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Development Of A Stability Indicating RP-RRLC Method For Determination Of Allopurinol And Its Degradation Products In Solid Oral Dosage

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Abstract: A simple, sensitive and reproducible reversed phase rapid resolution liquid chromatography (RP-RRLC) coupled with a photodiode array detector method was developed and validated for determination of Allopurinol and its related substances in pharmaceutical dosage forms. The chromatographic separation was achieved on Zorbax SB C8 (1.8µm, 4.6mm X 50mm) column using gradient elution of potassium dihydrogen phosphate buffer (pH 2.50, 0.025M) and methanol at flow rate of 1.0 ml/min. UV detection was performed on 230 nm. Total run time was 10 min within which main compound and other known and unknown impurities were separated. Stability indicating capability was established by force degradation experiments and separation of known degradation products. The method was validated for accuracy, repeatability, reproducibility and robustness. Linearity, LOQ and LOD were established for Allopurinol and its known impurities.
Key words: Allopurinol, impurities, RRLC, stability indicating method.

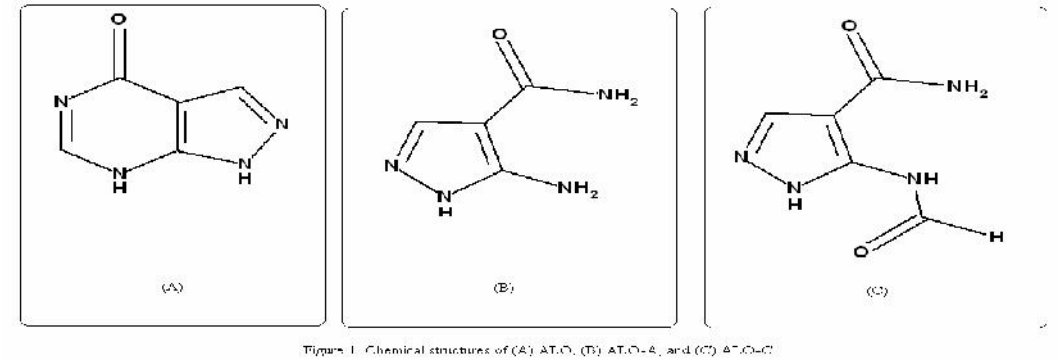
1. INTRODUCTION

Allopurinol (ALO) is a 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 purine analog; it is a structural isomer of hypoxanthine (a naturally occurring purine in the body) and is an inhibitor of the enzyme xanthine oxidase. 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 amidophos phoribosyl 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 ALO is given in figure 1(A).

ALO is an official drug in USP, EP, BP, and IP. Several analytical methods such as spectrophotometric [4-8], capillary zone electrophoresis [9], polarography [10-12], second derivative oscillopolarography [13], and room temperature phosphorescence [14] methods are reported for estimation of ALO in bulk drug, formulations and in biological matrices. Literature survey reveals that some HPLC methods are reported for estimation of ALO in its biological samples [15-22]. Estimation of ALO in pharmaceutical dosage form by HPLC methods are also reported [23]. To the best of our knowledge, none of the currently available analytical methods can separate all the known related compounds

Figure-1: Chemical structures of (A) ALO, (B) ALO-A, and (C) ALO-C



and degradation impurities in ALO dosage forms. Furthermore, there is no stability-indicating HPLC/RRLC method is reported in the literature for the determination of ALO and its impurities in solid oral dosage form. It is, therefore, felt necessary to develop a new rapid, stability-indicating method for the determination of assay and impurities in ALO solid oral dosage form. Two known degradation products Allopurinol impurity A (ALO-A), and Allopurinol impurity B (ALO-B) are reported in pharmacopoeia. Chemically ALO-A is 3-amino-4-carboxamidopyrazole hemisulfate, and ALO-B is 5-(formylamino)-1H-pyrazole-4-carboxamide.
Chemical structures of ALO-A and ALO-B are given in figure 1(B), and 1(C) respectively.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Reference standard of ALO was kindly gifted by Torrent Research Center, Gandhinagar, India with purity of 99.12%. USP reference standards of ALO-A and ALO-B were also gifted from them.

