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Original Research Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ASSAY OF ZILEUTON IN BULK DRUG AND PHARMACEUTICAL FORMULATIONS

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ABSTRACT

Zileuton is an antiasthmatic durg. It acts by inhibiting 5-lipoxygenase enzyme thereby preventing formation of leukotrienes that cause bronchoconstriction. The new, simple, accurate, sensitive robust, reproducible, isocratic RP-HPLC method was developed using Xterra C 18 column (5µm, 250 × 4.6mm), at 1ml/min flow rate with 10min run time using 20µl injection volume and 25°C temperature conditions. The detection wavelength used is 299.5nm. Retention time is obtained at 3.155min. Linearity was established in the range of 2 to 10µg/ml with 0.999 correlation coefficient and % recovery of 99.6 – 99.9%. The method is validated according to the specifications in ICH guidelines.

Key words: Zileuton, RP-HPLC, isocratic mode.

INTRODUCTION

Zileuton is an antiasthmatic[1-4] drug. It inhibits conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid, thereby preventing formation of leukotrienes. Leukotrienes are substances that induce numerous biological effects including augmentation of neutrophil and eosinophil migration, neutrophil and monocyte aggregation, leukocyte adhesion, increased capillary permeability, and smooth muscle contraction. These effects contribute to inflammation, edema, mucus secretion, and bronchoconstriction in the airways of asthmatic patients. The literature survey revealed a HPTLC[8], UV[6] spectroscopic method, and HPLC[5], preparative hplc[7] were reported. The proposed method is HPLC method with new combination of mobile phase with better results which was validated according to ICH guidelines.

MATERIALS AND METHODS

Reagents and Chemicals: Zileuton bulk drug is a gift sample from RA Chem Pharma Ltd, Hyderabad. And formulation is GRILUTO CR, 600mg tablet by Cadila Health Care Ltd (PHARMA), GOA. Methanol and Acetonitrile are HPLC grade Merck Chemicals. Xterra C 18 column, 5µm, (250×4.6mm) is used for analysis. Millipore water obtained from milli-Q is used for all experimental work.

Preparation of Mobile Phase: Methanol and acetonitrile were taken in the ratio of 70:30(V/V) for 1000ml. i.e. 700ml of methanol and 300ml of acetonitrile were mixed and vacuum filtered through 0.45µm membrane. Filtrate was collected into mobile phase bottles and sonicated for about 5min.

Preparation of Standard Solutions: 50mg of drug was weighed and taken into 50ml volumetric flask. About 10ml of methanol was added to the flask and the drug is dissolved by placing the volumetric flask in the sonicator for around 5min and filtered through 41 whatmann filter paper. The filtered contents are then filled upto volume using methanol to make 1000µg/ml stock solution. Then 100µg/ml working standard solution was prepared by pipetting 10ml of 1000µg/ml solution into 100ml volumetric flask and filling the remaining volume with methanol.

Preparation of Sample Solutions: 600mg tablet was taken and crushed into powder using motor and pestle. Powder equivalent to 50mg of drug was weighed and taken into 50ml volumetric flask. About 20ml of methanol was added to the flask and the drug was dissolved by placing the volumetric flask in the sonicator for around 15min. The contents of the flask were filled

into centrifuge tubes and centrifuged at 2000rpm for 5 min. the supernatant was collected and filtered through the whatmann filter paper 41. Filtered contents were then made upto the volume using methanol to make 1000µg/ml stock sample solution. Then 100µg/ml working sample solution was prepared by pipetting 10ml of 1000µg/ml solution into 100ml volumetric flask and filling the remaining volume with methanol.

Preparation of Dilutions for Calibration curve construction: Dilute the working standard solution (100µg/ml) by pipetting 10ml of working standard solution into 100ml volumetric flask and filling up the volume with methanol to make 10µg/ml concentration solution. Now pipette 2, 4, 6 and 8ml of 10µg/ml solution into 10ml volumetric flasks and fill up the volume to mark with diluents. This gives dilutions of 2, 4, 6, 8 and 10µg/ml solutions respectively.

