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Analytical method development and validation of allopurinol and related substances by using RP-HPLC in bulk form

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***Abstract*---**A new simple, rapid, specific, economical, precise and accurate method for the estimation of Allopurinol by using reverse phase high performance liquid chromatography (RP-HPLC), has been developed and validated according to the ICH guidelines. The seperation was achieved by the Kromasil 100-5,C18,250x 4.6mm and potassium dihydrogen orthophosphate and Methanol used as mobile phase, at a flow rate of 1 ml /min and the column temperature was 30°C. Detection was carried out at 220nm. Retention time of allopurinol was found to be 5.6 mins.The developed method was validated in terms of system suitability, selectivity, linearity, precision, accuracy, limits of detection and quantification for the impurities following the ICH guidelines. Linearity observed for the allopurinol is within the limits, % RSD for repeatability was found to 0.525 .The mean recovery was found to be within the limits 96.1-96.6%. Developed method was found to be simple, rapid, accurate, precise and specific for the estimation of allopurinolin pure and their Pharmaceutical dosage form.

***Keywords*---**allopurinol, RP-HPLC, accuracy, precision, robustness.

Introduction

Allopurinol is a classified as a xanthine oxidase inhibitor. This means that it stops the enzyme xanthine oxidase from functioning correctly. Xanthine oxidase

converts oxypurines (hypoxanthine and xanthine) to uric acid. It is found in many organs such as the liver, stomach, heart, brain, kidneys, and blood plasma. Xanthine oxidase converts hypoxanthine to xanthine and then xanthine to uric acid. Uric acid is a product of broken down foods and cells that are excreted by the kidneys. A decreased level of xanthine oxidase, an increased amount of hypoxanthine and xanthine, or a decreased kidney function can all increase the level of uric acid in the blood.(Kasture A.V et., al).Too much uric acid in the blood builds up around joints and causes the pain and swelling associated with gout. An increase in uric acid is also associated with organ damage and failure. It is 1, 5-dihydropyrazolo[3,4-d]pyrimidin-4-one. Its Molecular Weight is 136.11. (Gerberdin SJ et., al 1998)

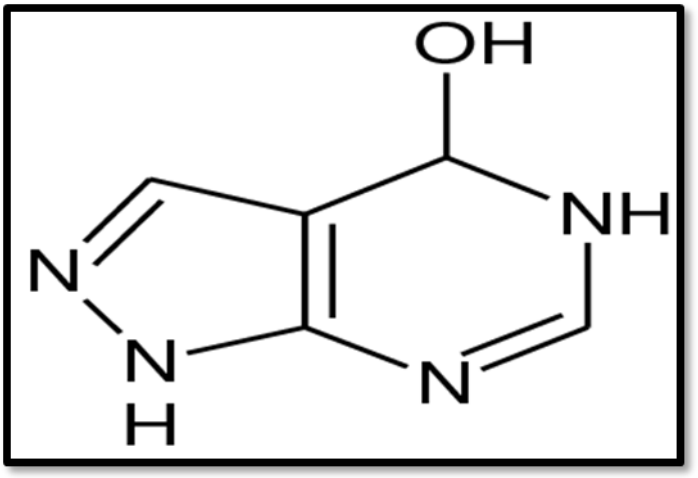

Figure 1: Structure of Allopurinol

In the literature, it was observed that there are not many approaches for measurement of Allopurinol. The work aims were to find an optimized new way to determine Allopurinol on a reduced period and with less solvent used. This research aims to improve simple, reliable, and effective methods for the determination of Allopurinol. (Hardwood Lm et., al 1989)

Materials and Methods

Table 1 Instruments used

S.No.	Name of the instrument	Inst. ID. No.	Make	Model
1	HPLC	AD/LC/128	Waters	2695 Pump&2489 Uv
2	HPLC	AD/LC/126	Waters	2695 Pump &2998 Uv
3	HPLC	AD/LC/100	Agilent	2695 Pump & 2489 Uv
4	BALANCE	AD/MB/04	Mettler	XP205DR
5	BALANCE	AD/MB/05	Mettler	XP56
6	BALANCE	AD/MB/05	Sartorius	MSA6.6S-000-DM
7	BALANCE	HCLI056	Sartorius	MSA2.7S-000-DM

HPLC Method Development
Preparation of Standard solution

Weigh accurately about 5.0 mg of each Allopurinol reference standard, Allopurinol Related compound-A (or) ALPRC-A, Allopurinol Related Compound-B,Allopurinol Related compound-C Allopurinol Related Compound-D and Allopurinol Related Compound-E into a 100 ml volumetric flask, Add 2.0 ml of 0.1 N NaoH solution and Sonicate for additional 5 minutes dilute to the volume with diluent. Dilute 1ml of the above prepared stock solution into a 100ml Volumetric flask with diluent. (Donald P.L et., al 2006) (Determann et., al 2012)

Mobile phase optimization
Initially the mobile phase tried was Phosphate buffer

Methanol in varying proportions. Finally the mobile phase was optimized to Potassium di hydrogen orthophosphate: Methanol in a gradient manner.

Optimization of Column

The method was performed with various columns like Hypersil ODS, 250x4.6mm,5 micrometer and Hypersil BDS, 50x4.6mm, 3micrometer. Kromasil 100-5, C18,250x4.6mm column was found to be ideal as it elute all related compounds with a good peak shape and resolution at 1ml/minute flow.

Table 2: Optimized Chromatographic Conditions

Column	Kromasil 100-5,C18,250x4.6mm
Detector Wavelength	220nm
Injection Volume	40microlitre
Flow Rate	1.0 ml/m
Column Temperature	30^0C
Sample Temperature	8^0C
Run Time	46min
Diluent	Solvent A: Solvent B(90:10) v/v

Table :3 Gradient program:(mobile phase)

Time(min)	Solvent-A (%)	Solvent B(%)
0.01	90	10
30	70	30
35	70	30
36	90	10
46	90	10

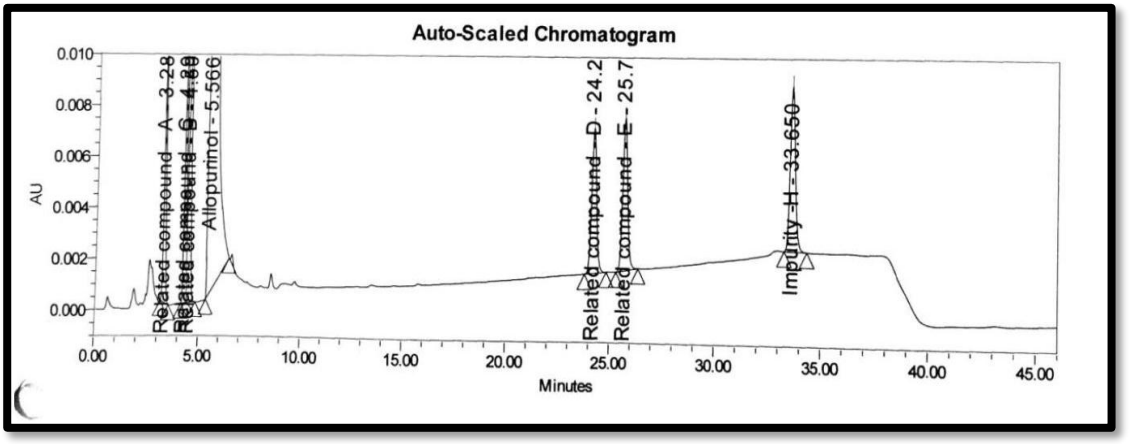

Fig 2: Standard Chromatogram

Table 4: Peak results of optimized chromatogram

S.no	Name	Rt	Area	%Area
1	Related Compound -A		4137	10.82
2	Related Compound -c	21.47	3649	10.07
3	Related Compound -B	17.56	3751	9.81
4	Allopurinol	28.13	3874	10.13
5	Related Compound-D	5.6	3591	9.38
6	Related Compound -E	14.79	3480	9.10
7	Impurity -H	16.11	4144	10.64

Method validation

Preparation of Solvent A

Dissolve about 1.25gm of Potassium di hydrogen orthophosphate in 1000 ml of water and mix. Filter and degas through 0.45 micrometer membrane filter paper.

