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Method development and validation for the estimation of Allopurinol and alpha lipoic acid by RP-HPLC method

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ABSTRACT

A Simple, specific and sensitive an isocratic Estimation by RP-HPLC analytical Method were developed and validated for the quantification allopurinol and alpha lipoic acid. Quantification was achieved by by using the mobile phase (Phosphate buffer Ph3.5: Acetonitrile) in the ratio of 55:45. Inertsil ODS, C-18,250×4.6mm ID, 5µm Particle size was used as stationary phase. The flow rate was 1.2 ml/min. Measurements were made at a wavelength of 230nm. The average retention times for Allopurinol and alpha Lipoic acid was found to be 2.457 & 6.320 min. The proposed method was validated for selectivity, precision, linearity and accuracy. The assay methods were found to be linear from 60-140µg/ml & 60-140 µg/ml for Allopurinol and alpha Lipoic acid respectively. All validation parameters were within the acceptable range. The developed methods were successfully applied to estimate the amount of Allopurinol and alpha Lipoic acid.

Keywords: Allopurinol and alpha Lipoic acid, RP-HPLC method, Inertsil ODS,C-18, 250×4.6mm ID, 5µm Particle size potassium di hydrogen phosphate, acetonitrile and Validation.

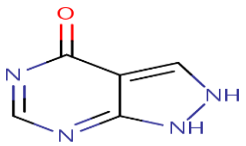
INTRODUCTION

Allopurinol

Allopurinol (Zyloprim, and generics) is a drug used primarily to treat hyperuricemia (excess uric acid in blood plasma) and its complications, including chronic gout.[3] It is a xanthine oxidase inhibitor which is administered orally. It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system. [4] A xanthine oxidase inhibitor that decreases uric acid production. It also acts as an antimetabolite on some simpler organisms.

Structure of Allopurinol

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

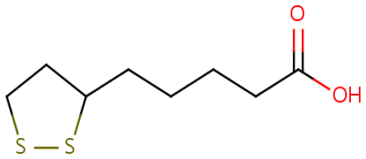


Alpha lipoic acid

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. ALA is made in animals normally, and is essential for aerobic metabolism. It is also manufactured and is available as adietary supplement in some countries where it is marketed as an antioxidant, and is available as a pharmaceutical drug in other countries.[6] A vitamin-like antioxidant.

Structure of Alpha Lipoic acid

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



MATERIALS AND METHODS

Instruments The chromatographic technique performed on a Nicolet evolution 100 Liquid chromatography with Shimadzu (LC 20 AT VP) UV-visible detector and Spinchrom software, reversed phase Inertsil ODS 3V (250x4.6mm) 5µm as stationary phase, Electron corporation double beam UV-visible spectrophotometer (vision pro-software), Ultrasonic cleaner, Shimadzu analytical balance AY-220, Vacuum micro filtration unit with 0.45µ membrane filter was used in the study.

Pharmaceutically pure sample of Allopurinol and alpha Lipoic acid were obtained as gift samples from Chandra lab, Prashanthinagar, Kukatpally, Hyderabad, India. The purity of the drugs were evaluated by obtaining its melting point and ultraviolet (UV) and infrared (IR) spectra. No impurities were found. The drugs were used without further purification. HPLC-grade Acetonitrile and OPA ware from standard reagents pvt ltd. Potassium Phosphate dibasic (AR grade) sodium acetate AR Grade were from Merck.

Determination of working wavelength (λmax) for allopurinol & alpha lipoic acid

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. [5]

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.

RESULTS

The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The resulting spectra are shown in the figure 1, 2 and the absorption curve shows characteristic absorption maxima at 250 nm for allopurinol, 220 nm for alpha lipoic acid and nm for the combination. [6]

Observation

The Isosbestic point was found to be 230nm for allopurinol and alpha lipoic acid in combination.

