AMERICAN JOURNAL OF
PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

Method development for Determination of Related substances of Zafirlukast and validation by RP-RRLC method

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ABSTRACT

Zafirlukast, Cyclopentyl (3-(2-methoxy-4-((o-tolylsulfonyl) carbamoyl) benzyl)-1-methyl-1H-indol-5-yl) carbamate (I). A high-performance liquid chromatographic (HPLC) reversed-phase rapid resolution method has been developed and validated for estimation of zafirlukast in a pharmaceutical active pharmaceutical ingredient, which was chromatographed on reversed-phase Hypersil Gold C_{18} , 2.1 x 100 mm , 1.9 µ column using mixtures of acetonitrile/water and the eluents were monitored at different wavelengths. The method was validated statistically for its linearity, accuracy, robustness and precision. The method employed same chromatographic condition as above and validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and solution stability. Present work also describes the development and validation of RP- RRLC method for the determination of impurities of zafirlukast.

Keywords: Zafirlukast, Reversed phase high performance liquid chromatography, Validation, Precision, Related substances (Impurities)

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Received 29 January 2018, Accepted 09 February 2018

Please cite this article as: Mohan K *et al*., Method development for Determination of Related substances of Zafirlukast and validation by RP-RRLC method. American Journal of PharmTech Research 2018.

INTRODUCTION

Zafirlukast belongs to the general class of leukotriene receptor antagonists(1), chemically known as Cyclopentyl (3-(2-methoxy-4-((o-tolylsulfonyl) carbamoyl)benzyl)-1-methyl-1H-indol-5-yl)carbamate(2)(I), exists in a fine white to pale yellow amorphous powder. Solubility includes slightly soluble in methanol and freely soluble in tetrahydrofuran, acetone, and dimethyl sulfoxideonitrile and is insoluble in water (3-5). Numerous analytical techniques were reported for the determination of drug and metabolites in plasma by NP-HPLC with fluorescence detection (6), RP-HPLC method (7) and LC-MS(8) .Some other analytical techniques such as Spectrophotometric method (9), RP-HPLC method (10) and HPLC method (11) with chiral stationary phase were used for analysis of drug, impurities and isomer present in the bulk drug and formulation. While preparation of products and during storage of the drug substances can found Organic impurities and the criteria for their acceptance up to certain limits are based on pharmaceutical studies or known safety data (12, 13). As per regulatory guidelines, the pharmaceutical studies using sample of the isolated impurities can be considered for safety assessment. Present RP-RRLC eventually a new technique was developed for the analysis of four impurities present in the laboratory sample of title compound. The impurities isolated from the filtrates upon crystallization of desired product. The accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) and robustness of the method were determined in accordance with ICH guidelines (12-13)

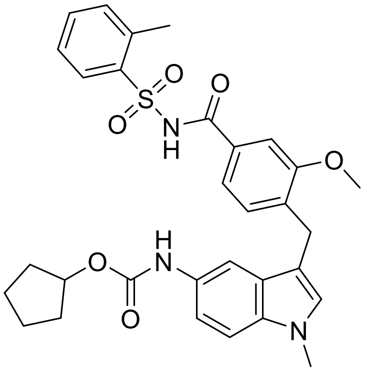

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Figure 1: Structure of Zafirlukast

MATERIALS AND METHOD

Instruments: Rapid Resolution liquid chromatogram (Agilent 1200 series) equipped with Binary pump SL with maximum pressure 600bar, Auto sampler micro cell) and data handling system **(**temptation software)., UV-Detector(Semi Analytical balance, pH meter, Sonicator, Filtration unit and 0.22µ membrane filters.

Standard, Chemicals and Reagents:

Zafirlukast(ZAF1)working standard, Zafirlukast(ZAF1) Sample, Zafirlukast(ZAF1) Impurities(MAM Imp-1, MAM Imp-2, ZAF Imp1,ZAF Imp2) Potassium dihydrogen orthophosphate , Sodium lauryl sulphate, Ortho Phosphoric acid (85%), Acetonitrile, HPLC grade, Methanol, HPLC grade, Milli Q Water, HPLC grade.