Optimization of chromatographic conditions: Various Physicochemical factors of drug from literature survey were taken into consideration and chromatographic conditions were set. Experiment was run at 25°C for 10minutes with 1ml/min flow rate and 20µl injection volume. Detection wavelength of 299.5nm was selected after running a scan in the range of 200 – 300nm with 10µg/ml solution. Trails were performed taking methanol:water, acetonitrile:water and methanol:acetonitrile as mobile phases. The best peak with good number of theorietical plates, less retention time and minimum tailing factor was observed with methanol:acetonitrile in the ratio of 70:30 respectively. Methanol was selected as solvent as drug is freely soluble in it.

RESULTS

Validation parameters were calculated according to ICH guidelines - validation of analytical procedures: text and methodology Q2(R1), Food and Drug Administration (FDA) regulations which are reviewed by center for drug evaluation and research (CDER) last updated on 02/21/2013.

Linearity and range: The calibration plots were constructed between concentrations $v_{s.}$ peak areas for prepared dilutions of working standard solution. The Linearity was found in the range of 2µg/ml to 10µg/ml. The linearity parameters such as regression equation, slope, correlation coefficient and Y-intercept calculated were tabulated in table no. 1. The correlation coefficient was found to be within acceptable range.

Precision: According to ICH guidelines three different concentrations in the range of 2.0-10.0µg/ml were taken and their readings are noted in triplicates from which percentage assay is calculated for all the

values. Statistical parameters such as mean, standard deviation and percentage relative standard deviation were calculated for each of the three concentrations both within a day (intraday precision) and between two days, by different analysts (intermediate precision). The percentage assay of each individual sample is between 97% - 102% and percentage RSD is NMT 2%. Hence, the results were found within the limits as shown in the tables 2, 3.

Accuracy: The accuracy test was performed at three different concentration levels of 50%, 100% and 150% i.e. 2.0, 4.0 and 10.0µg/ml solutions with three replicates at each level. Percentage recovery was calculated for all the nine reading from the ratio of amount of drug added by amount of drug found. Further statistical parameters such as mean, standard deviation and percentage relative standard deviation were calculated for percentage recovery data and the results were found to be within the required limits i.e. the mean percentage recovery was found to be within 97% - 103% and %RSD was less than 2% as shown in the table 5.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): Limit of Detection (LOD) was the minimum concentration at which the analyte can be detected without actually being quantitated where as Limit of Quantitation (LOQ) was the minimum concentration at which the analyte response can be taken for quantitation i.e. being able to measure various chromatographic parameters like area of curve, peak height, theatrical plates, %RSD with reliable accuracy and precision. These were obtained by comparing the signal to noise ratio (S/N) of blank and drug at different concentrations. The LOD value was found at 0.02µg/ml concentration where the signal to noise ratio is found to be 3:1 and the LOQ value was found at 0.08µg/ml with a signal to noise ratio of 10:1.

Robustness: The robustness of this method was tested by varying various analytical conditions and comparing the parameters such as retention time, Theoretical plate count and tailing factor.

Stability of Standard and Sample preparations: The standard and sample preparations prepared for assay method were checked for their stability when exposed to normal bench top environmental conditions. These preparations were checked for their stability after 24 hours of preparation of solutions by running the method at optimized chromatographic conditions. The standard preparation is considered stable as symmetry factor is within range of 0.98 to 1.02 and sample preparation is considered stable as the percentage assay was within range of 97% - 103%.

Stability of Mobile phase: The mobile phase was exposed to normal bench top environmental conditions for a period of three days and the assay method was carried out at optimized chromatographic conditions with freshly prepared standard and sample preparations. The percentage assay was calculated for obtained values and was found within the range of 97% - 103%.

Change in wavelength: wavelength was varied by ±1nm of selected wavelength for the optimized conditions and experiment was carried out changing other chromatographic conditions. The change in parameters is reported in the table 6.

Change in temperature: temperature was varied by +5°C of selected temperature for the optimized conditions and experiment was carried out changing other chromatographic conditions. The change in parameters is reported in the table 6.