Preparation of Solvent B

Use Methanol as a Mobile phase-B. Filter and degas through 0.45micrometer membrane filter paper.

Specificity
Preparation of Allopurinol Related Compound-A Solution (0.20%)

Weigh about 5mg of Allopurinol related compound-A into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and sonicate for one minute, add 80ml of diluent and Sonicate for additional 5 min. Dilute to the volume with diluent. Take 1ml of above prepared stock solution into a 100ml volumetric flask, dilute to the volume with diluent and mix.

Preparation of Allopurinol Related Compound-B Solution (0.20%)

Weigh about 5mg of Allopurinol related compound-B into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and sonicate for one minute, add 80ml of diluent and sonicate for additional 5 min. Dilute to the volume with diluent. Take 1ml of above prepared stock solution into a 100ml volumetric flask, dilute to the volume with diluent and mix.

Preparation of Allopurinol Related Compound-C Solution (0.20%)

Weigh about 5mg of Allopurinol related compound-C into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and sonicate for one minute, add 80ml of diluent and sonicate for additional 5 min. Dilute to the volume with diluent. Take 1ml of above prepared stock solution into a 100ml volumetric flask, dilute to the volume with diluent and mix.

Preparation of Allopurinol Related Compound-D Solution (0.20%)

Weigh about 5mg of Allopurinol related compound-D into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and sonicate for one minute, add 80ml of diluent and sonicate for additional 5 min. (KarlssonE et., al 1998) Dilute to the volume with diluent. Take 1ml of above prepared stock solution into a 100ml volumetric flask, dilute to the volume with diluent and mix.

Preparation of Allopurinol Related Compound-E Solution (0.20%)

Weigh about 5mg of Allopurinol related compound-E into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and Sonicate for one minute, add 80ml of diluent and sonicate for additional 5 min. Dilute to the volume with diluent. Take 1ml of above prepared stock solution into a 100ml volumetric flask, dilute to the volume with diluent and mix.

Preparation of Impurity-H Solution (0.20%)

Weigh about 5mg of Impurity-H into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and Sonicate for one minute, add 80ml of diluent and Sonicate for additional 5 min. Dilute to the volume with diluent. Take 1ml of above prepared stock solution into a 100ml volumetric flask, dilute to the volume with diluent and mix.

Preparation of Allopurinol Standard Solution (0.10%)

Weigh about 5mg of Allopurinol standard solution into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and sonicate for one minute, add 80ml of diluent and Sonicate for additional 5 min. (Walls D et., al 2011) Dilute to the volume with diluent. Take 1ml of above prepared stock solution into a 100ml Volumetric flask, dilute to the volume with diluent and mix.

Preparation of Blend solution

Weigh about 5mg of Allopurinol sample into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and sonicate for one minute, add 80ml of diluent and sonicate for additional 5 min then add each 1ml of Allopurinol Related Compound-A, Allopurinol Related compound-B, Allopurinol related compound-C. Allopurinol Related Compound D, Allopurinol Related compound-E and Impurity –H from individual stock solutions. Dissolve and dilute to the volume with diluent and mix.

DL, QL and Accuracy at QL

Detection Limit (DL)

Preparation of DL solution Based on the signal to noise ratio obtained from reference DL solution, prepare the DL solution by diluting each individual stock solution to obtain the DL concentration of each component, which should give signal to noise ratio about 3:1 to 5:1.

Quantitation Limit

Preparation of QL Solution

Prepare the QL solution by diluting each individual stock solution to obtain QL concentration, the resulting solution of each component signal to noise ratio should be about 10:1.

Accuracy at QL

Preparation of Accuracy at QL solution
Prepare the solution having of 100% sample and spike each known impurity at QL level should prepare in triplicates

Linearity and range
Linearity
Preparation of Linearity Stock Solution

Weigh about 5mg of Allopurinol sample into a 100ml Volumetric flask, add 2ml of 0.1N NaOH solution and sonicate for one minute, add 80ml of diluent and sonicate for additional 5 min then add each 1ml of Allopurinol Related Compound-A, Allopurinol Related compound-B, Allopurinol related compound-C. Allopurinol Related compound-D, Allopurinol Related compound-E and Impurity –H from individual stock solutions. Dissolve and dilute to the volume with diluent and mix.

Preparation of Linearity level-1 Solution

Consider first 3 injections of QL level

Preparation of Linearity level-2 Solution (50%)

Take 0.5ml of Linearity stock solution into a 100ml volumetric flask and dilute to the volume with the diluents and mix.

Preparation of Linearity level-3 Solution (80%)

Take 0.8ml of Linearity stock solution into a 100ml volumetric flask and dilute to the volume with the diluents and mix.

Preparation of Linearity level-4 Solution (100%)

Take 1ml of Linearity stock solution into a 100ml volumetric flask and dilute to the volume with the diluents and mix

Preparation of Linearity level-5 Solution (120%)

Take 1.2ml of Linearity stock solution into a 100ml volumetric flask and dilute to the volume with the diluents and mix.

Preparation of Linearity level-6 Solution (150%)

Take 1.5ml of Linearity stock solution into a 100ml volumetric flask and dilute to the volume with the diluents and mix.

Range
Lower level: (Linearity level-1)

Reproduce the peak area response of each component from linearity level-1(QL level) and calculate % RSD for the peak area response of each component.

Upper level :(Linearity level-6)

Reproduce the peak area response of each component from linearity level-6(QL level) and calculate % RSD for the peak area response of each component

Precision

Perform the analysis by spiking the sample with corresponding impurity at 100% level for 6 times and determine the method precision. Calculate the % RSD of the results obtained from method precision study Calculate the 95% confidence interval of mean.

Accuracy
Preparation of Impurity stock solution

Weigh about each 5mg of Allopurinol Related compound-A,Allopurinol Related compound-B, Allopurinol Related Compound-C, Allopurinol Related compound-D, Allopurinol related compound-E and impurity-H into a 100ml volumetric flask.

Add 2ml of 0.1N NaOH solution and sonicate for minute, add 80ml of diluent and sonicate for additional 5min. Dilute to the volume with the diluents and mix.

Preparation of Accuracy level-1

solution Consider 3 preparations of accuracy at QL

Preparation of accuracy level-2 solution (50%)

Weigh about 25 mg of the test sample into a volumetric flask Add 2ml of 0.1N NaOH solution and sonicate for minute, add 80ml of diluent and sonicate for additional 5min. Dilute to the volume with the diluents and mix.

Preparation of accuracy level-3 solution (100%)

Weigh about 25 mg of the test sample into a volumetric flask Add 2ml of 0.1N NaOH solution and sonicate for minute, add 80ml of diluent and sonicate for additional 5min, then add 1ml of impurity stock solution, Dilute to the volume with the diluents and mix.

Preparation of accuracy level-4 solution (150%)

Weigh about 25 mg of the test sample into a volumetric flask Add 2ml of 0.1N NaOH solution and sonicate for minute, add 80ml of diluent and sonicate for additional 5min, then add 1.5ml of impurity stock solution,(Regnier J et., al 1983) Dilute to the volume with the diluents and mix. Procedure Inject each Accuracy level-2 level-4 in triplicates and Accuracy level-3 for six times into system and record the chromatograms. Calculate the %recovery of each impurity.

Intermediate precision

Carry out the intermediate precision study on a different day, different analyst, with different instrument using fresh preparations Perform the analysis by spiking the sample with corresponding impurity at 100% level for 6 times and determine the method precision. Calculate the % RSD of the results obtained from intermediate precision study. Calculate the 95% confidence interval of mean.

Robustness

Mobile phase stability Prepare the mobile phase as per the method and store at room temperature for 2-7days and carry out the analysis using above mobile phase. Establish the system suitability as per the method. Helmut d et., al 1969) Prepare and inject the spiked test sample solution in triplicate preparations and record the chromatograms. Calculate the % of known impurities, AUI and total impurities as per the method. Calculate the % RSD of the results obtained from the study.

