Impurity profiling and method development of API and related substances of Allopurinol by LC-MS

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ABSTRACT:

The drug Allopurinol is used to treat kidney stones and some forms of gout. In the drug material used to make Allopurinol, many contaminants were found. LC-MS/MS was used to identify impurities, while IR and NMR were used to characterise them following synthesis. For the separation of contaminants, a Kromasil C18, 150 mm 4.6 mm, 5 m particle size column was employed using a reverse phase gradient system. The employment of a Q-TOF mass spectrometer with an electrospray ionisation (ESI) source and ESI positive mode, which provides fragmentation with mass precision and accurate mass up to four decimal places, was important for the detection of contaminants. The medicine Allopurinol included five contaminants, it was discovered.

***KEY WORDS*:** *LC-MS, low level, Allopurinol, Impurities.*

INTRODUCTION:

A drug called allopurinol is used to lower elevated blood uric acid levels. [1,2] It is used primarily to treat excessive uric acid levels that can happen with chemotherapy as well as to avoid gout, certain forms of kidney stones, and gout. [3] [4] It can be injected into a vein or given orally. Itchiness and redness are frequent adverse reactions when taken by mouth [4]. When administered by injection, nausea and renal issues are frequent adverse effects. [4] Allopurinol looks to be safe to begin taking during a gout episode, despite it not traditionally being advised. [5] [6] The medicine should be continued even during an acute gout attack in patients who are already taking it. [5] [3] Use during pregnancy hasn't been well studied, although it doesn't seem to be dangerous. The class of drugs known as xanthine oxidase inhibitors includes allopurinol. [4] In the United States, allopurinol was given the go-ahead for medicinal usage in 1966. It is listed as one of the Essential Medicines by the World Health Organization [4]. [7] [8] It is possible to get generic allopurinol. [4] With more than 15 million prescriptions written in 2019, it was the 43rd most often prescribed drug in the US. [9] [10]

Pharmacology

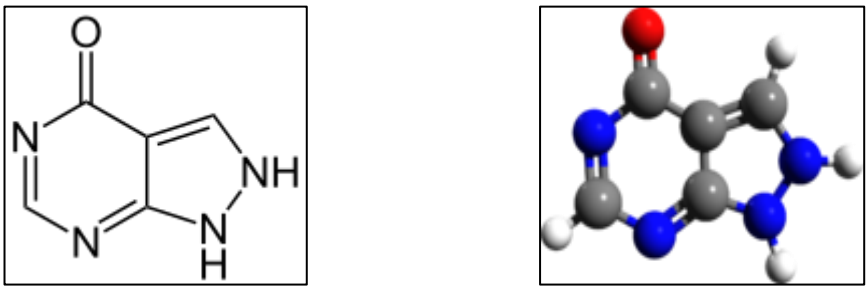
Contrary to popular belief, aldehyde oxidase is primarily responsible for the metabolism of allopurinol rather than its target, xanthine oxidase. [11] Allopurinol's active metabolite, oxipurinol, is likewise a xanthine oxidase inhibitor. Within two hours of oral treatment, allopurinol nearly entirely transforms into oxipurinol, whereas oxipurinol is slowly eliminated by the kidneys over a period of 18 to 30 hours. Because of this, the bulk of allopurinol's effects are thought to be mediated by oxipurinol. [12]

Mechanism of action

A purine analogue, allopurinol is an inhibitor of the xanthine oxidase enzyme and a structural isomer of hypoxanthine, a purine that occurs naturally in the body. [13] Uric acid, a byproduct of human purine metabolism, is produced as a result of the sequential oxidation of hypoxanthine and xanthine by xanthine oxidase. [14] Inhibition of xanthine oxidase results in an increase in hypoxanthine and xanthine in addition to inhibiting the synthesis of uric acid. While hypoxanthine may be recovered to the purine ribotides adenosine and guanosine monophosphates, xanthine cannot be converted to purine ribotides. The first and rate-limiting enzyme of purine biosynthesis, amidophosphoribosyl transferase, may experience feedback inhibition as a result of elevated amounts of these ribotides. As

a result, allopurinol lessens the production of uric acid and could also prevent the synthesis of purines. [15]

