Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 1, 2014

Research Article

REVERSE PHASE HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS QUANTITATIVE ESTIMATION OF ALPHA LIPOIC ACID AND ALLOPURINOL IN TABLETS

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Received: 03 Oct 2013, Revised and Accepted: 25 Oct 2013

ABSTRACT

Objective: To develop a simple, accurate, precise, linear and rapid Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for the simultaneous quantitative estimation of Allopurinol 100 mg and Alpha Lipoic acid 100 mg in tablets as per ICH guidelines.

Methods: The optimized method uses reverse phase column, Enable C18G (250 X 4.6 mm; 5µ), a mobile phase of acetonitrile: 0.02M ammonium acetate buffer adjusted to pH 4.6 in the proportion of 50:50 v/v, flow rate of 0.8 ml/min and a detection wavelength of 210 nm using a UV detector.

Results: The developed method resulted in allopurinol eluting at 3.01 min and alpha lipoic acid at 8.42 min. Both the drugs exhibited linearity in the range 50-175 µg/ml. The precision is exemplified by relative standard deviations of 0.83 % for allopurinol and 1% for alpha lipoic acid. Percentage Mean recoveries were found to be in the range of 98-102, during accuracy studies. The limit of detection was obtained as 3 ng/ml for allopurinol and 0.5 µg/ml for alpha lipoic acid, while the limit of quantitation was obtained as 10 ng/ml for allopurinol and 1µg/ml for alpha lipoic acid.

Conclusion: A simple, accurate, precise, linear and rapid RP-HPLC method was developed and validated for the simultaneous quantitative estimation of Allopurinol 100 mg and Alpha Lipoic acid 100 mg in tablets as per ICH guidelines and hence can be used for the routine analysis of allopurinol and alpha lipoic acid in tablets in various pharmaceutical industries.

Keywords: RP-HPLC, Allopurinol, Alpha Lipoic acid, Validation.

INTRODUCTION

Alpha Lipoic acid (**Figure 1,** 6,8-thioctic acid or 1,2-dithiolane-3-pentanoic acid or 1,2-dithiolane-3-valeric acid) is a natural antioxidant present in prokaryotic and eukaryotic cells. Alpha lipoic acid supplementation is advocated in the treatment of Acquired immune deficiency syndrome (AIDS), chaga, diabetes, heavy metal poisoning, ischemia reperfusion injury, liver diseases (alcoholic liver disease, mushroom poisoning), neurodegenerative disorders, radiation injury, wilson's disease and the effect of cigarette smoking [1-2]. Allopurinol (**Figure 2,** 4-hydroxypyrazolo[3,4-d] pyrimidine) is used in the treatment of medical conditions associated with elevated levels of uric acid, such as gout and the prevention of tumor lysis syndrome. Allopurinol is effective for the treatment of both primary hyperuricemia of gout and secondary hyperuricemia related to hematological disorders or anti-neoplastic therapy [3-5].

Fig. 1: Structure of Alpha lipoic acid

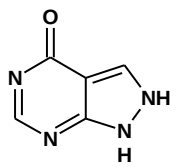

Fig. 2: Structure of Allopurinol

A detailed literature survey reveals that RP-HPLC methods have been reported for the quantitative estimation of allopurinol and alpha lipoic acid individually in various pharmaceutical dosage forms and in plasma [6-8]. RP-HPLC methods are reported for the analysis of alpha lipoic acid with other substances and similarly allopurinol with other drugs in combination. [9-13]. As per our detailed literature survey as on date, there are no RP-HPLC methods reported for the simultaneous quantitative estimation of allopurinol and alpha lipoic acid in any matrix either of pharmaceutical dosage forms, plasma, etc. In addition there exist no pharamcoepial RP-HPLC methods available for analysis of these two drugs in combination. Hence we here report a simple, sensitive, precise, accurate, linear and isocratic RP-HPLC method for the simultaneous quantitative estimation of allopurinol and alpha lipoic acid in tablets.

MATERIALS AND METHODS

Chemicals and Reagents

Analytically pure sample of allopurinol and alpha lipoic acid with purities greater than 99% were obtained as gift samples from Biophore pharmaceuticals, Hyderabad, India and tablet formulation [ALUNO-A] was procured from Apollo pharmacy, Hyderabad, India with labelled amount 100mg each of alpha lipoic acid and allopurinol. Acetonitrile (HPLC grade) was obtained from Sigma aldrich (Hyderabad, India), water (HPLC grade), ammonium acetate (AR grade), acetic acid (AR Grade) were obtained from SD Fine chemicals (Hyderabad, India), 0.45µm Nylon membrane filters were obtained from Spincotech Private Limited, Hyderabad, India.