Impurity Chemical Names:

MAM (Imp-1) **:** 3-methoxy-4-((1-methyl-5-nitro-1H-indol-3-yl)methyl)benzoic acid

MAM (Imp-2) **:** 4-((5-amino-1-methyl-1H-indol-3-yl)methyl)-3-methoxybenzoic acid

ZAF-1(Imp-1) **:** 3-methoxy-4-((1-methyl-5-nitro-1H-indol-3-yl)methyl)-N-(o-tolylsulfonyl)benzamide

ZAF-1(Imp-2): 4-((5-amino-1-methyl-1H-indol-3-yl)methyl)-3-methoxy-N-(o-tolylsulfonyl)benzamide

Preparation of Mobile phase :

Mobile phase-A:

Dissolved 1.36g (0.01M) of Potassium Di hydrogen phosphate in 1000ml of water and mixed, pH adjusted to 3.0 with diluted 85% of $H3PO4$ and added 0.29 g (0.001M) of Sodium lauryl sulphate. Filter the solution through 0.22µ membrane filter and sonicated to degas the buffer.

Mobile phase-B:

Transferred 850ml of Acetonitrile, 100 ml of Methanol and 50ml of Water into a 1000ml Mobile phase bottle and mixed. Filtered the solution through 0.22µ membrane filter and sonicated to degas the diluent.

Preparation of Diluent:

Transferred 800ml of Acetonitrile and 200 ml of Water into a 1000ml Mobile phase bottle and mixed. Filtered the solution through 0.22µ membrane filter and sonicated to degas the diluent

Preparation of Standard:

Accurately weighed about 10mg working standard (ZAF1) and transferred into a 10ml volumetric flask, added 5ml of diluent, and sonicated to dissolve. and diluted to volume with diluent.

Preparation of Sample:

Accurately weighed about 10mg Sample (ZAF1) and transferred into a 10ml volumetric flask, added 5ml of diluent, and sonicated to dissolve. and diluted to volume with diluent.

Preparation of impurity blend stock solution

Accurately weighed about 10mg each of Impurity-A, MAM (imp-1), MAM (imp-2), ZAF-1(imp-1), and ZAF (imp-2) and transferred into a 10ml volumetric flask, added 5ml of diluent. And diluted to volume with diluent

Table 1: Chromatographic Conditions:

Time (min.)	Solution A (%v/v)	Solution B (%v/v)
0	40	60
8	20	80
12.0	40	60
14.0	40	60

Mobile Phase **:** Solution A+ Solution B

Elution **:** Gradient

Column **:** Hypersil Gold C18, 2.1 x 100 mm, 1.9 µ

Temperature **:** Ambient

Flow rate **:** 0.4 ml/min **Wave length** **:** 215 nm

Injection Volume: 3µL **Runtime** **:** 14 mins

Validation of Method:

Accuracy and precision: Recovery samples were prepared using solutions at concentrations spanning from 50%, 100%, 150% of the all impurities and analyte with respect to the specification limit. Three preparations were made at each level except the 100% level, where six preparations are made. Each solution was injected once and analyzed.

Linearity:

Linearity(14) was performed to assess whether a linear relationship is obtained between the response and the concentration of ZAF1 related substances over the intended operating range of the method.

For related substances, linearity was performed for ZAF1 related compounds (impurities) from LOQ level to 150.0% level with respect to individual specification of ZAF1 and its impurities. A single injection of each linearity solution was analyzed.

The percentage recovery was calculated for related compounds and for the individual preparation at each level and a mean of the recovery was determined at each level and %RSD was calculated.

Table 2: Solution preparation for Range (accuracy, precision and linearity) study

Stock	Preparation
Impurity stock solution(1.0 mg/ml)	Accurately weighed 10 mg of individual impurity A, ,MAM(imp-1),MAM(imp-2),ZAF-1(imp 1), and ZAF-1(imp-2), each in 10ml volumetric flasks & makeup with diluent..
Sample solution (1.0 mg/ml)	Accurately weighed 10 mg of ZAF 1 Sample in 10ml volumetric flasks & makeup with diluent.
LOQ solution	Accurately weighed 10.2 mg of ZAF1 in 10ml diluent + 0.5µl of MAM(imp-1)+ 0.3 µl MAM(imp-2) + 3µl of ZAF-1(IMP-1).

