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Development and validation of RP-HPLC method for simultaneous estimation of allopurinol and alphalipoicacid in bulk and tablet dosage form

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ABSTRACT

A new simple ,Rapid selective , precise and accurate gradient reversed phase high performance liquid chromatographic method (RP-HPLC)method has been developed And validated for simultaneous estimation of allopurinol and alphalipoic acid in bulk and tablet dosage form. Chromatographic analysis was performed on a c-18 column (250×4.6×5 µ)at ambient temperature .the column used was as BDS in Isocratic mode, with mobile phase containing tetrabutylammoniumhydroxide buffer and acetonitrile(70:30v/v) Adjusted to Ph 6.6 with dilute orthophosphoric acid solution . The flow rate was 0.8 Ml/min and effluents were monitored at 230nm. The retention times of allopurionol and alpha lipoicacid were found to be 2.33 min and 6.32 min, respectively. The method was validated as per ICH guidelines. The recoveries of allopurinol and alpha lipoic acid were found to be 98.53 to 100.03 and 98.5 to 99.9% respectively. The proposed method was found to be accurate reproducible and consistent. It was successfully applied for the analysis of these drugs in marketed formulations and could be effectively used for the routine analysis of formulations containing any one of the above drugs or a combination, without any alteration in the chromatographic conditions.

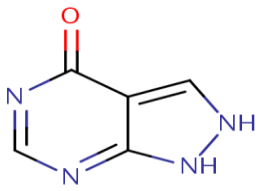
Keywords: Allopurinol, Alphalipoicacid , RP-Hplc, Validation.

INTRODUCTION

The present study has been undertaken in order to develop a new simple, rapid, efficient and reproducible RP-HPLC method for the analysis of Allopurinol and Alpha Lipoic acid [1].

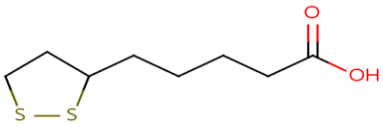
An attempt was made in a stepwise manner to device a simple, rapid, selective, validated and sophisticated method, like, High Performance Liquid Phase Chromatography (Reverse Phase) for Allopurinol and Alpha Lipoicacid [2]. **Allopurinol** is a drug used primarily to treat hyperuricemia and its complications, including chronic gout [3]. A xanthine oxidase inhibitor that decreases uric acid production [4]. It also acts as an antimetabolite on some simpler organisms [5]. Allopurinol and its active

metabolite, oxypurinol, inhibit the enzyme xanthine oxidase, blocking the conversion of the oxypurines hypoxanthine and xanthine to uric acid [6]. Elevated concentrations of oxypurine and oxypurine inhibition of xanthine oxidase through negative feedback results in a decrease in the concentrations of uric acid in the serum and urine [7]. Allopurinol also facilitates the incorporation of hypoxanthine and xanthine into DNA and RNA, leading to a feedback inhibition of de novo purin synthesis and a decrease in serum uric acid concentrations as a result of an increase in nucleotide concentration [8].



1H, 2H, 4H-pyrazolo [3, 4-d] pyrimidin-4-one

Lipoic acid (LA), also known as α-lipoic acid and alpha lipoic acid (ALA) and thiotic acid is an organosulfur compound derived from octanoic acid. Lipoic Acid is generally involved in oxidative decarboxylations of keto acids and is presented as a growth factor for some organisms. Lipoic acid exists as two enantiomers, the R-enantiomer and the S-enantiomer. Normally only the R-enantiomer of an amino acid is biologically active, but for lipoic acid the S-enantiomer assists in the reduction of the R-enantiomer when a racemic mixture is given. Some recent studies have suggested that the S-enantiomer in fact has an inhibiting effect on the R-enantiomer, reducing its biological activity substantially and actually adding to oxidative stress rather than reducing it. Furthermore, the S-enantiomer has been found to reduce the expression of GLUT-4s in cells, responsible for glucose uptake, and hence reduce insulin sensitivity.



5-(1,2-dithiolan-3-yl)pentanoic acid.

