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Stability Indicating UHPLC-PDA Assay for Simultaneous Determination of Antazoline Hydrochloride and Naphazoline Hydrochloride in Ophthalmic Formulations

Amir Ali,¹ Umar Farooq,¹ Mahmood Ahmed,^{1,*} Muhammad Makshoof Athar,¹ Kashif Nadeem² and Ghulam Murtaza²

¹ Institute of Chemistry, University of the Punjab, Lahore-Pakistan 54590

² Schazoo Pharmaceutical Laboratories, Lahore-Jaranwala Road, Sheikhpura

* Corresponding author: E-mail: mahmoodresearchscholar@gmail.com
tel.:0092 300 8819844

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Abstract

In the present study, a newly developed method based on ultrahigh performance liquid chromatography (UHPLC) was optimized for the simultaneous determination of antazoline hydrochloride (ANZ) and naphazoline hydrochloride (NFZ) in ophthalmic formulations. Isocratic separation of ANZ and NFZ was performed at 40 °C with an ACE Excel 2 C18-PFP column (2 µm, 2.1 × 100 mm) at a flow rate of 0.6 mL min⁻¹ whereas the mobile phase consisted of acetonitrile/phosphate buffer (60:40, v/v, pH 3.0) containing 0.5% triethylamine. Both analytes were detected at a wavelength of 285 nm and the injection volume was 1.0 µL. The overall run time per sample was 4.5 min with retention time of 0.92 and 1.86 min for NFZ and ANZ, respectively. The calibration curve was linear from 0.500–100 µg mL⁻¹ for ANZ and NFZ with a correlation coefficient ≥ 0.9981 while repeatability and reproducibility (expressed as relative standard deviation) were lower than 1.28 and 2.14%, respectively. In comparison with high-performance liquid chromatography (HPLC), the developed UHPLC method had remarkable advantages over HPLC as the run time was significantly reduced by 3.4-fold with a 5-fold decreased solvent consumption. Forced degradation studies indicated a complete separation of the analytes in the presence of their degradation products providing high degree of method specificity. The proposed UHPLC method was demonstrated to be simple and rapid for the determination of ANZ and NFZ in commercially available ophthalmic formulations providing recoveries between 99.6 and 100.4%.

Keywords: Antazoline; naphazoline; allergic conjunctivitis; vasoconstrictor; UHPLC

1. Introduction

Antazoline hydrochloride (ANZ), chemically known as *N*-benzyl-*N*-(4,5-dihydro-1*H*-imidazol-2-yl-methyl)aniline hydrochloride (C₁₇H₂₀ClN₃) is an imidazoline ligand which acts as histamine H1 receptor antagonist. It has not only a high affinity for 2-adrenoceptors in various tissues, but also binds to non-adrenergic imidazoline sites. ANZ is classified as first generation antihistamine used to reduce congestion of nasal passages and in eye drops in combination with naphazoline (NFZ) to reduce allergic conjunctivitis symptoms.^{1–3} Another drug belonging to the imidazole group is naphazoline hydrochloride

(NFZ, C₁₄H₁₅ClN₂) which is α-sympathomimetic drug with decongestive and vasoconstrictor properties reducing the mucous membrane swelling rapidly.^{4–6} The chemical structures for both drugs are depicted in Fig.1. Until now, NFZ was only analyzed in combination with other drugs than ANZ in ophthalmic formulations using capillary electrophoresis,⁷ gas chromatography,⁸ spectrofluorimetry,⁹ phosphometric,¹⁰ electrochemical¹¹ detectors coupled to HPLC.^{12,13}

To the best of our knowledge, only one first-derivative spectrometric assay for the simultaneous determination of ANZ and NFZ in ophthalmic formulations was published in the literature. ANZ in combination with NFZ

was determined by first derivative spectrometry.⁶ However, our goal was to optimize and validate an ultrahigh performance liquid chromatography (UHPLC) based method for the direct simultaneous analysis of ANZ and NFZ in ophthalmic preparations. High performance liquid chromatography (HPLC) is prominent technique in laboratories for the last 30 years but did not keep the pace with the growing demand for a reliable analysis in short time. More samples can be analysed in very short time with reliability and accuracy by UHPLC. Eddy and longitudinal diffusion coefficients in Van Deemter equation play an important role regarding the separation of analytes. These coefficients are directly related to particle size of column packing which results in better resolution.^{14–17} The developed method was compared with HPLC to prove its adequacy for pharmaceutical studies with minimum consumption of solvents, high resolution and symmetrical peaks. The UHPLC-based method was validated in accordance to guidelines from the International Conference on Harmonization (ICH)^{18–21} and forced degradation studies were conducted to demonstrate the applicability of the proposed UHPLC method.