Detection and Quantification limit:

The detection limit (15) was determined by the analysis of a standard solution with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected. The limit of quantification was determined by the analysis of standard solutions with known concentration of analyte and by establishing the minimum level at which the analyte can be quantified with an acceptable precision.

Table 3: LOD and LOQ sample preparation (Related substances).

Impurity Names	Impurity stock	LOQ	LOD
MAM-Imp1	1mg/ml	From the stock solution take 0.5µl in 10ml volumetric flasks & makeup with diluent.	From the stock solution take 0.9µl in 50ml volumetric flasks & makeup with diluent
MAM-Imp2	1mg/ml	From the stock solution take 0.3µl in 10ml volumetric flasks & makeup with diluent	From the stock solution take 0.5µl in 50ml volumetric flasks & makeup with diluent
ZAF-Imp1	1mg/ml	From the stock solution take 0.8µl in 10ml volumetric flasks & makeup with diluent	From the stock solution take 5µl in 50ml volumetric flasks & makeup with diluent
ZAF-Imp2	1mg/ml	From the stock solution take 0.4µl in 10ml volumetric flasks & makeup with diluent	From the stock solution take 0.7µl in 50ml volumetric flasks & makeup with diluent

The limit of detection and limit of quantification for ZAF1 and It's related compound (impurities) was determined using the S/N Ratio method.

The LOQ and LOD results for ZAF1 and Its related compound are presented

Precision at Quantification (16) (Related substances).

Test preparation at LOQ level was prepared and analyzed in replicates (six injections) and the % RSD of the peak area of individual impurity is calculated and reported.

Table 4: Solution preparations for Precision at LOQ study

Sample	Preparation
Impurity solution(1mg/ml)	Accurately weighed 10 mg of individual impurity A, MAM(imp-1),MAM(imp-2),ZAF-1(imp 1), and ZAF-1(imp-2), each in 10ml volumetric flasks & makeup with diluent..

Accuracy at limit of quantification (17) (Related substances).

Recovery solutions of ZAF1 sample spiked with the It's related compounds (impurities) at the estimated LOQ level was prepared and analyzed to establish the accuracy at LOQ. Three preparations were made and a single injection was given from each preparation. The percentage recovery was calculated for the individual preparation at each level and a mean of the recovery was determined at each level.

Table 5: Solution preparation for accuracy at LOQ

Stock	Preparation
Related compound standard stock solution	Accurately weighed 10 mg of individual impurity A, MAM(imp-1),MAM(imp-2),ZAF-1(imp 1), and ZAF-1(imp-2), each in 10ml volumetric flasks & makeup with diluent..
Sample solution (0.03mg/ml)	Accurately weighed 3 mg of ZAF1 in 100ml volumetric flask and make up with diluent.
LOQ	Accurately weighed 3.0mg ,3.1 mg and 3.2 mg of ZAF1 each in three different flask containing 100ml diluent + 0.5µl of MAM(imp-1)+ 0.3 µl ZAF(imp-2) +3µl of ZAF-1(imp-1)+0.4 µl of MAM (imp-2).

Solution stability and mobile phase stability:

One of the standard , sample preparation and spiked sample at 100% level of the method concentration for related substances were stored at room temperature and on bench top and were analyzed periodically to establish the stability.

One of the standard , sample preparation for assay were prepared and stored on bench top over a period of 24 hours and were analysed to establish the solution stability and mobile phase stability.

The solution stored at room temperature was analyzed against a freshly prepared standard solution on Day-0&1 to establish the stability.

Intermediate Precision (Ruggedness)

In order to demonstrate ruggedness, six different sample preparations at the 100% level were analyzed by another chemist on another system. The two chemists analyzed the different sample preparations in different days using different standard preparations, different mobile phase lots, columns, and different HPLC systems. The % related substances were determined for each of the sample preparation. .