CHEMISTRY:

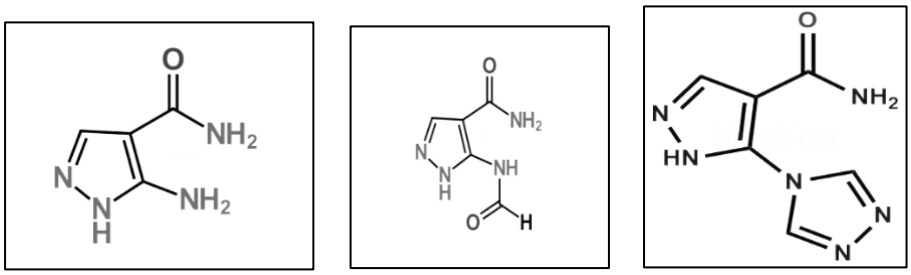


a b

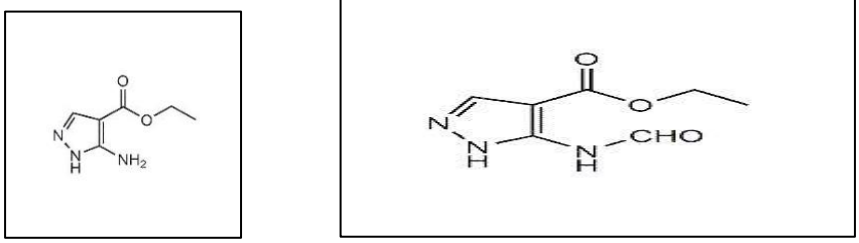
Fig 1: a and b structure of alloperinol having molecular formula C5H4N4O and molecular mass 136.114 g·mol−1 [16]

Related substance Impurities:

API	SR NO	Impurity
Allopurinol	1	Allopurinol EP Impurity A
	2	Allopurinol EP Impurity B
	3	Allopurinol Impurity C
	4	Allopurinol EP Impurity D
	5	Allopurinol EP Impurity E



A **B** **C**



D **E**

Fig 2: Related Substances of Alloperinol [16]

The present study focuses on impurity profiling and method development of API and related substances of allopurinol by LC-MS.

MATERIALS AND METHODS:

Materials:

We bought the allopurinol medicinal material and its impurities from Sigma Aldrich and Chemieliva, both in India. Acetonitrile, LC-Ms grade water, and formic acid were purchased from Fisher Scientific as HPLC grade solvents.

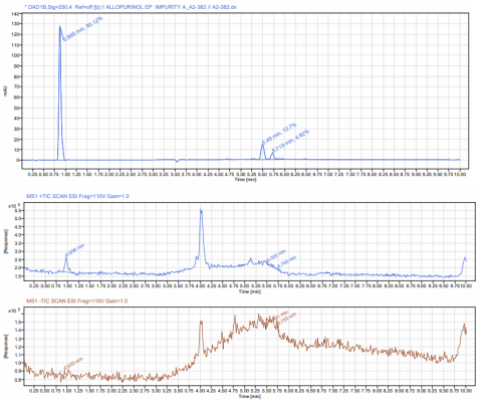
Instrumentation and Method Conditions

A LC-MS system with a Waters Alliance HPLC system (Waters, Milford, MA, USA) that includes a 2996 photodiode array detector and a 2695 quaternary pump and auto sampler. an electrospray ionisation (ESI) source attached to a hybrid quadrupole-time-of-flight mass spectrometer (Q-TOF micro; Waters, Milford, MA, USA) that was used in positive ESI mode. The MassLynx V4.1 programme (Waters) was employed. With flow rates of 50 l/h for cone gas flow and 500 l/h for desolvation gas flow, nitrogen was used for both. Temperatures at the source and desolvation were 120 and 230 C, respectively. The sample cone voltage was 15V, and the capillary voltage was 3500V. There was a 6V collision energy. Mass spectra were collected with a resolution of around 5000 at full-width half-maximum spanning a m/z range of 100-1000. Argon was employed as a collision gas in the MS/MS procedure. Infusion of leucine enkephalin (m/z of the protonated molecule 556.2771) into the lock spray reference channel served as a reference substance for accurate mass measurements.