Instrument

HPLC analysis was performed on Shimadzu LC-20AD Prominence Liquid Chromatograph comprising a LC-20AD pump, Shimadzu SPD-20A Prominence UV-VISIBLE detector and a reverse phase C18 column, Enable C18G (250 X 4.6 mm; 5µ). A manually operating Rheodyne injector with 20 µL sample loop was equipped with the HPLC system. The HPLC system was controlled with "Lab solutions lite" software. An electronic analytical weighing balance (0.1mg sensitivity, Shimadzu AY 220), digital pH meter (DELUX model 101), a sonicator (sonica, model 2200 MH) and UV-Visible Spectrophotometer (Shimadzu UV-1800 series, software-UV probe version 2.42) were used in this study.

Method

Selection of Wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of alpha lipoic acid and allopurinol. Suitable wavelength selected was 210 nm (**Figures 3-4**).

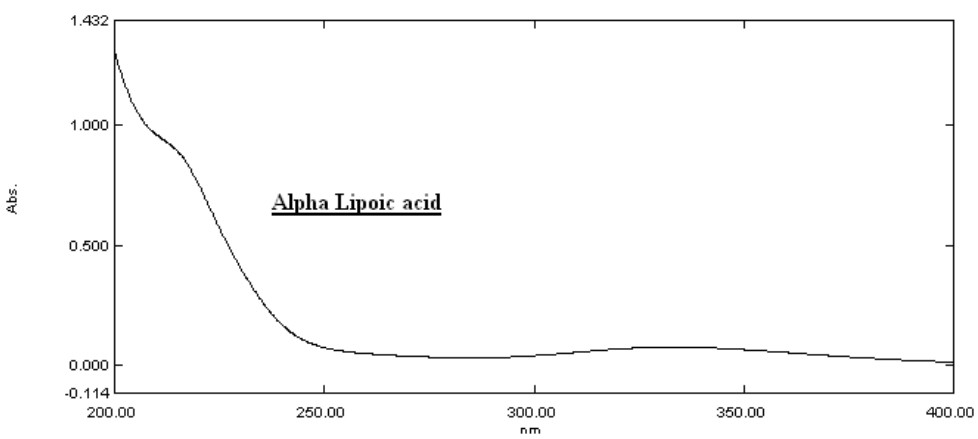

Fig. 3: UV spectrum of Alpha lipoic acid

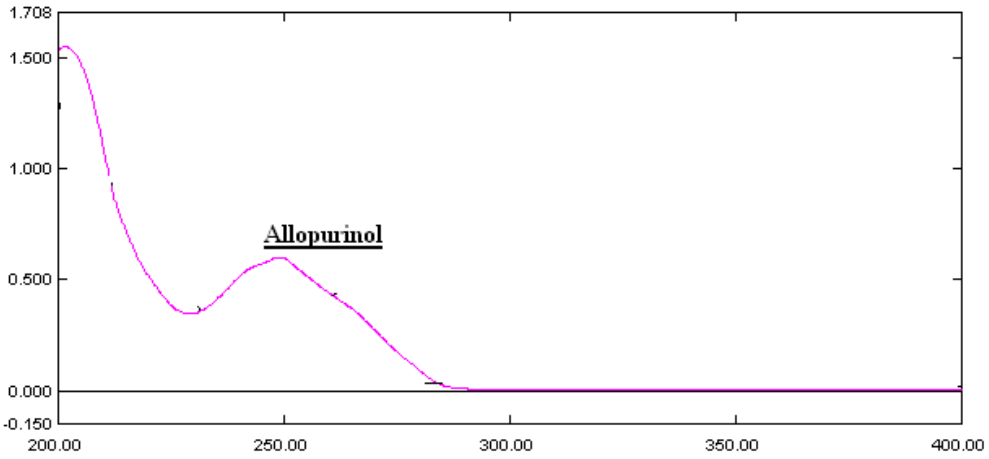

Fig. 4: UV spectrum of Allopurinol

Chromatographic conditions

The separation of the drugs was achieved on a reverse phase C18 column, Enable C18G (250 X4.6 mm; 5µ), mobile phase consisting of a mixture of acetonitrile and ammonium acetate buffer (20 mM, adjusted pH with 30% v/v of glacial acetic acid to 4.6) in the ratio of 50:50, v/v. The mobile phase was set at a flow rate of 0.8 ml/min and the volume injected was 20 µl for every injection. The detection wavelength was set at 210 nm.

