Original Article

Novel Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Alfuzosin and Dutasteride in Pharmaceutical Dosage Form

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ABSTRACT

Aim: The present study was to develop a simple, accurate and stable reverse phase liquid chromatographic method and validate in bulk drug and pharmaceutical dosage form for the simultaneous determination of Alfuzosin Hydrochloride (ALF) and Dutasteride (DUT). **Materials and Methods:** Chromatographic separation has been accomplished using an XTerra C_{18} Column (150 × 4.6mm, 5μm particle size) as the stationary phase, with an isocratic system of mobile phase Ammonium dihydrogen phosphate buffer (pH 6.5) and methanol (25:75 proportions) at a flow rate of 1.0 mL/min, detection was executed at 246 nm using an UV detector. The optimized method was validated in accordance to International Conference on Harmonization guidelines. **Results:** The method developed was found to be linear as regression analysis showed good correlation (R^2 = 0.999) with a linear curve at concentration range of 25-150 μg/mL for ALF and 1.25-7.5 μg/mL for DUT. The approach was unique as it was free of degradants in spite of subjecting the drugs to forced degradation. The percentage recovery was in the range of 99.55 and 99.23 % for ALF and DUT respectively from the pharmaceutical dosage form. The developed method showed accurate, precise, robust results with an LOD and LOQ of 0.41 and 0.71 μg/mL and 4.27 and 2.14 μg/mL respectively. **Conclusion:** Due to the flexibility, accuracy and high precision, the developed method can be employed in routine analysis of bulk and dosage forms.

Key words: Alfuzosin Hydrochloride, Dutasteride, RP-HPLC, Method development, Validation, Degradation.

INTRODUCTION

Alfuzosin Hydrochloride (ALF) chemically known as (R,S)-N-[3[(4-amino-6,7-dimethoxy-2-quinazolinyl) methylamino] propyl] tetrahydro-2-furancarboxamide hydrochloride[1] with an empirical formula $C_{19}H_{27}N_5O_4$.HCl (Figure 1), is a selective α-1 adrenergic receptor blocker in the lower urinary tract that induces smooth muscle relaxation in the bladder neck and prostate, thereby convalescing urine flow and block bladder outlet in benign prostate hyperplasia.[2] ALF also has a vasodilation effect due to catecholamine's (epinephrine and norepinephrine). ALF has oral bioavailability of 60% under fasting conditions. It is a white to off-white crystalline powder which melts at approximately 240°C with molecular weight 425.91 g/mol is freely soluble in water, sparingly soluble in alcohol and practically insoluble in dichloromethane. ALF is used in humans with benign prostatic hyperplasia to enhance urination.

Dutasteride (DUT), chemically known as (5α, 17β)-N-{2,5bis (trifluoromethyl) phenyl}-3-oxo4- azaandrost-l-ene-17-carboxamide, belongs to class of drugs called 5α-reductase inhibitors with an empirical formula $C_{27}H_{30}F_6N_2O_2$ (Figure 2)

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molecular weight of 528.5 g/mol.[3] DUT is a competitive and selective inhibitor of Type 1 and Type 2 isoforms of 5α-reductase enzyme that converts testosterone to 5α-dihydrotestosterone thereby inducing enlargement of prostate gland, is used in treatment of benign prostatic hyperplasia, which occurs frequently in men over the age of 50 years.[4] DUT blocks 5α-reductase enzyme, thus inhibiting testosterone conversion to dihydrotestosterone.[5-7] DUT is used in the treatment of benign prostatic hyperplasia, observed in men with enlarged prostate gland.

For the diagnosis of benign prostatic hyperplasia ALF and DUT are currently available in combined dosage form. Stability of pharmaceutical dosage forms is of great concern as it affects the drug product's safety and efficacy. Under the impact of various environmental factors, the FDA and ICH recommendations stated the stability testing data on the medication. The samples generated from forced degradation can be used to develop a stability indicating method that can be used to analyze accelerated and long term stability studies.