Robustness of Experimental parameters:

The method robustness measures the ability of the analytical method to tolerate minor variations in the method recommended parameters, demonstrating the reliability of the method under normal use. The following parameters were studied for this particular method.

Mobile phase composition:

The effects of change in mobile phase composition were evaluated by analyzing the standard solution, a sample solution, and related compounds spiked solution using two different mobile phase composition (90% and 110%).

Column temperature:

The effects of column temperature were evaluated by the standard solution, a sample solution, and ZAF1 related compound spiked solution using two different column temperatures, viz., 27°C (Control) ,22°C and 32°C.

Mobile Phase pH:

The effects of change in mobile phase pH were evaluated by analyzing the standard solution, a sample solution, and a ZAF1 related compounds spiked solution using two different mobile phase pH values with 2.8and 3.2.

Flow rate:

The effects of change in mobile phase flow rates were evaluated by analyzing the standard solution, a sample solution, and a related compound spike solution using two different flow rates with 0.3 mL/min and 0.5 mL/min

RESULTS AND DISCUSSION:

Table-6: Range Study (Related substance)

S.No	Impurity Name	RRF	Test (1 &2Avg)	% area obtained for known impurity		
				50% (avg)	100%(avg)	150%(avg)
1	MAM Imp-1	1.24	0	0.0682	0.135	0.1922
2	MAM Imp-2	1.18	0	0.066	0.1286	0.1912
3	ZAF1 Imp-1	0.23	0.045	0.070	0.1175	0.1695
4	ZAF1 Imp-2	0.97	0.2	0.456	0.723	0.9942

Table 7: %Recovery

S.No	Impurity Name	%impurity at spiked level in blank	Recovery			% Recovery		
			50%	100%	150%	50%	100%	150%
1	MAM Imp-1	0.1	55	109	155	110	109	103.3
2	MAM Imp-2	0.1	56	109	162	112	109	108
3	ZAF1 Imp-1	0.5	48	93.2	138.4	96	93.2	92
4	ZAF1 Imp-2	0.5	54	109	165	108	109	110

Table 8: Spiked Impurity

% Impurity(spiked)		
50% (Avg)	100% (Avg)	150% (Avg)
0.055	0.109	0.155
0.175	0.340	0.542
0.056	0.109	0.162
0.105	0.142	0.207
0.057	0.113	0.178
0.305	0.511	0.737
0.47	0.745	1.025

Table 9: Precision results for related substances:

Preparation	MAM IMP-1	MAM IMP-2	ZAF1 IMP-1	ZAF1 IMP-2
1	0.109	0.109	0.509	0.753
2	0.109	0.109	0.514	0.758
3	0.109	0.109	0.512	0.752
4	0.109	0.111	0.517	0.757
5	0.110	0.110	0.517	0.760
6	0.109	0.110	0.517	0.758
Mean	0.109	0.110	0.514	0.756
SD	0.00041	0.001	0.003	0.003
%RSD	0.37	0.680	0.590	0.379

Table 10: Linearity results of MAM Imp-1:

Level	Concentration(%)	Area
LOQ	0.00005	0.4045
25%	0.0250	9.1000
50%	0.0500	15.0010
75%	0.0750	23.1014
100%	0.1000	29.5064
125%	0.1250	36.5010
150%	0.1500	42.6909
Correlation		0.9990
Y-intercept		1.3050
Y-intercept at 100% level		4.4