Preparation of mobile phase

A mixture of 550 volumes of Phosphate buffer pH 3.5 and 450 volumes of Acetonitrile was prepared. The mobile phase was sonicated for 10min to remove gases. [7]

Analysis of formulation

Preparation of mixed standard solution

weigh accurately 100 mg of Allopurinoland 100 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.

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 100 µg/ml of allopurinol and 100 µg/ml of alpha lipoic acid was made by adding 1 ml of stock solution to 10 ml of mobile phase. [8]

Calculation

The amount of alpha lipoic acid and allopurinol present in the formulation by using the formula given below, and results shown in above table:

$$\% \text{ Assay} = \frac{\text{AT}}{\text{AS}} \times \frac{\text{WS}}{\text{DS}} \times \frac{\text{DT}}{\text{WT}} \times \frac{\text{P}}{100} \times \frac{\text{AW}}{\text{LC}} \times 100$$

Where,
AS: Average peak area due to standard preparation
AT: Peak area due to assay preparation
WS: Weight of alpha lipoic acid /allopurinol in mg
WT: Weight of sample in assay preparation
DT: Dilution of assay preparation

Method validation
Linearity and range
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. Calibration curve with concentration verses peak areas was plotted by injecting the above prepared solutions and the obtained data were subjected to regression analysis using the least squares method.

Method precision (repeatability)

Prepared sample preparations of Allopurinol and Alpha lipoic acid as per test method are injected 6 times in to the column.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on standard deviation of the y-intercept and the slope of the calibration curve by using the equations [2] and [3], respectively.

LOD = 3.3 δ/S ……. (3)
LOQ =10 δ/S …….. (4)
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.

Accuracy (recovery study)

The accuracy of the method was determined by calculating the recoveries of Allopurinol and Alpha lipoic acid Known amounts of standard solutions of Allopurinol and Alpha lipoic acid was added at 50% concentration to pre quantified sample solutions of Allopurinol and Alpha lipoic acid (50,100,125µg/ml)andInternal standard (50,100,125 µg/ml). The amount of Allopurinol and Alpha lipoic acid recovered was estimated by using the following formulas.

$$\% \ Recovery = \frac{amount\ found}{Amount\ added} \times 100$$

$$Amount\ Found(mcg/ml) = \frac{\text{Mean test area}}{\text{Mean standard area}} \times \text{Standard concentration}$$

Specificity

In an assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay results are unaffected by the presence of these extraneous materials. There should be no interference of the diluents, plasma at retention time of drug substances. [9]

Robustness

Robustness is the measure of a method remain unaffected by small, deliberate changes in method parameters like flow rate and detection wavelength on assay of the analyte of interest. Here the detection wavelength varied ±2nm and flow rate was varied ±0.2 ml/min. The results were shown in (Table 6)

Ruggedness

The ruggedness of the method was studied by analysing the sample and standard preparations by two analysts. The %RSD assay values between two analysts was calculated i.e., (limit <2%).

This indicates the method was rugged. The results were shown in Table 7.

RESULTS AND DISCUSSION

In Analytical RP HPLC method, the primary requirement for developing a method for analysis is that the using different solvents and buffers and columns to get better retention time and theoretical plates for both Standard and Internal standard, and better cost effective and time saving method than the previously developed methods. The Maximum uv absorbance was found to be 230 nm by scanning in UV region. The chromatographic method was optimized with mobile phase consisting of Phosphate buffer: Acetonitrile (55:45) and Inertsil ODS, C-18,250×4.6mm ID, 5µm Particle size. All the validation parameters were studied at a the wavelength 230nm. Accuracy was determined by calculating the recovery and the results were in acceptable range (limit 98-102%). The method was successfully used to determine the amount of allopurinoland alpha lipoic acid. The results obtained were in good agreement with the corresponding labelled amount (Table 8). The method was linear in the concentration range of 50 to 100 µg/ml for allopurinoland alpha lipoic acid (Table 1 and 2). Precision was calculated as repeatability for the drugs (Table No.9). Robustness and ruggedness results were in acceptable range (Table 6 and Table7). As the allopurinoland alpha lipoic acid peaks were well separated, the method is more specific. Summary of all validation parameters for method is given in Table 10. By observing the validation parameters, the method was found to be simple, sensitive, accurate and precise. Hence the method can be employed for the routine analysis allopurinol and alpha lipoic acid in formulation.