Literature survey disclosed a narrow research on analytical method and estimations have been reported on the combination of these two drugs in pharmaceutical preparations and human plasma in single or in mixed forms with different medications yet uncovered a few strategies on drugs independently. The analytical techniques gave an account of Literature survey uncovered not many RP-HPLC methods for simultaneous estimation of Alfuzosin Hydrochloride and Dutasteride.[8-15] The focus of the present study is to develop a simple, precise and accurate reversed-phase HPLC method for the estimation of ALF and DUT in bulk and pharmaceutical dosage form according to ICH guide lines.

By considering the impediments of the reported methods such as lack of broad linearity range, greater run time and retention time, present work was aimed to develop and validate a simple, sensitive, precise and accurate stability indicating RP-HPLC with UV detection method, without using any internal standard, for simultaneous estimation on ALF and DUT in bulk and in pharmaceutical dosage forms according to ICH guidelines.[16]

MATERIALS AND METHODS

Chemicals and Reagents

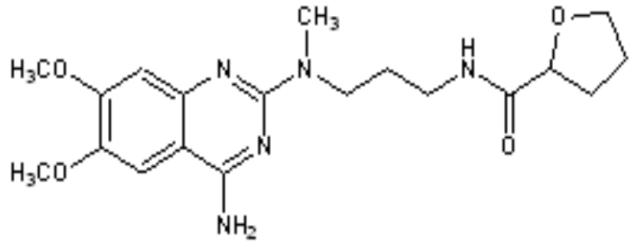
The reference drugs Alfuzosin Hydrochloride and Dutasteride were acquired as generous gift s amples from Dr. Reddy's Laboratories, Hyderabad (Andhra Pradesh, India). All of the Chemicals utilized were Ammonium dihydrogen Phosphate (AR) Thomas Baker, HPLC grade acetonitrile (Merck), as a mobile phase. The water used in buffer preparation was freshly prepared from Milli-Q and filtered using a nylon 0.45 microns membrane filter. The marketed formulation, Alfuzosin and Dutasteride combination tablets (10 mg/0.5 mg) manufactured by Dr. Reddy's Laboratories Ltd., brand name of Dutalfa were purchased from the local drug store.

Equipment

Waters HPLC Aliance 2695 with data processing software Empower-2 and 2489 UV/Vis dual absorbance detector, Intelligent LC pump with sampler customized at 20 µL capacity per injection, column used for separation was XTerra C_{18} (150×4.6mm, 5µm). UV Spectrophotometer (Labindia, UV3000+ with UV win 5 software), Analytical Balance (Sartorius CP224S), Ultra Sonicator (Frontline FS 4), pH Meter (ADWA, AD102U), nylon filter Paper 0.45 microns (Milli Pore) were used during the study.

Chromatographic conditions:

The optimised conditions for the simultaneous estimation of ALF and DUT were performed using XTerra C_{18} Column (150×4.6mm, 5µm particle size) as stationary phase at ambient temperature. The elution was isocratic and the mobile phase comprising of a blend of Ammonium dihydrogen phosphate buffer



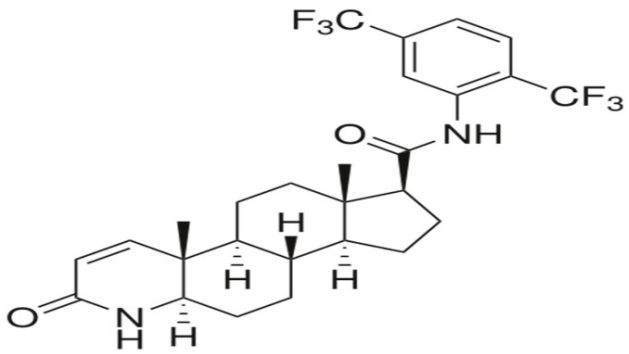


Figure 1 and 2: Chemical structures of Alfuzosin HCl and Dutasteride.

(pH 6.5) and methanol in the proportion of 25:75 % v/v at a flow rate of 1.0 mL/min. The instrument was controlled at an ambient temperature. The eluent was monitored at 246nm with run time of 6 min and purity analysis was performed over a wavelength range of 200-400 nm. The injection volume was 20µL capacity. Prior to inject the solutions, column was equilibrated using mobile phase for a minimum of 30min through the system. The retention times for ALF and DUT under the optimized chromatographic conditions was found to be 2.14 and 4.26 min as shown in Figure 3.