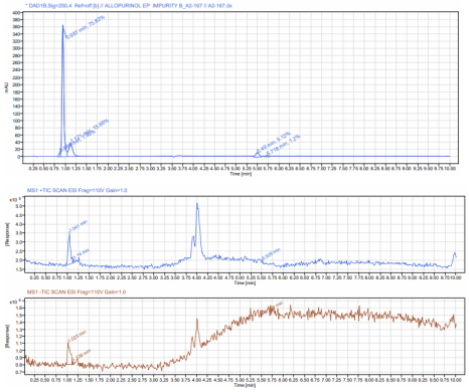
Analytical methods

HPLC method used for Alloperinol API:

The buffer solution used for the preparation of Mobile phase A consists of 0.01 M aqueous ammonium acetate and its pH was adjusted to 3.5 with Trifluoro acetic acid. Acetonitrile was used as Mobile phase B. Kromasil C18, 150 mm × 4.6 mm, 5 m particle size column was used with a time gradient program of T (min)/% of Mobile phase B (v/v). Initial gradient of Mobile phase B starts with 32% and at 15 min it was 48%, and changed to 54% at 32 min and reached 85% at 40 min. The ratio being continued up to 50 min and at 53 min it was brought back to initial composition (32%), which was continued up to 60 min with a flow rate of 1.0 ml/min and column eluent, was monitored by UV detector at 315 nm. Column oven temperature was 30 ◦C. The injection volume was 10 l. Diluent was the mixture of water and acetonitrile in the ratio of (20:80) and sample concentration was 1 mg/ml.



A



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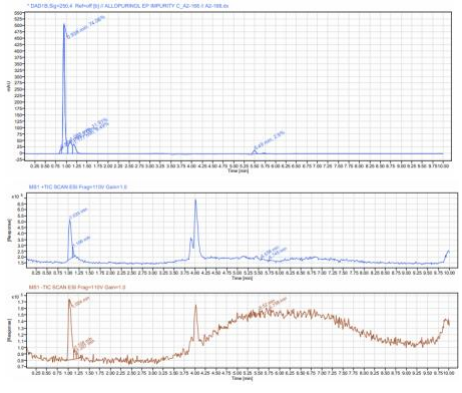

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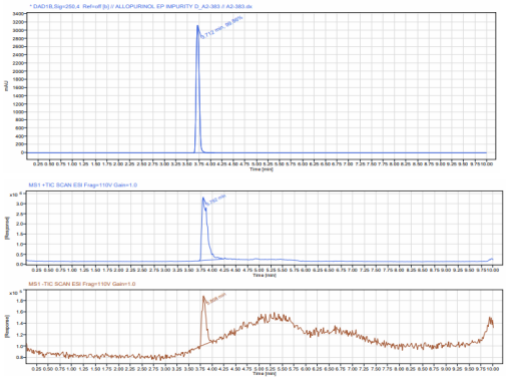
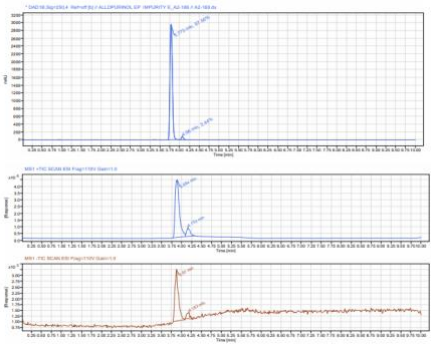


