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Structural evaluation of degradation products of Loteprednol using LC-MS/MS: Development of an HPLC method for analyzing process-related impurities of Loteprednol

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Abstract: The current investigation entails the characterization of five degradation products (DPs) formed under different stress conditions of loteprednol using liquid chromatography–tandem mass spectrometry (LC-MS/MS). In addition, this study developed a stable high-performance liquid chromatography (HPLC) method for evaluating loteprednol along with impurities. The method conditions were meticulously fine-tuned which involved the exploration of the appropriate solvent, pH, flow of the mobile phase, columns, and wavelength. The method conditions were carefully chosen to successfully resolve the impurities of loteprednol and were employed in subsequent validation procedures. The stability profile of loteprednol was exposed to stress degradation experiments conducted under five conditions, and DPs were structurally characterized by employing LC-MS/MS. The chromatographic resolution of loteprednol and its impurities along with DPs was effectively achieved using a Phenomenex Luna 250 mm C18 column using 0.1 % phosphoric acid, methanol, and acetonitrile in 45:25:30 (v/v) pumped isocratically at 0.8 mL/min with 243 nm wavelength. The method produces an accurate fit calibration curve in 50-300 μg/mL for loteprednol and LOQ (0.05 μg/mL) – 0.30 μg/mL for its impurities with acceptable precision, accuracy, and recovery. The stress-induced degradation study revealed the degradation of loteprednol under basic, acidic, and photolytic conditions, resulting in the formation of seven distinct DPs. The efficacy of this method was validated through LC-MS/MS, which allowed for the verification of the chemical structures of the newly generated DPs of loteprednol. This method was appropriate for assessing the impurities of loteprednol and can also be appropriate for structural and quantitative assessment of its degradation products.

Key words: loteprednol, stress degradation products, characterization, impurity analysis

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1. Introduction

The pharmaceutical product that exhibits therapeutic activity comprises both active pharmaceutical ingredients (API) and excipients. API was tasked with exhibiting pharmacological effects upon absorption into the systemic circulation within the living body.[1] However, there are situations in which both the active ingredient and the excipients might lack complete purity. These impurities can stem from diverse origins such as synthesis, excipients, residual solvents, or degradation products. Termed as impurities, these additional substances are distinct from API and excipients.[2]

Presently, impurities pose a significant public health concern, exposing the safety and efficacy of treatments. The confirmation and quantitative evaluation of impurities in raw materials is crucial for ensuring safe and effective therapies.[3] Because of the presence of N-nitrosodimethylamine impurity, which has carcinogenic activity, the Valsartan drug was withdrawn in 2018.[4] From this, impurity control has emerged as a pivotal and challenging aspect in pharmaceutical industry.[5] ICH (International Conference on Harmonization) categorizes impurities into three groups by their origin and nature: organic, elemental, and residual solvent impurities.[6] Organic impurities refer to organic chemical compounds that emerge in the preparation process or preservation stage. These includes starting products, synthesis intermediates, and by-products formed during the synthesis of pharmaceutical raw materials, and degradation products primarily resulting from inadequate storage conditions.[7]

Impurity profiling requires analytical techniques that are highly sensitive, selective, and efficient in