Preparation of Analytical solutions

Preparation of Ammonium dihydrogen phosphate buffer solution

In a 1000 mL volumetric flask, a weighed quantity of 0.57515 gm Ammonium dihydrogen phosphate $(NH_3)_4H_2PO_4$ was taken. To this, 500ml of HPLC grade water was added, sonicated for 10 min and then filtered through 0.45µ membrane filter. Resulting solution was adjusted to 6.5 pH using 1N sodium hydroxide.

Preparation of Mobile phase

A mixture of 250 mL ammonium buffer (30%) and 750 ml of HPLC grade methanol was taken, degassed for 5 min in ultrasonic water bath and filtered under vacuum filtration through 0.45µ filter.

Preparation of standard stock solution

The standard stock solution was prepared by dissolving 10 mg of standard ALF and 0.5 mg of standard DUT taken in to 100 mL to which 15 mL of mobile phase was added and sonicated for about 10 min then the final volume was made up to 100 mL with mobile phase, mixed properly and then filtered using 0.45µ membrane filter. 10 mL of this solution was pipetted out so as to achieve concentrations of 100 µg/mL and 5 µg/mL for ALF and DUT respectively by making it up to 100 mL with mobile phase.

Preparation of sample solution (Marketed formulation)

10 marketed Dutalfa tablets were taken and powdered. Tablet powder equivalent to 10mg of ALH and 0.5mg of DUT was accurately weighed and transferred to the 50ml volumetric flask with the mobile phase and sonicated for 15 min. The resulting solution was filtered using 0.45µm nylon filter and the filtrate was again diluted to obtain the final concentration levels of 100 µg/ml of ALF and 5 µg/ml of DUT. Under optimized chromatographic conditions the final prepared solutions were analyzed.

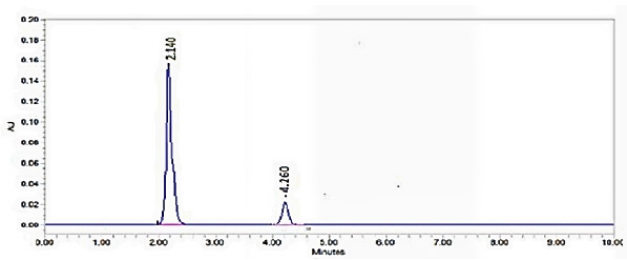


Figure 3: Chromatogram of Standard.

Selection of analytical wavelength

ALF and DUT were prepared separately by sufficient dilution of each standard solution with mobile phase, while the blank solution taken is mobile phase and tested at 200-400 nm range for absorbance.

Development and validation of HPLC method

The current research was investigated in order to obtain a new, reliable, cost-effective and convenient method for ALF and DUT simultaneous determination using HPLC in tablet dosage form that can be employed in routine analysis. The RP-HPLC method developed has been validated for the parameters such as system suitability, specificity, linearity, detection limit (LOD), quantitation limit of (LOQ), accuracy, precision, robustness, ruggedness and stability studies as per ICH guidelines.[17-21] Forced degradation studies were conducted to elucidate the characteristics of the active substance's intrinsic stability. For all the parameters %RSD was determined.

System Suitability

System suitability tests on freshly prepared standard solutions of ALF and DUT were carried out by injecting in six replicates. The standard chromatogram evaluated parameters like plate number (N), retention time, resolution, tailing factor.

Specificity

Specificity was ascertained whether method is affected by the presence of other components or not, for the developed method by analyzing under normal conditions for standard drug, marketed formulation, blank and placebo solutions. To determine specificity, ALF and DUT were studied with parameters like retention time, resolution factor and peak purity data.

Linearity

Linearity of the method developed was elucidated by linear regression analysis and is measured using least square method. A series of standard solutions of ALF and DUT were prepared and injected into the HPLC system at six different concentration levels (25%, 50%, 75%, 100%, 125% and 150%) i.e., 25-150 µg/mL (ALF)

and 1.25-7.5 µg/mL (DUT). Calibration curves for the standard solutions were plotted against respective concentrations with their response ratios (ratios of the peak area of the analytes). Slope-a, intercept-b and correlation coefficient-R^2 were determined by applying linear regression equation.