D



E

Fig 3 : Chromatogram of impurities

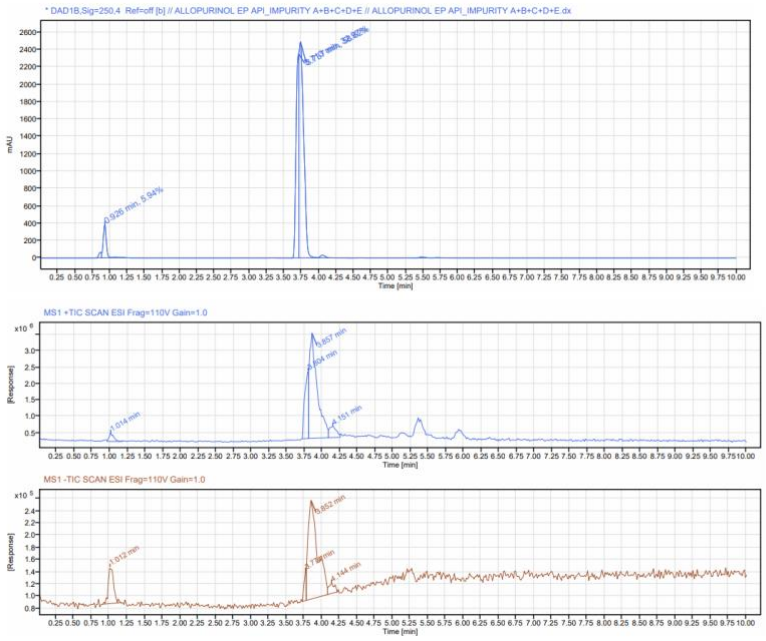


Fig 4: Chromatogram of API spiked with known impurities

Table 1 : Details of Analysis of Impurities

IMPURITIES	RT [min]	Area	Height	Area%
ALLOPURINOL EP IMPURITY A	0.865	445.8309	128.4159	80.1230
	4.684	1.7843	0.4091	0.3207
	5.268	4.2484	0.6979	0.7635
	5.490	70.6447	13.6936	12.6960
	5.719	26.8435	5.5638	4.8242
	5.841	3.3606	0.6366	0.6039
	5.841	3.3606	0.6366	0.6039
	6.465	1.8422	0.3609	0.3311
ALLOPURINOL EP IMPURITY B	0.869	29.5713	10.8901	1.9864
	0.937	1128.8159	360.9841	75.8246
	1.121	236.1556	36.4536	15.8630
	5.490	76.2688	9.9685	5.1231
	5.718	17.9080	1.2977	1.2029
ALLOPURINOL EP IMPURITY C	0.869	44.6772	17.7436	2.0904
	0.938	1583.1877	510.5686	74.0757
	1.088	254.4795	46.4318	11.9068
	1.177	181.4440	35.0226	8.4896
	5.490	61.9592	12.6320	2.8990
	5.719	11.5083	2.9301	0.5385
ALLOPURINOL EP IMPURITY D_A	3.712	14509.0892	3141.0007	99.8564
	3.998	20.8650	3.4671	0.1436
ALLOPURINOL EP IMPURITY E	3.773	97.5550	12860.2992	2959.4316
	4.060	322.3084	79.3230	2.4450

