase A, acetonitrile as mobile phase-B and column temperature 20 $^{\circ}$C, was significant in achieving the desired resolution of allopurinol Imp-2 and Imp-3. Different experiments revealed that that resolution between imp-2 and imp-3 can be achieved only in 100% aqueous buffer in the above mentioned UPLC stationary phases. Addition of 1% organic modifier in the mobile phase Aresults rapid elution of allopurinol and co elution between imp-2 and imp-3.After several trials for gradient profile, chromatographic conditions were finalized as described under chromatographic conditions. Autosampler temperature was maintained at 8 $^{\circ}$C throughout analysis to enhance the solution stability of the sample.

Results of forced degradation

Allopurinol was found to be stable under stress conditions such as thermal and photolytic conditions. Significant degradation of the drug substance was observed under oxidative stress conditions, acidic and basic hydrolysis conditions. The major degradation products of allopuriol under these conditions were Imp-A and Imp-B respectively. The mass balance of stressed samples was close to 99%. Degradation studies and peak purity test results derived from PDA detector confirmed that the allopurinol peak was homogenous and pure in all the stress samples. The developed UPLC method was found to be specific in the presence of

Table 1: Potential impurities of allopurinol

Sl. No.	Structure	Mol. Wt.	IUPAC name	Code	Origin
1		126.1	5-amino-1*H*-p yrazole-4-carboxamide	IMP-1	Process
2		154.1	5-(formylamino)-1*H*-pyrazole-4-carboxamide	IMP-2	Process
3		178.2	5-(4*H*-1,2,4-triazol-4-yl)-1*H*-pyrazole-4-carboxamide	IMP-3	Process
4		155.2	ethyl 5-amino-1*H*-pyrazole-4-carboxylate	IMP-4	Process
5		183.2	ethyl 5-(formylamino)-1*H*-pyrazole-4-carboxylate	IMP-5	Process

Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and their degradation products confirmed the stability indicating power of the newly developed method. Chromatograms of forced degradation study have been depicted in Figure 3 and summary report of forced degradation studies are depicted in Table 3.

Table 2: Method validation summary report

Parameter	Imp-1	Imp-2	Imp-3	Allopurinol	Imp-4	Imp-5
System suitability						
RT	1.20	2.94	3.15	4.20	8.12	8.31
RRT	0.29	0.70	0.75	1.00	1.93	1.98
Rs	-	11.33	1.39	6.50	32.50	2.91
N	7853	5589	9303	13140	245688	270454
T	1.08	1.35	1.28	1.46	1.10	1.05
Linearity						
r	0.9948	0.9982	0.9982	0.9982	0.9984	0.9991

Slope	16.34	39.83	35.70	49.04	24.89	44.45
Intercept	0.2828	0.3095	0.2272	0.6502	0.3487	0.3651
Detection limit (%)	0.003	0.006	0.006	0.002	0.003	0.002
Quantitation limit (%)	0.009	0.018	0.018	0.006	0.009	0.006
Precision (QL) % RSD (n 6)	2.6	4.5	3.9	2.2	3.6	2.9
Repeatability (intra day) % RSD (n 6)	2.5	1.5	2.6	0.3	3.1	3.2
Intermediate precision (inter day) % RSD (n 12)	2.1	3.3	2.4	0.2	3.4	2.9
Accuracy at QL level (n 3)						
Amount added (%)	0.0090	0.018	0.018		0.0090	0.0060
Amount recovered (%)	0.0085	0.0171	0.0191	-	0.0096	0.0055
% Recovery	94.4	95.0	106.1		106.6	91.7
Accuracy at 80% level (n 3)						
Amount added (%)	0.16	0.16	0.16		0.16	0.16
Amount recovered (%)	0.1589	0.1566	0.1639	-	0.1656	0.1563
% Recovery	99.31	97.9	102.4		103.5	97.7
Accuracy at 100% level (n 3)		.				
Amount added (%)	0.20	0.20	0.20		0.20	0.20
Amount recovered (%)	0.1941	0.1925	0.2086	-	0.2036	0.1952
% Recovery	97.1	96.3	104.3		101.8	97.6
Accuracy at 120% level (n 3)						
Amount added (%)	0.24	0.24	0.24		0.24	0.24
Amount recovered (%)	0.2285	0.2310	0.2501	-	0.2489	0.2322
% Recovery	95.2	96.3	104.2		103.8	96.8

n, number of determinations; RT, retention time; RRT, relative retention time; Rs , USP resolution; N, number of theoretical plates; T, USP tailing factor; r, correlation coefficient.