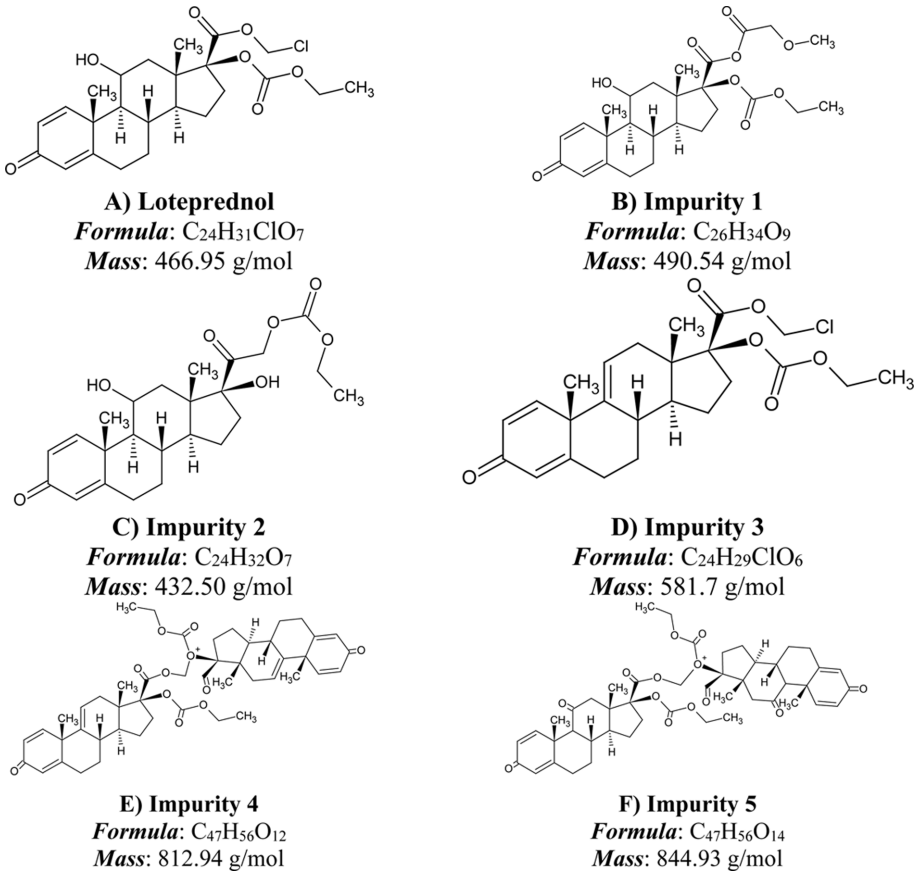


Fig. 1. Systemic details of loteprednol and its impurities.

detecting and quantifying trace amounts of impurities. Conventional methods, due to their lower sensitivity and accuracy, are insufficient for quantification given the nominal quantities in which impurities may exist within drug substances.[8] Furthermore, the structural similarities shared by many impurities with the parent drug molecule underscore the necessity for advanced hyphenated analytical techniques. HPLC and LC-MS/MS are versatile methods extensively employed for trace level impurity evaluation in pharmaceutical compounds.[9]

Loteprednol is a corticosteroid, especially glucocorticoids, prescribed to eliminate various inflammatory conditions, particularly those affecting the eyes. It has potent anti-inflammatory and immunosuppressive properties.[10] Loteprednol is primarily used in ophthalmology, meaning it is designed for eye-related conditions. It is available in various forms, including eye drops and ointments. Mild irritation or stinging in the eyes, light sensitivity, blurred vision, and in rare cases, more severe side effects such as eye pain or changes in vision are possible side effects of loteprednol.[11]

A literature review was undertaken to identify diverse analytical methods for the evaluation of loteprednol. In the literature, few HPLC,[12-16] one UHPLC[17] and UV[18] method reported for analyzing loteprednol in dosage forms. The available literature indicates the absence of any reported analytical method for evaluating process-related impurities of loteprednol. Additionally, no author has characterized the stress degradation compounds of loteprednol. Therefore, this paper introduces an optimized HPLC procedure for the evaluation of the impurities of loteprednol and the LC-MS/MS characterization of stress degradation compounds. This study focuses on process-related impurities, specifically impurities 1, 2, 3, 4, and 5, selected based on their availability. The systemic details along with the structure of loteprednol and impurities are depicted in *Fig.* 1.

2. Experimental

2.1. Chemicals and equipment

Pure API of loteprednol (98.75 %) along with impurities 1-5 was procured from Sun Pharmaceutical Industries. Ltd., Mumbai. The Inveltys® brand ophthalmic formulation with 1 % dosage was obtained from a local pharmacy. Methanol (HPLC purity), acetonitrile (HPLC purity), water (milli-Q®), and membrane filters (0.2 μ) along with laboratory reagent grade chemicals such as potassium dihydrogen phosphate, orthophosphoric acid, hydrogen peroxide, sodium hydroxide (NaOH), and hydrochloric acid (HCl) were purchased from Merck Limited, Mumbai. This study utilized HPLC (Waters, Japan) system with Agilent ChemStation and a triple quadrupole LC-MS (Waters, Japan) system with MassLynx software for HPLC and LC-MS/MS analysis of loteprednol and its impurities.

2.2. Standard solution preparation

A precisely measured 1 mL (10 mg) of loteprednol API and impurities was placed into 10 mL flask containing 5 mL pure methanol as a diluent. The mixture was then fully dissolved using a sonicator. After dissolution, the undissolved compound if any, was eliminated by filtration. To obtain separate solutions of loteprednol and impurities, each with a concentration of 1000 μg/mL, the volume in the flask was adjusted with the diluent.