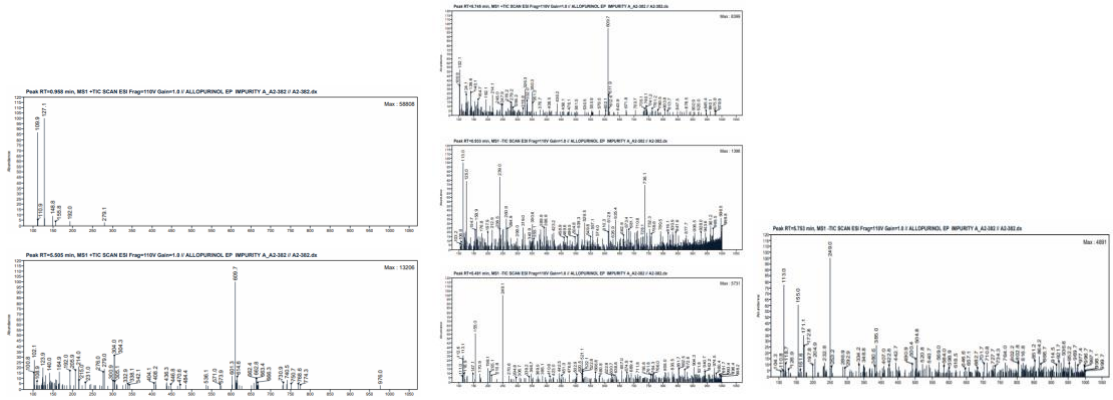


Fig 5: MS/MS fragmentation pattern of impurities **A**

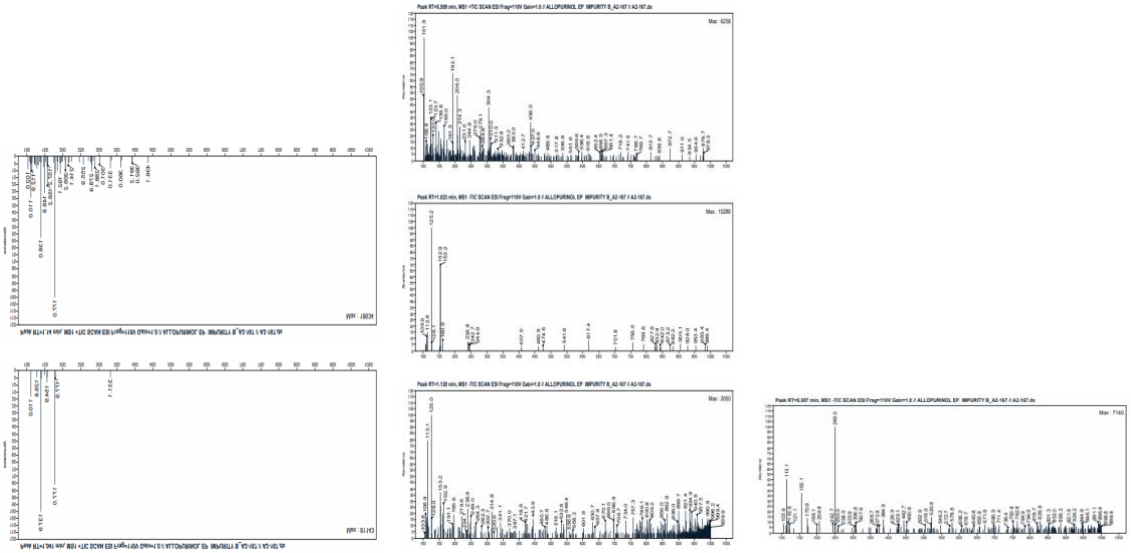


Fig 6: MS/MS fragmentation pattern of impurities B

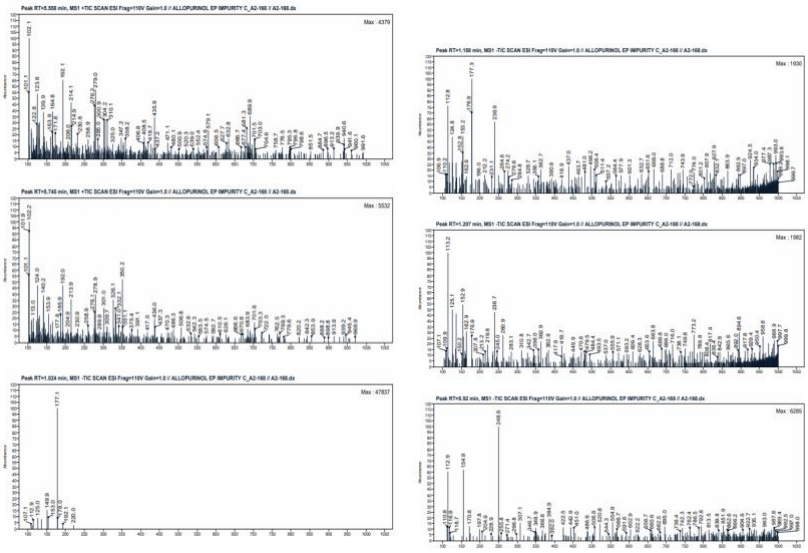


Fig 7 : MS/MS fragmentation pattern of impurities C

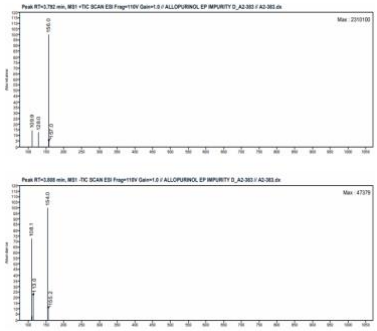


Fig 8: MS/MS fragmentation pattern of impurities D

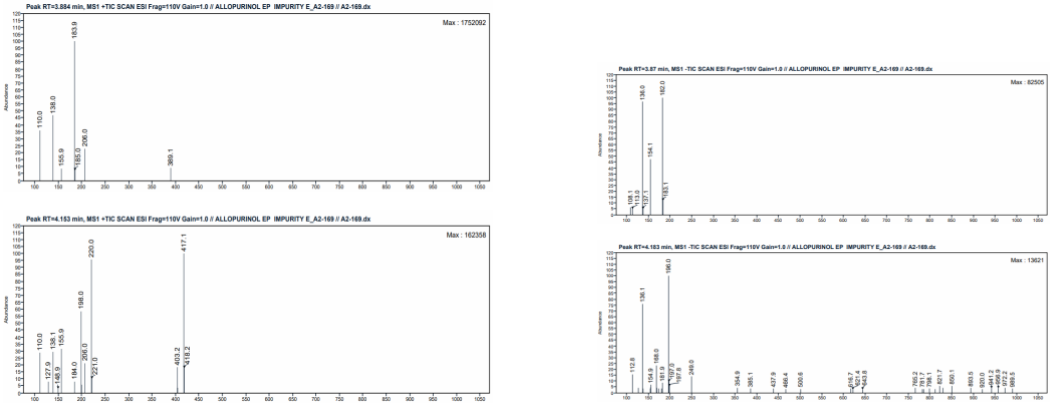


Fig 9: MS/MS fragmentation pattern of impurities E

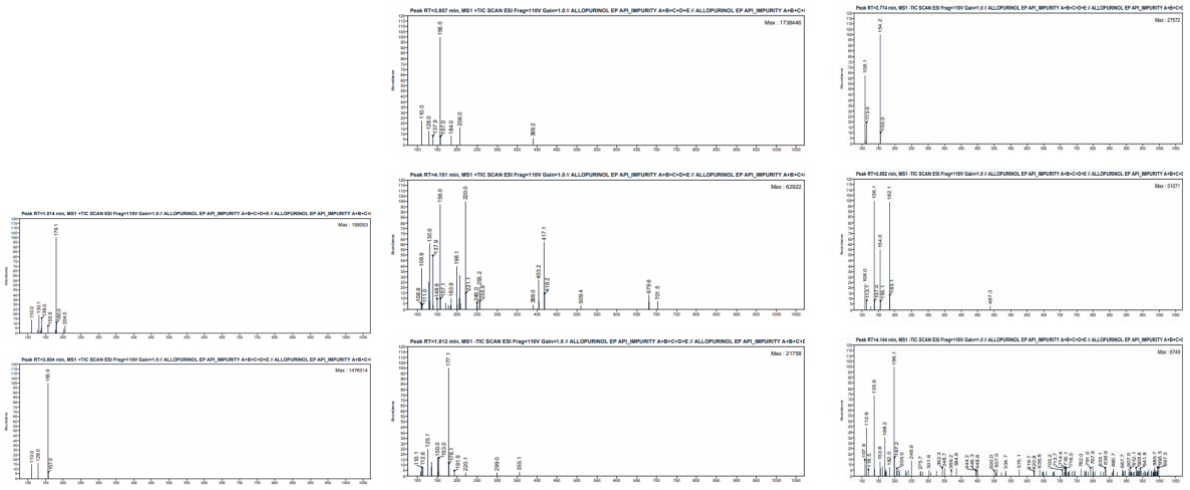


Fig 10: MS/MS fragmentation pattern of API spiked with known impurities

METHOD VALIDATION

Specificity

The individual preparation of Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E solutions at a concentration in the diluent together with the preparation of an Allopurinol sample solution spiked with five impurities served to establish the method's specificity. With satisfactory separation of Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E at the retention periods of 2.61 min, 7.47 min, 5.54 min, and 4.77 min, respectively, the allopurinol peak was eluted at 9.2 min.