Precision

System precision was achieved using six standard concentration replicates (100 µg/mL of ALF and 5 µg/mL of DUT). Six different sample preparations of marketed sample from same homogenous blend (100 µg/mL of ALF and 5 µg/mL of DUT) were used for method precision. Intermediate precision/ Ruggedness is obtained by analyzing the sample under modifying normal test conditions such as analyst and equipment. Retention time and peak area were determined and expressed as mean and %RSD from the data collected.

Accuracy

Accuracy in terms of assay and percent recovery was evaluated for the developed method. The study was performed by standard addition method at 50, 100 and 150 % levels. Known amounts of standard solutions of drugs ALF and DST were spiked to pre-analyzed sample solutions, injected in to HPLC system in triplicate and percentage recoveries were calculated. % recovery was calculated using area observed for each level.

Robustness

Robustness of the optimized method was examined by evaluating the effect of small deliberate variations in procedural variables such as flow rate (±2%), shift in wave length (±2 units) and proportion of the organic content in the mobile phase (±2%) and % RSD.

Sensitivity

The detection limit (LOD) and quantification limit (LOQ) were determined as the quantities for which the signal-to-noise ratios were 3:1 and 10:1, respectively. Based on calibration curve standard deviation (SD) of the peak area and the slope (S) were calculated to determine LOD and LOQ.

Forced Degradation Study

According to International Conference on Harmonisation (ICH) guidelines, stability testing of new drug substance and products recommend that stress testing be carried out to elucidate the active substance's intrinsic stability characteristics. The forced degradation studies of ALF and DUT were attempted to elucidate the intrinsic stability characteristics of the active substance under stress conditions such as acid, alkaline, humidity, thermal and photolytic conditions (n=3) as per ICH guidelines. For acidic degradation, 2 mL of standard solution was refluxed at 60°C for 1hr with 3N HCl. Later, using NaOH the solution was neutralised. 2 mL standard solution was combined with 2N NaOH at 60°C for 1hr for alkaline degradation after which the solution was neutralised with 2N HCl. Working standard solution was refluxed with 30% solution of H_2O_2 at 60°C for 1hr for oxidative degradation. For photolytic degradation, 2 mL of working standard solution was exposed to ultra violet (UV) (200 watt hr/m^2) as per ICH Guidelines. For thermal degradation, 2 mL of working standard solution was exposed to temperatures at 105°C for 3 days. All these solutions were prepared in amber volumetric flasks, except for photo degradation. Once the degradation treatments were finished, the samples were cooled to room temperature, diluted with the diluent and injected for chromatographic analysis.[22-24]

Procedure for Assay

The optimized chromatographic conditions were reported with a steady base line. 20µL standard (pure drug) and sample (extracted from Dutalfa tablets) solutions were injected into the HPLC system separately and the chromatogram was recorded. The amount of the drug in the sample may be determined from the peak area of ALF and DUT.

RESULTS AND DISCUSSION

An effort has been made for a simple, rapid, accurate and precise method for the simultaneous estimation of ALF and DUT in pure form and in formulation by an isocratic RP – HPLC method. The absorption maximum (λ_{max}) of ALF and DUT was found to be 246 nm and the same was selected as detection wavelength in the method development and validation process of the RP-HPLC system. By changing the chromatographic parameters such as mobile phase composition, pH and buffers used in the mobile phase, an optimized method was developed. A satisfactory separation and good peak symmetry for ALF and DUT were attained with a mobile phase consisting of Ammonium dihydrogen phosphate buffer (pH 6.5) and methanol in the proportion of 25:75 % v/v with isocratic elution program at a flow rate of 1 mL/ min, using XTerra C_{18} Column (150×4.6mm, 5µm particle size) as stationary phase at ambient temperature to improve reproducibility and repeatability with an injection volume of 20µL. Complete resolution with smooth baseline with retention time of 2.14 min for ALF and 4.26 min for DUT were eluted at lesser time

compared to the results of reported methods (Figure 3) and none of the impurities were found to be interfering with its assay.