Figure 1. Molecular structures of analytes

2. Experimental

2.1. Chemicals and Reagents

ANZ, NFZ reference standard were provided by Schazoo Labs (Pakistan). Acetonitrile (ACN), potassium dihydrogen phosphate (KH_2PO_4), phosphoric acid (H_3PO_4 , 85%) and triethylamine (TEA) were supplied by Honeywell (USA). All the chemicals and reagents were of analytical grade while GenPure water system (Thermo Scientific, USA) was used to obtain ultrapure water ($18 \text{ M}\Omega\cdot\text{cm}^{-1}$).

2.2. Chromatography

For HPLC analysis, Shimadzu Japan liquid chromatographic system (LC-20A) with diode array detector (SPDM20A), online degasser (DGU-20A5), and equipped with ACE 5 C18 column ($5 \mu\text{m}$, $4.6 \times 250 \text{ mm}$) was used. For UHPLC analysis, Shimadzu Japan, liquid chromatographic system (Nexera 2, LC-30AD), with diode array detector (*i*-DReC, SPD-M30A), online degasser (DGU-20A5), and equipped with ACE Excel 2 C18-PFP column ($2 \mu\text{m}$, $2.1 \times 100 \text{ mm}$) was used. Both systems were equip-

ped with auto sampler (SIL-20AXR) with injection volume ranges between $0.1\text{--}50 \mu\text{L}$. Mobile phase comprised of ACN and phosphate buffer pumped in ratio of 60:40 (v/v) at pH 3.0 adjusted by TFA (pH meter, Orion 5 Star, Thermo Scientific, UK). $5.0 \mu\text{L}$ and $1.0 \mu\text{L}$ injection volume was injected and flow rate set at 1.0 and 0.6 mL min^{-1} for HPLC and UHPLC, respectively. The detection was carried out at 25°C and 40°C , respectively, for HPLC and UHPLC with best selected wavelength of 285 nm by *i*-DReC (detector). Shimadzu LC program (Lab Solutions software) was used to record chromatograms, peak quantification and integration. Mobile phase, standard solutions and samples were filtered through nylon filter ($0.45 \mu\text{m}$, Sartorius, Germany) before injection into chromatographic system.

2.3. Standard and Working Solutions

Individual stock standard solutions of ANZ and NFZ ($1000 \mu\text{g mL}^{-1}$) were prepared in methanol and placed in an ultrasonic bath for 15 min. Subsequently, working solutions were prepared by serial dilution of the individual stock standard solutions with the mobile phase. Mixed standard solution of ANZ ($2.50 \mu\text{g mL}^{-1}$) and NFZ ($50.0 \mu\text{g mL}^{-1}$) were also prepared in the same manner.

2.4. Solution for Forced Degradation Studies

Forced degradation studies were conducted with ANZ or NFZ solutions at a final concentration of $50.0 \mu\text{g mL}^{-1}$. For mild stress conditions the solutions were treated with 0.1 M HCl and 0.1 M NaOH for acidic and basic hydrolysis for 1 h, $3\% \text{ H}_2\text{O}_2$ was employed for oxidative stress (1 h), while photolytic and thermal stress tests were performed after exposing the solid form for 6 h under UV (254 nm) or kept at 100°C , respectively. In contrast for drastic stress conditions, 1 M HCl and 1 M NaOH were utilized for acidic and basic hydrolysis for 10 h, $30\% \text{ H}_2\text{O}_2$ was employed for oxidative stress (10 h), whereas photolytic and thermal degradation were evaluated after exposing the solid form for 24 h under UV (254 nm) or kept at 100°C , respectively.