Method validation

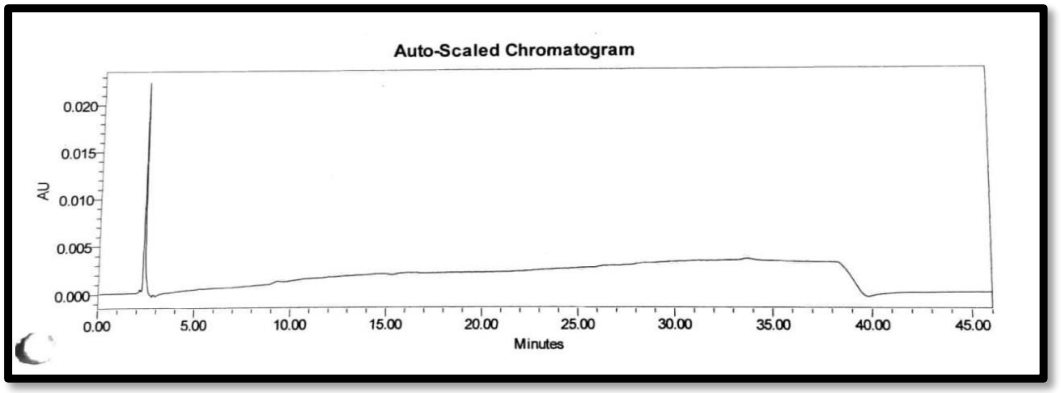

Fig 3 : chromatogram showing blank (mobile phase preparation)

