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Research Article

Analytical Method Development and Validation for the analysis of Alfuzosin Hydrochloride and its Related Substances using Ultra Performance Liquid Chromatography

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ABSTRACT

A low-cost reverse phase ultra-performance liquid chromatography (RP-UPLC) method for analyzing alfuzosin hydrochloride in the presence of impurities and degradation products from forced decomposition products has been developed. Several method development trials for the separation of drug from impurities were carried out. Furthermore, the best chromatographic separation was attained on a Waters Acquity HSS T3 C18, 100 mm x 2.1 mm, particle size 1.8, UV detection at 254 nm and gradient elution of perchloric acid (pH 3.5 with sodium hydroxide) with a mixture of organic solvents. (acetonitrile, tetrahydrofuan) as mobile phase for drugs. The method has been assessed for specificity/selectivity, linearity/range, repeatability, recovery, and reliability, which can be utilized for quality control during production, as well as to assess the stability of alfuzosin hydrochloride samples. The total elution time was about6.5 minutes, and the equilibration time was approximately 1.5 minutes, allowing for the analysis of more than 100 samples per day.Besides that, the pH sensitive analytical method addressed in the US Pharmacopoeia and the European Pharmacopoeia was discussed.

KEYWORDS

UPLC method Validation, Alfuzosin Hydrochloride; Degradation products; Impurities.

INTRODUCTION

Alfuzosin hydrochloride is termed as (±)-N-[3-[(4-amino-6,7-dimethoxy-2-

quinazolinyl)methylamino]propyl]tetrahydro-2-furamide monohydrochloride with the empirical formula $C_{19}H_{27}N_5O_4 \cdot HCl$ and a molecular weight of 425.91 g/mol. Men primarily use alfuzosin to treat symptoms of an enlarged prostate (benign prostatic hyperplasia or BPH), such as difficulty urinating (leakage, hesitation, insufficient bladder emptying and poor flow), increased urination, painful urination, and urge to urinate. Moreover, alfuzosin is an alpha blocker, which relaxes the muscles of the prostate and bladder, designed to allow urine to drain quite easily.

This paper provides a simple step gradient reverse phase UPLC technique for separating impurities reported in the US Pharmacopeia and the European Pharmacopeia, as well as possible impurities and degradants during the synthesis process established in our laboratory. Figure 1 illustrates the structure of Alfuzosin and

the aforementioned related compounds. Organic impurities can be formed during the manufacturing and storage of an API. To a certain extent, International Conference on Harmonization (ICH) acceptance criteria are based on pharmaceutical research or known safety data as defined in the ICH guidelines (1) [ICH guidelines for Impurities in New Medicinal Substances Q3A (R2), 2006]. Most of the analytical procedures for alfuzosin hydrochloride are isocratic methods using the traditional HPLC method. However, in this experiment, the analytical technique proposed is compatible with LC-MS and has a total runtime of roughly 8.0 minutes, with an elution time of about 6.5 minutes and a 1.5-minute equilibration time. The DL, QL, accuracy, precision and reliability were defined in accordance with the ICH recommendations (2) [ICH guidelines, Verification of analytical procedures: text and methodology, Q2 (R1), (2005)]. Here, for the first time in this article, a new, effective, and proven stability is reported that indicates the separation of eight potential contaminants and degradation products in a "one-shot" analysis using the UPLC method.

MATERIAL AND METHODS

Chemicals

Perchloric acid 70%, Sodium Hydroxide procured from Sigma-Aldrich, Hydrogen peroxide (30%) purchased from Thermo Fisher scientific. LC grade methyl alcohol and ethanenitrile were used, while remaining were analytical grade. Throughout the experiments, HPLC-grade water was purified using a Milli-Q Reagent Water system (Millipore, Bedford, MA) and used to prepare the aqueous solutions and the mobile phase. Alfuzosin Hydrochloride sample was procured locally and Impurities A-F (Figure 1) were procured from European pharmacopeia.

Instrumentation

Liquid Chromatography was achieved on an Acquity^{TM} UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary pump solvent management system, micro degasser, an autoplate-sampler, and thermostatic column compartment. Chromatographic separation was performed on a Waters Acquity UPLC HSST3 C18, 10 cm x 2.1mm, 1.8µ particle size LC column with an in-line filter (0.22 µm) prior to the column. pH meter model 744Metrohm AG Switzerland, FS110D water bath were equipped with MV controller from Fisher Scientific (US), electro-thermostatic blast oven (Heratherm model, Thermo Scientific, US), Hundred Thousandth Balance XPE205DR, Mettler Toledo, Switzerland. A 50-W clear xenon lamp was used as the light source for evaluating the photolytic experiment (Newtronics photostability chamber(model NLPS4SI).

