Development and validation of a regioselective, specific, stability indicating LC and assay method for zafirlukast

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ABSTRACT

Zafirlukast is a selective and competitive orally-administered inhibitor of the cysteinyl leucotrienes and is currently indicated for the prophylaxis and treatment of chronic asthma. A simple, rapid, and reliable LC method for the determination of ZAF in pharmaceutical drug substances and drug products was developed and validated. The influence of buffer concentration, buffer pH, organic modifier, column temperature, ion-pair reagent and injection volume was systemically investigated in octadecyl columns (ID 46 mm, length 25 cm, practical size 5 μm). Optimum results were obtained with 10 mM potassium buffer, 5 mM of 1-decane sulphonic sodium salt at pH 4.0, and a column temperature of 27 °C. The detection wavelength was set at 220 nm. The method was suitably validated with respect to linearity, limit of detection and quantification, accuracy, precision, selectivity, robustness and ruggedness. The linear calibration range had a LOQ of 1.5 μg mL⁻¹, and the limit of detection and quantification were 0.009 and 0.06 μg mL⁻¹, respectively, with a relative standard deviation of 6.7%. The proposed method was applied for the determination of ZAF in its pharmaceutical drug substances and drug products. The results obtained from the developed method were compared to an LC method reported in the literature, and no significant statistical differences were found. Assay and RS methods were fully validated and a comparison was made. The results confirm that the methods are highly suitable for their intended purpose.

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INTRODUCTION

Zafirlukast is chemically described as 4-(5-cyclopentyloxy-carbonylamino-1-methyl-indol-3-yl-methyl) methoxy-N-o-tolylsulfonylbenzamide. It is used in the treatment of asthma, often in conjunction with an inhaled steroid and/or long-acting broncho dilator. Its empirical formula is $C_{31}H_{33}N_3O_6S$ and its molecular weight is 575.7 g/mol.

Zafirlukast is an oral leucotriene receptor antagonist (LTRA) that blocks the action of the cysteinyl leucotrienes on the CysLT1 receptors, thus reducing constriction of the airways, build-up of mucus in the lungs, and inflammation of the breathing passages.

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Zafirlukast is available in tablet form under the brand name Accolate and is usually dosed twice daily.

Limited LC methods have been reported in the literature. Furthermore, T. Radhakrishna et al. developed an LC method and derived spectrophotometric methods for the determination of zafirlukast[1]. Ficarra et al. described an LC method for the analysis of zafirlukast in its pharmaceutical formulation[2]. Khanh H. Bui et al. reported a normal phase LC method for the determination of zafirlukast in human plasma using fluorescence detection[3]. J.D. Fischer et al. reported on the quantitative determination of zafirlukast using HPLC in human plasma during the comparison of zafirlukast absorption after oral and colonic administration[4].

Attempts were made to develop a single LC method that could be used to determine nineteen process-related

impurities and metabolites in bulk samples of zafirlukast. This manuscript deals with the development of a stability-indicating analytical method using samples generated from forced degradation studies. Although nineteen impurities can be detected and separated with reasonable resolution by this single method, nevertheless only five process-related impurities are monitored as known impurities due to the absence of remaining impurities in the finished product[5-8]. The developed method was validated to meet the stipulations of the ICH guidelines[9-12].