Specificity

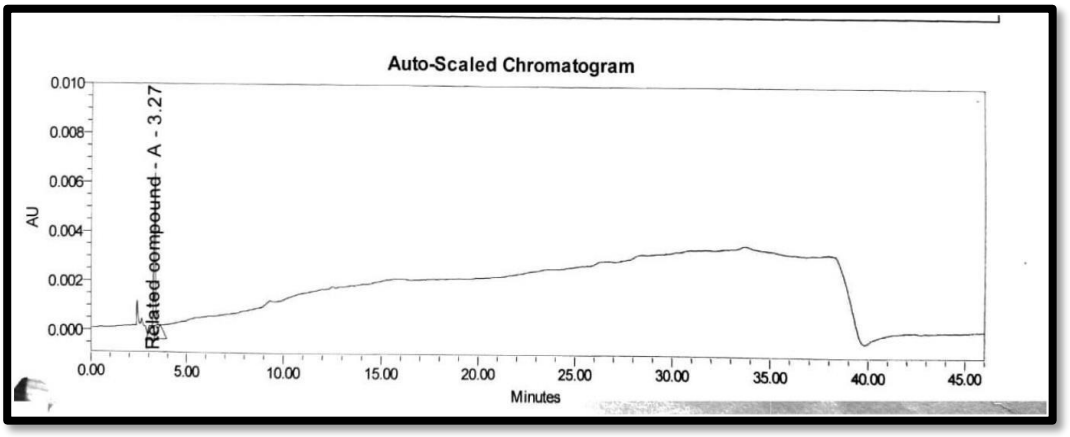

Fig 4: Chromatogram for Allopurinol Related Compound –A

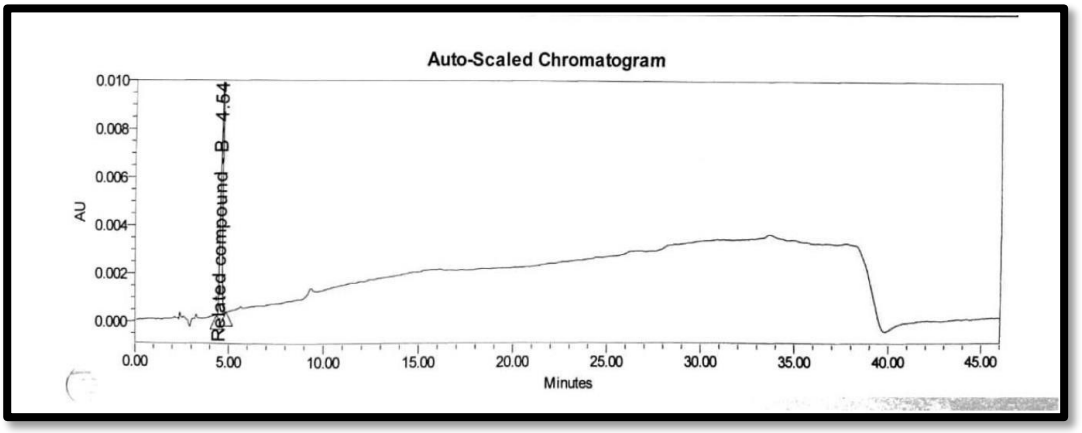

Fig 5: Chromatogram for Allopurinol Related Compound –B

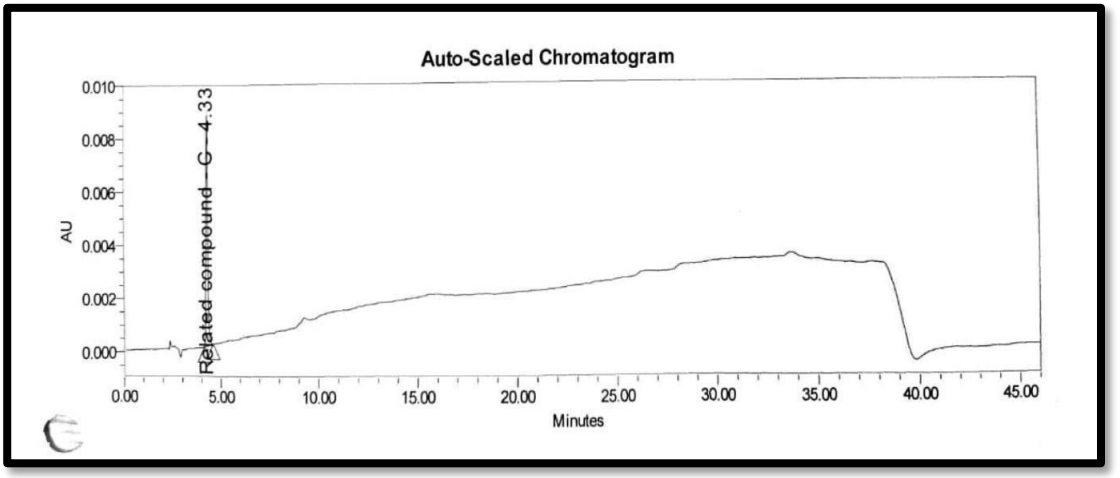

Fig 6: Chromatogram for Allopurinol Related Compound –B

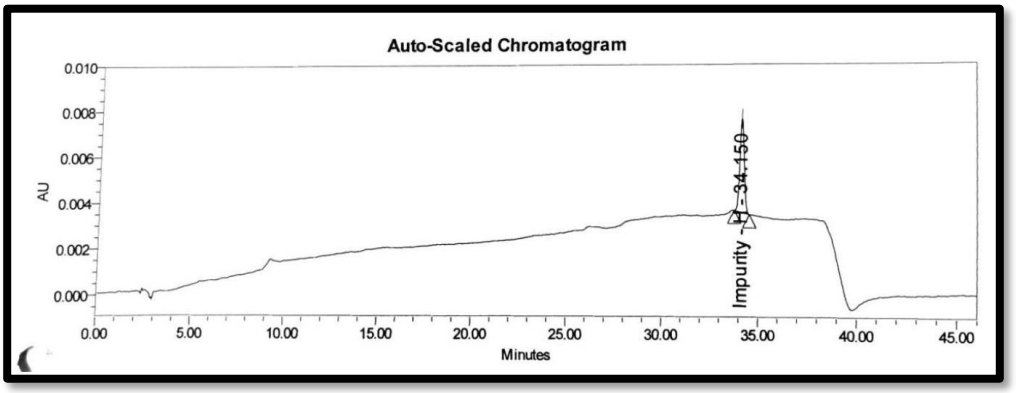

Fig 7: Chromatogram for Impurity H

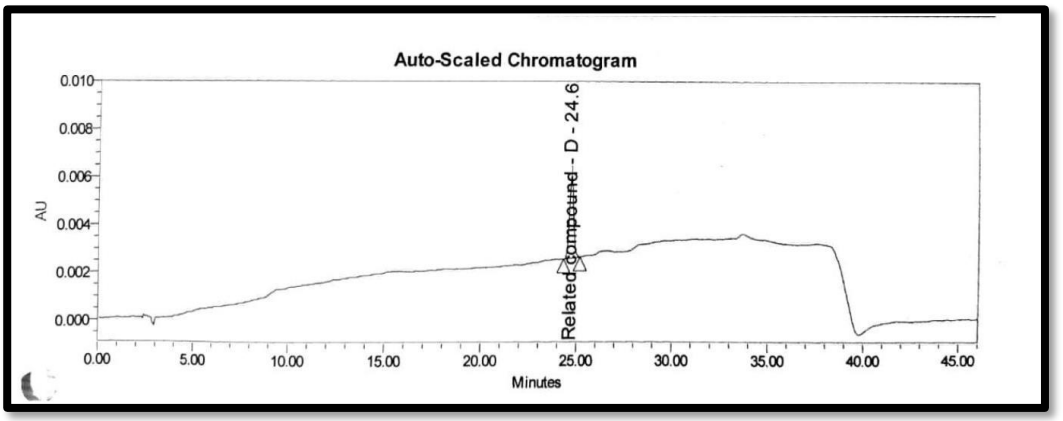

Fig 8: Chromatogram for Allopurinol Related Compound-D

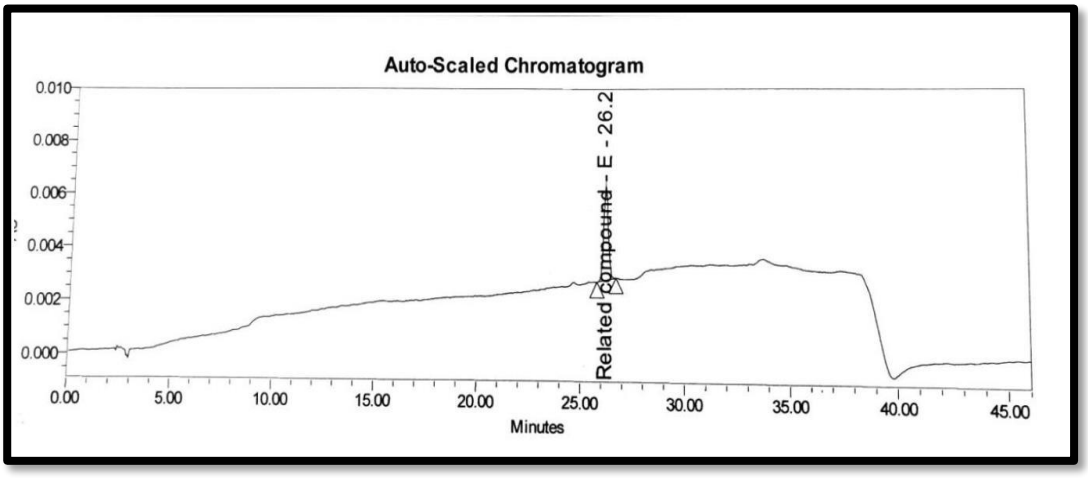

Fig 9: Chromatogram for Allopurinol Related Compound-E

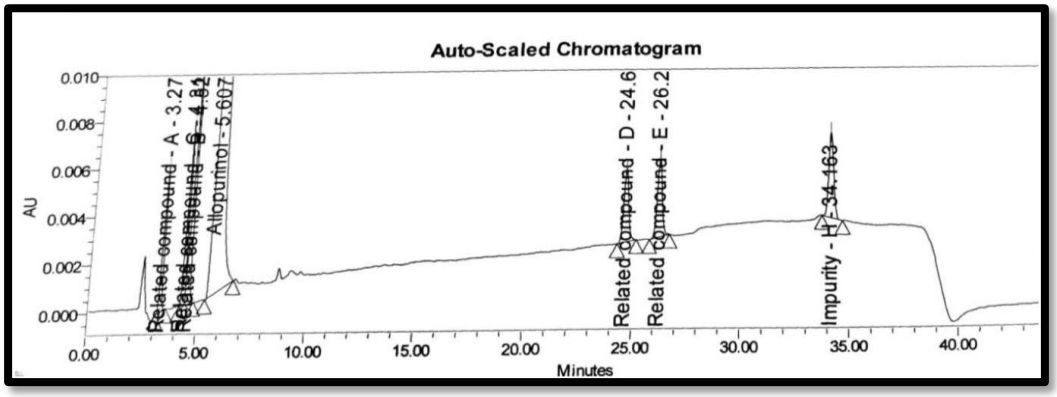

Fig 10: Chromatogram for blend solution

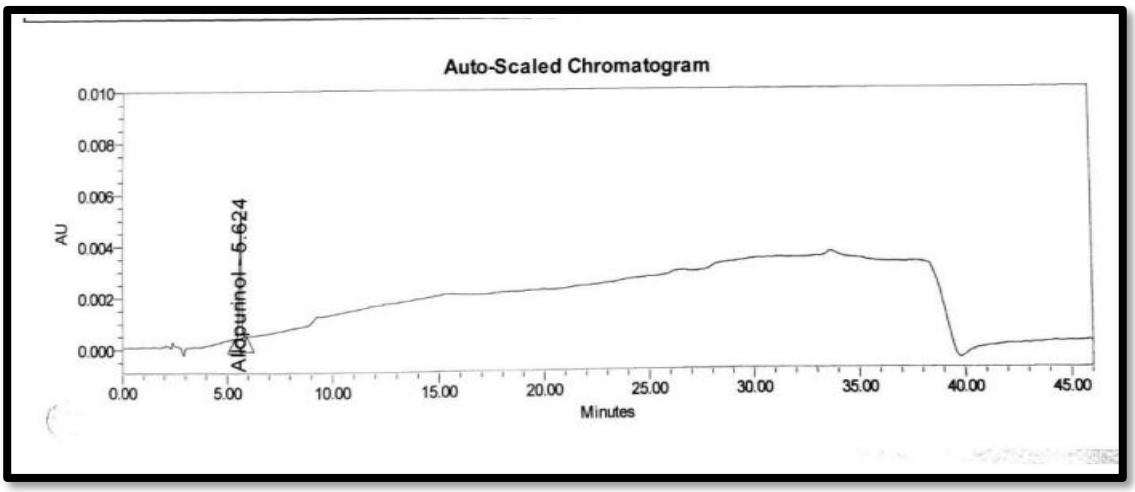

Fig 11: Chromatogram for Allopurinol

Table 5: Specificity results

Name Of the Component	Rt	From Blend Solution Rt
Related Compound -A	3.276	3.272
Related Compound -c	4.330	4.312
Related Compound -B	4.542	4.524
Allopurinol	5.624	5.607
Related Compound-D	24.690	24.664
Related Compound -E	26.690	26.236
Impurity -H	34.190	34.163

Detection limit (DL)

The detection limit is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantitated. The detection limit was determined as the lowest concentration for which the response is approximately three times greater than the base line.

Table 6: DL Results

S.no	Name of the component	DL in (%)	S/N ratio
1	Related Compound -A	0.004	3.8
2	Related Compound -c	0.004	4.3
3	Related Compound -B	0.003	4.0
4	Allopurinol	0.003	4.7
5	Related Compound-D	0.007	4.2
6	Related Compound -E	0.007	3.8
7	Impurity -H	0.007	4.6

Observation: The Signal to Noise ratio of each component was within the limit.

Quantitation limit (QL)

Table 7: QL Results

S.no	Name of the Component	QL IN (%)	S/N ratio
1	Related Compound -A	0.012	3.8
2	Related Compound -c	0.012	4.3
3	Related Compound -B	0.011	4.0
4	Allopurinol	0.010	4.7
5	Related Compound-D	0.024	4.2
6	Related Compound -E	0.020	3.8
7	Impurity -H	0.024	4.6

Observation: The Signal to Noise ratio of each component was within the limit.