2.5. Analysis of Ophthalmic Preparation

Curall-A[®] and Orbaclear[®] eye drops both contain 0.5% of ANZ (5.00 mg mL^{-1}) and 0.025% of NFZ (0.250 mg mL^{-1}). Suitable aliquots of each commercially available product was diluted with methanol, sonicated for 15 min and was further diluted with mobile phase prior to UHPLC analysis.^{22,23}

2.6. Validation Studies

Validation studies were performed to characterize the proposed analytical method including specificity, linearity, accuracy, precision, limit of detection (LOD), li-

mit of quantitation (LOQ) and conformity of chromatographic parameters (tailing factor, selectivity factor, resolution, theoretical plates). Conformity of chromatographic conditions are basically system suitability tests which are the foremost part of validation studies.

Specificity

Analysis of placebo (aqueous based sodium chloride and benzalkonium chloride) was performed to assess the specificity of the proposed method. Moreover, the forced degradation study is also another parameter which was performed to prove the specificity of chromatographic method.^{21,24}

2. 6. 1. Linear Dynamic Range

For both LC-based methods (HPLC and UHPLC), the linear dynamic range was selected within 0.500–100 $\mu\text{g mL}^{-1}$ for both analytes. A linear calibration curve in the form of $y = ax + b$ was obtained by plotting the peak area y against the nominal concentration x of seven concentrations (0.500, 2.50, 12.5, 25.0, 50.0, 75.0 and 100 $\mu\text{g/mL}$) whereas a represented the slope of the calibration curve and b indicated the intercept.

2. 6. 2. Accuracy and Precision

The accuracy of each method was determined in triplicate by spiking a known amount of analyte standard solution in the dosage form resulting in final concentrations of 2.50, 5.00 and 7.50 mg mL^{-1} for ANZ, whereas NFZ was spiked at 0.125, 0.250 and 0.375 mg mL^{-1} . This represented 50, 100 and 150% of each analyte in the dosing formulation. For precision determination, ANZ as well as

NFZ were spiked at 4.00, 5.00, 6.00 mg mL^{-1} and 0.200, 0.250, 0.300 mg mL^{-1} , respectively, representing 80, 100 and 120% of each analyte. The intra-day precision (repeatability) was evaluated by replicates of five on one day whereas the inter-day precision (reproducibility) was determined over three consecutive days.

2. 6. 3. Method's LOD/LOQ

ANZ and NFZ standard solution was injected in replicates of six. The resultant parameters of the linear regression including the standard deviation (SD) of the response based upon the slope a and intercept b determined the LOD and LOQ of the UHPLC method. The LOD and LOQ were defined as three times and ten times the SD of the intercept, respectively.^{25–28}

2. 6. 4. Method Robustness

Small but deliberate changes in chromatographic conditions such as mobile phase, pH, column temperature and flow rate were used to evaluate the robustness of proposed UHPLC method.

3. Results and Discussion

3. 1. Optimization of Chromatographic Conditions

System suitability tests were performed to optimize both LC-based methods. In the first step, a UV absorption spectrum was acquired in the range of 200–400 nm resulting in a maximum absorbance at 285 nm for both analy-

Table 1. Results of tested stationary phases

HPLC					
Column	Drug	R_s	T_r	α	N
Hypersil ODS	ANZ	3.66	1.27	2.14	4521
(250 × 4.6 mm, 5 μm)	NFZ	–	1.36	–	5043
Venusil XBP C18	ANZ	4.66	1.21	2.35	3203
(250 × 4.6 mm, 5 μm)	NFZ	–	1.34	–	4597
ACE 5 C18	ANZ	8.92	1.17	2.32	5432
(250 × 4.6 mm, 5 μm)	NFZ	–	1.13	–	6162
Purespher® RP-18	ANZ	0.89	2.27	1.13	3576
(250 × 4.6 mm, 5 μm)	NFZ	–	1.66	–	3278
UHPLC					
ACE Excel 2 C18-PFP	ANZ	3.43	0.97	2.27	17289
(100 × 2.1 mm, 2 μm)	NFZ	–	0.94	–	18521
Waters ACQUITY 1.7 BEH C 18	ANZ	1.09	2.07	0.87	13098
(100 × 2.1 mm, 2 μm)	NFZ	–	1.83	–	11832
Agilent Poroshell 2.7 120 EC C18	ANZ	1.01	2.23	0.88	12094
(100 × 2.1 mm, 2 μm)	NFZ	–	1.45	–	11095
Phenomenex Kinetex 2.6 C18	ANZ	0.92	2.07	0.93	10654
(100 × 2.1 mm, 2 μm)	NFZ	–	1.44	–	13294