The method developed was validated by the following procedures as per ICH guidelines for system suitability, linearity, specificity, accuracy, precision, robustness, sensitivity and ruggedness. Parameters such as theoretical plate (N), tailing factor (T) and retention time calculated from the standard chromatogram to evaluate system suitability as shown in Table 1. The number of theoretical plates for ALF and DUT was 5942 and 5264, the tailing factor is 1.13 and 1.06 for ALF and DUT and the retention periods were 2.14 min and 4.26 min for ALF and DUT, respectively. The process was therefore deemed suitable as the parameters studied lie in the rage of acceptance criteria. The specificity of the method suggests that no interferences of mobile phase and placebo or any other excipients co-eluted with the drug and the drug's peak presented in Figure 4, indicating it to be pure in nature and hence the developed method is specific without any interences of impurities or excipients. The method developed showed linear correlation at different concentration levels of 25-150 µg/mL for ALF and 1.25-7.5 µg/mL for DUT. The calibration curve obtained by plotting peak area versus concentration is presented in Figure 5 and 6 was linear and the correlation coefficient for ALF and DUT was found to be 0.9997 and 0.9998 with regression equations, y = 11946x + 27309 and y = 21926x – 3188 respectively and the results were tabulated in Table 2. The precision of the method was assesed from peak area determinations of six sample solution replicates and % RSD for system precision tabulated in Table 3 was found to be 0.72 and 0.53, % RSD for method precision was found to be 0.67 and 0.54 and that for ruggedness was found to be 0.66 and 0.64 for ALF and DUT respectively. The results reveal that the proposed method is repeatable, reproducible and precise as %RSD was found to be less than 2%. The accuracy of the optimized method was calculated by assay and recovery studies at three concentration levels 50%, 100% and 150%. The mean percentage recovery values for ALF and DUT were found to be 99.55 and 99.23 % respectively which showed better recovery values compared to the reported recovery

Table 1: System suitability parameters.

Parameter	ALF	DUT
Theoretical plates (n)	5942	5264
Tailing factor (T)	1.13	1.06
Retention time (R_t)	2.140	4.260

Table 2: Linearity data of ALF and DUT by the proposed Method.

ALF		DUT	
Concentration (µg/mL)	Mean Peak Area (*n*=6)	Concentration (µg/mL)	Mean Peak Area (*n*=6)
25	324638	1.25	23220
50	621504	2.5	52658
75	918633	3.75	78955
100	1225462	5	106538
125	1546213	6.25	134739
150	1798967	7.5	160304
Standard Deviation (s)	510216.57	Standard Deviation (s)	46813.14
Slope (a)	11946	Slope (a)	21926
Intercept (b)	27309	Intercept (b)	-3188
Correlation coefficient	0.9997	Correlation coefficient	0.9998
Regression equation	y = 11946x + 27309	Regression equation	y = 21926x - 3188

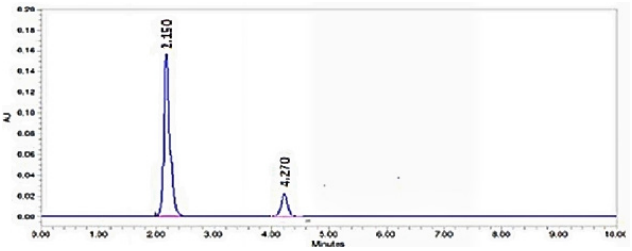


Figure 4: Chromatogram of Sample.

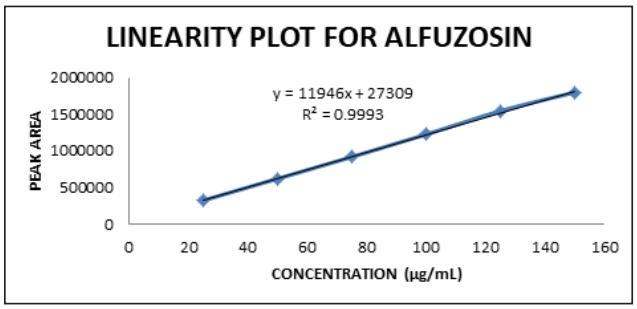


Figure 5: Linearity plot for Alfuzosin.