2.3. Sample solution preparation

The pharmaceutical formulation with the Inveltys® brand, which contained 1 % of loteprednol was used for preparing the formulation solution. 10 mg loteprednol equivalent Inveltys® solution was taken in a 10 mL flask, which was half filled with the diluent. The formulation analytes were dissolved using a sonicator, and any undissolved formulation excipients were eliminated through filtration. Subsequently, the final volume was adjusted to 1000 μg/mL for loteprednol. Additional dilution was performed as necessary to achieve a loteprednol concentration equivalent to a 100 % precision level, and the solution was promptly analyzed after preparation.

2.4. Method optimization

The method optimization started by evaluating the

optimal wavelength for detecting loteprednol and impurities and was assessed using a UV detector. Standard solutions, each containing loteprednol and impurities at 10 µg/mL concentration, underwent individual scanning across 200 to 400 nm. The overlaid spectra of the analytes revealed an isoabsorption wavelength appropriate for accurately detecting both substances. The stationary phase that produces significantly high analyte resolution was assessed by optimizing columns of various configurations and manufacturers. The composition of the mobile phase was meticulously fine-tuned which involved exploration of various solvents, pH levels, and flow rates. The method conditions were carefully chosen to successfully resolve the impurities of loteprednol and were subsequently employed for validation.

2.5. Method validation

The guidelines prescribed by ICH[19-20] and those authors reported in the literature were used for conducting a validation study of the proposed method.[21-28]

The method sensitivity for detecting the impurities was assessed using the signal (s)/noise (n) ratio approach. The s/n results of 10 and 3 were established as the quantification limit (LOQ) and detection limit (LOD), respectively. The construction of a linear plot involved considering the LOQ of each impurity as the lower limit in the calibration curve, and the concentration of loteprednol was finalized to ensure that the standard contained 0.1 % of the impurities. The correlation and regression data in the calibration curve were used to confirm the range of analysis.

The reproducibility of the method was evaluated through precision experiments, including interday (n=6), intraday (n=3 on each day), and ruggedness (n=3 for each analyte). In every precision study, the area response was recorded and tabulated. % RSD (relative standard deviation) was determined and results of <2 considered acceptable according to guidelines.

Accuracy was assessed through a recovery study conducted at 50 %, 100 %, and 150 % levels within linearity. The % RSD at every studied level was calculated for each analysis, with recovery results falling within the range of 98-102 % and a % RSD of less than 2 deemed acceptable. Reliability was evaluated by varying the proposed method conditions, and analyses were performed under each altered condition. Changes in area response were assessed, and a % change of less than 2 was considered acceptable.

2.6. Stability studies

The assessment of drug stability encompassed a comprehensive examination across a various conditions, ranging from photolytic exposure, dry heat, oxidative stress, hydrolytic stress in both acidic and basic environments. To scrutinize these aspects, a meticulous approach was adopted. Initially, an ultraviolet (UV) detector was meticulously configured to meticulously capture absorbance data, facilitating insight into potential degradation. Additionally, a highly sensitive and precise LC-MS/MS technique employed which allows thorough characterization of DPs that emerged under these stressors. Specifically, to discern the impact of hydrolytic, oxidative, and photolytic influences, a meticulously prepared loteprednol solution, carefully calibrated to a specific concentration, was utilized. This solution was meticulously prepared using methanol, ensuring the integrity of the investigative process and the accuracy of the subsequent analyses.

Hydrolytic degradation testing was conducted under acidic and basic conditions to evaluate the drug's susceptibility to hydrolytic breakdown. In 10 mL flasks, 1 mL of loteprednol formulation solution was carefully measured, followed by the addition of 1 mL of the respective stress inducing solution (0.1 N NaOH or 0.1 N HCl) and waiting for 24 h. Additionally, the drug was heated at 70 °C in presence of respective stress inducing solution for 7 hours to induce degradation. Then, the sample was neutralized and volume adjusted to the mark, and samples were analyzed.

Oxidative degradation was performed using 15 % hydrogen peroxide as the oxidizing agent. In 10 mL flask, 1 mL of loteprednol formulation solution was carefully measured, followed by addition of 1 mL of 15 % hydrogen peroxide and wait untill 24 h. Additionally, the drug was heated at 70 °C in water

bath in the presence of the respective stress-inducing solution for 7 hours to induce degradation. Then, neutralized and volume adjusted to the mark and samples were analyzed. Thermal degradation was performed by exposing 70 °C for 7 days in an oven. Photolytic degradation was conducted by exposing loteprednol powder to UV light for 7 days in a photostability chamber. The stress-induced loteprednol samples were brought to a standard concentration and assessed using the proposed method.