tes. Different compositions (60:40, 50:50, 40:60 and 30:70, v/v) of the mobile phase (ACN: phosphate buffer) were examined to optimize the chromatographic conditions including tailing factor, selectivity factor, resolution, theoretical plates to get compliance with the ICH guidelines. Different columns were also tested for HPLC analysis including ACE 5 C18, Venusil XBP C18, Hypersil ODS and Purespher® RP-18 while ACE Excel 2 C18-PFP, Waters ACQUITY 1.7 BEH C 18, Agilent Poroshell 2.7 120 EC C18 and Phenomenex Kinetex 2.6 C18 were employed with UHPLC at different pH (3.0, 4.0, 5.0 and 6.0). Free silanol in column packing could be interacting with drugs of both acidic as well as of basic nature. In order to improve the peak shapes, TEA, a silanol blocker,

was added to the mobile phase (0.5%, v/v).²⁹ Silanol blocker provided additional selectivity by π - π and dipole interaction which resulted in achieving the better overall resolution. The summary of the tailing factor, selectivity factor, resolution and theoretical plates for each of the tested columns are presented in Table 1.

In the end, the mobile phase consisting of ACN and phosphate buffer (10 mM) in a ratio of 60:40 (v/v) with the addition of 0.5% TEA was found to be excellent using ACE 5 C18 and ACE Excel 2 C18-PFP columns for UHPLC and HPLC analysis, respectively. The chromatographic parameters under final conditions are summarized in Table 2 exhibiting an excellent peak shape, resolution and higher number of theoretical plates.

Table 2. System suitability test parameters

Parameters	Analyte/Technique			
	HPLC		UHPLC	
	ANZ	NFZ	ANZ	NFZ
Retention time (t_R in min)	6.45	3.21	1.86	0.92
Tailing factor (T)	1.17	1.13	0.97	0.94
Resolution (Rs)	8.92	–	3.43	–
Selectivity factor (α)	2.32	–	2.27	–
Theoretical plates (N)	5432	6182	17289	18521
% RSD of Retention Time (t_R)	0.003	0.002	0.002	0.004

Figure 2. Typical HPLC and UHPLC Chromatograms of mobile phase, placebo, ANZ and NFZ

3. 2. Validation Studies

The specificity of the optimized UHPLC method was examined with NFZ and ANZ at 2.50 and 50.00 $\mu\text{g mL}^{-1}$, respectively, relative to the blank mobile phase (Fig. 2).

The presence of placebo did not interfere during the determination of ANZ and NFZ as the components were baseline separated. For both chromatographic methods over a dynamic range of 0.5–100 $\mu\text{g mL}^{-1}$, seven concen-

trations (0.5, 2.5, 12.5, 25, 50, 75, 100 $\mu\text{g mL}^{-1}$) were employed to construct a calibration graph for ANZ and NFZ. The calibration curves were linear for ANZ and NFZ with a coefficient of determination (r^2) ≥ 0.9981 regardless of the LC-based method (Table 3). Accuracy of methods by both the techniques under investigation was performed by evaluating the recovery studies after spiking the known amount of standard drugs in commercial products.