Different analytical methods have been reported in the literature for the assay of allopurinol and alpha-lipoic acid in bulk and tablet dosage form and includes spectrophotometry, TLC, HPLC, HPTLC, LCMS. The present study was to establish a simple, sensitive and low cost RP-HPLC method for simultaneous estimation of allopurinol and alpha lipoic acid in bulk and tablet dosage form.The developed method was validated as per ICH guidelines.

MATERIALS AND METHODS

Reagents

Reagents used Water(HPLC grade), Sodium Acetate(Sodium Acetate), Methanol(HPLC Grade), Potassium Phosphate(AR Grade), Acetonitrile(HPLC Grade),Ammonium acetate(Ammonium acetate),Triethylamine(AR Grade).

Drugs

Allopurinol and Alpha Lipoic acid drugs (Gift Samples obtained from Chandra labs, Hyd), Aluno A (100+100)(Allopurinol-100 mg Lipoic Acid-100 mglabel claims).

Instruments

UV-Visible Spectrophotometer (Nicolet evolution 100), HPLC (Shimadzu (LC 20 AT VP), Ultra sonicator (Citizen, Digital Ultrasonic Cleaner), pH meter (Global digital), Electronic balance (Shimadzu), HPLC Column (Inertsil ODS 3V(250x4.6mm) 5µm), Syringe(Hamilton).

Mobile phase

A mixture of 55 volumes of Phosphate Buffer pH3.5 and 45 volumes of Acetonitrile was prepared. The mobile phase was sonicated for 10min to remove gases.

Preparation of Phosphate Buffer 20mm

2.72gm of potassium di hydrogen phosphate (KH2PO4) was weighed and dissolved in 1000ml of water and volume was made up to 1000ml with water. Adjust the pH to 3.5 using ortho phosphoric acid. The buffer was filtered through 0.45µ filters to remove all fine particles and gases.

Preparation of standard stock solution of ALLOPURINOL

10 mg of ALLOPURINOL was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Preparation of standard stock solution of ALPHA LIPOIC ACID

10mg of ALPHA LIPOIC ACID was weighed in to 100ml volumetric flask and dissolved in Methanol and then dilute up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Preparation of mixed standard solution

Weigh accurately 100 mg of Allopurinoland100 mg of AlphaLipoicacid in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 100 µg/ml of Allopurinol and 100 µg/ml of AlphaLipoicacid is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram

Assay

Preparation of samples for Assay

Preparation of mixed standard solution

Weigh accurately 100 mg of Allopurinoland100 mg of AlphaLipoicacid in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 100 µg/ml of Allopurinol and 100 µg/ml of AlphaLipoicacid is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram

Preparation of standard stock solution

Standard stock solutions of ALLOPURINOL and ALPHA LIPOIC ACID (µ/ml) were prepared by dissolving 2.5 mg of ALLOPURINOL and 100 mg of ALPHA LIPOIC ACID in 100 ml of mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min. and dilute 100ml with mobile phase.

Tablet sample

20 tablets (each tablet contains 100 mg of ALLOPURINOL and 100 mg of ALPHA LIPOIC ACID) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of ALLOPURINOL and ALPHA LIPOIC ACID (µg/ml) were prepared by dissolving weight equivalent to 100 mg of ALLOPURINOL and 100 mg of ALPHA LIPOIC ACID and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 100µg/ml of ALLOPURINOL and 100µg/ml of ALPHA LIPOIC ACID was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Method validation

System suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, tailing factor, retention time resolution and asymmetric factor were evaluated.

Accuracy

Accuracy of the method was determined by Recovery studies. To the formulation (preanalysed sample), the reference standards of the drugs were added at the level of 80%, 100%, 120%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 80%, 100%, 120%.