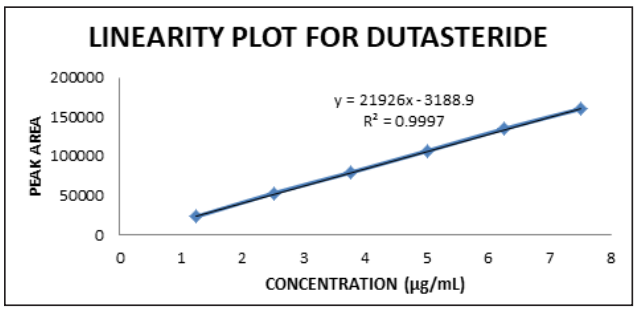


Figure 6: Linearity plot for Dutasteride.

Table 3: Precision data of ALF and DUT by the proposed Method.

Injection	System precision		Method precision			Intermediate precision
	ALF	DUT	ALF	DUT	ALF	DUT
Injection 1	1216532	103193	1216293	103244	1216269	103293
Injection 2	1216262	103206	1216341	103169	1216321	103284
Injection 3	1216698	103253	1216698	103217	1216501	103242
Injection 4	1216498	103248	1216436	103292	1216297	103277
Injection 5	1216353	103237	1216592	103236	1216386	103251
Injection 6	1216392	103292	1216233	103258	1216414	103229
Average	1216456	103238.2	1216432	103236	1216365	103262.7
S D	153.9	35.5	180.9	41.3	86.2	25.6
% RSD	0.72	0.53	0.67	0.54	0.66	0.64

Table 4: Accuracy data of ALF and DUT by the proposed Method.

	Conc of spiked level	Amount added (µg)	Amount found (µg)	Percent Recovery	Mean (%) Recovery	% RSD
ALF	50%	50.65	50.58	99.86	99.61	0.30
		50.93	50.82	99.78		
		50.14	49.73	99.18		
	100%	100.15	99.86	99.71	99.38	0.38
		100.04	99.34	99.30		
		100.23	99.36	99.13		
	150%	150.12	150.03	99.94	99.66	0.16
		150.26	149.54	99.52		
		150.58	149.85	99.51		
DUT	50%	2.58	2.56	99.22	99.21	0.16
		2.56	2.24	99.21		
		2.53	2.15	99.21		
	100%	5.12	5.09	99.41	99.32	0.16
		5.56	5.52	99.28		
		5.38	5.34	99.26		
	150%	7.52	7.46	99.20	99.16	0.16
		7.56	7.50	99.20		
		7.58	7.51	99.08		

Table 5: Robustness data of ALF and DUT by the proposed Method.

Variations		ALF				DUT
% Assay*		Theoretical plates	Tailing factor	% Assay*	Theoretical plates	Tailing factor
-2% of pH in the mobile phase	100.08	5849	1.18	100.17	5263	1.02
+2% of pH in the mobile phase	100.14	5932	1.27	100.22	5148	1.09
Flow rate at 0.9 mL/min	100.15	5625	1.09	100.13	5792	1.11
Flow rate at 1.1 mL/min	100.18	5643	1.15	100.18	5045	1.08
Wavelength at 244 nm	100.29	5698	1.12	100.04	5312	1.10
Wavelength at 248 nm	100.20	5642	1.15	100.01	5275	1.04