Table 3. Statistical evaluation of regression data of ANZ and NFZ by HPLC and UHPLC

Parameters	Analyte/Technique			
	HPLC		UHPLC	
	ANZ	NFZ	ANZ	NFZ
Linearity range ($\mu\text{g/mL}$)	0.5–100	0.5–100	0.5–100	0.5–100
Slope	6.0615	0.7585	0.5854	0.6854
Intercept	23.303	32.745	134.52	104.52
Standard error of slope	1.01×10^{-2}	5.41×10^{-3}	3.1×10^{-2}	4.21×10^{-2}
Standard error of intercept	0.163	0.087	1.132	1.932
Coefficient of determination (r^2)	0.9991	0.9989	0.9984	0.9981
Limit of detection ($\mu\text{g mL}^{-1}$)	0.078	0.041	0.052	0.029
Limit of quantification ($\mu\text{g mL}^{-1}$)	0.261	0.137	0.174	0.097

Table 4. Accuracy studies of ANZ and NFZ by HPLC and UHPLC

Drugs	Spiked concentration (mg mL^{-1})	HPLC		UHPLC	
		^a Concentration found (mg mL^{-1}) $\pm$ SEM; RSD	Recovery (%)	^a Concentration found (mg mL^{-1}) $\pm$ SEM; RSD	Recovery (%)
ANZ	2.500	2.531 ± 0.021 ; 1.18	101.2	2.552 ± 0.022 ; 1.48	102.0
	5.000	5.071 ± 0.018 ; 0.71	101.4	5.081 ± 0.021 ; 0.71	101.6
	7.500	7.483 ± 0.017 ; 0.43	99.7	7.572 ± 0.014 ; 0.33	100.9
NFZ	0.125	0.127 ± 0.002 ; 2.08	101.6	0.126 ± 0.002 ; 2.55	100.8
	0.250	0.256 ± 0.001 ; 0.98	102.4	0.258 ± 0.002 ; 1.16	103.2
	0.375	0.379 ± 0.004 ; 1.98	101.1	0.379 ± 0.001 ; 0.40	101.1

^a All measurements were made in triplicate SEM: Standard error mean RSD: Relative standard deviation

Table 5. Precision studies of ANZ and NFZ by HPLC and UHPLC

Technique: HPLC					
Drugs	Repeatability (n = 5)		Reproducibility (n = 5)		
	Concentration (mg mL^{-1})	Concentration found (mg mL^{-1}) $\pm$ SEM; RSD	Concentration found (mg mL^{-1}) $\pm$ SEM; RSD		
			Day 1	Day 2	Day 3
ANZ	4.0	3.941 ± 0.031 ; 1.45	3.922 ± 0.01 ; 0.61	3.931 ± 0.020 ; 1.41	4.013 ± 0.021 ; 1.03
	5.0	4.910 ± 0.011 ; 0.42	4.951 ± 0.02 ; 0.84	4.991 ± 0.020 ; 1.00	5.021 ± 0.032 ; 1.19
	6.0	5.982 ± 0.061 ; 1.70	5.992 ± 0.03 ; 1.20	5.992 ± 0.020 ; 0.80	6.041 ± 0.031 ; 1.01
NFZ	0.20	0.192 ± 0.002 ; 1.88	0.198 ± 0.002 ; 2.30	0.205 ± 0.003 ; 2.23	0.204 ± 0.002 ; 1.98
	0.25	0.246 ± 0.001 ; 1.02	0.258 ± 0.002 ; 1.72	0.253 ± 0.002 ; 1.97	0.252 ± 0.001 ; 1.29
	0.30	0.291 ± 0.001 ; 0.72	0.299 ± 0.003 ; 2.49	0.306 ± 0.003 ; 1.88	0.301 ± 0.001 ; 0.98
Technique: UHPLC					
ANZ	4.0	4.050 ± 0.021 ; 1.28	4.000 ± 0.021 ; 1.02	4.010 ± 0.021 ; 1.19	4.030 ± 0.011 ; 0.54
	5.0	5.020 ± 0.020 ; 0.80	5.030 ± 0.022 ; 0.79	5.030 ± 0.012 ; 0.42	5.010 ± 0.012 ; 0.39
	6.0	5.990 ± 0.022 ; 0.84	6.010 ± 0.021 ; 0.78	5.980 ± 0.011 ; 0.42	5.990 ± 0.022 ; 0.74
NFZ	0.20	0.201 ± 0.001 ; 1.20	0.203 ± 0.002 ; 1.67	0.204 ± 0.001 ; 1.64	0.203 ± 0.002 ; 2.14
	0.25	0.252 ± 0.001 ; 0.93	0.249 ± 0.001 ; 1.08	0.251 ± 0.001 ; 1.08	0.253 ± 0.001 ; 1.23
	0.30	0.301 ± 0.001 ; 0.97	0.301 ± 0.001 ; 0.54	0.299 ± 0.001 ; 0.81	0.301 ± 0.002 ; 1.35