MATERIALS AND METHODS

Chemicals

Samples of zafirlukast and its five impurities (Figure 1) were received from the Research and Development
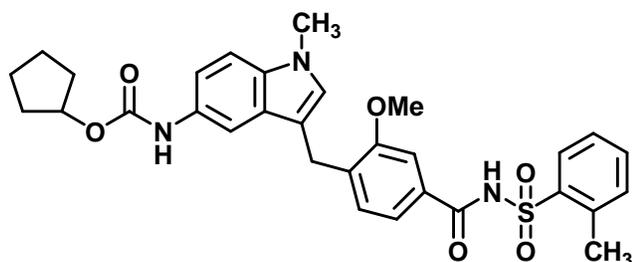
Zafirlukast structure (a)
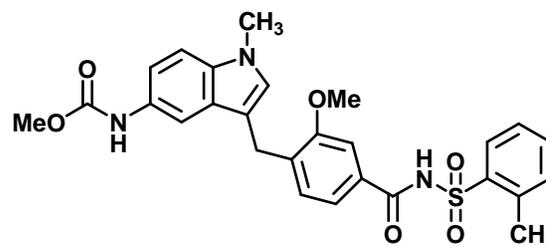
Imp-1(b)
N- {4-[5-(methoxy carbonyl) amino-1-methylindol-3-yl-methyl]-3-methoxy benzoyl}-2-methyl benzene sulpho- namide.
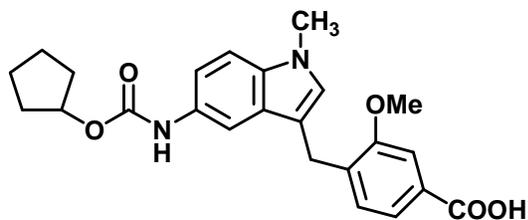
Imp-2 (c)
Methyl 4-[5-(Cyclopentyloxy carbonyl) amino-1- methylindol-3-yl-methyl]-3- methoxy benzoic acid
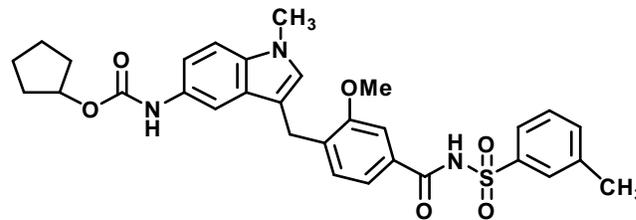
Imp-3 (d)
N- {4-[5-(Cyclopentyloxy carbonyl) amino-1-methylindol -3-yl-methyl]-3- methoxy benzoyl}-3-methyl benzene sulphonamide.
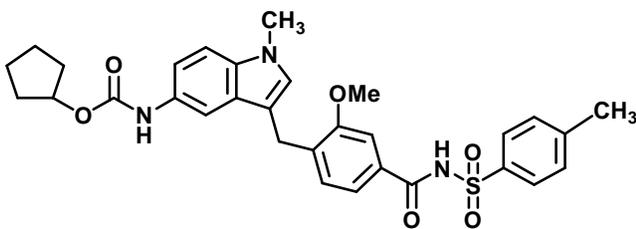
Imp-4 (e)
N- {4-[5-(Cyclopentyloxy carbonyl) amino-1-methylindol -3-yl-methyl]-3- methoxy benzoyl}-4-methyl benzene sulphonamide.
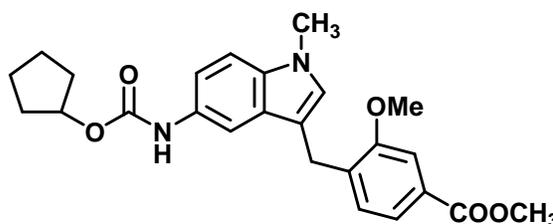
Imp-5 (f)
Methyl 4-[5-(Cyclo pentyloxy carbonyl) amino-1- methyl indol-3-yl-methyl]-3-methoxy benzoate

Figure 1 : Chemical names and structure

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Department of Integrated Product Development of Dr. Reddy's Laboratories Limited, Hyderabad, India. HPLC-grade acetonitrile and methanol were procured from Ranchem, India. Analytical reagent grade potassium dihydrogen orthophosphate and orthophosphoric acid were purchased from Merck, India. 1-Decane sulphonic acid sodium salt was purchased from S.D. Fine Chem. Limited, India. High pure water was prepared by using a Millipore Milli-Q Plus purification system.

Apparatus

The LC system used for the forced degradation method development studies was an Agilent 1100 series (manufactured by Agilent Technologies, Waldbronn, Germany) LC system with photodiode array and variable wavelength detectors. The output signal was monitored and processed using Empower software (designed by Waters) on a Digital Equipment Co. computer.

Chromatographic conditions

The chromatographic column used was a Zodiac 100, C 18,250 mm x 4.6 mm, with a 5 μm particle size. The buffer was a mixture of 10 mM potassium dihydrogen orthophosphate and 5 mM 1-decane sulphonic acid sodium salt. The pH was adjusted to 4.0 with dilute orthophosphoric acid (solvent A), acetonitrile (solvent B) and methanol in the ratio (85:10:5)(*v/v/v*). The flow rate of the mobile phase was kept at 0.8 mL min^{-1}. The LC gradient was set as time / % B: 0.01/40, 5/40, 17/62, 33/62, 35/60, 45/74, 55/74, 60/86, 65/93, 75/40, 85/40. The column temperature was maintained at 27 °C and the wavelength was monitored at 220 nm. The injection volume was 20 μL. The diluent acetonitrile and water in the ratio 800:200 (*v/v*) was used to prepare the standard, blend and system-suitability solutions.