Gradient grade acetonitrile and methanol (Rankem, India), orthophosphoric acid, (Rankem, India), potassium dihydrogen phosphate (Rankem, India), are also gifted by Torrent Research Center. The nylon filters with pour size of 0.22µm (Waters, Milford, USA) were used to filter sample preparation.

Tablet formulation containing 10 mg of ALO and placebo were also gifted by Torrent Research Center.

2.2 Buffer preparation

Solution of phosphate buffer (0.025M) was prepared by dissolving 3.4 gm of potassium dihydrogen phosphate in one liter of water for HPLC. The pH of this solution was adjusted to 2.50 with orthophosphoric acid. The buffer preparation was found stable with respect to pH and visual clarity for about 70 hours.

2.3 Chromatographic system

Analyses were performed on 1200 SL system (Agilent, USA), consisting of binary solvent manager, auto sampler manager, PDA detector. The output signal was monitored and processed by Chemstation software. The detection was set at a sampling rate of 40 points/s. The separation of ALO, its known impurities and its degradation products was achieved on column Zorbax SB C8 50mm x 4.6mm, 1.8µm particle size Agilent, USA). The finally selected and optimized conditions were as follows: injection volume 5 µl, gradient elution (as shown in table 1), at a flow rate of 1.0 ml/min at 25.0°C column oven temperature, detection wavelength 230 nm.

2.4 Standard solution preparation

Standard solution was prepared for assay by dissolving standard substance in diluent (mixture of buffer solution 80% and acetonitrile 20%) to obtain solution containing 100µg/ml of ALO.

Standard solution was prepared for related impurities by dissolving standard substance in diluent to obtain solution containing 2µg/ml of ALO.

2.5 Sample solution preparation

Ten tablets were weighed and crushed to the fine powder. An accurately weighed portion of the powder equivalent to 100 mg of ALO was taken in 100 ml volumetric flask. Ten milliliter of 0.1N sodium hydroxide solution was added to this flask and sonicated with shaking in an ultrasonic bath for 2 minutes. Further about 60 ml of diluent was added to the flask and sonicated with shaking for 10 minutes. This solution was diluted to the mark with diluent and mixed. It was filtered through 0.22µm PVDF filter paper discarding first 3 ml of the filtrate. This filtrate was used for the estimation of

impurities. For the estimation of ALO, 10 ml of this filtrate was diluted to 100 ml with diluent.

2.6 Method validation

2.6.1 System suitability

System suitability parameters were measured so as to verify the system performance. System precision was determined on five replicate injections of standard preparation. All important characteristics including relative standard deviation (% RSD), theoretical plate number and asymmetry were measured.

2.6.2 Specificity

Forced degradation studies were performed to demonstrate selectivity and stability indicating capability of the proposed method. The powdered sample of tablets was exposed to acidic (5N HCl, 100°C, 2 hours), alkaline (5N NaOH, 100°C, 90 min), strong oxidizing (10% H_2O_2, 100°C, 3 hours), thermal (100°C, 6 Hours), and photolytic (1.2 million lux hours) degradation conditions. Also, standard of ALO was exposed to above stress conditions, to identify source of degradation peaks. All the exposed tablet samples and standards were then analyzed by the proposed method.

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

The LOD and LOQ of ALO and its impurities were determined by using signal to noise approach as defined in International Conference on Harmonization (ICH) guideline. Increasingly dilute solution of drug and each impurity was injected into the chromatograph and signal to noise (S/N) ratio was calculated at each concentration.

2.6.4 Linearity

Linearity was determined over the range from 25% to 150% of specification concentration (0.2%) using six calibration levels (25%, 50%, 75%, 100%, 125%, and 150%) for ALO, ALO-A an for ALO-B. In order to study linearity of response, a series of working standard solutions (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 µg ml^{-1} for ALO, ALO-A and for ALO-B) were prepared. The linearity of peak area responses versus concentration was studied. The correlation graph was constructed by plotting the peak areas obtained at optimized conditions.

For assay method, linearity was demonstrated from 50% to 150% of sample concentration using seven calibration levels (50%, 60%, 70%, 90%, 100%, 110% and 150%) for ALO. Each linearity solution was injected in duplicate. The mean area at each level was determined and a graph of mean area versus concentration was plotted.

2.6.5 Precision

Precision was investigated using sample preparation procedure for six real samples of tablets and analyzed by proposed methods. Intermediate precision was studied using different column, and performing the analysis on different day.