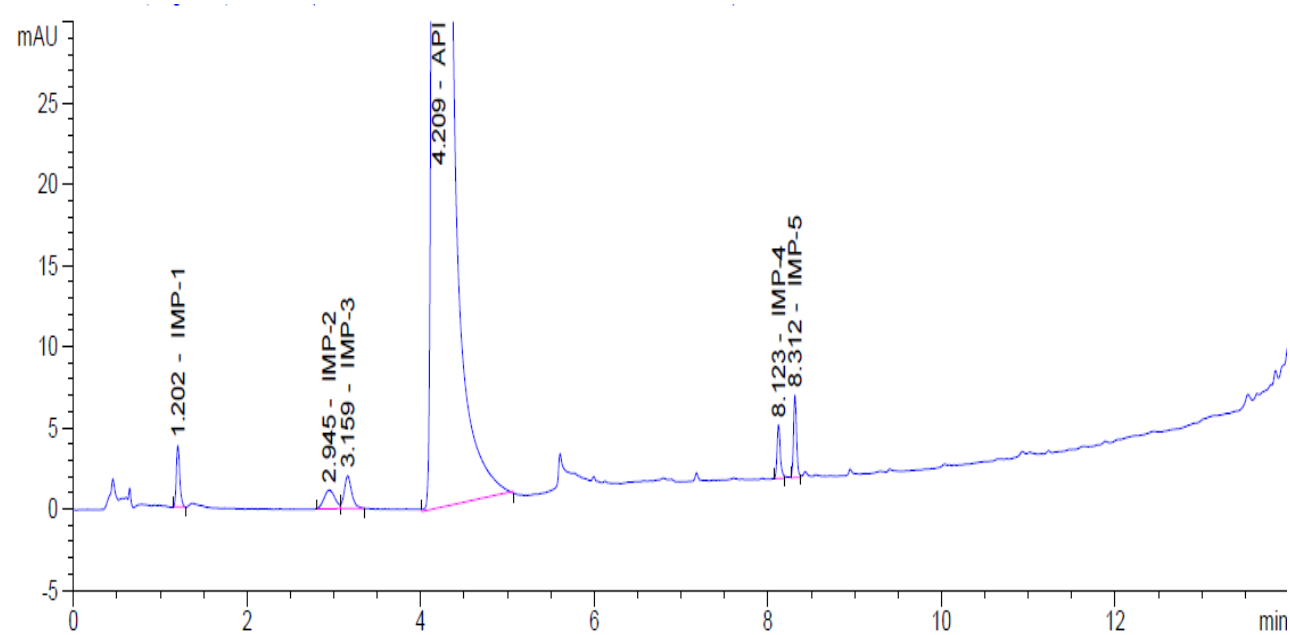


Figure 2: Chromatogram of allopurinol spiked with impurities.

Table 3: Summary report offorced degradation study.

Stress condition	Time (h)	% Assay of active substance	% of degradation products	Mass balance* (%)
Acid Hydrolysis (1N HCl)	2	94.1	4.4	98.5
Basic Hydrolysis (1N NaOH)	2	89.2	9.2	98.4
Oxidation (5% H2O2)	2	87.3	11.5	98.8
Dry Heat (105 °C)	48	99.6	-	99.6
Wet Heat (70 °C)	48	99.2	-	99.2
UV (254 nm)	48	99.1	-	99.1

*It is the summation of assay of active substance and % of degradation products

Method Validation

The newly developed method was validated for sensitivity, linearity, precision and accuracy, robustness and system suitability according to ICH guidelines[18]. Validation study was carried out for Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5. The system suitability and selectivity were checked by injecting 200 µg/mL of allopurinol solution containing 0.4µg/mL of all impurities monitored throughout the validation. Method validation results are summarized in Table 2.

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection and limit of quantitation were determined for allopurinol and for each of the related substances as per ICH $Q2R_1$ guideline. The LOD and LOQ for Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 and allopurinol were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of diluted solutions with known concentration. The limit of detection and the limit of quantitation for imp-1 and imp-4 were about 0.003% and 0.009%, imp-2 and imp-3 were about 0.006% and 0.018%, imp-5 and allopurinol were about 0.002% and 0.006% of analyte concentration i.e. 200 µg/mL respectively. Precision study was also carried at the LOQ level by injecting six individual preparations of all impurities and the relative standard deviation for LOQ concentration for all impurities were below 5%.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample. A linearity test solution for related substance method was prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 150% of the permitted maximum level of the impurity (i.e. LOQ, 0.10 µg/mL, 0.20 µg/mL 0.32 µg/mL, 0.40 µg/mL and, 0.60 µg/mL) was subjected to linear regression analysis with the least squares method. Calibration equation obtained from regression analysis was used to calculate the

corresponding predicted responses. The residuals and sum of the residual squares were calculated from the predicted responses. The correlation coefficient obtained was greater than 0.99 for all impurities. The result showed an excellent correlation between the peak and concentration of all impurities. The range of the method was from LOQ to 0.60 µg/mL of the analyte concentration (200 µg /mL).