Buffer Preparation

The buffer solution is prepared by weighing 1.54g of ammonium acetate and transferring to 1000 ml of HPLC grade water to get 20 mM buffer strength and later pH was adjusted to 4.6 using 30%v/v acetic acid in water. The buffer was then filtered through 0.45 µm nylon membrane filter.

Mobile phase Preparation

The mobile phase was prepared by mixing acetonitrile and buffer in the ratio of 50:50, v/v and later sonicated for 10 minutes for the removal of air bubbles.

Preparation of stock and working standard solution

100 mg of allopurinol and 100 mg of alpha lipoic acid were accurately weighed and taken in 100 ml clean and dry volumetric flask containing 50 ml of diluent (same as mobile phase) and then sonicated for 5 minutes to dissolve. Later the solution was made up to the mark using the mobile phase. This is considered as standard stock solution (1mg/ml). 1 ml of the stock solution was pipetted out and made up to 10 ml to get a concentration, each drug of 100 µg/ml, treated as working standard, 100% target concentration.

Preparation of stock and working sample solution

Sample solution containing both the drugs was prepared by dissolving tablet powder into diluent (mobile phase). Ten tablets were weighed separately and their average weights were determined. The average weight was weighed from the ten tablets grinded in a pestle and mortar and then transferred to a 100 ml volumetric flask containing 80 ml diluents. Sonication was done for five minutes and later the volume was made up to 100 ml using mobile phase. Then the sample preparation was filtered through 0.45µ nylon membrane filter to get sample stock solution of 1mg/ml. 1 ml of the above stock solution was pipetted out and made up to 10 ml to get working sample solution equivalent to a concentration of working standard, each drug of 100 µg/ml.

RESULTS AND DISCUSSION

Method Development

A Reverse phase HPLC method was developed keeping in mind the system suitability parameters i.e. resolution factor (Rs) between peaks, tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of allopurinol at 3.01 min and alpha lipoic acid at 8.42 min. **Figures 5** and **6** represent chromatograms of blank solution and mixture of standard solution respectively. The total run time is 9 minutes. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (*Rt*), number of theoretical plates (*N*), peak resolution (Rs) and peak Tailing factor (T) were evaluated for six replicate injections of the standards at working concentration. The results given in **Table 1** were with in acceptable limits.