2.6.6. Accuracy

To confirm the accuracy of the proposed methods, recovery experiments were carried out by standard addition technique. The accuracy of the method for ALO-A, ALO-B and for unknown impurity was evaluated in triplicate at four different concentration levels, LOQ, 50%, 100%and 150% of the target specification concentration for all compounds. The mean of percentage recoveries (n = 9) and the relative standard deviation was calculated.

The accuracy of the method for ALO was evaluated in triplicate at three different levels, 50%, 100%, and 150% with respect to sample concentration of ALO. The samples were prepared by mixing known amount of ALO standard with placebo powder. All preparations were injected in duplicate and % recovery was evaluated at each level.

2.6.7. Robustness

The robustness as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions was studied by testing influence of small changes in pH of buffer (±0.2 units), change in column temperature (25°C+5) and change in flow rate (1 ml±5%).

2.6.8. Stability of sample preparation

Stability of sample solution was established by storage of sample solution at ambient temperature for 48 hours. Sample solutions were re-analyzed after 24 hours, after 48 hours and ALO and impurities were determined and compared against fresh samples.

Table-1: Gradient Program for elution of ALO and impurities

Time (min)	% A, phosphate buffer (0.025M, pH 2.5)	% B, Methanol
0.0	100	0
6.0	60	40
7.0	100	0
10.0	100	0

3. RESULTS AND DISCUSSION

3.1. Method development and optimization

For successful method validation, preliminary tests were performed with the objective to select adequate and optimum condition. Parameters, such as choice of analytical column, pH of buffer, mobile phase composition and proportion, detection wavelength and other factors were exhaustively studied. Quantification was achieved with PDA detection at 230nm based on the peak area. Experiments were performed on isocratic mobile phase system, with methanol. To develop a stability indicating method, first the retention behavior of these three compounds with change in percentage of methanol and with change in pH of buffer was studied on Zorbax SB C8 column (1.8µm, 4.6mm X 50mm) column. While assessing the effect of change of proportion of methanol in mobile phase, the pH of buffer was set to 2.5 and while assessing the effect of pH of buffer, the mobile phase composition was buffer-methanol (95:5, v/v). As Zorbax SB column is best suitable for pH less than 3.0, all studies were done on Zorbax SB C8 (50 X 4.6), 1.8µ column. ALO-A was relatively retained for shorter time and well separated from all other compounds. ALO-B and ALO were found co-eluting. Thus, the critical separation to achieve was between ALO-B, and ALO. Different experiments indicate that separation between ALO-B, and ALO enhances with decrease in percentage of methanol in mobile phase also the retention of all compounds are not pH dependant To achieve successful separation between ALO-B and ALO, and to ensure that all possible non-polar degradation products are eluted, it was decided to use a gradient run. ALO, its impurities and all degradation products were all resolved in reasonable time of 10 minutes.

3.2 Analytical parameters and validation

After satisfactory development of method, it was subjected to method validation which was covering all criteria of ICH guideline [24]. The method was validated to demonstrate that it is suitable for its intended purpose by the standard test procedure to evaluate adequate validation characteristics.

3.2.1 System suitability

The percentage R.S.D. of area count of five replicate injections was below 2.0%. Low values of R.S.D. of replicate injections indicate that the system and chromatography are precise. Results of other system suitability parameters such as asymmetry and theoretical plates are presented in **table 2.** As seen from that data, the acceptable system suitability parameters would be: relative standard deviation of replicate injections is not more than 2.0%, tailing factor of peak of ALO is not more than 2.0 and theoretical plates for ALO peak is not less than 5000.

3.2.2 Specificity

The results of force degradation study are given in **table 3.** ALO was found very sensitive to acid hydrolysis. The assay value of ALO was dropped to 85.2%. The major degradation product of ALO in acid hydrolysis was ALO-A, and an unknown impurity with area of 8.1% and 5.4% respectively. In base hydrolysis, the assay value of ALO was dropped to 86.8%. The major degradation product of base hydrolysis was ALO-A with area of 10.2%. ALO was found sensitive in oxidation degradation also. The major degradation product of oxidation degradation of ALO was ALO-A, ALO-B and an unknown with area of 8.08%, 4.03% and 1.1% respectively. ALO was found stable in thermal degradation and photolytic degradation. Chromatographs of acid, base and oxidation degraded samples are presented in **figure 2, figure 3 and figure 4 respectively. Chromatograph of as such sample preparation is presented in figure 5.** All major degradation peaks were investigated for their spectral purity, all peaks were found spectrally pure. Also spectra of known impurities in degraded tablet samples were similar to its respective impurity standard substances, indicating that there was no co-elution of unknown degradation peak at retention time of respective known impurities.