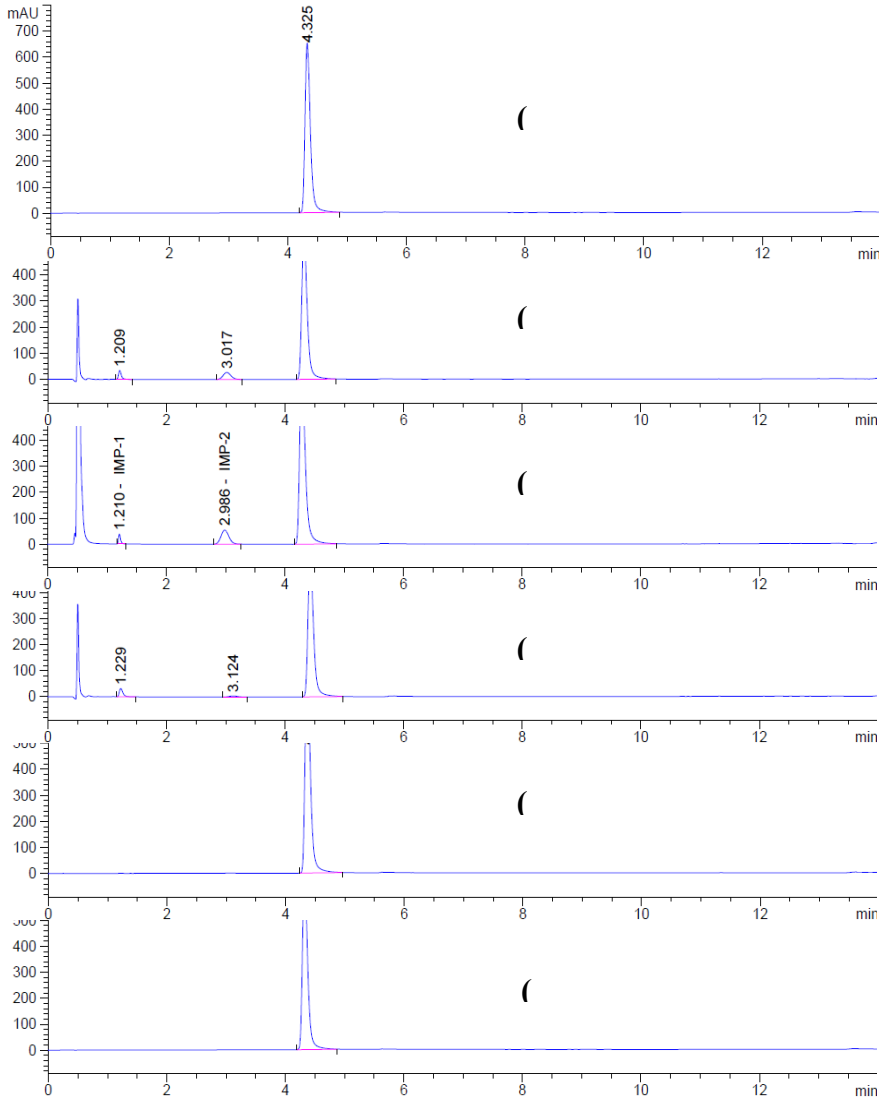


Figure 3: Typical chromatogram of allopurinol under stress conditions: (a) thermal degradation, (b) photolytic degradation, (c) acid hydrolysis, (d) oxidative degradation, (e) base hydrolysis and (f) in water

Precision

Precision of the method was studied for method precision and intermediate precision. Method precision was checked by injecting six individual preparations of (200 µg/mL) allopurinol spiked with 0.4µg/mL of each impurity. In the intermediate precision study, the similar procedure of

method precision was carried out by a different day. % RSD of areas of each impurity was within 5.0, confirming good precision at low level of the developed analytical method.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the method was evaluated in triplicate at LOQ, 80% level (0.32 µg/mL) , 100% level (0.40 µg/mL) and120% level (0.48µg/mL). The percentage recovery of all impurities in drug substance has been calculated. Chromatogram of allopurinolspiked with five impurities was depicted in Figure 2.

Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and the resolution between closely eluting peaks, Imp-2 and Imp-3 was evaluated. Close observation of analysis results of deliberately changed chromatographic conditions viz; flow rate (0.5±0.05 mL/min), mobile phase pH (3.0±0.1), and column temperature (20±2 $^{\circ}$C) revealed that resolution between Imp-2 and Imp-3 was greater than 1.2 and no significant change in relative retention time for all impurities in spiked sample illustrating the robustness of the method. However addition of 1% acetonitrile in mobile phases A deteoriated the resolution between imp-2 and imp-3. So it is strongly recommended that no solvent should be mixed with mobile phase-A which will diminish the resolution between Imp-2 and Imp-3.

Solution stability and mobile phase stability

The solution stability of allopurinol and its related impurities was carried out by leaving both spiked and unspiked sample solutions in tightly capped HPLC vials at 8°C for 48 h in an auto sampler. Content of each impurity was determined against freshly prepared standard solution. No significant changes were observed in the content of any of the impurities. The solution stability and mobile phase stability experiment data confirms that the sample solutions and mobile phase used during related substance determination were stable for at least 48 hour.

CONCLUSION

In this paper, a sensitive, selective, specific, accurate, validated and well-defined stability indicating UPLC method for the quantification of allopurinol process related impurities and degradation products were described. The behavior of allopurinol under various stress conditions was studied and presented. All the degradation products and process impurities were well separated from the drug substance demonstrates the stability indicating power of the method.This

newly developed method has been validated as per regulatory requirements and can be used for routine and stability studies for the quantitative determination of potential impurities in allopurinol drug substance.

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