Accuracy at QL

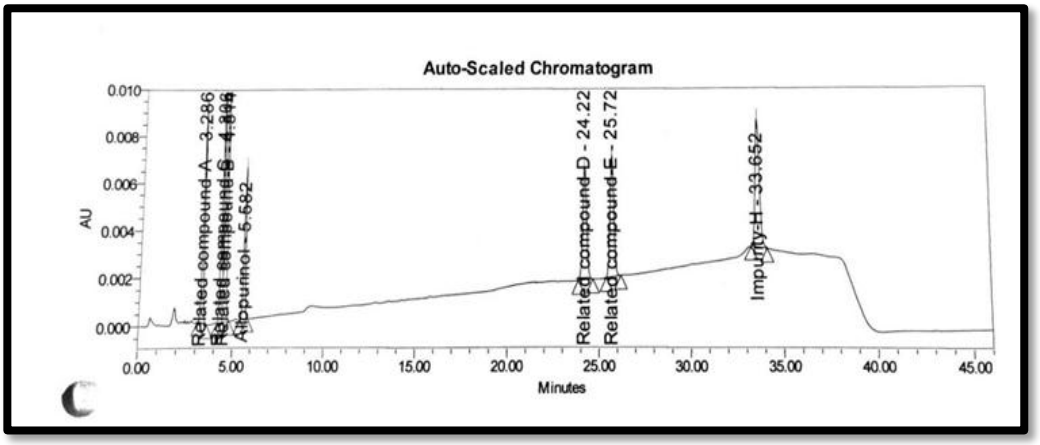

Fig 12: Chromatogram showing QL solution 1

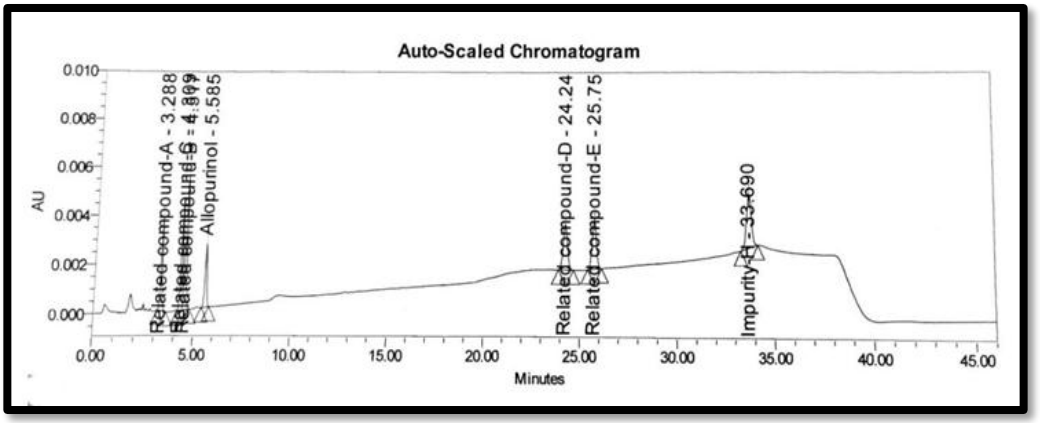

Fig 13: Chromatogram showing QL solution 2

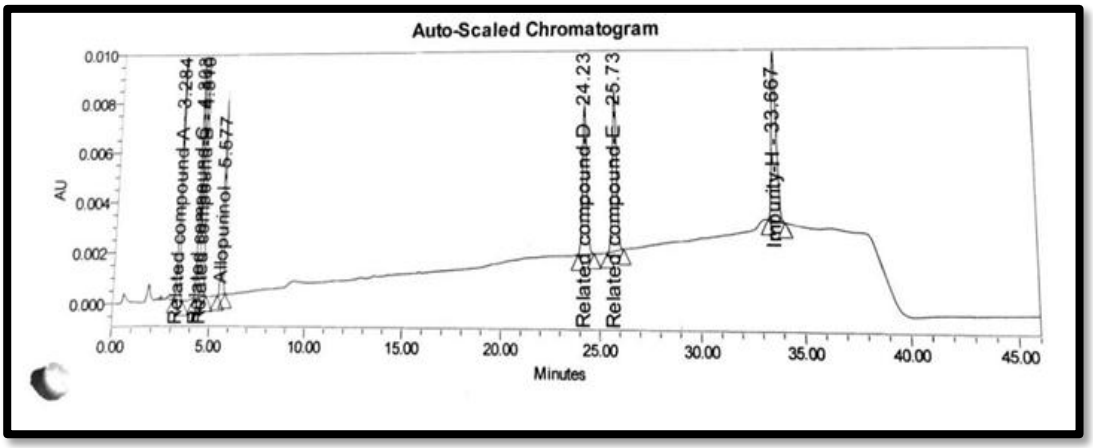

Fig 14: Chromatogram showing QL solution 3

Table :8 Accuracy at QL results

Accuracy at QL	% Impurity added	% of impurity found	% Recovery	% RSD
Allupurinol Related Compound -A	0.012	0.0414	89.3	6.48
Allupurinol Related Compound -C	0.0118	0.0400	84.7	1.95
Allupurinol Related Compound -B	0.0107	0.00983	91.9	2.32
Allupurinol Related Compound -D	0.0243	0.02186	90	0.68
Allupurinol Related Compound-E	0.0201	0.0181	90.2	2.73
Impurity	0.0236	0.0224	95.2	1.27

Linearity

Linearity study was conducted for each known impurities and Allopurinol standard in the range of QL level to 150%level.