Linearity

The method's linearity test is carried out in accordance with ICH recommendations. The effectiveness of this method is tested at six different concentrations between LOQ and 150%. The calibration curve was created by plotting peak areas against analyte concentration. From least squares linear regression analysis, the correlation coefficient, intercept for four potential genotoxic impurities, and slope values were obtained. All potentially genotoxic impurities had correlation coefficients of >0.998. The linearity experiment showed that the mass spectrometric responses within the range of 0.04-1.8 ppm were proportional to their concentration.

Recovery Studies

Recovery studies were determined by spiking the five impurities at LOQ level, 50%, 100% and 150% of the specification concentrations, i.e 0.02, 0.4, 1.1 and 1.6 ppm with respect to the sample concentration . The recovery of impurities at LOQ level should be in the range of 70.0% to 130.0%. The recovery of impurities at three levels (50%, 100% and 150%) should be in the range of 80.0% to 120.0 %. Well recovery values of 97.38% to 107.90% for Imp-A, 97.67% to 102.34% for Imp-B, 99.30 % to 102.92 % for Imp-C, 98.25% to 103.23% for Imp-D and 97.23% to 102.33% for Imp-E were obtained.

Limit of Quantification and Limit of Detection

Limit of quantification (LOQ) and Limit of detection (LOD) were determined by analyzing different concentrations of impurities at low concentration. In this process, the concentrations of standard solutions were reduced sequentially to obtain limit of quantification, such that they yield S/N ratio as 10.1, 10.4, 10.1 and 10.0 for Imp-A, Imp-B, Imp-C, Imp-D and Imp-E respectively. The Limit of

quantification (LOQ) of 0.03 ppm is general for all the impurities with a LOD of 0.01 ppm and is nearly three times less than LOQ. This analysis was carried out in MRM mode and LOD was determined as 0.01ppm. The precision of six injections containing 0.03 ppm of each potential genotoxic impurities at LOQ level was below 5.0% RSD.

Precision and Ruggedness

To determine precision of the method through repeatability and ruggedness, we prepared six fresh preparations of standard mixture solutions containing five impurities at a concentration of 1.0 ppm of each one on the same day and their recoveries were checked. Ruggedness was evaluated by injecting the six freshly prepared solutions containing 1.0 ppm of each impurity at different days and their recoveries with % RSD values. The lower values of % RSD (below 4 %) values confirm that the precision of the developed method is good and well suited for different laboratory conditions.

Robustness

The robustness of the method was evaluated by making deliberate changes in experimental conditions including column temperature, flow rate and source temperature in mass source. Actual flow rate of the mobile phase was 0.5 mL/min and the same was altered by 0.2 units i.e. 0.3 mL/min and 0.7 mL/min. The effect of column temperature on the analysis was studied at 43° C and 47° C (temperature altered by 2 units). The robustness of the proposed method also evaluated by the changing the temperature in mass source with ± 20°C. No significant change in the chromatographic performance was observed for all the above deliberately varied experimental conditions, which indicated the robustness of the method.