Stress degradation tests

The stress degradation of alfuzosin hydrochloride was investigated using hydrolysis (acid and base), photolytic, oxidation, and thermal forced factors. The various degradation studies, the 10 mg of alfuzosin hydrochloride was dissolved in 10 mL of diluent in a 25 mL volumetric flask. The acid/base hydrolysis tests were performed with 5 mL of 3N hydrochloric acid (3.0 mol·L^{-1}), and 5 mL of 3M sodium hydroxide (3.0 mol·L^{-1}) and both acid and base hydrolysis process were carried out at 60°C for 4 hours,respectively. Furthermore, oxidative hydrolysis was obtained by adding 5mL of 30% hydrogen peroxide at RT for 6 hours and made up to volume with dissolving solvent. In addition, the photolytic and thermal tests were performed with a few milligrams of alfuzosin hydrochloride placed on the watch glasses and maintained at

105°C for 3 days in a hot air oven, and 0.4 mg ml^{-1} sample solution was kept at 80°C for 8 h in hot water medium for the thermal experiment. Similarly Alfuzosin HCl was stressed to Photolytic exposure guidelines (3) (International Conference on Harmonization (ICH), photo stability testing of new drug substances and products, Q1B, (1996)) to light with an overall illumination of not less than 1.2 million lux hours and an developed near ultraviolet energy of not less than 200 watt hours/square meter radiation in photostability chamber. Humidity degradation was carried out at 95% humidity/25°C / 24 h. In each of the above experiments, diluent was prepared without analytes.

Preparation of sample solution

The acid/base hydrolysis samples were diluted to the mark with dissolving solvent. The oxidative degradation solutions were diluted to volume with dissolving solvent, as were the photolytic exposure and thermal degradation solutions.Before UPLC analysis, all solutions were filtered through 0.2 m membrane filters and kept at room temperature.

Chromatographic conditions

The analysis was conducted by waters Acquity UPLC equipped with a photodiode array detector (PDA) at 254 nm. The waters acuity UPLC HSS T3 Octadecyl 10 cm x 2.1mm, 1.8µ particle size fast LC column was used for separation. The Waters Empower software was used for data collection and mobile phase-A is a 99:1 v/v mixture of buffer and tetrahydrofuran. The buffer is composed of 0.5%perchloric acid in water and a pH of 3.50 adjusted with sodium hydroxide.

Mobile phase-B is a 99:1 mixture of acetonitrile and tetrahydrofuran. The time gradient program was (T min/A: B; T0.01/95:5; T1.5/85:15;T5.0/30:70, T6.0/30:70 ;) thus the time analysis is 6.01 min, and the original composition of the eluent was restored after 8.0 min (95:5) and maintained for another 1.50 min. The mobile phase flow was set at 0.3mL min^{-1}, the stationary phase was kept at 30°C and the injection volume was set at 2.00 µL. As a dissolving solvent, a 20:80:1 mixture of buffer, acetonitrile, and tetrahydrofuran was used to prepare working solutions of reference and sample preparations. Both the mobile phase and the diluent were filtered using a nylon membrane filter with a pore size of 0.2m.

A standard was prepared containing of 0.0006 mg/mL of all impurities together with alfuzosin hydrochloride. A sample solution was prepared consisting of 0.4mg/mL alfuzosin hydrochloride.

EXPERIMENTAL

System suitability

A standard solution consisting of alfuzosin hydrochloride along with impurities mix at the maximum allowable concentration (0.0006 mg/ml) was inserted in six replicates. The RSD was analyzed for the areas of all impurities and alfuzosin hydrochloride. The resolution between impurities A and D was calculated.

Specificity

During the specificity study, alfuzosin hydrochloride and impurities ranging from impurity-A to impurity-H were injected one at a time. The alfuzosin hydrochloride sample preparation (0.4mg/mL) was also injected, which was added with impurities at 0.15% level (total impurity mixture at 0.0006 mg/mL). The spectra and purity plots for each ingredient in the added sample were obtained using a DAD detector. In addition, forced degradation studies have beencarried out to prove the method stability. The sample solution was added to acid and base hydrolysis , oxidation with 30% H_2O_2, photolytic degradation, humidity(95%) and thermal(105°C). A PDA was used to determine peak purity.