Table 1: Linearity of Allopurinol

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

Table 2: 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

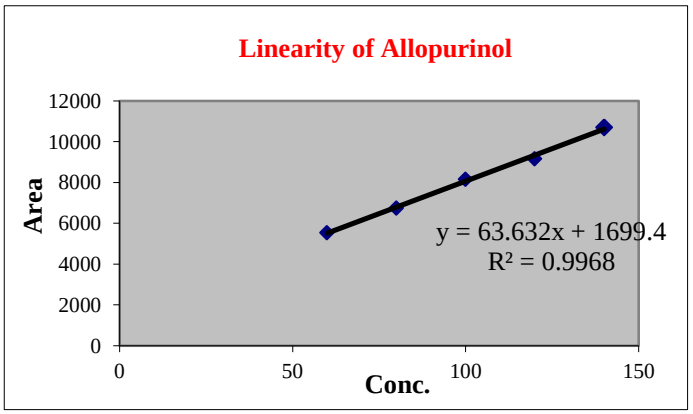


Fig 1: Linearity graph of Allopurinol

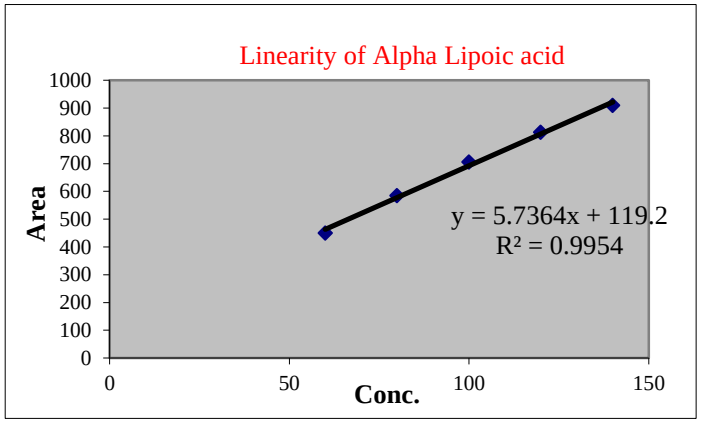


Fig 2: Linearity graph of alpha lipoic acid

Table 3: LOD &LOQ data for Allopurinol and alpha Lipoic acid

Parameter	Micro grams		Area	
	Allopurinol	Alpha Lipoic acid	Allopurinol	Alpha Lipoic acid
LOD	1.64	18.21	104.53	104.71
LOQ	4.97	55.19	316.75	317.30

Table 4: 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 5: Recovery results for Alpha lipoic acid

Recovery level	Accuracy Alpha lipoic acid					Average % Recovery
	Amount taken (mcg/ml)	Area	Average area	Amount recovered (mcg/ml)	% 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				
	120	832.824				98.79%
120%	140	949.944	942.123	139.11	99.36	

140	943.091			
140	933.335			

Table 6: Result of Robustness study

	Allopurinol		Alpha lipoic acid	
Parameter	**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 7: Results for Ruggedness

Allopurinol	**% Assay**	**Alpha lipoic acid**	**% Assay**
Analyte 01	99.86	**Analyte 01**	100.16
Analyte 02	99.02	**Analyte 02**	102.15
% RSD	0.20	**% RSD**	0.21

Table 8: 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	

Acceptance criteria: The percentage assay should be in the limits of 98-102%.