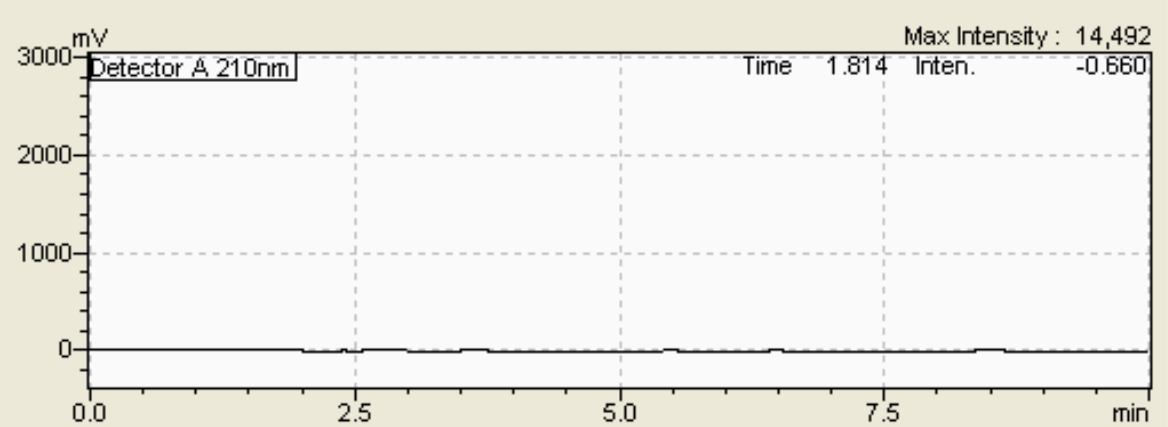

Fig. 5: Typical Chromatogram of Blank solution

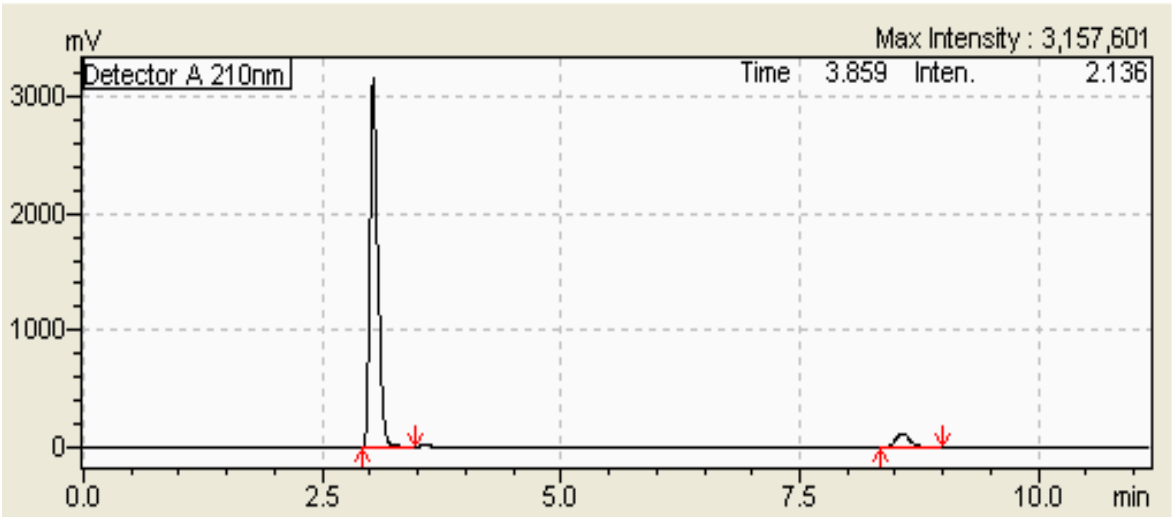

Fig. 6: Typical chromatogram of the mixture of Standard solutions

Table 1: System suitability studies results.

Parameters*	Acceptance Limits	Allopurinol	Alpha lipoic acid
Retention time (min)	% RSD < 1	3.01	8.42
Resolution factor (Rs)	Not less Than 2	23.081	
Number Of Theoretical plates (N)	Not less Than 2000	5340	12280
Tailing factor (T)	Not More Than 2	1.2	1.1

* Mean of six injections

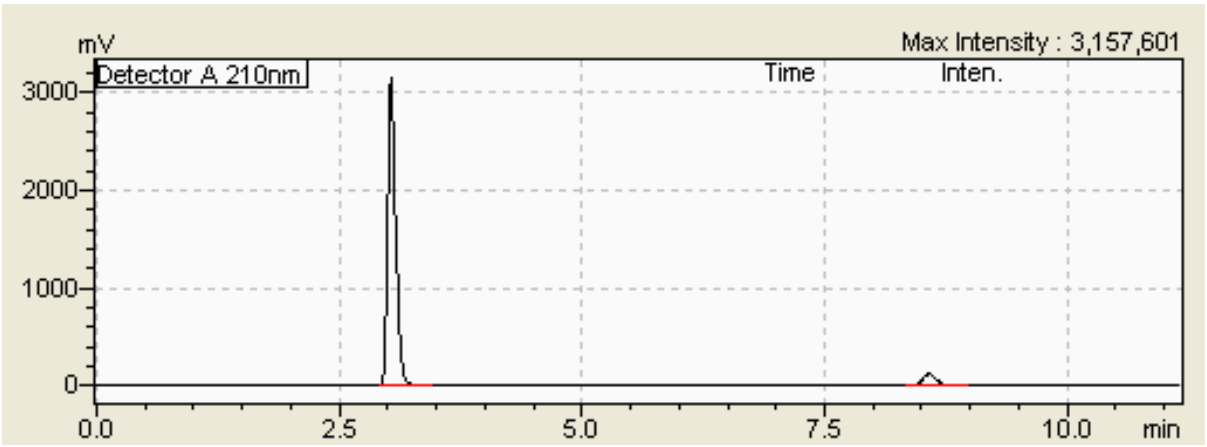

Fig.7: Typical chromatogram for the sample (tablet)

In order to test the applicability of the developed method to a commercial formulation, 'ALUNO A' was chromatographed at working concentration and it is shown in **Figure 7.** The sample peaks were identified by comparing the relative retention times with the standard drugs mixture (**Figure 6**). System suitability parameters were within the acceptance limits, ideal for the chromatographed sample. Integration of separated peak area was done and each drug concentration was determined by using the peak area concentration relationship obtained in the standardization step. The protocol affords reproducible quantification of the two drugs with error less than 10%, which is the standard level in any pharmaceutical quality control.