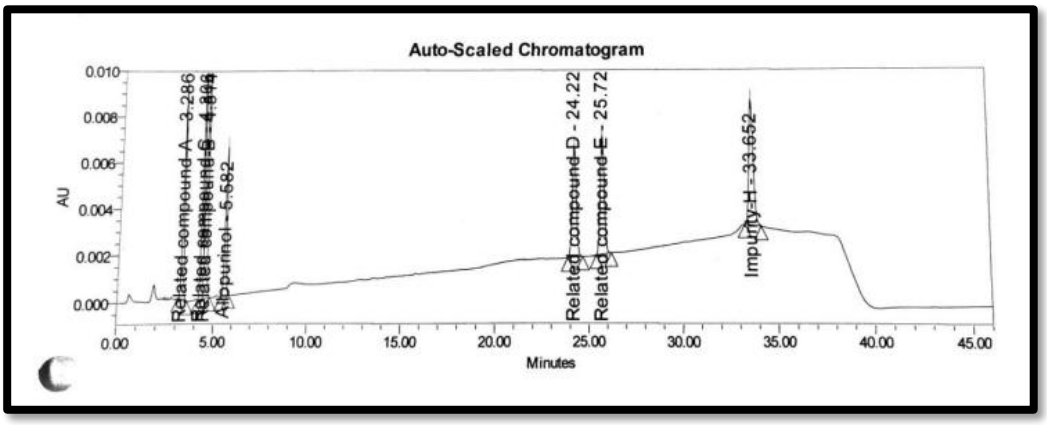

Fig 15: Chromatogram showing linearity level 1

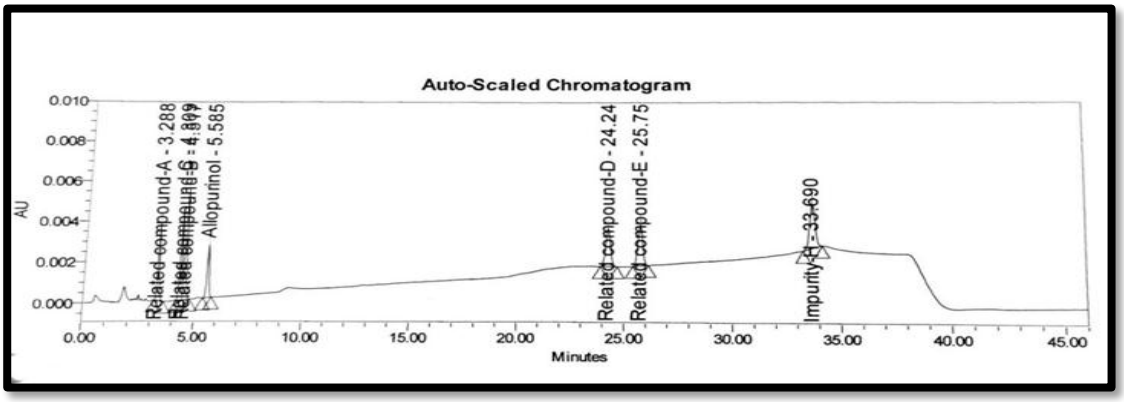

Fig 16: Chromatogram showing linearity level 2

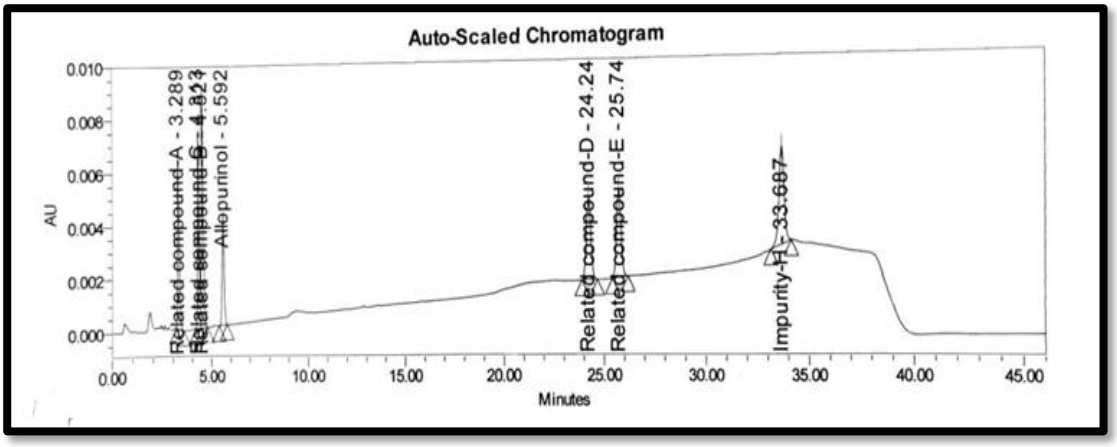

Fig 17: Chromatogram showing linearity level 3

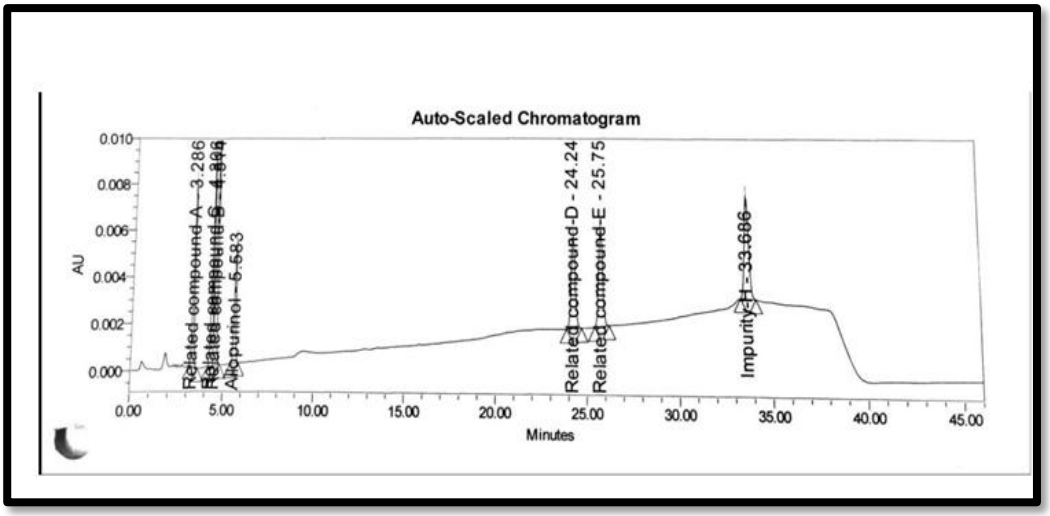

Fig 18: Chromatogram showing linearity level 4

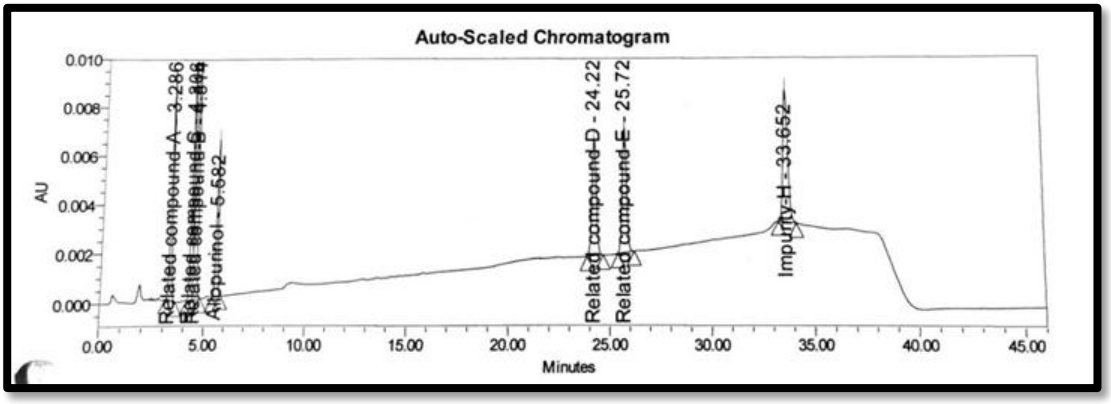

Fig 19: Chromatogram showing linearity level 5

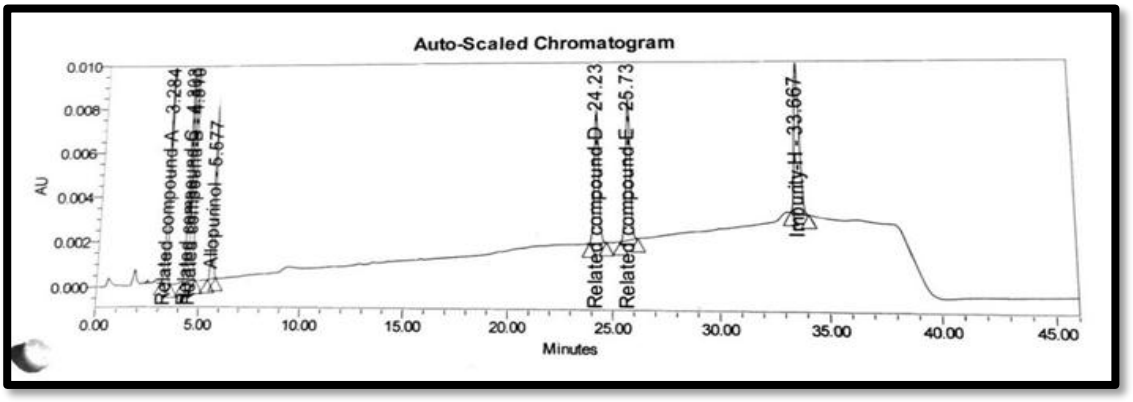

Fig 20: Chromatogram showing linearity level 6

Table: 9 Linearity for Allopurinol Related compound-A

Concentration (%)	Area
0.012	2565
0.101	22131
0.161	35830
0.202	44533
0.242	54035
0.302	66488

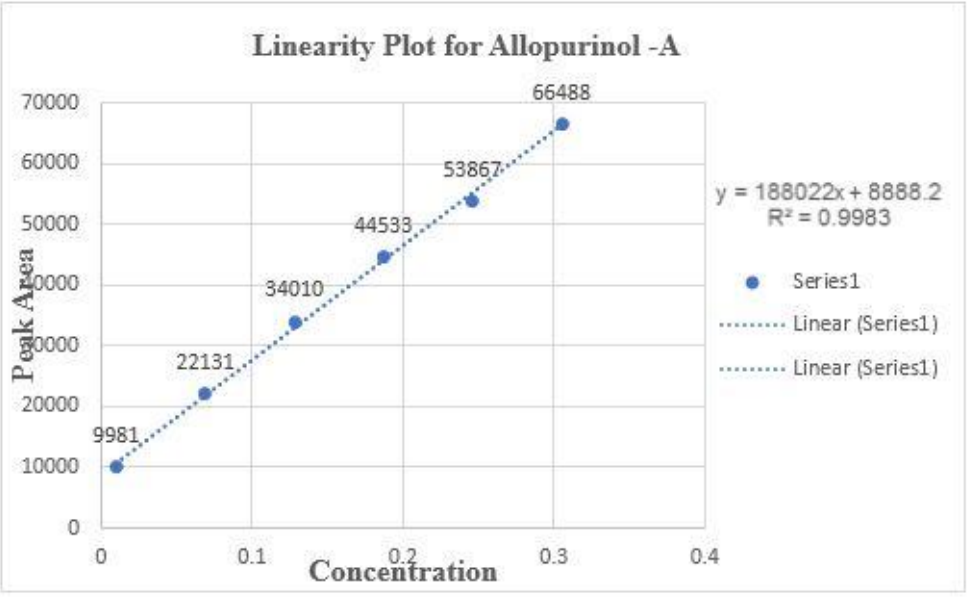

Fig 21: Linearity curve of Allopurinol RC-A

Table: 10 Linearity for Allopurinol Related compound-C

Concentration (%)	Area
0.012	3308
0.101	27845
0.161	45262
0.202	56260
0.242	67828
0.302	83443