Sample Preparation

Standard and test solutions with concentrations of 500 μg mL^{-1} were prepared individually, while a stock solution of impurity blend (mixture of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5) with a concentration of 50 μg mL^{-1} was prepared as the diluent. A mixture containing 7.5 μg mL^{-1} of each impurity and 500 μg mL^{-1} of zafirlukast standard was prepared as a system suitability solution.

METHOD VALIDATION

The developed method is validated to meet the stipulations of the ICH guidelines.

Specificity

Specificity is the ability of a method to measure the analyte response in the presence of its potential impurities and degradation products. The specificity of a developed LC method for zafirlukast was carried out in the presence of its impurities: namely, Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5.

Forced degradation studies were performed on a bulk drug to provide an indication of the method's stability indicating property and specificity. Intentional degradation was attempted to stress the conditions of photolytic degradation (as per the ICH recommended condition), thermal degradation (drug substance exposed at 105 °C), acid hydrolysis (using 0.5 N HCl), base hydrolysis (using 1 N NaOH), water hydrolysis (reflux at 100 °C) and oxidative degradation (using 2% H_2O_2) to evaluate the proposed method's ability to separate zafirlukast from its degradation products. For heat and light studies the study period was seven days, whereas for acid, base, water hydrolysis and oxidative degradation it was 12 h, 1.5 h, 48 h and 1.5 h, respectively. PDA was employed to check and ensure the homogeneity and purity of the zafirlukast peak in the stressed sample solutions. Assessment of mass balance in the degraded samples was checked to see whether the amount of impurities detected in the stressed sample matched the amount present before the stress was applied. Assay studies were carried out on the stressed sample against a zafirlukast-qualified reference standard and the mass balance (% assay + % sum of all impurities + sum of all degradants) was tabulated. An assay was also calculated for the bulk sample by spiking all five impurities (Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5) at concentrations of 0.75 μg mL^{-1}.

Precision

The assay method precision was evaluated by carrying six independent assays of zafirlukast test samples against the qualified reference substance, and calculating the %RSD and confidence intervals. The precision was checked by injecting six individual (*n*=6)

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preparations of (0.5 mg mL^{-1}) zafirlukast spiked with 0.75 µg mL^{-1} of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 with respect to the analyte concentration. The %RSD was calculated for the percentage areas of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5.

The intermediate precision of the method was also evaluated using different analysts, different days, and different instruments in the same laboratory.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 were estimated at a S/N ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision and accuracy studies were also performed at the LOQ level by injecting six individual preparations (*n*=6) of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 and calculating the %RSD for each area.

The accuracy was carried out by standard addition at the LOQ level, and a recovery study was done in order to calculate the % recovery.

Linearity

Linearity of the test solutions for the assay method were prepared from a stock solution at five concentration (*n*=5) levels from 50% to 150% assay analyte concentration (50, 75, 100, 125 and 150%). The peak area versus concentration data was calculated by a least-squares linear regression analysis.

Linearity test solutions for the RS method were prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at seven concentration (*n*=7) levels from LOQ to 150% with respect to the impurities specification level of 0.75 µg mL^{-1} (i.e., LOQ, 0.1875 µg mL^{-1}, 0.375 µg mL^{-1}, 0.5625 µg mL^{-1}, 0.75 µg mL^{-1}, and 1.125 µg mL^{-1}). The calibration curve was drawn by plotting the peak areas of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 against their corresponding concentrations. The %RSD and Y-intercept of the calibration curve was calculated.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three concentration levels (i.e. 25, 50, and 75 µg mL^{-1}) in the bulk drug sample.

Standard addition and recovery experiments were conducted to determine the accuracy of the related substance methods for quantification of all five impurities in the bulk drug samples.

The study was carried out in triplicate at 0.375 µg mL^{-1}, 0.75 µg mL^{-1} and 1.125 µg mL^{-1} of the analyte concentration (500 µg mL^{-1}). The percent recoveries of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 were calculated.

Robustness

To determine the robustness of the developed method, the chromatographic conditions were deliberately altered and the system suitability criteria were verified. The selectivity factors in the robustness studies for each impurity were established and compared with regular experiments.

The flow rate of the mobile phase was 0.8 mL min^{-1}. To study the effect of flow rate on the resolution, the same was altered by 0.2 units, i.e. from 0.6 to 1.0 mL min^{-1}. The effect of pH on the resolution of impurities was studied by varying ± 0.2 pH units (at 3.8 and 4.2 buffer pH).

The effect of column temperature was studied from ± 5 °C of 27 °C.