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. RP-HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines [14] for validation of analytical procedures. The method was validated for the parameters like system suitability, specificity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitiation (LOQ).

Specificity

Figures 5-7 for blank, mixture of standard drug solution and sample chromatogram reveal that the peaks obtained in mixture of standard solution and sample solution at working concentrations are only because of the drugs as blank has no peaks at the retention times of allopurinol and alpha lipoc acid. Accordingly it can be concluded that, the method developed is said to be specific.

Precision

System precision

Six replicate injections of the mixture of standard solution at working concentration showed % RSD (Relative Standard Deviation) less than 2 concerning peak areas for both the drugs, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in **Table 2**.

Method precision

Method precision was determined by performing assay of sample under the tests of (i) repeatability (Intra day precision) and (ii) Intermediate precision (Inter day precision) performed during 3 consecutive days by three different analysts, at working concentration.

Table 2: System precision results

Injection number	Allopurinol		Alpha Lipoic acid	
(n)	Rt	Peak Area	Rt	Peak area
1	3.02	20523526	8.42	1623115
2	3.01	20623432	8.41	1622123
3	3.04	20423326	8.44	1631280
4	3.02	20523526	8.41	1613173
5	3.03	20523412	8.43	1638128
6	3.01	20623221	8.41	1629131
Average		**20540074**		**1626158.33**
SD		**75256.056**		**8631.405**
% RSD		**0.366**		**0.53**

Repeatability (Intra day precision)

Six consecutive injections of the sample at working concentration showed % RSD less than 2 concerning % assay for both the drugs which indicate that the method developed is method precise by the test of repeatability and hence can be understood that the method gives consistently reproducible results (**Table 3**).

Ruggedness (Intermediate Precision / Inter day precision/)

Six consecutive injections of the sample solution at working concentration on three consecutive days by three different analysts, showed % RSD less than 2 for % assay for both the drugs within and between days, which indicate the method developed is inter day precise / rugged (**Table 4)**.

Linearity

Standard solutions of allopurinol and alpha lipoic acid at different concentrations level (50%, 75%, 100%, 125%, 150% and 175%) were prepared in triplicate. Calibration curves were constructed by plotting the concentration level of drugs versus corresponding mean peak area. The results show an excellent correlation between mean peak area and concentration level of drugs within the concentration range (50-175 µg/ml) for both the drugs and the results are given in **Tables 5-6** and **Figures 8-9.** The correlation coefficient of alpha lipoic acid and allopurinol are 0.9994 & 0.9996 respectively, which meet the method validation acceptance criteria and hence the method is said to be linear in the range of 50-175 µg/ml.

Accuracy

Accuracy was determined by means of recovery experiments, by addition of active drug to preanalyzed sample at different spiked levels (50-150%). At each level, three determinations were performed. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay. The amounts recovered and percent mean recovery were calculated as shown in **Tables 7 and 8.** The accepted limits of recovery are 98% - 102% and all observed data are within the required range which indicates good recovery values and hence the accuracy of the method developed.

Table 3: Intra day precision results

n	Allopurinol	Alpha Lipoic acid
	% Assay	% Assay
1	99.5	99.38
2	99.03	99.75
3	99.49	100.1
4	100.04	99.23
5	101.23	101.9
6	99.02	100.8
Average	99.72	100.19
S.D.	0.83	1
% R.S.D.	0.83	1

Table 4: Inter day precision results

n	% Assay -Allopurinol			% Assay- Alpha lipoic acid		
	Day 1	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
1	99.5	99.1	99.5	99.38	99.18	99.32
2	99.03	100	99.03	99.75	99.2	100.1
3	99.49	99.49	99.1	100.1	101.2	101.4
4	100.04	100.2	100.04	99.23	99.25	99.32
5	101.23	101.1	100.23	101.9	101.1	99.1
6	99.02	99.02	99.43	100.8	99.2	99.4
Average	**99.72**	**99.81**	**99.55**	**100.19**	**99.85**	**99.77**
SD	**0.83**	**0.78**	**0.488**	**1**	**1**	**0.86**
% RSD	**0.83**	**0.78**	**0.48**	**1**	**1**	**0.86**

Table 5: Linearity of the chromatography system

Drugs	Linearity range (µg/ml)	R^2	Slope	Intercept
Allopurinol	50 – 175	0.9994	209626	-455746.45
Alpha lipoic acid	50 – 175	0.9996	15961.2	39842.11