Linearity/Range, Quantitation limit (QL) and Detection limit (DL)

As prepared six various concentrations of standard solutions of linearity with alfuzosin hydrochloride with all impurities ranging from LOQ to 200%of the limit concentration specified in the specification.Each linearity standard solution was injected three times, and each ingredient was exposed to a linear regression analysis.

System precision-repeatability-standard solution

The precision of the system was tested in six replicates using studying standard solution incorporating alfuzosin HCl and the aforementioned related compounds at 0.0006 mg/mL concentration.

Method precision-repeatability-sample solution

The repeatability of the system was investigated by studying alfuzosin hydrochloride samples in six preparations and the RSD was evaluated for individual and total impurity values.

Ruggedness- intermediate precision

Correctness was tested again with a different analyst, on various days, with different instruments, and with a different column. The RSD for individual and total impurities values was analyzed overall.

Accuracy

Alfuzosin hydrochloride samples were prepared spiked with impurities at the level of 50 %, 100 % and 120 % were tested.

Robustness

Various parameters of the pertaining to instrument conditions intentionally changed to determine the sturdiness of the method. A standard solution containing alfuzosin hydrochloride with its impurities with a maximum allowable concentration was inserted in six replicates, and the RSD for the area of all impurities and peaks of alfuzosin hydrochloride was found to be below 10.0%. The resolution between impurities E and Impurity D was observed to be more than 1.5.

i) Difference in flow rate ± 10%
ii) Difference in column oven temperature ± 5ºC
iii) Difference in wavelength ± 5 nm

Solution stability

Sample solution was injected at various time intervals for about 24 h maintained at 25±2°C through spiking impurities at 0.15% level. The cumulative RSD was determined for the area of impurities and alfuzosin hydrochloride peak in the standard solution and area for individual and total impurities in sample solution.

METHOD VALIDATION RESULTS AND TABLES:

System suitability

The peak area of the alfuzosin hydrochloride, the resolution criteria between impurities peaks E and C from the preparation for method validation was greater than 1.5.RSD. All impurities from the repeated injections of standard preparations were less than 10.0%, and all parameters were met throughout the validation process (Table1).

Specificity

The alfuzosin Hydrochloride peak was not co-eluted from each other impurities as shown in Figure 3.No blank peaks were observed during retention. The purity angle for the alfuzosin hydrochloride peak in the spiked sample was less than the purity threshold. As a result, the method was selective and specific. In addition, forced degradation studies have confirmed by specificity of the method. Alfuzosin Hydrochloride exhibited degradation products throughout acid, alkali hydrolysis and oxidation. Thus, the peak purity angle in all the aforementioned degradation samples was less than the purity threshold for alfuzosin hydrochloride. The method demonstrated stability for determining impurities in alfuzosin hydrochloride. The results of forced degradation studies are shown in Table 2.

Linearity/range , Detection limit (DL) and Quantitation limit (QL) for related substance method

The regression curves were in good agreement between peak area response with over all the concentration range as shown in Table 3 based on linear regression analysis for each ingredient.The DL and QL are also obtained in the same table.

Precision-repeatability

The RSD for independent and total impurities was revealed to be less than acceptance value (Table 4).

Intermediate precision-ruggedness

The calculated RSD of individual and total impurities was less than 10.0%.The overall RSD between method accuracy and intermediate precision was less than 10.0%, which indicating that the method is rugged, as shown in Table 4.

Accuracy

The improvement of prepared three sample was investigated in the range from 84.6% to 106.0%. The results are shown in Table 4.

Robustness

The robustness study findings were well within the limit for associated substance method (RSD NMT 10.0%). The data are presented in Table 5.