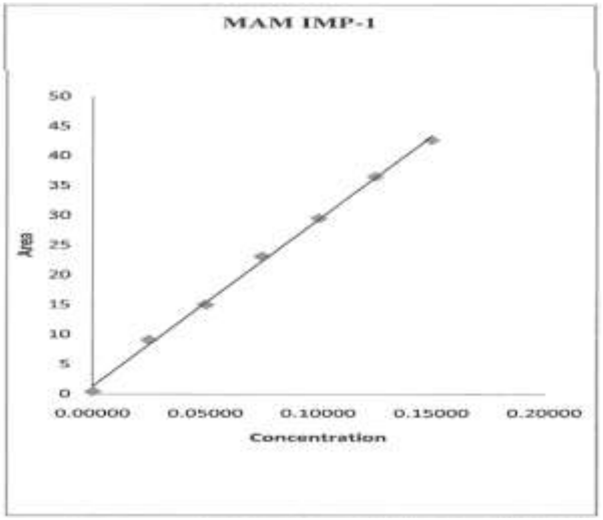

Figure 2: Linearity of MAM Imp-1

Table 11: Linearity results of MAM Imp-2

Level	Concentration	Area
LOQ	0.00003	1.1210
25%	0.0250	6.7934
50%	0.050	14.7206
75%	0.075	21.0376
100%	0.100	27.3579
125%	0.125	35.9598
150%	0.150	42.3832
Correlation		0.9991
Y-intercept		0.4683
Y-intercept at 100% level		

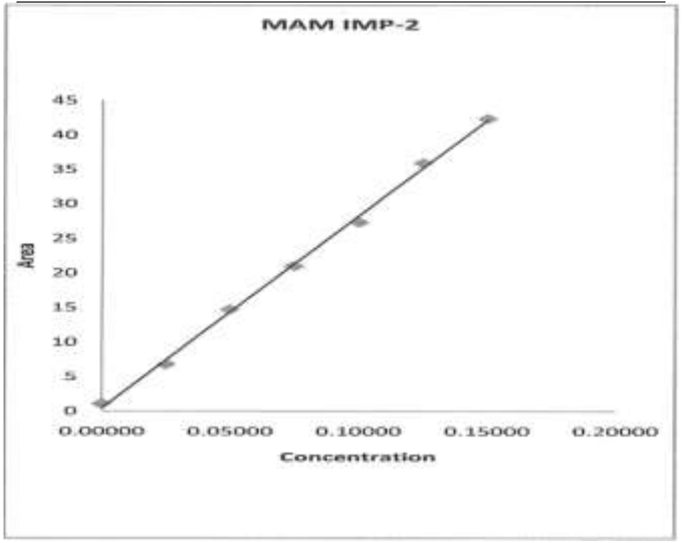

Figure-3: Linearity of MAM Imp-2

Table 12: Linearity results of ZAF1 Imp-1

Level	Concentration (%)	Area
LOQ	0.0003	1.3009
25%	0.125	7.4000
50%	0.250	12.8001
75%	0.375	19.0588
100%	0.500	24.9300
125%	0.625	30.7241
150%	0.750	37.1233
Correlation		0.9999
Y-intercept		1.2295
Y-intercept at 100% level		4.9

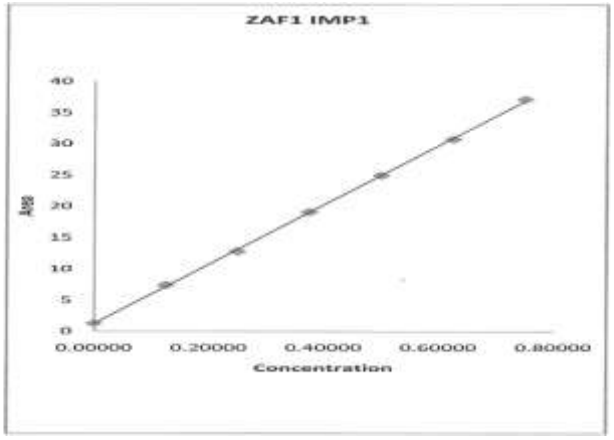

Figure 4: Linearity of ZAF Imp-1

Table 13: Linearity results of ZAF1 Imp-2

Level	Concentration (%)	Area
LOQ	0.00004	1.4662
25%	0.125	46.5500
50%	0.250	83.0000
75%	0.375	118.0000
100%	0.500	154.5000
125%	0.625	187.1912
150%	0.750	221.3333
Correlation		0.9990
Y-intercept		7.5309
Y-intercept at 100% level		4.9