The recovery results were obtained between the ranges 99.7–102.4% and 100.8–103.2% (Table 4) for HPLC and UHPLC respectively which justified the suitability of the techniques for their intended applications. For precision studies, the results of repeatability and reproducibility are presented in Table 5 by injecting three different concentrations (80, 100 and 120% level of analyte) of standard solutions of ANZ and NFZ ($n = 5$) on same day and three consecutive days, respectively.

RSD values for repeatability and reproducibility were obtained less than 1.88 and 2.49, respectively, for HPLC while below than 1.28 for repeatability and 2.14 for reproducibility assays with UHPLC.

3. 3. Forced Degradation Studies

Forced degradation studies under mild and drastic conditions were performed to demonstrate the applicability of our UHPLC method to assess the stability of the drug. For each forced degradation experiment, the corresponding chromatograms are depicted in Figure 3 and 4, whereas the percentage of initial drug substance is shown in Table 6. The ANZ and NFZ stock solutions were diluted in separate measuring flasks with HCl (0.1 M and 1.0 M), NaOH (0.1 M and 1.0 M) and H_2O_2 (3.0 and 30%) to a final concentration of $50.0 \mu\text{g mL}^{-1}$ and incubated for 1 h and 10 h for mild and drastic stress, respectively. For both mild and drastic conditions, no degradations (Figs. 3 and 4) were observed for ANZ and NFZ under acidic stress and the extent of degradation was declared as none (Table 6).

In contrast, basic stress was found more effective than acidic one. Under mild conditions, there was only a slight extent of degradation and the remaining concentration of ANZ and NFZ compared to their initial values were 96.1% and 95.2%, respectively. Two impurity peaks at retention times $t_R = 0.6$ min and $t_R = 1.35$ min (FD1 and

FD2, Fig. 3b) were observed after basic degradation of NFZ, while one impurity peak at $t_R = 2.25$ min (FD1, Fig. 4b) was observed for ANZ degradation under basic stress conditions. For drastic conditions, substantial amount of both ANZ and NFZ were degraded and remaining amount of 22.6% and 48.8% was found respectively under basic stress conditions. Two impurities peaks for each of ANZ and NFZ were observed at $t_R = 0.9$ min, $t_R = 2.35$ min (FD1 and FD2, Fig. 4g) and $t_R = 1.08$ min, $t_R = 1.35$ min (FD1 and FD2, Fig. 3g), respectively. The addition of 3% H_2O_2 did not show any effect on ANZ but NFZ was degraded substantially and remaining amount obtained was 74.6% with impurities at $t_R = 0.55$ min, $t_R = 0.71$ min (FD1 and FD2, Fig. 3c). After treatment with 30% H_2O_2 , slight degradation of ANZ (remaining amount 94.8%) and substantial degradation of NFZ (remaining amount 71.1%) were observed. Two peaks at $t_R = 0.55$ min, $t_R = 0.68$ min (FDD1 and FDD2, Fig. 3h) were obtained as degradation products of NFZ, while at $t_R = 0.95$ min degradation product of ANZ was obtained (FDD1, Fig. 4h). Solid form of both analytes was kept at 100°C in oven for thermal and under UV light at 254 nm for photolytic degradation.

NFZ degraded substantially under mild and drastic thermal stress conditions with two impurity peaks at $t_R = 0.65$ min, $t_R = 1.31$ min (Fig. 3d and 3i). In contrast, no ANZ degradation was observed under mild thermal stress, whereas substantial amount of ANZ degraded under drastic conditions (remaining amount 64.9%), indicated by an impurity at $t_R = 0.95$ min (FDD1, Fig. 4i). No photolytic degradation resulted for ANZ and NFZ under mild conditions. However under drastic photolytic stress, substantial degradation of ANZ and NFZ resulted in an impurity peak at $t_R = 0.95$ min and $t_R = 0.60$ min, respectively (Fig 3j and 4j). As a result of these experiments, the proposed UHPLC method was found to be specific for the analysis of ANZ and NFZ including their degradation products.