Solution stability

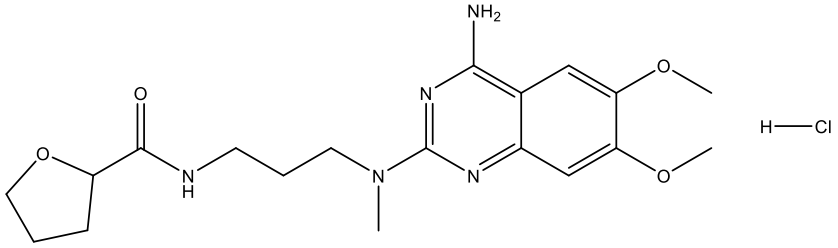
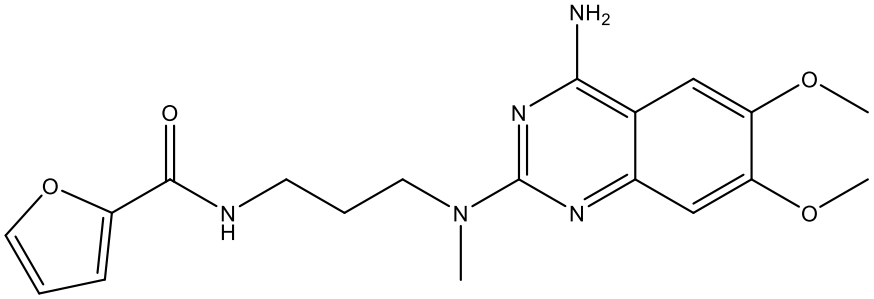
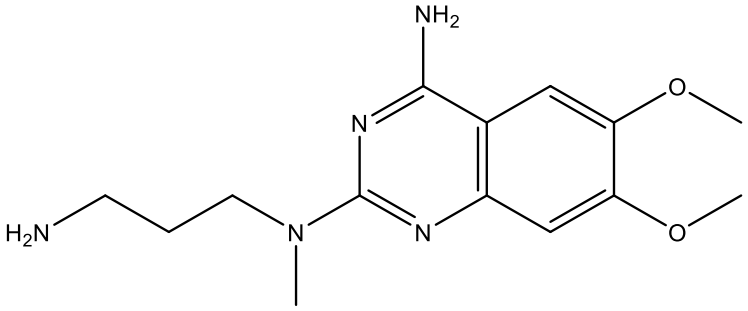
The cumulative percent RSD was estimated for individual and total impurities in the standard solution. It was found that the RSD should be less than 10.0%. The results were summarized in Table 6.

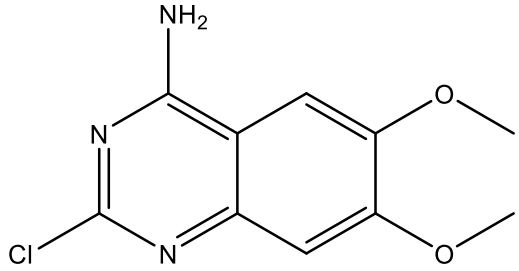
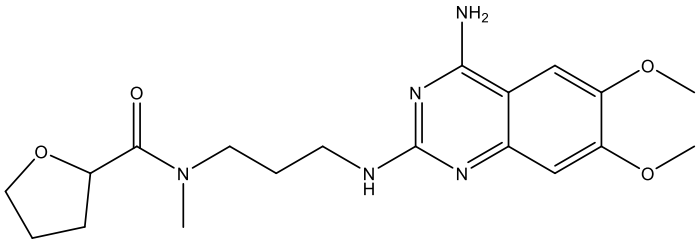
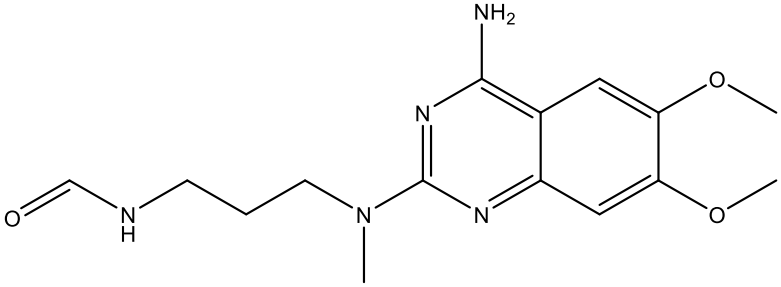
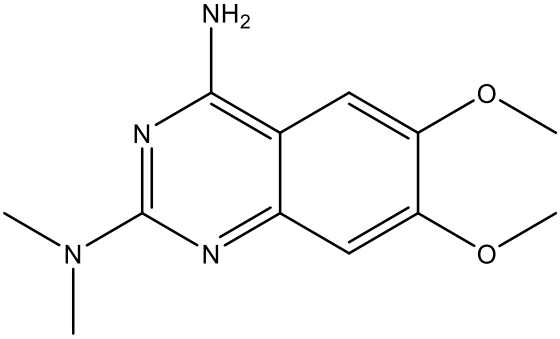
Figure captions:

Figure 1. Structure of Alfuzosin Hydrochloride and its impurities.