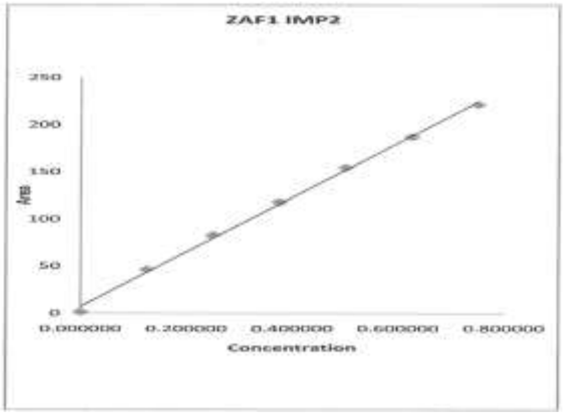

Figure-5: Linearity of ZAF1 Imp-2

LOQ results:

Table 14: Detection and Quantification limit:

S.No	Impurity Names	LOQ Solution		
		Conc W.r.t test (mg/ml)	Conc W.r.t test (%)	S/N ratio
1	MAM Imp-1	0.00005	0.005	10.1
2	MAM Imp-2	0.00003	0.003	10.1
3	ZAF1 Imp-1	0.0003	0.03	10.3
4	ZAF1 Imp-2	0.00004	0.004	9.9

Table 15*:* LOD results:

S.No	Impurity Names	LOD Solution		
		Conc W.r.t test (mg/ml)	ConcW.r.t test (%	S/N ratio
1	MAM Imp-1	0.000018	0.0018	2.9
2	MAM Imp-2	0.00001	0.001	2.9
3	ZAF1 Imp-1	0.0001	0.01	2.1
4	ZAF1 Imp-2	0.000014	0.0014	2.3

Table 16: Accuracy at limit of quantification:

S.No	Impurity Name	% impurity at spiked level in blank	% Recovery			Avg
			Preparation 1	Preparation 2	Preparation 3	
1	MAM Imp-1	0.425	107.95	126.16	116.16	116.75
2	MAM Imp-2	1.051	78.47	79.96	83.22	80.55
3	ZAF1 Imp-1	1.045	115.71	120.16	122.54	119.47
4	ZAF1 Imp-2	1.249	117.48	119.10	123.33	119.97

Table-17: Precision at Quantification:

Preparation	MAM IMP-1	MAM IMP-2	ZAF1 IMP-1	ZAF1 IMP-2
1	0.456	1.216	1.248	1.265
2	0.484	1.191	1.327	1.393
3	0.466	1.182	1.182	1.282
4	0.463	1.177	1.222	1.324
5	0.427	1.242	1.250	1.340
6	0.425	1.174	1.274	1.386

Mean	0.453	1.197	1.251	1.331
SD	0.02317	0.025	0.045	0.048
%RSD	5.11	2.048	3.56	3.59

Table-18: Solution stability results

Impurity	Solution stability		Acceptance criteria
	Initial	24 hours	
MAM Imp-1	0.109	0.11	If the specification limits of impurities is 0.1% to 0.5%the variation shall be within +/_20%
MAM Imp-2	0.110	0.109	
ZAF1 Imp-1	0.514	0.500	
ZAF1 Imp-2	0.753	0.74	

Table-19: Mobile phase stability results

Impurity	Mobile phase stability		Acceptance criteria
	Initial	24hours	
MAM Imp-1	0.109	0.11	If the specification limits of impurities is 0.1% to 0.5%the variation shall be within +/_20%
MAM Imp-2	0.110	0.11	
ZAF1 Imp-1	0.514	0.5	
ZAF1 Imp-2	0.753	0.75	

Table-20: Intermediate Precision results

Preparation	MAM imp-1	MAM imp-2	zaf1 imp-1	zaf1 imp-2
1	0.084	0.092	0.43	0.632
2	0.086	0.092	0.433	0.635
3	0.086	0.092	0.434	0.635
4	0.085	0.092	0.435	0.635
5	0.085	0.091	0.432	0.632
6	0.085	0.092	0.433	0.632
Mean	0.08517	0.09183	0.43283	0.633
SD	0.00069	0.00037	0.00157	0.001
%RSD	0.8069	0.4058	0.36326	0.2368

Table-21: Robustness results :