Table 9: Results for Method precision of Allopurinol and Alpha lipoic acid

Allopurinol			**Alpha lipoic acid**		
S. No.	**R.T**	**Area**	**S. No.**	**R.T**	**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.	2.440	8547.227	**Avg.**	6.301	721.597
S. D	0.0039	153.354	**S. D**	0.030	11.269
% RSD	**0.16**	**1.79**	**% RSD**	**0.47**	**1.56**

Table10: Validation parameters of evaluated method

S. No	Parameter	Value Obtained of Bosentan	Value Obtained of Internal Standrad
1.	ACCURACY(% Recovery)	98%	102%
2.	Linearity concentrations Range(µ g/mL)Regression coefficient (R2 value)	60-140µg/ml 0.996	60-140µg/ml 0.995
3.	LOD(mcg/mL)	1.64 µg/ml	18.21 µg/ml
4.	LOQ(mcg/mL)	4.97 µg/ml	55.19 µg/ml
3.	Precision (% RSD) Method precision(Repeatability) (%RSD, n = 6)	0.0-0.16	0.0-0.47
4.	Robustnes	Met with system suitability criteria	Met with system suitability criteria
5.	Ruggedness(%RSD analyst to analyst variation)	0.20	0.21

SD=Standard deviation, LOD = Limit of detection, LOQ = Limit of quantification, RSD = Relative standard deviation

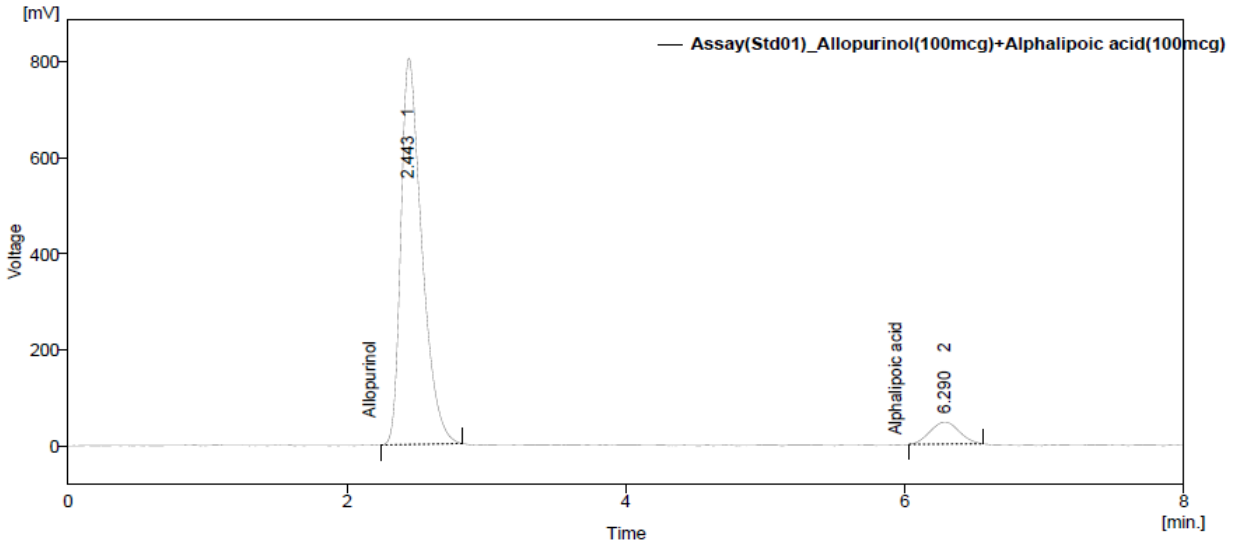


Figure 3: Chromatogram of Standard

CONCLUSION

The proposed isocratic estimation by RP-HPLC method was found to be simple, sensitive, accurate and precise for determination of allopurinol and alpha lipoic acid. The method utilizes easily available and cheap solvent for analysis of allopurinol and alpha lipoic acid hence the method was also economic for estimation of allopurinol and alpha lipoic acid in formulations.

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