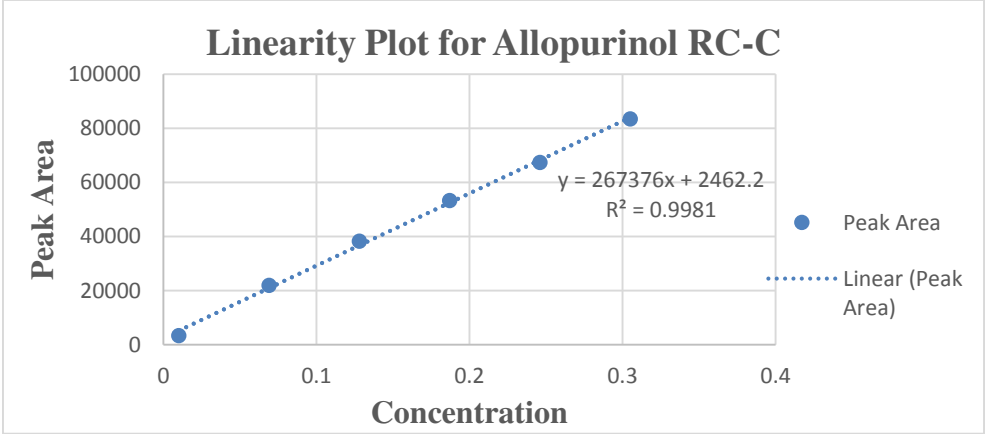

Fig 22 : Linearity curve of Allopurinol RC-C

Table :11 Linearity for Allopurinol Related compound-B

Concentration (%)	Area
0.011	3576
0.101	39953
0.161	64763
0.201	80828
0.242	97659
0.302	119897

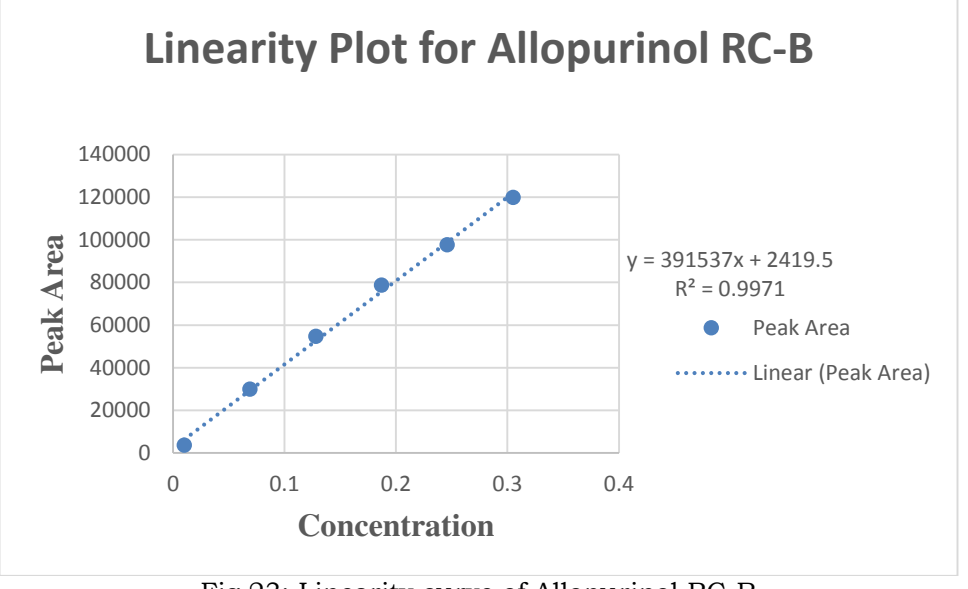

Fig 23: Linearity curve of Allopurinol RC-B

Table :12 Linearity for Allopurinol

Concentration (%)	Area
0.010	3808
0.050	18158
0.080	29338
0.100	36414
0.120	44023
0.150	54239

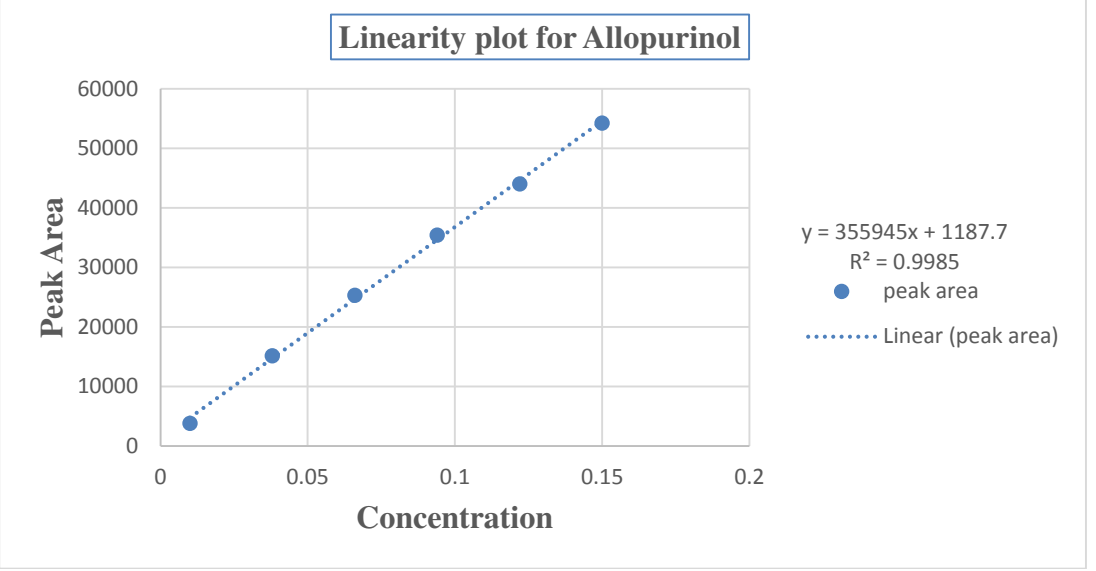

Fig 24: Linearity curve of Allopurinol

Table: 13 Linearity for Allopurinol Related compound-D

Concentration (%)	Area
0.024	7336
0.099	29607
0.159	48023
0.198	60228
0.238	72214
0.298	89029

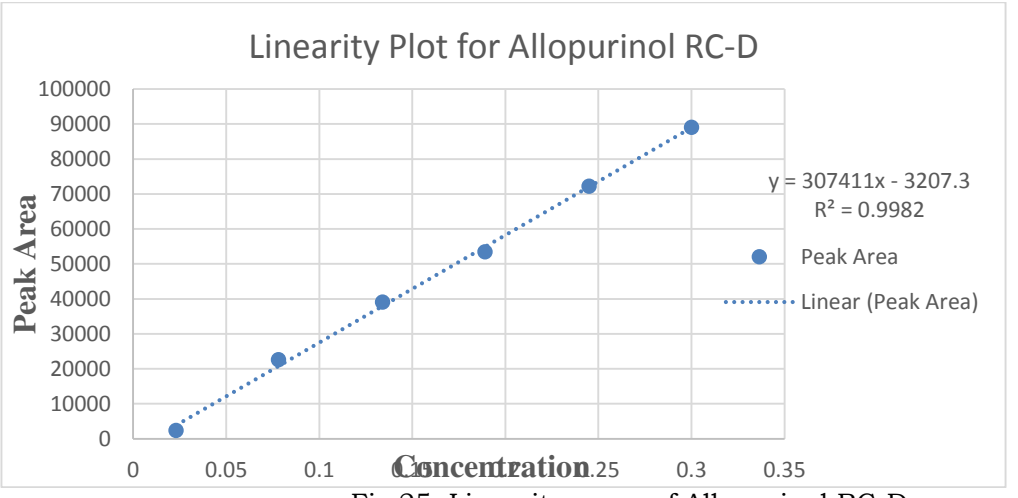

Fig 25: Linearity curve of Allopurinol RC-D

Table: 14 Linearity for Allopurinol Related compound-E

Concentration (%)	Area
0.020	6421
0.100	31353
0.160	50594
0.200	63436
0.240	76260
0.301	94397