Table-2: System suitability and robustness results

System suitability parameters	Robustness parameters	ALO (assay method)	ALO (related substance method)
%RSD	No change	0.32%	0.51%
	Flow rate (0.9ml/min)	0.10%	0.64%
	Flow rate (1.1ml/min)	0.13%	0.32%
	Column 1	0.32%	0.65%
	Column 2	0.42%	0.44%
	Column oven temperature 20.0°C	0.78%	0.81%
	Column oven temperature 30.0°C	0.06%	0.68%
Column efficiency	No change	11529	12529
	Flow rate (0.9ml/min)	17428	17168
	Flow rate (1.1ml/min)	14438	13967
	Column 1	15816	14163
	Column 2	13254	16124
	Column oven temperature 20.0°C	11284	11496
	Column oven temperature 30.0°C	14245	13863
Asymmetry	No change	1.02	0.98
	Flow rate (0.9ml/min)	1.1	1.27
	Flow rate (1.1ml/min)	0.97	1.23
	Column 1	1.02	1.27
	Column 2	1.06	1.18
	Column oven temperature 20.0°C	1.02	0.99
	Column oven temperature 30.0°C	1.1	1.20

Table-3: Force degradation study data

Degradation condition	% Area			
	ALO impurity A	ALO impurity B	Major unknown degradant	Total degradation
No degradation (controlled sample)	0.01%	0.04%	---	0.05%
Acid hydrolysis (5 N HCl, 100°C, 2 hours)	8.2%	0.05%	0.64%	14.7%
Base hydrolysis (5 N HCl, 100°C, 2 hours)	10.21%	0.19%	0.10%	13.1%
Oxidation degradation (5 N HCl, 100°C, 2 hours)	8.09	4.04	1.10%	13.8%
Thermal degradation (100°C, 6 hours)	0.01%	0.07%	0.01%	0.09%
Photolytic degradation (1.2 Million lux hours)	0.01%	0.03%	0.01%	0.05%

Figure-2: Chromatograph of acid degraded tablet sample. ALO-A and major degradant are main degradation product of ALO

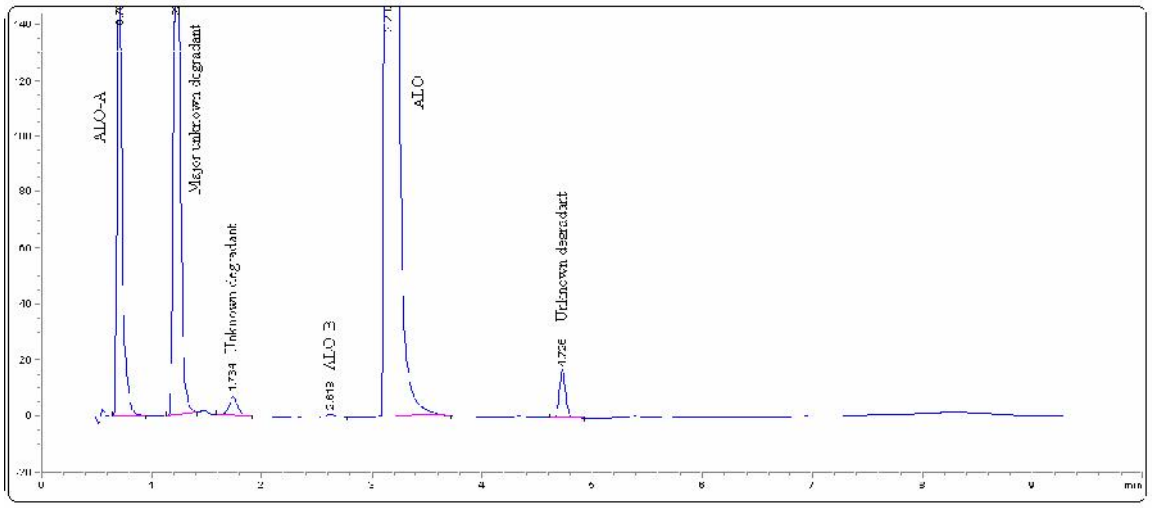


Figure 2. Chromatograph of acid degraded tablet sample. ALO-A and major unknown degradant are main degradation product of ALO