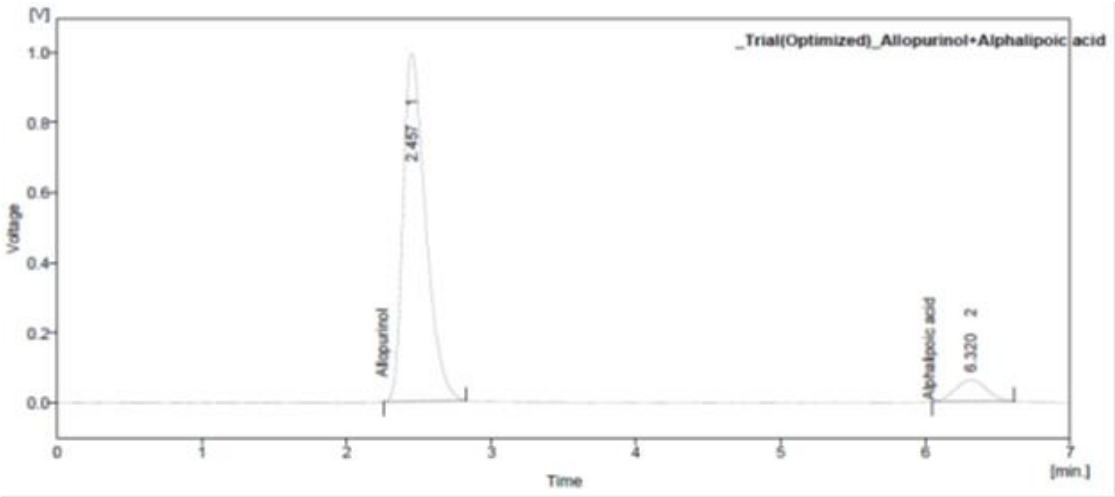


FIG-1: HPLC Chromatogram of Allopurinol and Alpha lipoic acid.

Precision

Method precision

For precision same concentration solution of Allopurinol and Alpha lipoicacid was injected 6times and observed for any peculiar change in the areas and % RSD was calculated for each drug

Limit of Detection

$$LOD = \frac{3.3\sigma}{S}$$

Where, σ = the standard deviation of the response
S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Observation

The LOD for this method was found to be 1.64 µg/ml & area 104.53 for ALLOPURINOL and 18.21 µg/ml &area 104.71 for ALPHA LIPOIC ACID

Limit of Quantification

$$LOQ = \frac{10\sigma}{S}$$

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve
The slope S may be estimated from the calibration curve of the analyte.

Observation

The LOQ for this method was found to be 4.97 µg/ml & area 316.75 for ALLOPURINOL and 55.19 µg/ml & area 317.30 for ALPHA LIPOIC ACID

Robustness

Robustness is generally done by changing the parameters like flow rate and organic phase of the mobile phase.

Ruggedness

The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts

Acceptance criteria

The % Relative standard deviation of Assay values between two analysts should be not more than 2.0%.

Assay

Twenty micro litres of sample and standard solutions were injected separately into the chromatographic system and the peak areas for the analyte peaks were measured. The % content of each drug was calculated

Table-1 Assay results

ALLOPURINOL			**ALPHA LIPOIC ACID**	
	Standard Area	**Sample Area**	**Standard Area**	**Sample Area**
Injection-1	8416.477	8796.606	670.532	744.549
Injection-2	8401.247	8486.572	789.759	727.439
Injection-3	8405.335	8424.324	718.683	723.860
Injection-4	8429.709	8362.688	767.403	779.117
Injection-5	8374.071	8612.064	761.668	788.958
Average Area	8405.368	8536.451	741.609	752.7846
Tablet average weight	625		625	
Standard weight	100		100	
Sample weight	625		625	
Label amount	100		100	
std. purity	99.2		99.3	
Amount found in mg	**100.75**		**100.80**	
Assay(%purity)	**100.75**		**100.80**	

Table-2 Results for system suitability of ALLOPURINOL

Injection	**Retention time (min)**	**Peak area**	**Theoretical plates (TP)**	**Tailing factor (TF)**
1	2.440	8732.758	4236	1.848
2	2.433	8496.782	4230	1.879
3	2.443	8434.962	4240	1.869
4	2.443	8424.539	4240	1.869
5	2.443	8442.276	4292	1.809
6	2.440	8752.046	4236	1.848
Mean	2.4403	8547.227	-	-
SD	0.0039	153.354	-	-
%RSD	**0.16**	**1.79**	-	-