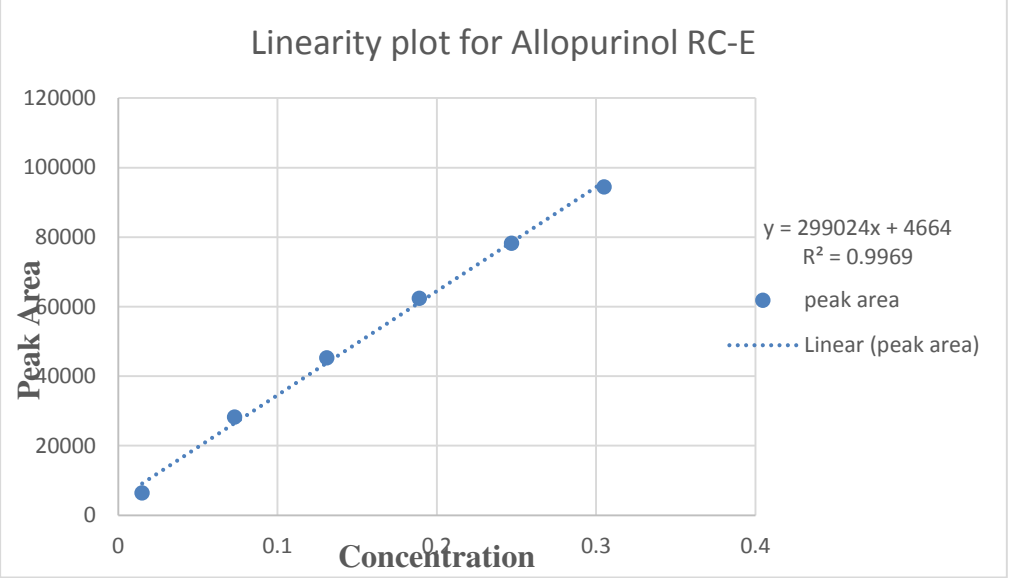

Fig 26: Linearity curve of Allopurinol RC-E

Table: 15 Linearity for Allopurinol Impurity-H

Concentration (%)	Area
0.024	8523
0.100	39788
0.160	60961
0.200	71578
0.240	86663
0.300	106736

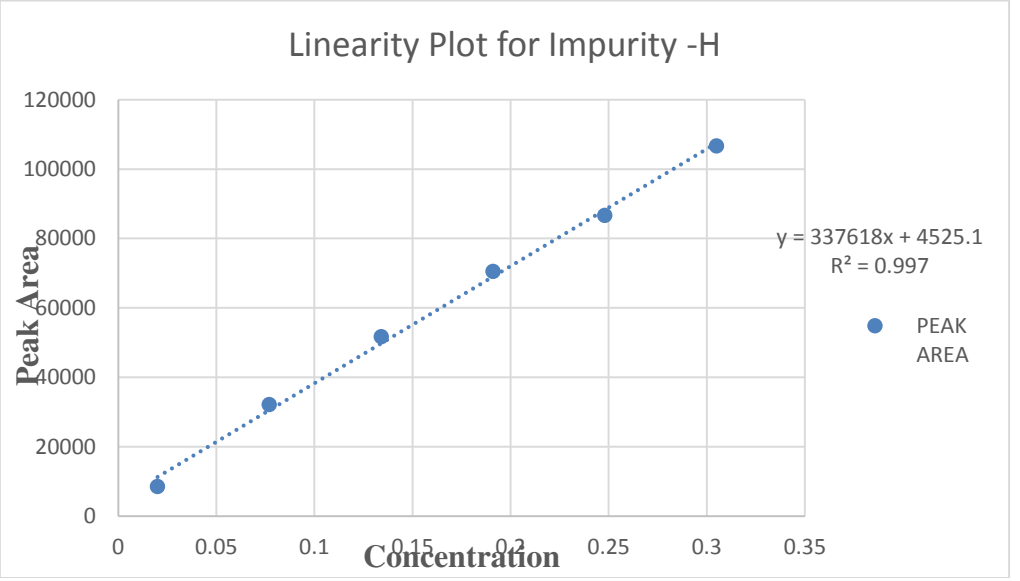

Fig 27: Linearity curve of Allopurinol Impurity-H

Precision

Table: 16 Precision results

S.no	Name of the component	Results in (%)	%RSD
1	Related Compound -A	0.199	0.66
2	Related Compound -c	0.193	0.21
3	Related Compound -B	0.206	0.19
4	Related Compound-D	0.194	0.52
5	Related Compound -E	0.194	0.52
6	Impurity -H	0.194	0.77

Accuracy

Accuracy of the method was proved by checking the % recovery of each impurity in test solution spiked with each impurity at 50%,100%,150% level. Each level solution wasprepared in triplicates and % recovery of each impurity was reported.

Table : 17 % of recoveries of all impurities of Allopurinol

Name of the Impurity	Level	% Impurity added	% Impurity found	% Recovery	%RSD
Allopurinol Related Compounds-A	50%	0.1006	0.0967	96.1	2.89
	100%	0.2012	0.1949	96.9	0.18
	150%	0.3019	0.2899	96.0	0.06
Allopurinol Related Compounds-C	50%	0.099	0.6755	96.0	0.22
	100%	0.1998	0.1942	97.2	0.21
	150%	0.2996	0.2890	96.5	0.10

Allopurinol Related Compounds-B	50%	0.1007	0.0981	97.4	0.16
	100%	0.2014	0.1933	96	0.10
	150%	0.3022	0.2868	94.9	0.11
Allopurinol Related Compounds-D	50%	0.0997	0.0983	98.3	0.42
	100%	0.1994	0.1946	97.6	0.67
	150%	0.2990	0.2875	96.4	0.33
Allopurinol Related Compounds-E	50%	0.1007	0.0972	96	0.47
	100%	0.2014	0.1972	96.3	0.52
	150%	0.3021	0.2877	95	0.34
Impurity -H	50%	0.1007	0.0978	97.5	0.21
	100%	0.2003	0.1935	97	0.86
	150%	0.3004	0.2875	97.5	0.39

Robustness
Mobile Phase Stability

Table: 18 Robustness results

S.no	Name of the compound	Results in %	% RSD
1	Allopurinol Related compound -A	0.195	0
2	Allopurinol Related compound -C	0.197	0.30
3	Allopurinol Related compound -B	0.224	0.27
4	Allopurinol Related compound -D	0.200	0.31
5	Allopurinol Related compound -E	0.194	0.29
6	Impurity-H	0.205	0.46

Observation

The % RSD results Obtained from mobile phase stability study were within limit.

Conclusion

A New method was established for the estimation of Allopurinol Related substances by RP-HPLC method. The Chromatographic conditions were successfully developed for the estimation of Allopurinol by using Kromasil 100-5, C18,250x4.6mm 5 µm particle size column, flow rate was 1 mL/min, mobile phase ratio was potassium dihydrogen orthophosphate: Methanol, detection wavelength was 220 nm. The instrument used was Agilent 1200 series, separation module, Empower software version-3. The Retention time was found to be 35 minutes. The system suitability parameters for Allopurinol such as theoretical plates and tailing factor were found to be within the limits. The analytical method was validated according to the ICH guide The Linearity study for Allopurinol was found in the concentration range was within the limits and Correlation coefficient of the impurities was found to be 0.9999, 0.9998, 0.9999, 0.9999, 0.9999 and 0.9985, respectively. % RSD for repeatability was 0.19. The precision study was precise and repeatable. Accuracy of the components was found to be within the limits of 85-115%. DL and QL of the signal to noise ratio are within the limits. Hence the suggested RP- HPLC method can be used for the

routine Analysis of Allopurinol of related substances in the pharmaceutical dosage forms.

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