2.7. LC-MS characterization of the DPs

DPs generated during stress exposure were recognized by LC-MS/MS. The detected eluents from the UV detector were connected to the mass detector for producing mass spectra, and the resultant mass data were carefully observed for characterization. During this process, it is crucial to ensure that 40 % of the eluents are directed into the mass detector with the assistance of a splitter. The resulting mass spectra and mass fragmentation patterns were meticulously summarized to assess the stress-induced changes in the DPs.

2.8. Method applicability

The optimized method was evaluated for quantifying impurities in the loteprednol ophthalmic solution. The sample solution was prepared from Inveltys® ophthalmic solution, and the method was tested by direct analysis as well as by spiking it with known concentrations of the studied impurities. The obtained chromatograms and their corresponding responses were then employed to evaluate the method's applicability.

3. Results and Discussion

This study aims to propose an HPLC procedure for the evaluation of process-related impurities of loteprednol. To attain the best resolution of the analytes, various optimization experiments were conducted using diverse column configurations. The optimization process also involved fine-tuning the mobile phase. Different solvent compositions were explored, encompassing various buffer strengths, to determine the most suitable mobile phase. This approach allowed for the effective resolution of loteprednol impurities. Consequently, various buffers with varying pH levels were examined to achieve optimal resolution.

Loteprednol impurities were resolved effectively resolved on Phenomenex Luna C18 column (250 mm × 4.6mm; 5 μm id) at 35 °C. The chromatographic conditions involved a mobile phase composed of 0.1 % phosphoric acid, methanol, and acetonitrile in 45:25:30 (v/v) at pH 5.7, employing an isocratic elution at 0.8 mL/min flow. Detection at 243 nm was chosen based on observations indicating an optimal detector response compared to other wavelengths for all analytes. The individual UV scanning spectra along with overlay spectra of loteprednol and its impurities was presented in supplementary note attached to this manuscript. Various mass operating conditions were optimized and the conditions that produce high intense fragments with significantly less noise was finalized as appropriate for the analysing of loteprednol, its impurities and DPs. *Table* 1 presents the mass operating conditions finalized in this study. The blank and system suitability chromatogram noticed in the developed method is presented in *Fig*. 2.

The blank chromatogram observed while analyzing the diluent as a sample is presented in *Fig*. 2A, which clearly shows no chromatographic detections in the entire run time. The 0.1 % impurity spiked loteprednol formulation solution was also assessed using this method. The chromatogram shows well-

Table 1. Mass operating conditions optimized in this study

S No	Parterres	Condition
1	Ionization mode	Positive ionization
2	Electron Multiplier Voltage (V)	500
3	Collision energy (eV)	35
4	Fragmentor (V)	250
5	Nebulizer (Nitrogen) gas and	4 bar
6	MS1 RES	Wide
7	drying gase (Nitrogen) flow	8 mL/min at 200 ºC
8	capillary voltage	3.5 kV
9	Extractor voltage	3 V
10	Source lamp temperature	300 ºC

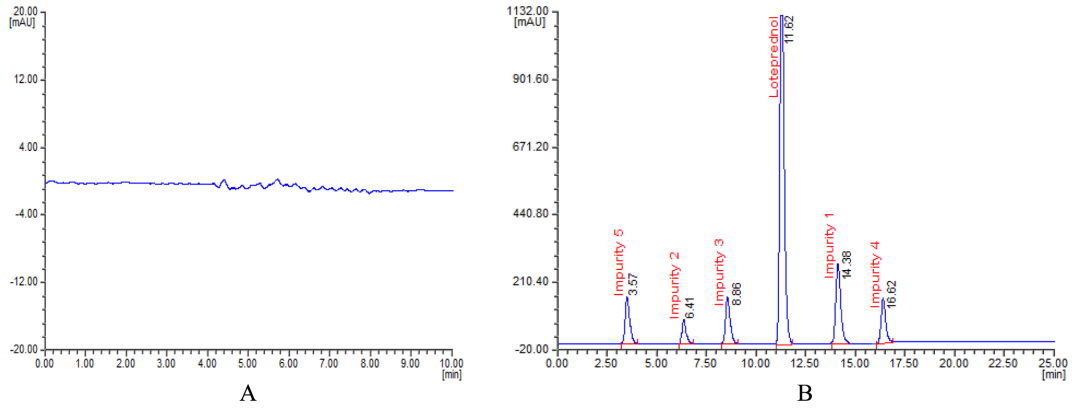


Fig. 2. Specificity chromatograms obtained using the optimized method. Chromatogram observed while analysing blank solution (A) and standard solution containing known concentration of loteprednol with 0.1 % impurity (B).

resolved and retained peaks representing loteprednol and impurities. The retention time (t_R) was noticed as 11.62 min for loteprednol and 14.38 min, 6.41 min, 8.86 min, 16.62 min and 3.57 min for impurities 1, 2, 3, 4, and 5 respectively. Chromatogram does not visualize any additional detection over the entire run time (*Fig*. 2B) suggesting that the method was specific for analyzing impurities of loteprednol.