Figure-3: Chromatograph of base degraded sample. ALO-A as major degradation product of ALO

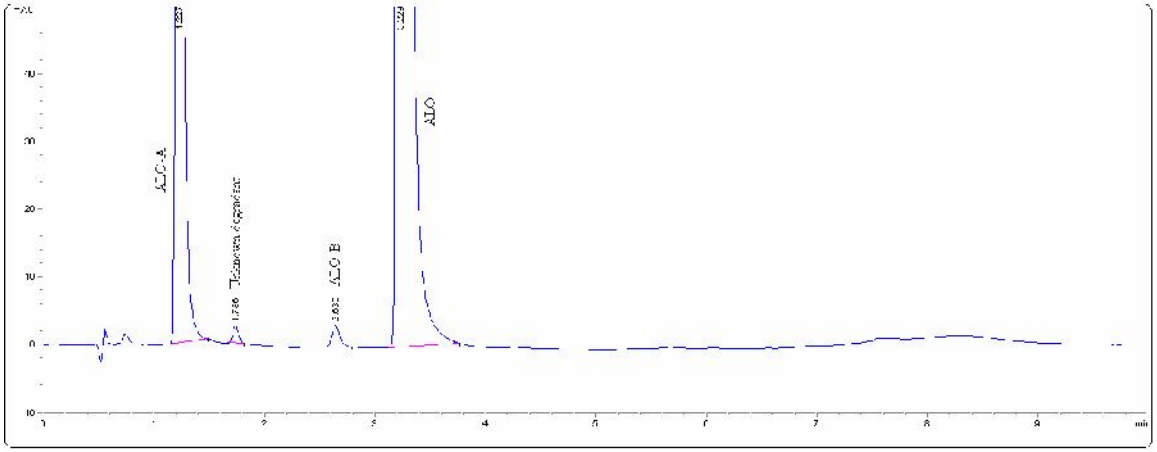


Figure 3. Chromatograph of base degraded tablet sample. ALO-A is major degradation product of ALO.

Figure-4: Chromatograph of oxidation degraded tablet sample. ALO-A and ALO-B are major degradation product of ALO

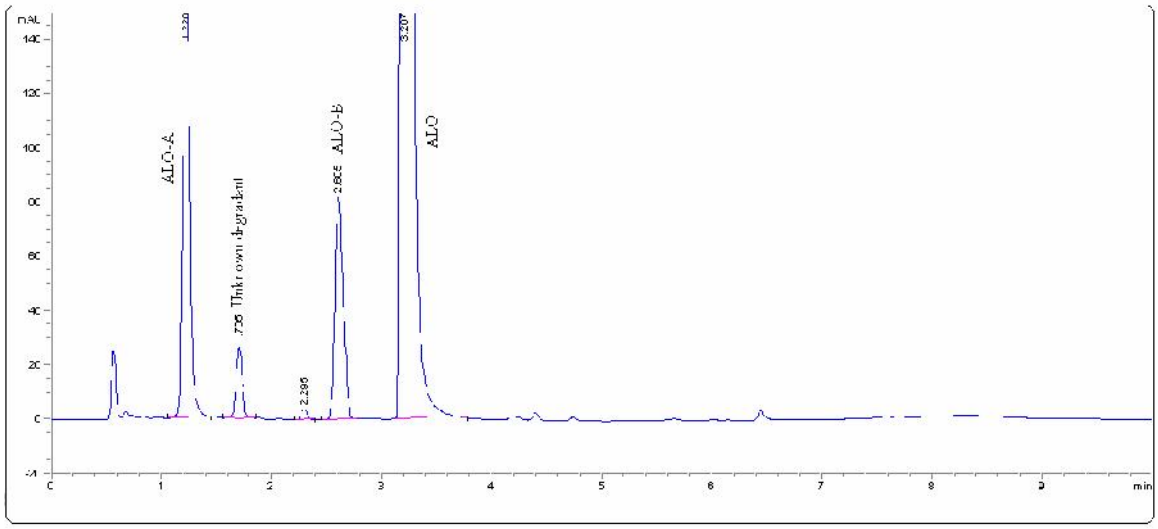


Figure 4. Chromatograph of oxidation degraded tablet sample. ALO-A and ALO-B are major degradation product of ALO