Table 6. Forced degradation results of ANZ and NFZ by UHPLC

Stress conditions	Amount remaining ^a Mean ± SEM (%)			Extent of degradation	
	ANZ	NFZ	ANZ	NFZ	
Mild conditions	0.1M HCl (1 h)	99.9 ± 0.04	99.8 ± 0.04	None	None
	0.1M NaOH (1 h)	96.1 ± 0.09	95.2 ± 0.25	Slight	Slight
	3% H ₂ O ₂ (1 h)	98.6 ± 0.09	74.6 ± 0.05	None	Substantial
	6 h under 100 °C	99.6 ± 0.01	63.6 ± 0.02	None	Substantial
	6 h under UV-254	99.1 ± 0.01	99.0 ± 0.19	None	None
Drastic conditions	1.0 M HCl (10 h)	99.4 ± 0.04	99.7 ± 0.01	None	None
	1.0 M NaOH (10 h)	22.6 ± 0.16	48.8 ± 0.14	Substantial	Substantial
	30% H ₂ O ₂ (10 h)	94.8 ± 0.19	71.1 ± 0.09	Slight	Substantial
	24 h under 100 °C	64.9 ± 0.15	63.3 ± 0.18	Substantial	Substantial
	24 h under UV-254	89.1 ± 0.11	87.7 ± 0.05	Substantial	Substantial

^a All measurements were made triplicate

Figure 3. Typical UHPLC chromatograms of pure bulk sample of NFZ ($50 \mu\text{g mL}^{-1}$) under stress conditions. Mild stress conditions: (a) acidic (b) basic (c) oxidative (d) thermal (e) photolytic. Drastic stress conditions: (f) acidic (g) basic (h) oxidative (i) thermal (j) photolytic.

Figure 4. Typical UHPLC chromatograms of pure bulk sample of ANZ ($50 \mu\text{g mL}^{-1}$) under stress conditions. Mild stress conditions: (a) acidic (b) basic (c) oxidative (d) thermal (e) photolytic. Drastic stress conditions: (f) acidic (g) basic (h) oxidative (i) thermal (j) photolytic.

3. 4. Method Robustness

The robustness of proposed the UHPLC method was evaluated by slight changes of the chromatographic parameters including the flow rate ($\pm 0.1 \text{ mL min}^{-1}$), the mobile phase ratio ($\pm 5.0 \text{ mL}$) including its pH (± 0.1), the column temperature ($\pm 5 \text{ }^{\circ}\text{C}$) and the detection wavelength ($\pm 2 \text{ nm}$). Afterwards, the drug content besides chromatographic parameters like retention time, tailing factor, number of theoretical plates and resolution were determined. The results summarized in Table 7 demonstrated that the effects of the deliberate changes in chromatographic conditions are neglectable and that the proposed UHPLC method was robust for its intended applications.¹⁷

3. 5. Analysis of Commercial Ophthalmic Formulations

The applicability of proposed UHPLC method was evaluated by examining the commercial ophthalmic solutions (Curall-A[®] and Orbaclear[®]) with reported concentration of 5.0 mg mL^{-1} of ANZ and 0.25 mg mL^{-1} of NFZ. It was ensured that the removal of the excipients with an extraction step before analysis was unnecessary. It was concluded that the proposed UHPLC method was sufficiently accurate and precise (Table 8) with recovery and RSD ranges found between 99.6–100.4% and 1.03–1.20%, respectively.

4. Conclusion

In literature, UHPLC method for simultaneous determination of ANZ and NFZ in pharmaceutical formulations is not available. For this reason, stability indicating UHPLC method was fully validated according to ICH guidelines for determination of ANZ and NFZ in ophthalmic formulations. Remarkable advantages of UHPLC over HPLC were found, such as rapidity, ease of operation, minimum amount of solvents and high selectivity. Good recoveries, interference free and highly reproducible chromatograms were achieved. The proposed method was optimized and found suitable for quality control laboratories where time and economy are essentially required. Good recovery results showed that the proposed methods were free from interferences of commonly used excipients and additives in the formulations.