RESULTS AND DISCUSSION

Optimisation of chromatographic conditions

Imp-3 and Imp-4 are the regiomers to zafirlukast and thus form the primary potential impurities. The main target of the chromatographic method is the separation of Imp-3, Imp-4 and any degradants generated from the analyte peak. Due to the structure similarities between zafirlukast, Imp-3, and Imp-4, the separation became difficult in the initial stages of development. Impurities were co-eluted using different stationary phases (i.e. C18, C8 and phenyl), different mobile phases containing buffers like phosphate, sulphate and acetate with varying pH (2.5-7), and using organic modifiers like acetonitrile and methanol in the mobile phase. Introduction of a negative ion pair reagent into the mobile phase resulted in very good selectivity between the non-polar impurities, but the late-eluting impurities were observed even with a gradient elution at 90% of the organic ratio. The change in ion pair reagent from sodium lauryl sulfate to 1-decane sulphonic acid sodium salt given a

reasonable run time by adjusting the last peak capacity factor (k) value to ~45 min. The pH of the buffer was found to be critical in achieving separation between the zafirlukast peak and its meta isomer (Imp-3). At acidic pH values (< 3.0), co-elution of the Imp-3 peak was observed. Interestingly, good resolution was obtained between zafirlukast and Imp-3 at a buffer pH of 4.0. Various gradient trials were taken to obtain a maximum resolution between zafirlukast and its Imp-3 meta isomer at a 60% organic ratio. Satisfactory chromatographic separation was achieved using a solution (solvent A) of 10 mM potassium dihydrogen orthophosphate with 4 mM 1-decane sulphonic acid sodium salt (pH 4.0 adjusted with dilute orthophosphoric acid). Solvent B was a mixture of acetonitrile, methanol, and water in the ratio 850:100:50 (*v/v/v*). The LC gradient of solvent B was kept as T/% B: 0/40, 5/40, 17/62, 33/62, 35/60, 45/74, 55/74, 60/86, 65/93 and 75/40, with a post run time of ten minutes. Under optimised conditions, zafirlukast and the five impurities were well-separated with a resolution of > 2. The typical relative retention times of Imp-1, 2, 3, 4 and 5 were about 0.56, 0.71, 1.03, 1.05 and 1.21, respectively. Various C18 columns (i.e. Zodiac C18, Symmetry C18, Symmetry shield C18 and Venusil C18) were checked under the optimised conditions, and it was found that the Zodiac C18 achieved the best resolution. The system suitability results are given in TABLE 1. The developed LC method was found to be specific for zafirlukast and its five impurities.

TABLE 1: Linearity data for zafirlukast and related substances

Component	Calibration range (μg/ml)	Regression equation	SES[1]	SEI[2]	CC[3](r)	%intercept	LOD (μg/ml)	LOQ (μg/ml) (%RSD)
Imp-1	0.04 -1.13	$y = 118076x -0.28$	7176($\pm$4767)	4 ($\pm$ 3)	0.9999	0.3	0.0105	0.04 (5.9)
Imp-2	0.06 -1.13	$y = 114874x -1.23$	3064($\pm$4558)	0.7($\pm$1.7)	0.9998	1.4	0.010	0.06 (5.2)
Zafirlukast	0.06 -1.13	$y = 112977x -0.36$	11441($\pm$10802)	2.5($\pm$2.7)	0.9994	0.4	0.040	0.06(4.9)
Imp-3	0.06 -1.13	$y = 113446x -0.48$	8730($\pm$5801)	6.6($\pm$4.6)	0.9999	0.6	0.021	0.06 (5.5)
Imp-4	0.07 -1.13	$y = 100228x -1.26$	4806($\pm$3464)	2.1($\pm$1.4)	0.9999	1.7	0.009	0.06 (6.7)
Imp-5	0.06 -1.13	$y = 124196x -1.37$	5268($\pm$4013)	2.8($\pm$1.9)	0.9999	1.5	0.011	0.06 (5.3)

The selection of the 220 nm wavelength is highly appropriate for zafirlukast, not only for process-related impurities, but for degradants as well. The relative response factors of known impurities are quite close to a value of 1.0. While selecting the wavelength, the spectra and absorbance of degradants at 220 nm was also taken into consideration.