Figure 2. Various trials and conditions for method development.

Figure 3. Chromatographic separation of the Alfuzosin Hydrochloride and its impurities.

Alfuzosin hydrochloride	
Impurity A Furamide analog(USP) /EP Imp-A	 N-{3-[(4-Amino-6,7-dimethoxyquinazolin-2-yl)(methyl)amino]propyl}furan-2-carboxamide.
Impurity B USP Impurity Deacylatedalfuzosin Or EP Impurity-D	 N2-(3-Aminopropyl)-6,7-dimethoxy-N2-methylquinazoline-2,4-diamine
Impurity C EP Impurity-B	

	 2-chloro-6,7-dimethoxyquinazolin-4-amine
Impurity D EP Impurity-C	 *N*-(3-((4-amino-6,7-dimethoxyquinazolin-2-yl)amino)propyl)-*N*-methyltetrahydrofuran-2-carboxamide
Impurity E EP Impurity-E	 *N*-(3-((4-amino-6,7-dimethoxyquinazolin-2-yl)(methyl)amino)propyl)formamide
Impurity F EP Impurity-F	 6,7-dimethoxy-N^2,N^2-dimethylquinazoline-2,4-diamine

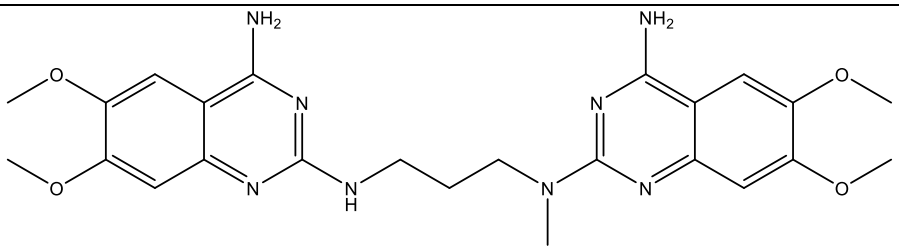
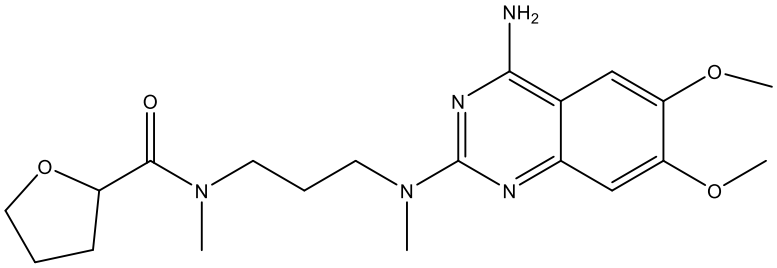
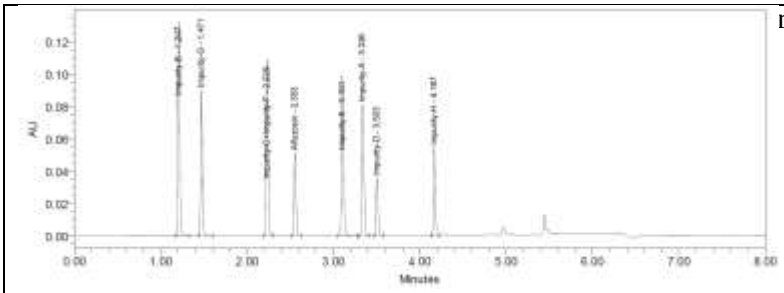
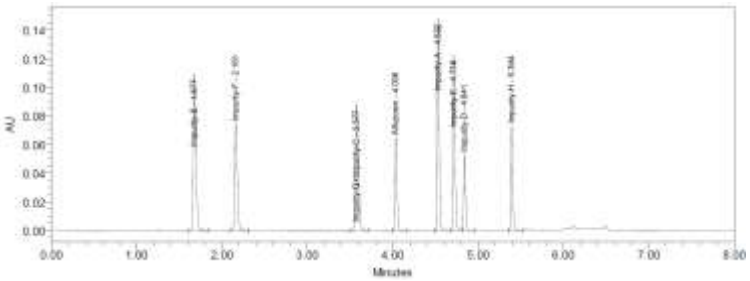
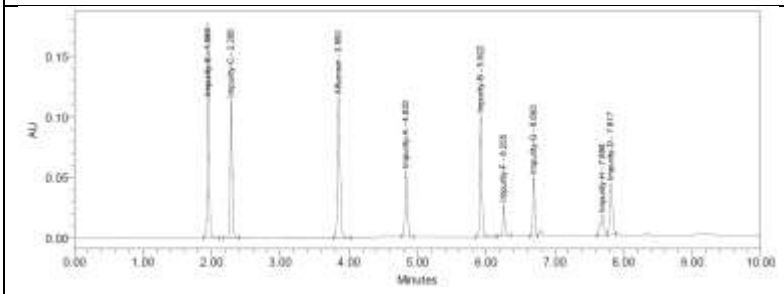
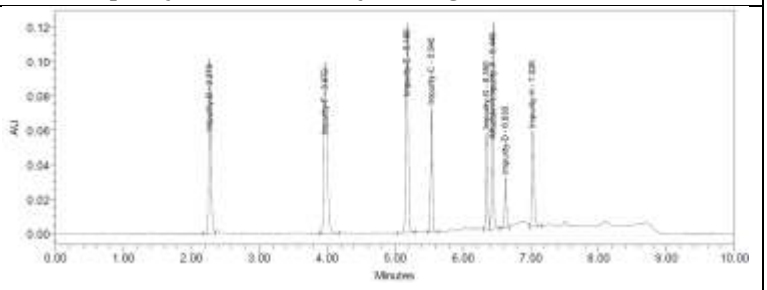
Impurity G EP Impurity-G	 N^2-(3-((4-amino-6,7-dimethoxyquinazolin-2-yl)amino)propyl)-6,7-dimethoxy-N^2-methylquinazoline-2,4-diamine
Impurity H (Process Impurity)	 *N*-(3-((4-amino-6,7-dimethoxyquinazolin-2-yl)(methyl)amino)propyl)-*N*-methyltetrahydrofuran-2-carboxamide