Sl. No.	Parameter Robustness	MAM imp-1	MAM imp-2	ZAF imp-1	ZAF imp-2
1	Ideal condition	0.190	0.301	1.48	1.60
2	90%Acetonitrile	0.190	0.303	1.47	1.60
3	110% Acetonitrile	0.192	0.301	1.49	1.62
4	90% Methanol	0.191	0.30	1.52	1.64
5	110% Methanol	0.188	0.30	1.47	1.60
6	Flow rate 0.3ml/min	0.194	0.30	1.45	1.60
7	Flow rate 0.5ml/min	0.185	0.31	1.52	1.64
8	Column temp 22^0C	0.183	0.30	1.46	1.60
9	Column temp 32^0C	0.194	0.310	1.50	1.62
10	Buffer pH 2.8	0.183	0.30	1.47	1.60
11	Buffer pH 3.2	0.191	0.310	1.46	1.60

HPLC Chromatograms:

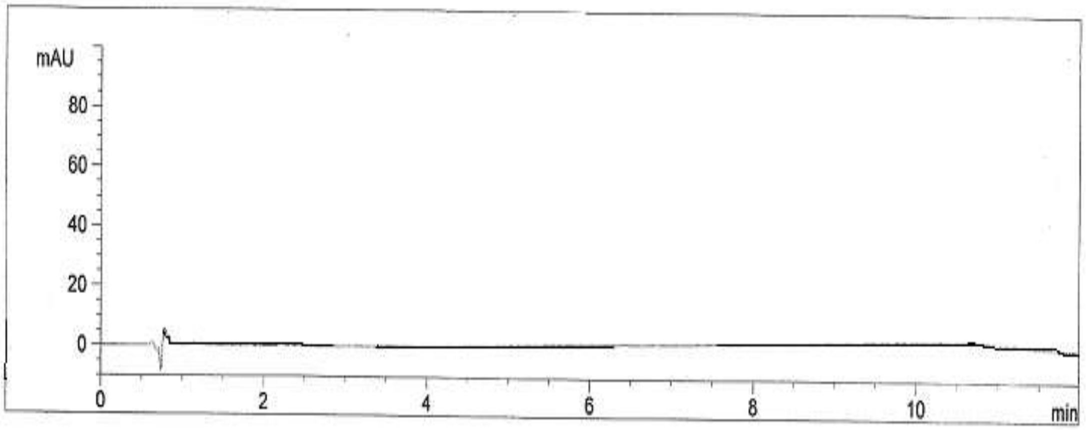

Figure 6: Typical Blank chromatogram of ZAF1

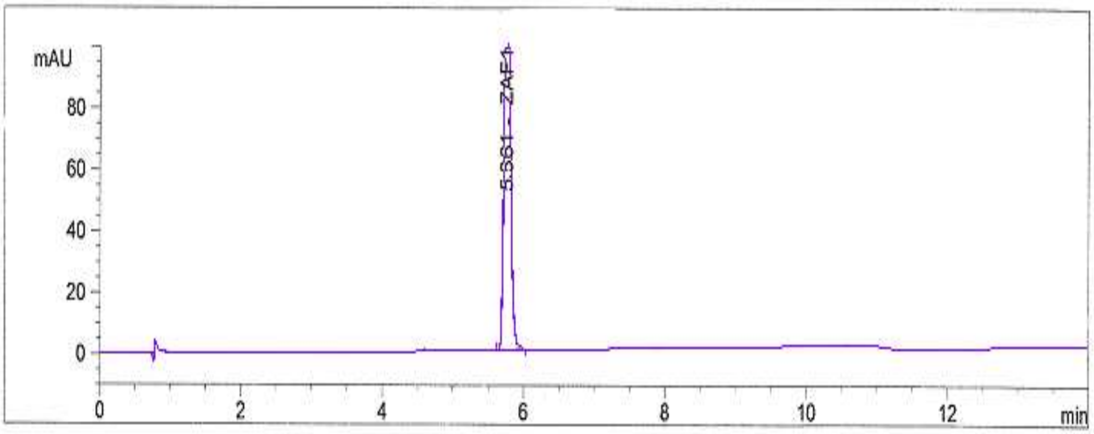

Figure 7: Typical standard chromatogram of ZAF1 standard