Table 6: Calibration data for Allopurinol and Alpha lipoic acid

% Level	Concentration (µg/ml)	Alpha lipoic acid	Allopurinol
50	50	848572	9906225
75	75	1238925	15365008
100	100	1623186	20623526
125	125	2022092	25582106
150	150	2436884	31170002
175	175	2843233	36116013

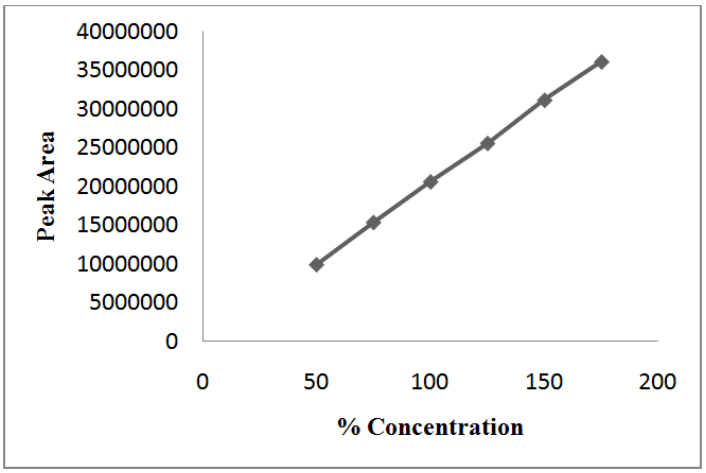

Fig.8: Calibration curve for Allopurinol.

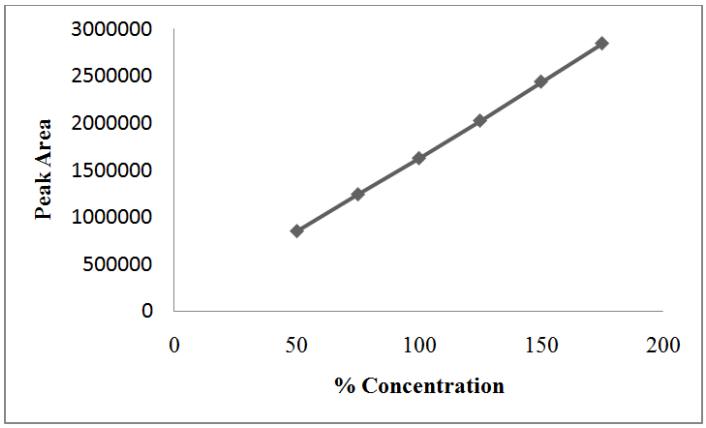

Fig.9: Calibration curve for Alpha lipoic acid.

Table 7: Results of Accuracy studies for Allopurinol

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*%Mean recovery
50	50	50.44	100.88
100	100	101.3	101.3
150	150	149.5	99.66

*Mean of three replicates

Table 8: Results of Accuracy studies for Alpha lipoic acid

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*%Mean recovery
50	50	50.86	101.72
100	100	100.1	100.1
150	150	149.25	99.5

*Mean of three replicates

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is concluded that the method is robust as it is found that the % RSD is less than 1 for both the drugs concerning % assay despite deliberate variations done concerning flow rate (± 0.2), pH (± 0.2) and % organic phase (± 5%).

Sensitivity

The sensitivity of measurement of allopurinol and alpha lipoic acid by use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and the limit of detection (LOD).The limit of detection (LOD) was obtained as 3 ng/ml for allopurinol and 0.5 µg/ml for alpha lipoic acid. The limit of quantitation (LOQ) was obtained as 10 ng/ml for allopurinol and 1µg/ml for alpha lipoic acid.

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity, ruggedness, robustness, limit of detection and limit of quantitation, for the simultaneous quantitative estimation of allopurinol and alpha lipoic acid in tablets. A good linear relationship was observed for both the drugs between concentration ranges of 50 and 175 µg/ml. The correlation coefficients were greater than 0.999 for both the drugs. The inter day and intraday precision results were good enough to say that the method developed is precise and reproducible. Accuracy studies revealed that mean recoveries after spiking experiments were between 98 and 102%, an indicative of accurate method. Accordingly it can be concluded that the developed reverse phase HPLC method is accurate, precise, linear, rugged and robust and therefore the method can be used for the routine analysis of allopurinol and alpha lipoic acid in tablets.

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