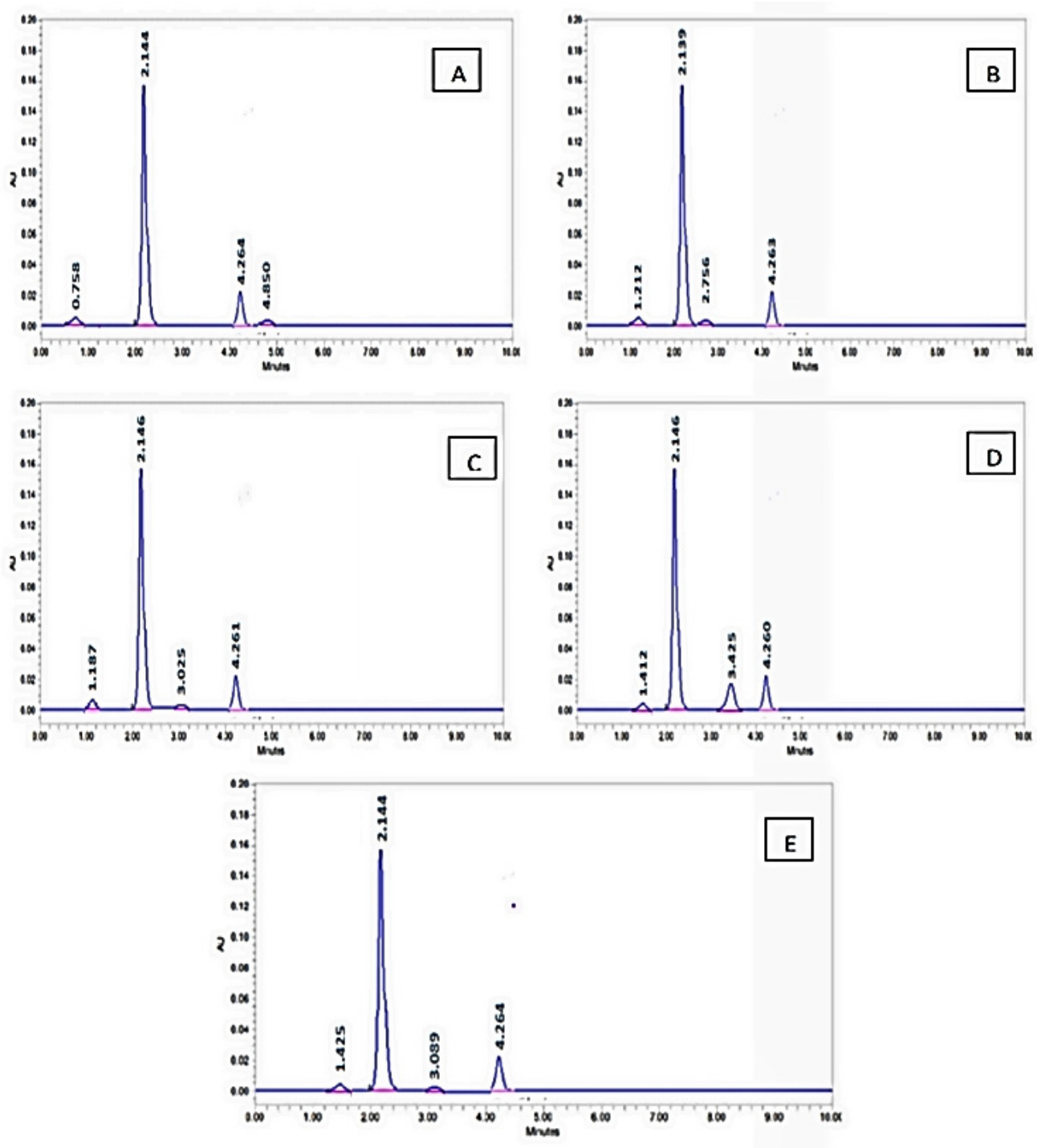


Figure 7: Chromatogram obtained after stress studies – A. acid degradation, B. alkali degradation, C. oxidative degradation, D. thermal degradation, E. photolytic degradation.

results and are outlined in Table 4 suggesting that the method was accurate and % recovery limits should be in range of 95-105% for ALF and 98-102% for DUT. Robustness has been studied with minor but deliberate modifications in parameters such as detection wavelength, pH of buffer in mobile phase and flow rate shown in Table 5. Inspite of the chromatographic changes, the responses lied within the limits of the assay without any major variations in each component's retention time and peak area. It was observed that the theoretical plates were 5731 and 5305 and tailing factor was 1.16 and 1.07 respectively for ALF and DUT were in acceptance limits. Small variations in these parameters did not alter the results which indicate the method is robust. ALF and DUT detection limit (LOD) and quantification limit (LOQ) were found to be 0.41 and 0.71 µg/mL and 4.27

Table 6: Sensitivity data of ALF and DUT by the proposed Method.

Parameter	ALF	DUT
LOD	1.41	0.71
LOQ	4.27	2.14

Table 7: Forced degradation data of ALF and DUT by the proposed Method.

Degradation Parameter	% Degradation	
	ALF	DUT
Acid	3.24	2.65
Alkali	5.62	6.37
Oxidative	2.42	2.38
Thermal	1.87	1.24
Photolytic	1.63	1.12

and 2.14 μg/mL, respectively. The data depicts that the method is sensitive (Table 6). Inspite of being exposed to stress conditions, minor degradation was observed with well resolved degradants showing acceptable results and the respective chromatograms shown in Figure 7. The assay limits for Alfuzosin and Dutasteride were in the range of 90-110% and the results obtained for ALF and DUT formulation were found to be 99.6%, 101.4% indicating the results were within the limits.