Change in flow rate: Flow rate was varied by ±1ml of selected flow rate for the optimized conditions and experiment was carried out changing other chromatographic conditions. The change in parameters is reported in the table 6.

System suitability: six replicates of 8µg/ml concentration are injected into the system which was set at optimized chromatographic conditions and parameters such as retention time, peak area, tailing factor and theoretical plate count were compared. The percentage relative standard deviation was found to be less than 2% for both the retention time and peak area as mentioned in the table 7.

Table.1.Linearity parameters and their values

S.No.	Parameters	Values
1.	Concentration Range	2.0 – 10.0 µg/ml
5.	Regression equation (Y)	Y = 37004.x + 7975
6.	Correlation Coefficient r^2	0.999
7.	Slope (m)	370040.8
8.	y-intercept (c)	7975.143

Table.2.linearity data for Zileuton

S.No.	Concentration (µg/ml)	Peak Area
1.	2.0	759246
2.	4.0	1498139
3.	6.0	2196789
4.	8.0	2996279
5.	10.0	3698621

Table.3.Intraday precision studies data

S.No	Concentration (µg/ml)	Sample Peak Area	% Assay	Statistical parameters
1.	2.0	456298	99.65	Mean=99.58
2.		454604	99.28	SD=0.271
3.		457031	99.81	%RSD=0.002
4.	6.0	1375353	100.12	Mean=99.89
5.		1370408	99.76	SD=0.195
6.		1371095	99.81	%RSD=0.001
7.	10.0	2294317	100.21	Mean=100.08
8.		2293172	100.16	SD=0.172
9.		2286990	99.89	%RSD=0.001

Table.4.Intermediate precision studies data

S.No	Concentration (µg/ml)	Sample peak area	% Assay	Statistical parameters
1.	2.0	456665	99.73	Mean=99.67
2.		456482	99.69	SD=0.077
3.		455978	99.58	%RSD=0.0007
4.	6.0	1370820	99.79	Mean=100.08
5.		1374392	100.05	SD=0.370
6.		1373155	99.96	%RSD=0.003
7.	10.0	2294317	100.12	Mean=99.95
8.		2288822	99.97	SD=0.1703
9.		2284472	99.78	%RSD=0.001

Table.5.Accuracy studies at three different concentration levels

S.No.	% Spike level	Amount added (µg/mL)		Amount found (µg/mL)	% Recovery	Statistical parameters
		Std. drug	Sample			
1	50	2.0	4.0	5.97	99.5	Mean=99.667 SD=0.134 %RSD=0.135
2		2.0	4.0	5.99	99.83	
3		2.0	4.0	5.98	99.67	
4	100	4.0	4.0	8.01	100.12	Mean=99.996 SD=0.165 %RSD=0.188
5		4.0	4.0	7.99	99.87	
6		4.0	4.0	8.00	100.0	
7	150	6.0	4.0	9.98	99.8	Mean=99.996 SD=0.125 %RSD=0.188
8		6.0	4.0	9.98	99.8	
9		6.0	4.0	10.02	100.2	

Table.6.Robustness data

Parameters	Variation in parameter	Retention time	Tailing factor	Theoretical plates
Wavelength (±1nm)	228.5nm	3.131	1.01	7869
	229.5nm	3.152	1.0	7751
	230.5nm	3.14	1.0	7579
Temperature (+5°C)	**25°C**	3.155	1.0	7669
	30°C	3.012	1.12	7186
Flow rate (±0.1ml/min)	0.9ml/min	3.26	1.0	7814
	1.0ml/min	3.15	1.01	7756
	1.1ml/min	3.01	1.1	6942

Table.7.System suitability parameters for proposed method

S.No.	Retention time	Peak area	Tailing Factor	Theoretical plate count
1	3.155	2996279	1.01	7669.59
2	3.151	2971942	1.01	7678.89
3	3.151	2986145	1.0	7834.53
4	3.153	2983385	1.0	7761.76
5	3.154	2995648	1.02	7689.41
6	3.151	2966894	1.0	7886.71
Mean	**3.1525**	**2983382**	-	–
SD	**0.0017**	**11008.7**	-	-
%RSD	**0.055**	**0.369**	-	-