Figure-5: Chromatograph of as such sample preparation

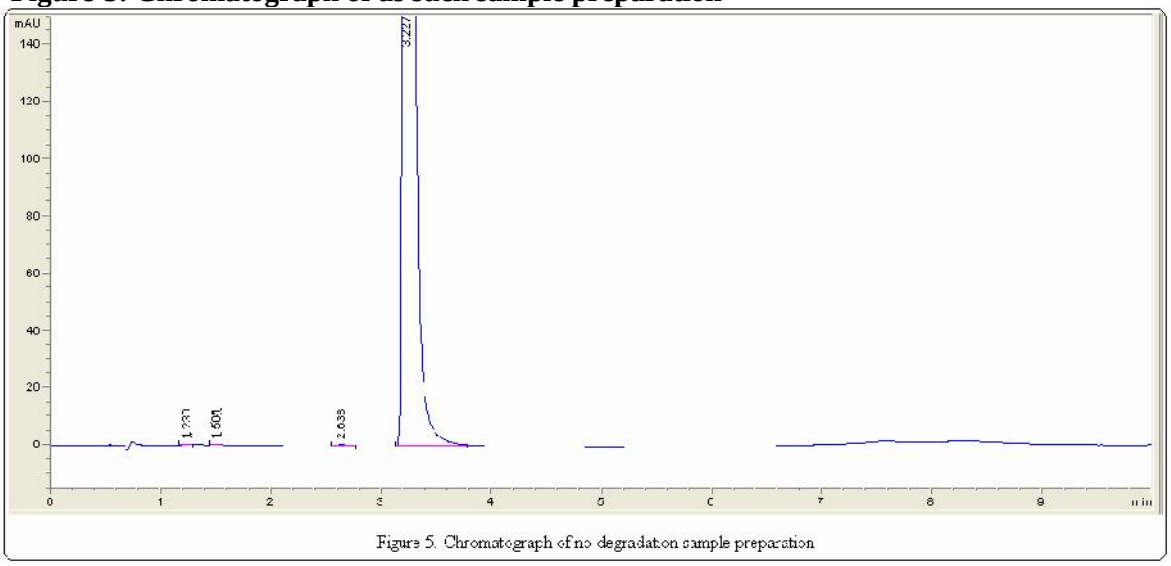


Figure 5. Chromatograph of no degradation sample preparation

3.2.3 LOD and LOQ

The concentration (in µg/ml) with signal to noise ratio of at lease 3 was taken as LOD and concentration with signal to noise ratio of at lease 10 was taken as LOQ, which meets the criteria defined by ICH guidance. The LOD and LOQ results of ALO and impurities are presented in table 4.

3.2.4 Linearity

The response was found linear from 25% to 150% of specification concentration limit. For all components coefficient was greater than 0.999. Linearity results of main compound and of impurities are presented in table 4.

For assay method, linearity was found linear from 50% to 150% of sample concentration. Linearity results for assay of ALO are presented in table 5.

3.2.5 Precision

The average % assay (n=6) of ALO was 99.8% with R.S.D. of 0.16%. Results are shown in table 5.

Impurities of ALO were not detected in samples hence, standard addition technique was used and impurities ALO-A and ALO-B were spiked to their specification concentration level. The average of ALO-A and ALO-B in six samples were 0.19% and 0.20% respectively. The R.S.D. value of ALO-A and ALO-B of six samples were found 2.72% and 0.00% respectively. The result of precision study for ALO-A and ALO-B are shown in table 6.

3.2.6 Accuracy

The amount recovered was within of amount added, which indicated that the methods are accurate. The results of recoveries for assay are shown in table 7 and for impurities are shown in table 8.

3.2.7 Robustness

No significant effect was observed on system suitability parameters such as asymmetry, theoretical plates, R.S.D. of interested compounds, when small but deliberate changes were made to chromatographic conditions. The results are presented in table 2 along with system suitability parameters of normal methodology. Thus, the methods were found to be robust with respect to variability in above conditions.