Table-3 Results for system suitability of ALPHA LIPOIC ACID

Injection	Retention time (min)	Peak area	Theoretical plates	Tailing factor	Resolution
1	6.277	714.994	3897	1.048	11.288
2	6.313	712.967	3729	1.047	11.228
3	6.280	725.037	3013	1.083	11.383
4	6.307	711.707	3721	1.013	11.180
5	6.353	723.164	3777	1.062	11.409
6	6.277	741.715	3789	1.063	11.195
Mean	6.301	721.597	-	-	-
SD	0.030	11.269	-	-	-
%RSD	**0.47**	**1.56**	-	-	-

Table-4 Linearity Preparations

Preparations	Volume from standard stock transferred in ml	Volume made up in ml (with mobile phase)	Concentration of solution(µg /ml)	
			ALLOPURINOL	ALPHA LIPOIC ACID
Preparation 1	0.6	10	60	60
Preparation 2	0.8	10	80	80
Preparation 3	1.0	10	100	100
Preparation 4	1.2	10	120	120
Preparation 5	1.4	10	140	140

Table-5 linearity of ALLOPURINOL

S.No.	Conc.(µg/ml)	Area
1	60	5546.444
2	80	6742.300
3	100	8163.224
4	120	9160.542
5	140	10700.547

Table-6 linearity of ALPHA LIPOIC ACID

S.No.	Conc.(µg/ml)	Area
1	60	449.912
2	80	585.233
3	100	706.534
4	120	812.710
5	140	909.810

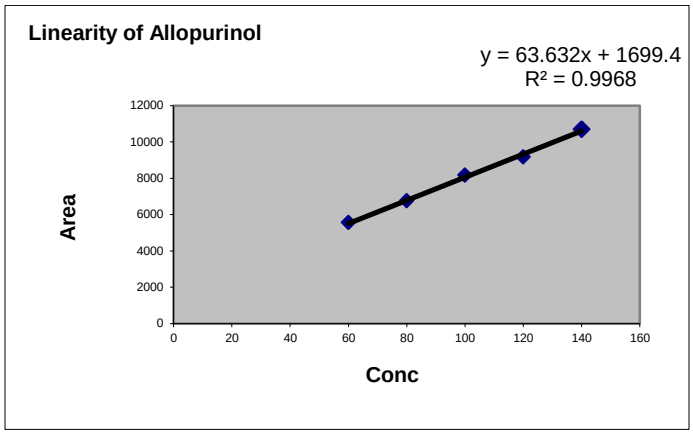


Fig. 2: Linearity graph of ALLOPURINOL

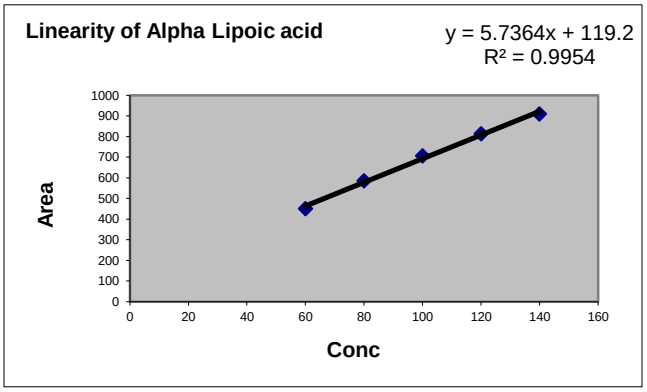


Fig. 3: Linearity graph of ALPHA LIPOIC ACID

Table-7 Recovery results for ALLOPURINOL

Recovery level	**Accuracy ALLOPURINOL**					Average %Recovery
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	
80%	100	**8381.268**	**8386.559**	**99.51**	**99.51**	
	100	**8389.732**				
	100	**8388.677**				
100%	120	**9596.193**	**9652.471**	**118.24**	**98.54**	
	120	**9691.402**				98.93%