Results of forced degradation studies

Considerable degradation was observed in the zafirlukast bulk samples under stress conditions such as photolytic stress, thermal stress, acid, base, and water hydrolysis. To achieve this level of degradation, the samples were tested in acid, base, 3% hydrogen peroxide and water for 2 h at 60 °C. Under these conditions, the degradation of the drug substance was observed during peroxide, acid and base hydrolysis (Figure 2 a-h). Zafirlukast was degraded into Imp-1 Imp-2 under acidic and basic conditions (treated with 0.5 N HCl at 70 °C for 12 h and 1 N NaOH at 70 °C for 1.5 h), confirmed by co-injection with qualified Imp-1 and Imp-2 standards. Moderate degradation of the drug substance was observed under water hydrolysis conditions (treated with water at 100 ºC for 48 h), leading to the formation of some unknown degradation peaks. Peak purity test results obtained from PDA confirmed that the zafirlukast peak is homogeneous and pure in all the analysed samples. The mass balance of stress samples was close to 99.7%. The assay of zafirlukast is unaffected in the presence of Imp-1, 2, 3, 4 and 5, which confirms the stability indicating power of the developed method.

Results of experimental method validation

Precision

The %RSD of zafirlukast during the assay method precision study was well within 0.5%, and the %RSD of Imp-1, 2, 3, 4 and 5 in the related substance method precision study was within 2%. The %RSD of the assay results obtained during the intermediate precision study was within 1.0%, and the %RSD of %area of Imp-1, 2, 3, 4 and 5 were within 2% confirming excellent precision of the method.

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Figure 2

a) Zafirlukast water degradation spiked

b) Zafirlukast acid degradation spiked

c) Zafirlukast peroxide degradation spiked

d) Zafirlukast thermal degradation spiked

e) Zafirlukast photo degradation spiked

f) Zafirlukast base degradation spiked

g) Zafirlukast sample
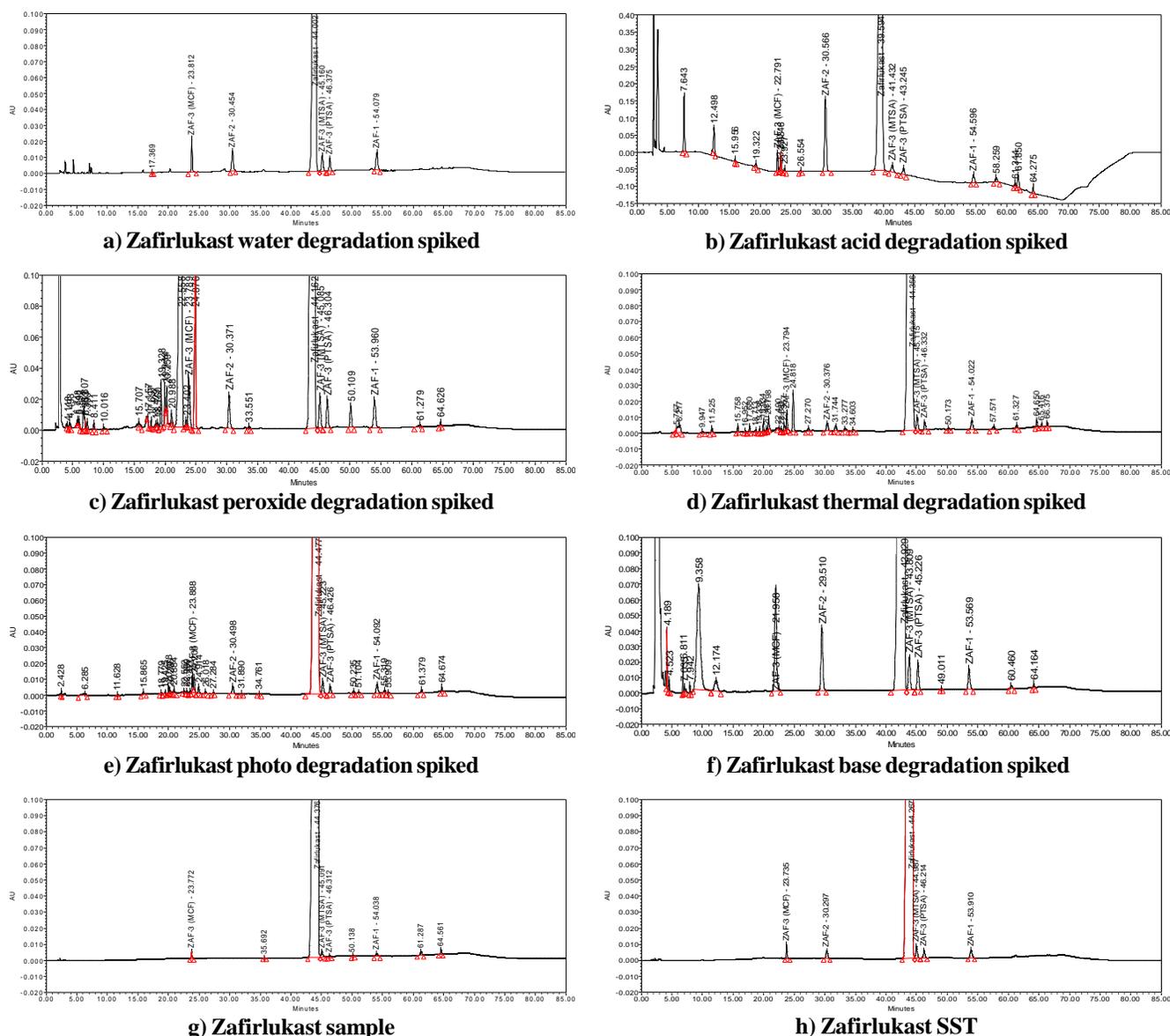
h) Zafirlukast SST