3.2.8 Stability of sample solution

Sample solution did not show any appreciable change in assay value and in impurities value when stored at ambient temperature. Stability data of sample solution are shown in table 9.

Table-4: LOD, LOQ and linearity results for impurities

Parameters	ALO-A	ALO-B	ALO
Linearity range(µg/ml)	0.52-3.12	0.495-2.97	0.500-3.00
Correlation coefficient (r)	0.9999	0.9995	0.9999
Slope of regression line	105.9542	179.2768	101.0194
Y-intercept	-0.4739	0.2758	-0.0391
LOD (µg/ml)[a]	0.1	0.2	0.1
Signal to noise ration at LOD[b]	4.6	4.0	3.1
LOQ (µg/ml)[a]	0.3	0.5	0.3
Signal to noise ration at LOD[c]	14.8	11.2	10.8
%RSD at LOQ[d]	1.05	1.17	1.34

[a]Based on signal to noise (S/N) ratio.
[b]Acceptance criteria, S/N > 3.
[c]Acceptance criteria, S/N > 10.
[c]Determined on six values.

Tablet-5: Precision and linearity results for assay method of ALO

Parameters	ALO
Method precision[a] (% assay)	99.8%
Method precision[b] (%RSD)	0.16%
Intermediate precision[a] (% assay)	99.1%
Intermediate precision[b] (%RSD)	0.21%
Linearity and range (%)	50% to 150%
Correlation coefficient (r)	1.000
Slope of regression line	10.0085
Y-intercept	-1.2465

[a]Average of six determinations.
[b]Determined on six values.

Tablet-6: Precision results for impurities

Parameters	ALO-A	ALO-B
Repeatability[a] (% impurity)	0.19%	0.20%
Repeatability[b] (%RSD)	2.72%	0.00%
Intermediate precision[a] (% impurity)	0.20%	0.20%
Intermediate precision[b] (%RSD)	0.00%	0.00%

[a]Average of six determinations.
[b]Determined on six values.

Table-7: Accuracy results for assay of ALO

Accuracy level[a]	ALO	
	% Mean recovery[b]	**% RSD[c]**
50%	100.0	0.2
100%	100.0	0.1
150%	100.4	0.1

[a]with respect to sample concentration.
[b]Average of three values.
[c]Determined on three values.

Table-8: Accuracy results for ALO-A, ALO-B and ALO

Accuracy level[a]	ALO-A		ALO-B		ALO	
	% Mean recovery[b]	% RSD[c]	% Mean recovery[b]	% RSD[c]	% Mean recovery[b]	% RSD[c]
LOQ	90.6	10.6	98.1	3.1	101.3	1.1
50%	96.2	0.6	91.4	0.7	99.7	0.5
100%	92.9	0.1	91.6	0.9	100.5	0.7
150%	91.9	0.3	90.7	0.8	99.9	0.6

[a]with respect to specification limit.
[b]Average of three values.
[c]Determined on three values.

Table-9: Stability data of sample solution of impurities and ALO

Parameter	Time interval		
	Initial	**24 hours**	**48 hours**
% ALO-A	0.19%	0.20%	0.20%
Absolute difference from initial value	-	0.01	0.01
% Difference	-	5.3	5.3
% ALO-B	0.20%	0.20%	0.20%
Absolute difference from initial value	-	0.00	0.00
% Difference	-	0.00	0.00
% Single maximum unknown impurity	Below LOQ	Below LOQ	Below LOQ
Absolute difference from initial value	-	-	-
% Difference	-	-	-
% Total impurity	0.39%	0.40%	0.40%
Absolute difference from initial value	-	0.01	0.01
% Difference	-	2.6	2.6
% Assay of ALO	100.0	99.7	99.2
Absolute difference from initial value	-	0.3	0.8

4.0 CONCLUSION

A RRLC method was successfully developed and validated for simultaneous determination of ALO and its impurities. The total run time was 10 min, within which ALO, its impurities and other degradation products are separated. Method validation results have proved the method to be selective, precise, accurate, robust, and stability indicating. The method can be implying for the routine analysis and stability study.

5.0 ACKNOWLEDGEMENT

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