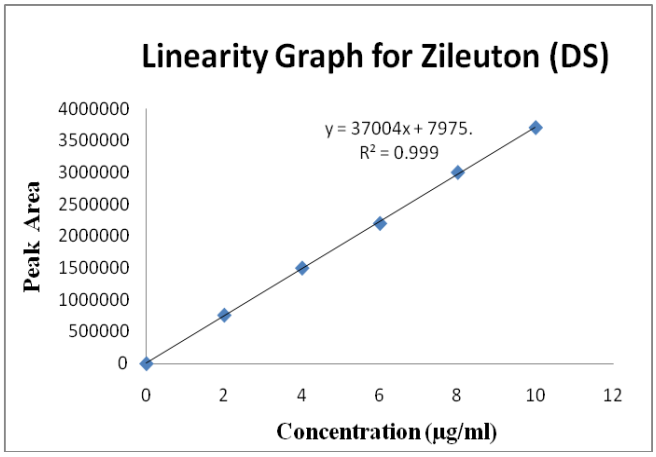

Fig.1.linearity plot of zileuton

Chromatograms for Linearity and Range calculation:

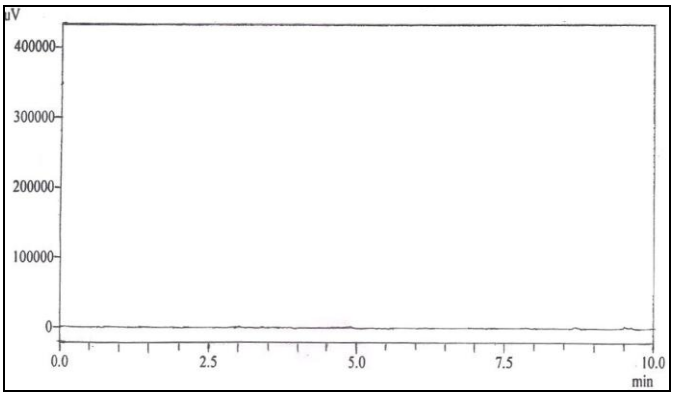

Fig.2.Blank Chromatogram

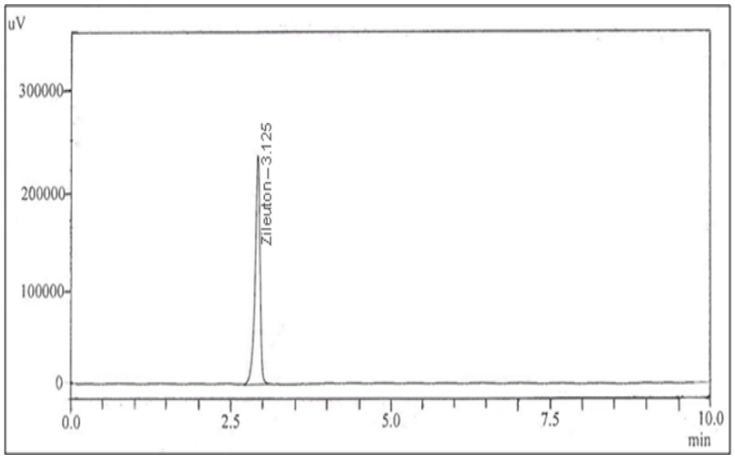

Fig.3.Chromatogram of 2µg/ml concentration

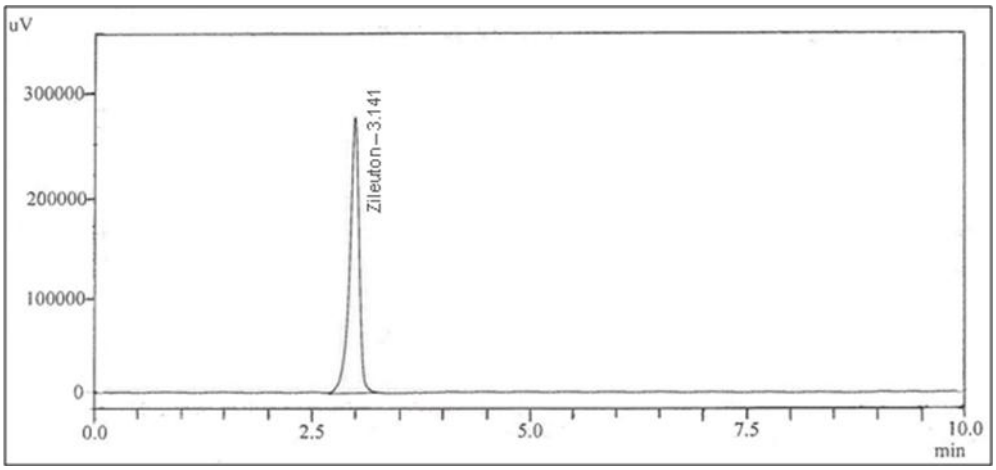

Fig.4.Chromatogram of 4µg/ml concentration

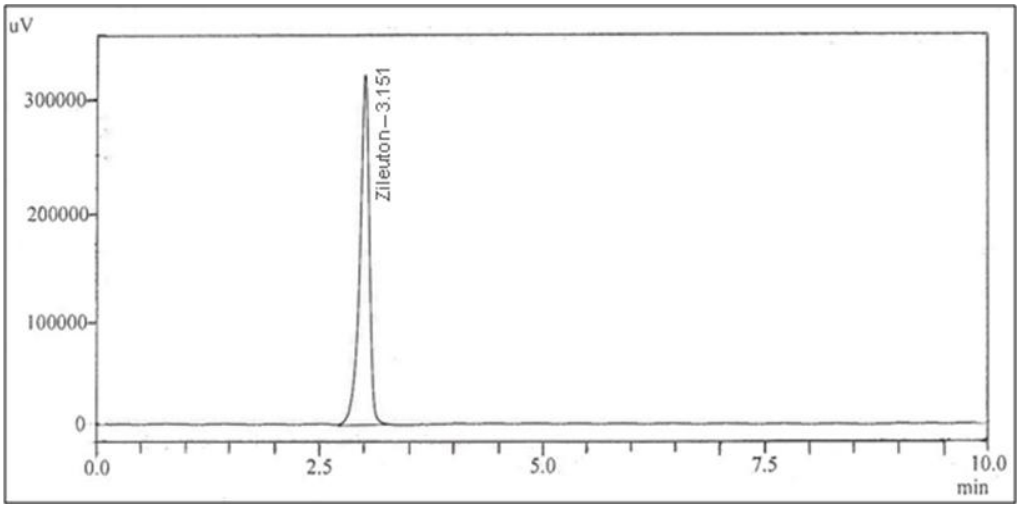

Fig.5.Chromatogram of 6µg/ml concentration

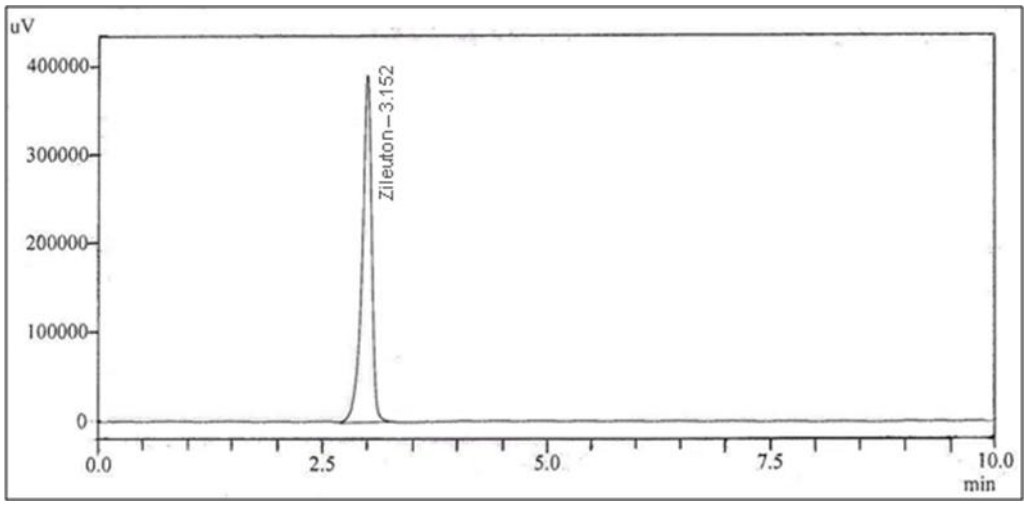

Fig.6.Chromatogram of 8µg/ml concentration

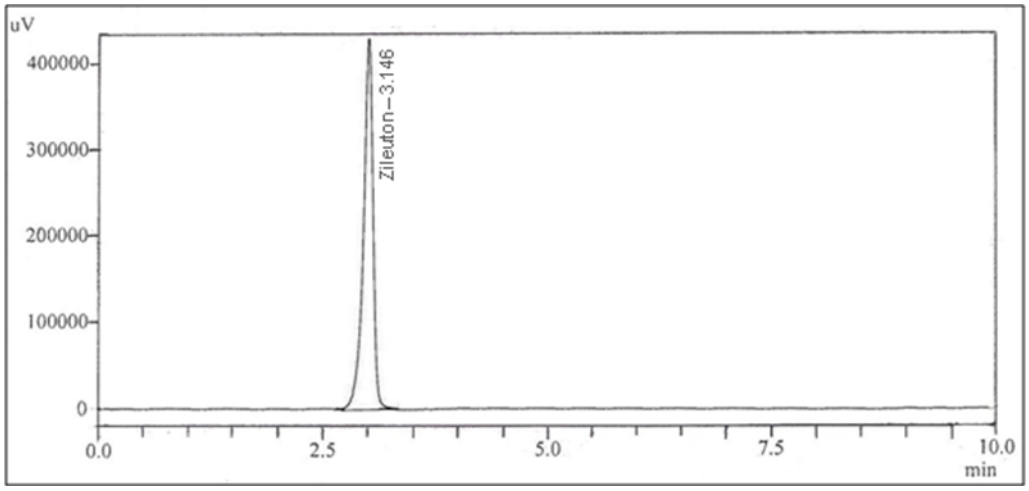

Fig.7.Chromatogram of 10µg/ml concentration

DISSCUSION

In HPLC method development for the drug zileuton the initial chromatographic conditions were selected hypothetically from the bookish knowledge and initial trail was conducted with 10minutes run time using XTerra RP 18 Column (5µm, 250×4.6mm) at room temperature conditions, 1ml/min