Figure 1. Structure of Alfuzosin Hydrochloride and its impurities.

Figure 2. Various trials and conditions for method development.

 Mobile phase-A: 0.1%Orthophosphoric acid in water Mobile phase-B: Acetonitrile Column : Acquity BEH C18 (50x 2.1mm,1.7µ) Initial B conc 30%, increased to 40% in 1.5 min, further to 90% at 6.0 min, %Revert back to initial conc at 6.01 min and maintained for 2.0 min. Column flow : 0.2mL/min, Column temp at 30°C and 2.00µL injection volume. **Result** Impurity-C and Impurity-F were closely eluting.	 Mobile phase-A: 0.2% Trifluoroacetic acid in water Mobile phase-B: Acetonitrile : Methanol in the ratio of 80:20 Column : Acquity BEH C8 (50x 2.1mm,1.7µ) Initial B conc 35%, increased to 55% in 2.5 min, further to 95% at 6.0 min Revert back to initial conc at 6.01 min and maintained for 2.0 min. Column flow : 0.2mL/min, Column temp at 30°C and 2.00µL injection volume. **Result** Impurity-G and Impurity-C were merging & Impurity-E and Impurity-D were Closely eluting.
 Mobile phase-A: 0.1%Dibasic sodium phosphate, pH adjusted to 7.5 with OPA Mobile phase-B: Acetonitrile Column : Acquity CSH C18 (50x 2.1mm,1.8µ) Initial B conc 30%, increased to 45% in 2.5 min, further to 75% at 6.0 min, maintained upto 8.0 min with 75%Revert back to initial conc at 8.01 min and maintained for 2.0 min. Column flow : 0.3mL/min, Column temp at 30°C and 1.00µL injection volume. **Result:** Peak splitting if Impurity-H and closely eluting with	 Mobile phase-A: 10mM Ammonuim acetate&Tetrahydrofuran(99:1) Mobile phase-B: Acetonitrile& Tetrahydrofuran(99:1) Column : Acquity BEHC18 (100x 2.1mm,1.7µ) Initial B conc 5%, increased to 70% in 8.0 min, revert back to initial conc at 8.01 min and maintained for 2.0 min. Column flow : 0.3mL/min, Column temp at 40°C and 1.00µL injection volume. **Result:** Alfuzosin and Impurity-A were merging.