RESULTS AND DISCUSSION

The goal of the current work was to create a sensitive and focused LC-MS/MS approach that can identify and count five contaminants in the active pharmaceutical constituent of Allopurinol. Due to the similar chemical structures of the five impurities and Allopurinol, it is crucial to establish effective separation between the two. Different reversed phase stationary phases have been evaluated in order to obtain a quick analysis time. These phases include C18, C8, and cyano phases columns like CSH C18 (15.0 cm x 3.0 mm, 1.7 micron), Hypersil BDS C8 (150 mm 4.6 mm, 3.5 m), Kromasil C8 (150 mm 4.6 mm, 3.5 m), and Symmetry C18 (100 mm 4.6. The Imp-B peak and Allopurinol peak overlapped when the Kromasil C8 column was utilised. With the Hypersil BDS C8 column, the peak forms and resolution between Allopurinol and the impurities (Imp-A, Imp-B, Imp-C, Imp-D, and Imp-E) were low. The responses and resolution for both the contaminants and Allopurinol on CSH C18 (15.0 cm x 3.0 mm, 1.7 micron) were deemed to be satisfactory. The analytes were effectively separated and kept from the drug component and from one another on this column. Using formic acid and trifluoroacetic acid in water and the solvent acetonitrile, several mobile phase compositions were evaluated. Finally, at a ratio of mobile phase A (0.1% formic acid in water), mobile phase B (acetone), and gradient ran, satisfactory separation and response were seen. The goal of the gradient programme was to achieve the best possible separation of impurities from one another with regard to the peak of the drug substance. For improved peak form and to prevent any movement in retention time, the column was thermostated at 45 °C. In order to prevent additional interference from contaminants and increase the sensitivity of the procedure, we employed a diverted valve in this method to divert the Allopurinol into garbage from retention time 6 to 10 min.

CONCLUSION:

The contaminants in the drug material used to make Allperinol were found utilising a Q-TOF mass spectrometer with the aid of the aforementioned investigation. structural identification was assisted by the Q-TOF method. This method worked well for quickly identifying contaminants. The findings demonstrate that contaminants in the drug ingredient used to make alloperinol are a result of both the synthesis pathway and carryover impurities from source components. The information provided is useful for comprehending Alloperinol's impurity profile and may also be useful for controlling contaminants in finished goods. Identification and backward integration of impurities present in active pharmaceutical components and drug product can both benefit from this study.

REFERENCES:

*[1] "Allopurinol Use During Pregnancy". Drugs.com. Archived from the original on 20 August **2016.***

*[2] Pacher P, Nivorozhkin A, Szabó C "Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol". Pharmacological Reviews. 58 (1): 87–114**(March 2006).***

*[3] World Health Organization **(2009).** Stuart MC, Kouimtzi M, Hill SR (eds.).*

*[4] WHO Model Formulary **2008**. World Health Organization. p. 39.*

*[5] "Allopurinol". The American Society of Health-System Pharmacists. Archived from the original on 29 April **2016.***

*[6] Robinson PC, Stamp LK ("The management of gout: Much has changed". Australian Family Physician. **May 2016).** 45 (5): 299–302.*

*[7] Satpanich, P; Pongsittisak, W; Manavathongchai, S "Early versus Late Allopurinol Initiation in Acute Gout Flare (ELAG): a randomized controlled trial". Clinical Rheumatology. **(2021).** 41 (1): 213–221*

*[8] World Health Organization (2019). World Health Organization model list of essential medicines: 21st list **2019.** Geneva: World Health Organization.*

*[9] World Health Organization **(2021).** World Health Organization model list of essential medicines: 22nd list (2021). Geneva: World Health Organization.*

*[10] "Allopurinol - Drug Usage Statistics". ClinCalc. 16 October **2021***

*[11] Reiter S, Simmonds HA, Zöllner N, Braun SL, Knedel M "Demonstration of a combined deficiency of xanthine oxidase and aldehyde oxidase in xanthinuric patients not forming oxipurinol". Clinica Chimica Acta; International Journal of Clinical Chemistry. **(March 1990).** 187 (3): 221–34.*

*[12] Day RO, Graham GG, Hicks M, McLachlan AJ, Stocker SL, Williams KM "Clinical pharmacokinetics and pharmacodynamics of allopurinol and oxypurinol". Clinical Pharmacokinetics. **(2007).** 46 (8): 623–44.*

*[13] Cameron JS, Moro F, Simmonds HA "Gout, uric acid and purine metabolism in paediatric nephrology". Pediatric Nephrology**(February 1993).**. 7 (1): 105–18.*

*[14] Q. Ye, Y. Huang, A. Rusowicz, Org. Process. Res. Dev. 14 **(2010)** 238–241.*

*[15] S. Vasanti, et al., Impurity profile – a review, Drug Invention Today 1 **(2009)** 81–88.*