flow rate and isocratic elution mode. The detection wavelength was fixed by scanning the working standard solution and noting the maximum absorbance wavelength which was found to be 299.5nm. The mobile phase composition (methanol: acetonitrile) was selected after conducting several trails when a good peak satisfying all performance parameters was observed. The retention time was found at 3.155. The quantitative linearity was found in the Concentration range of 2 to 10µg/ml.

The regression equation for linear range of concentrations was found to be Y = 37004.x + 7975. The limit of detection and limit of quantification were found at 0.02µg/ml and 0.08µg/ml respectively indicating the sensitivity of the method. The high levels of recovery found at three different proposed ranges indicate the accuracy of the method. No significant change was observed when the results for precision were checked from both within the day and between two days proving the proposed method to be precise. The method was checked for robustness at 5°C change in temperature, wavelength and flow rate difference of ±1 of proposed wavelength and flow rate. Accountable change in the results was not observed proving the proposed method to be robust.

CONCLUSION

The studies of results for proposed RP-HPLC method indicate it is simple as it does not involve any sophisticated treatments of samples or reagents. Its short run time makes the method a time saving process. Due to use of organic solvents the column life time does not deteriorate in long and repeated runs. Maximum recovery indicates the accuracy of method. It is also reproducible and robust. Thus, it can be used in both industrial and laboratory scale for estimation of zileuton quantitatively in both bulk and tablet dosage forms.

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