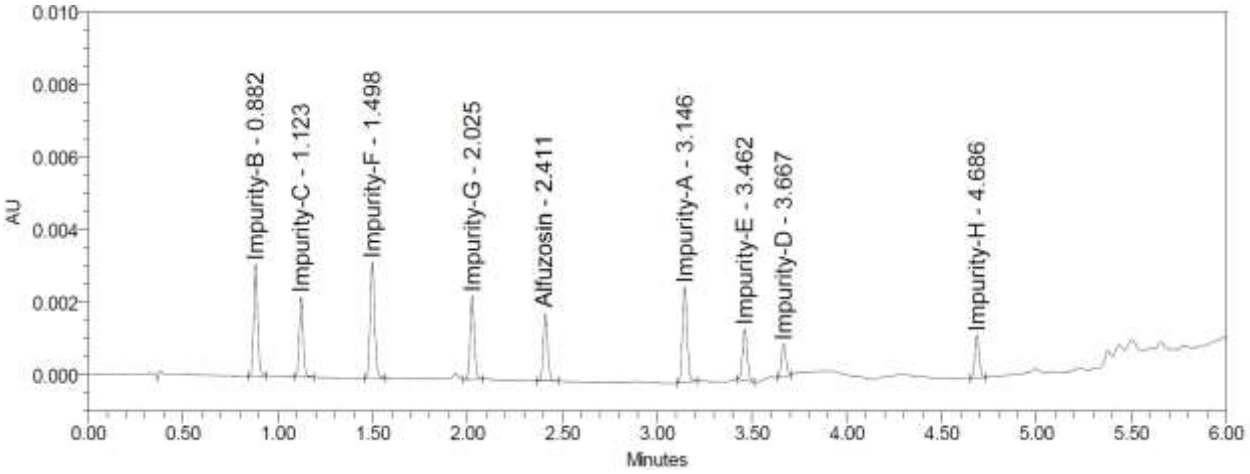


Figure 3. Chromatographic separation of Alfuzosin Hydrochloride and its impurities

Table 1. System Suitability Data

%Relative Standard Deviation for the area of Alfuzosin and its impurities in related substance validation (Acceptance criteria not more than 10.0%)

Parameters	**Alfuzosin**	**Impurities**							
		A	B	C	D	E	F	G	H
Specificity, Repeatability & Forced degradation	1.41	1.02	0.80	0.99	2.40	1.39	5.77	1.08	0.97
Intermediate Precision	3.06	1.88	1.12	1.45	2.89	2.06	4.55	2.29	2.01
Linearity	2.04	2.56	2.55	2.45	3.27	2.35	2.25	2.34	2.65
Accuracy	1.67	2.00	2.09	1.78	3.34	2.06	3.12	2.66	2.83
Solution stability	1.06	0.77	1.68	0.90	2.99	0.72	0.88	1.76	1.72
Robustness	1.77	1.32	1.38	2.54	2.63	1.39	1.17	2.23	1.87

Resolution between impurity-E and impurity D (Acceptance criteria not less than 1.5)

Parameters	**Resolution**
Specificity, Repeatability & Forced degradation	4.1
Intermediate Precision	4.5
Linearity	4.1
Accuracy	4.0
Solution stability	4.2
Robustness	4.3

Table 2. Results of Forced degradation.

Control sample (No treatment)	**Peak purity**	
	Purity	**Purity**

			0.417	1.259
Stress Study				
Samples	**Condition**	**% Degradatio**	**Peak Purity**	
			Purity	**Purity**
Acid Degradation	5ml 3N HCl/ 4 hours at	13.1	0.295	0.832
Alkali Degradation	5ml 3N HCl/ 4 hours at	11.2	0.321	0.759
Peroxide Degradation	5 ml 30% H_2O_2/ 25°C/6	9.8	0.396	0.991
Thermal Degradation-	105°C/3 days in hot air	1.8	0.263	1.581
Thermal Degradation-	80°C/8 Hrs in shaking	0.6	0.211	1.665
Humidity Degradation	25°C/95%RH/ 8 Hrs	0	0.438	1.043
Photodegradation	1.2 Million lux hrs and 200-watt hours/square	1.02	0.410	1.511

Table 3. Linearity, Limit of detection (LOD) and Limit of quantification (LOQ)

Componen	**Concentration range (µg/mL)**	**Regression equation**	**R^2**	**LOQ (µg/mL)**	**LOD (µg/mL)**
Alfuzosin Hydrochloride	0.069-1.199	y = 5351x-91	0.99684	0.069	0.029
Impurity-A	0.045-1.202	y = 8362x-200	0.99606	0.045	0.014
Impurity-B	0.057-1.203	y = 5754x-111	0.99844	0.057	0.017
Impurity-C	0.053-1.244	y = 6429x-65	0.99819	0.053	0.016
Impurity-D	0.093-1.224	y = 4479x-130	0.99632	0.093	0.028
Impurity-E	0.048-1.204	y = 8343x-198	0.99614	0.048	0.015
Impurity-F	0.122-1.207	y = 2865x-107	0.99493	0.122	0.037
Impurity-G	0.102-1.215	y=3509x-97	0.99391	0.102	0.031
Impurity-H	0.040-1.170	Y=10294x-184	0.99606	0.040	0.012