5. References

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Table 7. Robustness study of ANZ and NFZ by UHPLC

Chromatographic conditions	NFZ				ANZ				Rs
	Assay (%)	$t_R(\text{min})$	N	TF	Assay (%)	$t_R(\text{min})$	N	TF	
Flow rate:0.7	101.3	0.90	18540	0.95	101.2	1.85	17265	0.93	3.47
Flow rate:0.5	100.2	0.96	18351	0.99	99.8	1.88	17233	0.98	3.41
Mobile phase (65:35)	100.1	0.91	18040	0.93	99.1	1.87	17865	0.99	3.47
Mobile phase (55:45)	99.5	0.93	18259	0.98	99.8	1.87	17223	0.93	3.41
Column temp. (45 $^{\circ}\text{C}$)	99.3	0.95	18390	0.97	101.3	1.89	17444	0.96	3.41
Column temp. (35 $^{\circ}\text{C}$)	100.1	0.93	18287	0.96	100.1	1.81	17304	0.98	3.38
Wavelength (287 nm)	100.2	0.92	18289	0.99	100.2	1.87	17109	0.94	3.44
Wavelength (283 nm)	100.4	0.91	18401	0.96	100.3	1.86	17119	0.95	3.45
pH: 3.1	99.9	0.94	18540	0.96	100.4	1.88	17165	0.94	3.42
pH: 2.9	99.8	0.92	18387	0.93	100.3	1.89	17338	0.95	3.45

t_R : Retention time, N: theoretical plates, TF: tailing factor, Rs: resolution

Table 8. Assay result of ANZ and NFZ by UHPLC in commercial ophthalmic formulations

Product	Contents (mg mL ⁻¹)	Label claim (mg mL ⁻¹) $\pm$ SEM; RSD	^a Concentration found	Recovery (%)
Curall-A [®]	ANZ	5.00	5.010 ± 0.02 ; 1.20	100.2
	NFZ	0.25	0.249 ± 0.001 ; 1.12	99.6
Orbaclear [®]	ANZ	5.00	4.990 ± 0.02 ; 1.19	99.8
	NFZ	0.25	0.251 ± 0.001 ; 1.03	100.4

^aResults are expressed as average of ten measurements

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Povzetek

V predstavljeni študiji smo optimizirali novo metodo na osnovi ultravisokoločljivostne tekočinske kromatografije (UHPLC) za hkratno določanje antazolin hidroklorida (ANZ) in nafazolin hidroklorida (NFZ) v oftalmičnih pripravkih. Izo-kratska ločba ANZ in NFZ je potekala pri 40 °C na ACE Excel 2 C18-PFP koloni (2 µm, 2,1 × 100 mm) pri pretoku 0,6 mL min⁻¹, mobilna faza pa je bila sestavljena iz acetonitrila in fosfatnega pufru (60:40, v/v, pH 3,0), ki je vseboval 0,5 % trietilamina. Za oba analita je bila detekcija pri valovni dolžini 285 nm, volumen injiciranja pa je bil 1,0 µL. Skupni čas analize na vzorec je bil 4,5 min z retencijskim časom za NFZ 0,92 min in za ANZ 1,86 min. Kalibracijska krivulja je bila linearna od 0,500–100 µg mL⁻¹ za ANZ in NFZ s korelacijskim koeficientom ≥ 0,9981, medtem ko sta bili ponovljivost in obnovljivost (izraženi kot relativni standardni odklik) nižji od 1,28 % za ANZ in od 2,14 % za NFZ. V primerjavi z visokoločljivostno tekočinsko kromatografijo (HPLC) ima razvita UHPLC metoda znatne prednosti, saj je bil čas analize bistveno krajši za 3,4-krat, poraba topil pa manjša za 5-krat. Pri študiju prisilnega razpada smo dobili popolno ločbo analitov v prisotnosti njihovih razpadnih produktov, kar pomeni visoko stopnjo specifičnosti metode. Predlagana UHPLC metoda se je izkazala kot preprosta in hitra za določanje ANZ in NFZ v komercialno dostopnih oftalmičnih pripravkih z izkoristki med 99,6 in 100,4 %.