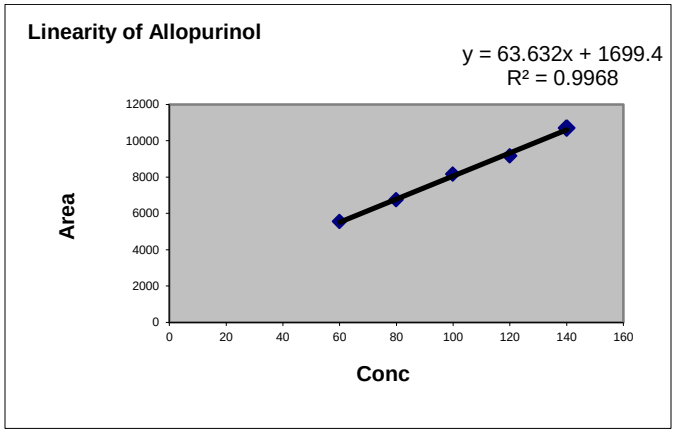
	120	**9669.818**				
120%	140	**10528.417**	**10554.907**	**138.27**	**98.76**	
	140	**10582.873**				
	140	**10553.432**				

Table-8 Recovery results for ALPHA LIPOIC ACID

Recovery level	**Accuracy ALPHA LIPOIC ACID**					Average
	Amount taken(mcg/ml)	Area	Average area	Amount recovered(mcg/ml)	%Recovery	%Recovery
80%	100	**729.979**	**720.578**	**98.50**	**98.50**	
	100	**712.701**				
	100	**719.054**				
100%	120	**842.827**	**835.360**	**118.23**	**98.53**	
	120	**830.429**				98.79%
	120	**832.824**				
120%	140	**949.944**	**942.123**	**139.11**	**99.36**	
	140	**943.091**				
	140	**933.335**				

Table-9 Results for Method precision of ALLOPURINOL and ALPHA LIPOIC ACID

ALLOPURINOL			**ALPHA LIPOIC ACID**		
S.No.	Rt	Area	S.No.	Rt	Area
1	2.440	8732.758	1	6.277	714.994
2	2.433	8496.782	2	6.313	712.967
3	2.443	8434.962	3	6.280	725.037
4	2.443	8424.539	4	6.307	711.707
5	2.443	8442.276	5	6.353	723.164
6	2.440	8752.046	6	6.277	741.715
avg	2.4403	8547.227	**avg**	6.301	721.597
stdev	0.0039	153.354	**stdev**	0.030	11.269
%RSD	**0.16**	**1.79**	**%RSD**	**0.47**	**1.56**



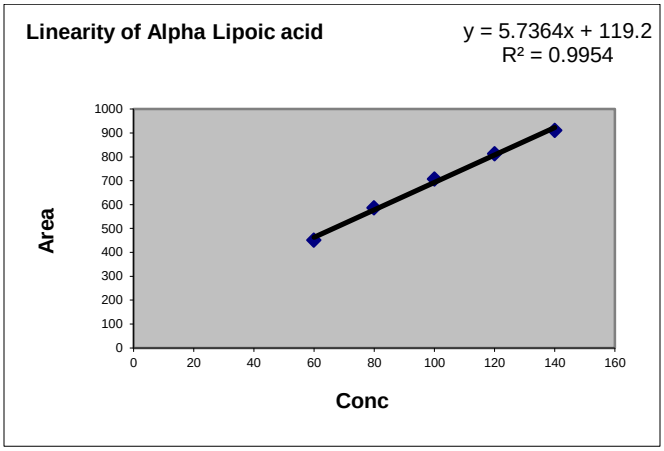


Fig. 4 & 5: Calibration graphs of ALLOPURINOL&ALPHA LIPOIC ACID

Table-10 Results for calibration graph

	ALLOPURINOL		**ALPHA LIPOIC ACID**	
S.No.	Concentration µg/ml	Peak Area	Concentration µg/ml	Peak Area
1	60	5546.444	60	449.912
2	80	6742.300	80	585.233
3	100	8163.224	100	706.534
4	120	9160.542	120	812.710
5	140	10700.547	140	909.810
S.D.	31.6	2015	31.623	182
Slope	63.6		5.73	