Linearity results (n=3), Acceptance criteria $R^2 > 0.98$

Table 4. Precision and Accuracy results

Validation step	**Parameter**	**Impurities**							
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Method precision	RSD	0.0	2.8	3.5	2.8	2.7	5.0	4.2	2.7

Intermediate precision	RSD	2.7	3.4	3.5	3.8	4.2	4.2	3.4	2.8
Accuracy (50%, 100% & 120%)	Average (% recovery)	93.7	95.3	96.7	96.3	95.6	94.5	92.9	95.4
	RSD (% recovery)	4.6	5.4	4.0	2.5	5.1	6.3	4.5	4.0

Acceptance criteria : 80 % to for Recovery 120% and not more than 10.0% for RSD

Table 5. Robustness

Parameter	**Impurities**							
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Overall RSD for individual impurities	5.5	5.7	6.2	4.5	3.3	7.2	6.5	5.7

Table 6. Solution stability (stored at 25°C ± 2°C)

	Parameter	**Area of Alfuzosin and its Impurities**							
		Alfuzosin	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Standard solution stability	**Cumulative RSD between initial to 24hrs**	0.8	0.4	0.6	1.8	3.2	0.7	0.7	0.9	1.2
Sample solution stability	**Cumulative RSD between initial to 24hrs**	1.3	0.6	1.2	2.1	2.9	0.9	1.2	1.6	1.7

RESULTS AND DISCUSSION

Several LC strategies that have shorter run times and higher throughput have been investigated to separate eight impurities along with alfuzosin hydrochloride, which includes various stationary phases, column dimensions and buffers. The various tests and their conditions are displayed in Figure 2. Furthermore, the Liquid chromatography system was improved by adopting a Waters ACQUITY UPLC® HSS T3 C18 10 cm x 2.1 milli meter fast LC column with a particle size of 1.8 µm. Consider, the ACQUITY UPLC® High strength silica Trifunctional Columns exploiting a Waters' advanced and patented stationary phase. It should be noted that T3 bonding with a trifunctional C18 alkyl phase can be associated with ligand density, which aids in aqueous mobile phase compatibility and polar compound retention. In addition, T3

endcapping is far superior to traditional trimethyl silane (TMS) endcapping. This special amalgamation of bonding and endcapping also improves the selectivity, lifetime, peak shape, column performance, method development, loading capacity, and stability. The mobile phase's initial gradient conditions are 95% of solvent A and 5% of solvent B, where a blend of 0.5% perchloric acid and water (99 : 1), pH adjusted to 3.5 with sodium hydroxide (buffer) with tetrahydrofuran, and a B is a mixture of acetonitrile and tetrahydrofuran (99 : 1). The gradient time program was extended to 85:15 for 1.5 min, then to 30:70 for 5.0 min and retained at 6.0 min. As a result, the effective separation time is 6.0 min. After 6.01 min, the eluent's original composition was restored and maintained for 2.0 min. And the parameters such as rate of mobile phase, column temperature, and injection volume were kept constant at 0.3 mL/min, 30 °C, and 2.00 µL, respectively, while the sample cooler was kept at 5 °C. As a diluent, a mixture (80:20:1) of buffer, acetonitrile, and tetrahydrofuran has been used to prepare reference solutions and test samples. All impurities and the alfuzosin hydrochloride peak were well isolated from one another, and there was no interference with the retention times of the known peaks in the blank. The LC-PDA studies were performed to ensure the purity of the prototype and the resolution of each degradation product peak in the UPLC-DAD chromatograms.

CONCLUSION

The UPLC method used to determine contaminants in Alfuzosin hydrochloride and active pharmaceutical ingredient is accurate, precise, and specific. The method has been evaluated and sufficient results have been obtained for all the verification parameters tested. The proposed approach can be used to easily measure the characteristics of Alfuzosin Hydrochloride in bulk pharmaceuticals. Because of the short analytical run time of 6.5 min, the consumption of lower solvent results is more cost-effective chromatographic method and greener chemistry.

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