CONCLUSION

For the determination of ALF and DUT from API and pharmaceutical dosage form a simple, precise, reliable, accurate, economical and rapid method was developed. The developed method that was validated to parameters such as specificity, linearity, accuracy, precision, robustness, LOD, LOQ and system suitability according to ICH guidelines showed values within limits. In terms of lesser analysis time, specificity and accuracy than previously disclosed reports, the present developed method has significant features. Comparing the obtained results with that of previously reported results, the present developed method showed better resolution at lesser retention time and is found to be economical. The validation study suggests that this method can be considered appropriate in performing quality control and routine procedural determinations of ALF and DUT in bulk and pharmaceutical dosage form.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

ABBREVIATIONS

ICH: International Council for Harmonization; **RP-HPLC:** Reverse phase high performance liquid chromatography; **LOD:** Limit of detection; **LOQ:** Limit of quantification, **RSD:** Relative standard deviation.

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SUMMARY

The RP-HPLC method developed for simultaneous estimation of Alfuzosin HCl and Dutasteride was validated in compliance with ICH guidelines and was confirmed to be simple and economical in terms of mobile phase. The method gave linear regression values and the sample recoveries in were in good agreement with their label claim. The drug showed no interference in degradation studies. Hence, in determining bulk as well dosage forms of Alfuzosin HCl and Dutasteride, the method can thus be easily adapted for routine analysis.

About Author

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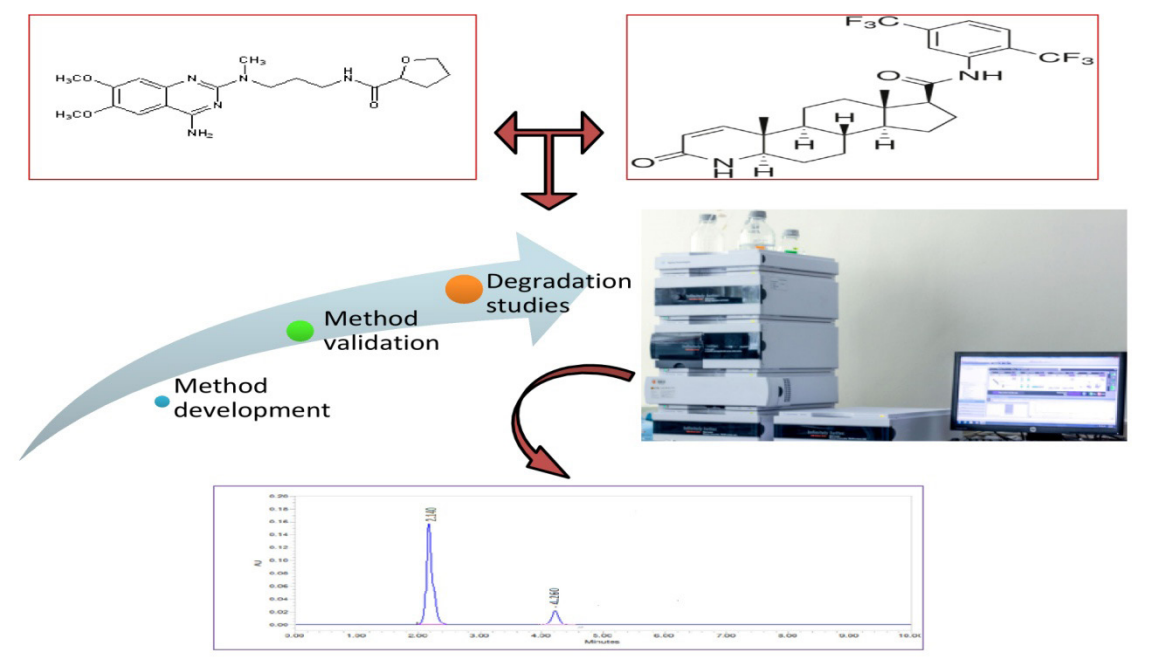
PICTORIAL ABSTRACT



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