Table-11 Result of Robustness study

Parameter	**ALLOPURINOL**		**ALPHA LIPOIC ACID**	
	Retention time(min)	**Tailing factor**	**Retention time(min)**	**Tailing factor**
Flow Rate				
0.8 ml/min	2.930	1.872	7.393	1.070
1.0 ml/min	2.440	1.848	6.277	1.048
1.2 ml/min	2.070	1.759	5.237	1.096
Wavelength				
210nm	2.413	1.818	6.123	1.033
212nm	2.440	1.848	6.277	1.048
214nm	2.417	1.818	6.123	1.068

Table-12 Results for Ruggedness

ALLOPURINOL	**%Assay**	**ALPHA LIPOIC ACID**	**%Assay**
Analyst 01	99.86	**Analyst 01**	100.16
Analyst 02	99.02	**Anaylst 02**	102.15
%RSD	0.20	**%RSD**	0.21

RESULTS AND DISCUSSION

To develop a new RP-HPLC method, several mobile phase compositions were tried. A satisfactory separation with good peak symmetry was obtained with C-18 (150mm X 4.6mm, i.d., 5µm,) column using mobile phase containing tetra butyl ammonium hydroxide buffer (pH 6.6): acetonitrile (70:30) v/v at a flow rate of 0.8 ml/min. Quantification was achieved at UV detection at 230 nm based on peak area. The retention time for alloperinolandalphalipoicacid were found to be 2.33 min and6.32 min respectively. The optimized method was validated as per ICH guidelines.

System suitability parameters like retention time, resolution, tailing and plate count were shown uniformity and %RSD was less than 1 and the results are given in and from the obtained results we can say that the system is suitable for analysis.

A linearity range was 250-750 µg/ml with correlation coefficient 0.998 was observed for both the drugs. In linearity plot the graph with three different concentrations versus areas to construct the linear regression equation and to calculate the value of correlation co-efficient. Linear correlation was found to be y = 63.63x + 169.9 for alloperinol and y = 5.736x + 119.2 for alphalipoicacid and calibration curve

The precision of the proposed method was carried in terms of the repeatability and the %RSD values was found to be 1.79 for allopurinol & 1.56 for alphalipoic acidwhich reveal that the proposed method is precise. Precision studies were tabulated.

The study of robustness in the present method shows no significant changes either in the peak area or Rt. Rubustness data is tabulated.

The method accuracy was evaluated by recovery studies. The percentage recovery of alloperinol and alphalipoicacid was found to be99.51% and 98.5 for 80% level; 98.53% and 98.5% for 100% level; 100.03% and 99.9.% for 150% level and results was shown.

Method specificity was concluded by those figures are alloperinol and alphalipoicacidstandard chromatogram and other one is formulation. There is no placebo and excipients peaks interference with standard and analytic peak so it proves method is selective.

SUMMARY AND CONCLUSION

A method was developed on trial & error basis by changing the variables wherever required. Finally a method was optimized and the conditions were determined. Method was developed by using RP HPLC Method During this optimization at every trial a new combination of mobile phase was tried to overcome the drawbacks of the previous run. Finally the method was optimized at trial 8, the optimized method was using Phosphate buffer pH 3.5: Acetonitrile (55:45v/v) at 212 nm and validated as per ICH guidelines. The method was validated for system suitability, linearity, precision, accuracy, specificity, robustness, LOD and LOQ. The system suitability parameters were within limit, hence it was concluded that the system was suitable to perform the assay. The method shows linearity between the concentration range of 60-140µg/ml for Allopurinol and 60-140µg/ml for Alpha Lipoic acid. The % recovery of were found to be in the range of 98.0 % - 102.0 %. As there was no interference due to mobile phase, the method was found to be specific. The method was robust as observed from insignificant variation in the results of analysis by changes in Flow rate and wavelength variation separately and analysis being performed by different analysts. The present method is validated and the results are better than previous methods which are performed on these drugs. Hence it can be concluded that the proposed method was a good approach for obtaining reliable results and found to be suitable for the routine analysis of Allopurinol and Alpha lipoic acid in Bulk drug and Pharmaceutical formulation.

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