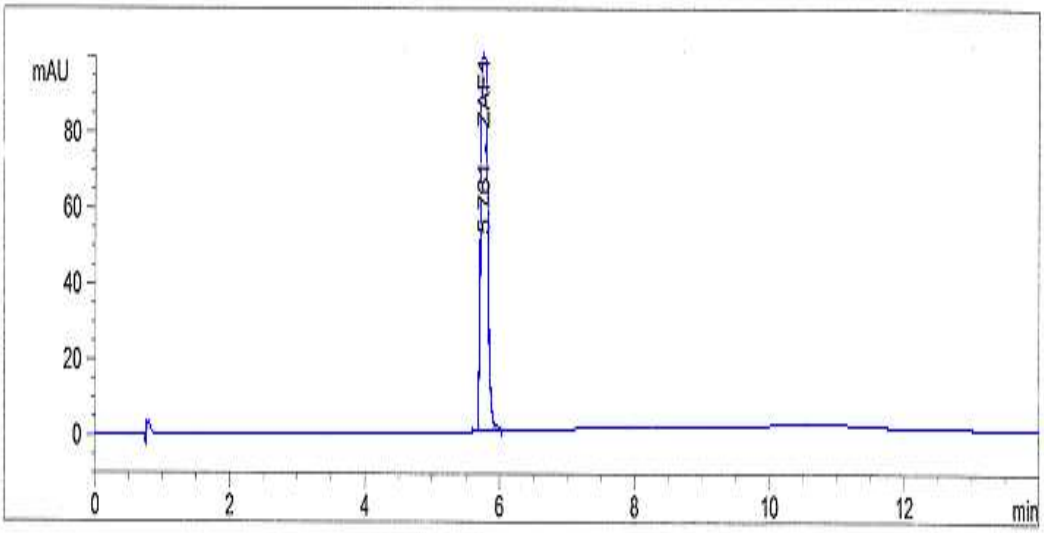

Figure 8: Typical chromatogram of ZAF1 sample

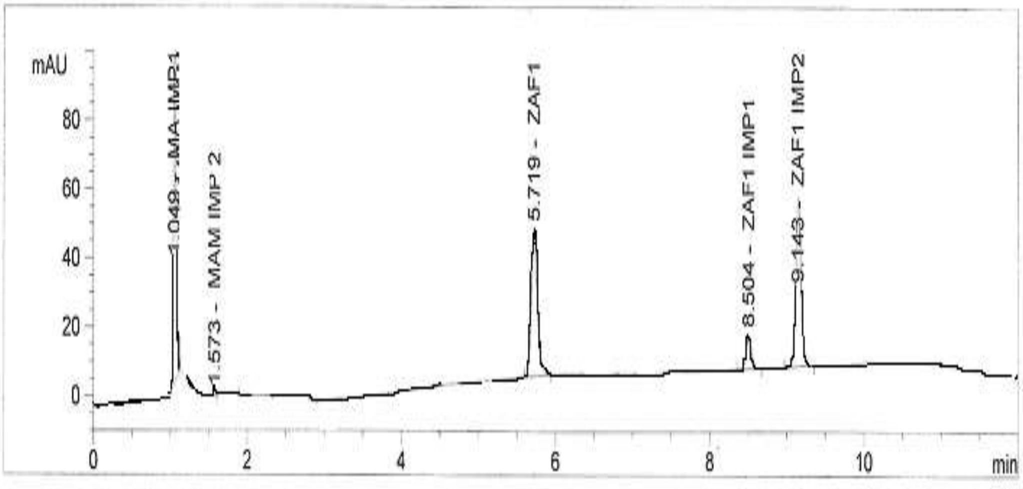

Figure 9: Typical blend chromatogram of ZAF1

CONCLUSION:

The developed method of reverse phase-rapid resolution analytical technique was selective, rapid, accurate and précised. Therefore, the method is used for the analysis of zafirlukast and its related substances and validated. The percentage RSD for all parameters was found within the guidelines limit, which indicates that validity of the method in fair agreement. The method is repeatable rugged and robust.

ACKNOWLEDGMENTS:

The Author is thankful to KL University for the support rendered to the Team.

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