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of zafirlukast, Imp-1, 2, 3, 4 and 5 were 0.0105, 0.004, 0.011, 0.021, 0.009 and 0.011 μg mL^{-1}, respectively. The LOQ of zafirlukast, Imp-1, 2, 3, 4 and 5 were 0.06, 0.03, 0.05, 0.05, 0.06 and 0.05 μg mL^{-1} (of analyte concentration, i.e. 500 μg mL^{-1}), respectively, for a 10 μL injection volume. The method precision for Imp-1, 2, 3, 4 and 5 at the LOQ level was below 10% RSD.

Linearity

Linear calibration plots for the assay method

were obtained over the calibration ranges tested, 125 to 750 μg mL^{-1}, and the correlation coefficient obtained was greater than 0.999. Linearity was checked for the assay method over the same concentration range for three consecutive days. The %RSD values of the slope and Y-intercept of the calibration curves were 3.1 and 4.3, respectively. The results show that an excellent correlation existed between the peak area and concentration of the analyte.

Linear calibration plots for the related substance method were obtained over the calibration ranges tested, LOQ to 1.5 μg mL^{-1}, for Imp-1, 2, 3, 4 and

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5. The correlation coefficient obtained had greater than 0.999 linearity over the same concentration range for three consecutive days. The %RSD values of the slope and Y-intercept of the calibration curves

were 4.6 and 5.5, respectively. The results show that an excellent correlation existed between the peak area and concentration of Imp-1, 2, 3, 4 and 5 (TABLE 1).

TABLE 2 : Accuracy data for Zafirlukast related substances

Componet	50% of specification level Amount (%w/w)			100% of specification level Amount (%w/w)			150% of specification level Amount (%w/w)		
	Added	Found	%Recovery	Added	Found	%Recovery	Added	Found	%Recovery
Imp-1	0.075	0.074	99.1± 0.8	0.151	0.15	99.3± 0.8	0.151	0.15	99.3± 0.8
Imp-2	0.075	0.074	99.1± 0.9	0.151	0.15	99.3± 0.9	0.151	0.15	99.3± 0.9
Imp-3	0.075	0.074	99.1± 0.10	0.151	0.15	99.3± 0.10	0.151	0.15	99.3± 0.10
Imp-4	0.075	0.074	99.1± 0.11	0.151	0.15	99.3± 0.11	0.151	0.15	99.3± 0.11
Imp-5	0.075	0.074	99.1± 0.12	0.151	0.15	99.3± 0.12	0.151	0.15	99.3± 0.12

Accuracy

The percent recovery of zafirlukast in the bulk drug samples ranged from 99.1% to 100.5%. The percent recovery of Imp-1, 2, 3, 4 and 5 in the bulk drug samples ranged from 91.8% to 102.3% (TABLE 2).

Robustness

In all the deliberate varied chromatographic conditions (flow rate, pH, and column temperature), the resolution between zafirlukast and Imp-1, 2, 3, 4 and 5 was greater than 2, illustrating the robustness of the method.

CONCLUSIONS

In this manuscript, the simple, accurate and well-defined stability indicating gradient for the LC method for the determination of zafirlukast in the presence of regiomers and degradation products was described for the first time. The behaviour of zafirlukast under various stress conditions was studied and presented. The information presented herein may be very useful for quality monitoring of bulk samples and finished dosage forms, as well as employed to check the quality during the stability studies.

ACKNOWLEDGEMENTS

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