System suitability is a crucial aspect of the chromatographic method validation process, serving to confirm the capability of the proposed method's to consistently generate well-resolved peaks with high reproducibility. This test is essential for assessing the HPLC system's performance and the procedure's ability to yield high-quality data. The evaluation of system suitability involved the assessment of several parameters, including resolution (Rs), USP tailing factor (T), plate count (N), and % RSD of area and elution time (t_R) and relative retention time (RRT). The calculated RSD of both the area and t_R of loteprednol and its impurities were less than 1 %, which falls within the acceptance limit. This suggests a high level of reproducibility of the peak representing loteprednol and its impurities, with approximately consistent area and retention time during each injection at the fixed concentration. Furthermore, Rs between the first eluted and subsequent peaks exceeded 2, indicating a satisfactory resolution of the proposed method. The system suitability results for loteprednol and its impurities were compared against the regulatory permissible limits and are presented in *Table* 2. These results affirm that the method successfully meets acceptable criteria.

The terms LOD and LOQ signify the method's capacity to accurately detect and quantitate the smallest analyte amount respectively. The LOD of impurities was identified as 0.015 µg/mL, and the LOQ was finalized as 0.05 µg/mL for impurities. To assess method linearity, a linear curve was derived by plotting the peak area against various standard concentrations of loteprednol and its impurities separately. The standard concentrations covered a range from 50 to 200 µg/mL for loteprednol and LOQ (0.05 µg/mL) – 0.2 µg/mL for its impurities. The calibration plot, which depicts analyte concentration versus peak area, exhibited a linear relationship across the specified concentration range. This linearity was established using the linear simple regression least squares method. The regression equation for the line is tabulated in *Table* 2. The linear graphs of loteprednol and its impurities observed in this study were presented in supplementary note attached to this manuscript.

Method precision was assessed by examining of parameters encompassing both repeatability and intermediate precision. A minimum of six determinations were conducted each day by injecting and analzsing freshly prepared concentrations. This rigorous testing allowed us to evaluate both the within-day (intraday) and, between-day (interday) variability and ruggedness. These results indicate acceptable levels

Table 2. System suitability results

Parameter	Experimental results for						Acceptance criteria
	Loteprednol	Impurity 1	Impurity 2	Impurity 3	Impurity 4	Impurity 5	
t_R (min)	11.62	14.38	6.41	8.86	16.62	3.57	--
RRT	--	1.23	0.55	0.76	1.43	0.30	< 2
RRF	--	0.096	0.069	0.058			--
R_S	5.49	6.37	5.66	4.39	4.36	--	> 2
A_S	1.02	0.99	1.07	0.96	1.07	0.95	
N	8155	9867	5541	6807	1142	3397	> 2000

of both intraday and interday variability. The mean RSD values for repeatability and reproducibility study were across the studied concentration level for loteprednol and its impurities were noticed to be acceptable (*Table* 2) suggesting that the method was reproducible.

To assess method accuracy, a recovery test was undertaken, deliberately spiking standard concentrations at three distinct levels corresponding to 50 %, 100 %, and 150 % of target concentration. These spiked samples, replicated three times, underwent analysis for the quantification of loteprednol and its impurities. Comparisons between the calculated analyte concentrations and the nominal concentrations allowed for

Table 3. Summary results noticed in linearity, precision, and accuracy study

Parameter	Results					
	Loteprednol	Impurity 1	Impurity 2	Impurity 3	Impurity 4	Impurity 5
			Linearity			
Range in µg/mL	50-200	0.05-0.2	0.05-0.2	0.05-0.2	0.05-0.2	0.05-0.2
Slope	7102.7	574917	241065	304174	266458	299605
Intercept	62481	290.59	99.64	710.58	649.92	190.54
r^2	0.9981	0.9988	0.9994	0.9998	0.9987	0.9988
			Precision[$$]			
Intraday	1.00	1.05	0.79	0.36	0.54	0.80
Interday (day 1)	0.57	0.32	0.88	0.32	0.87	0.66
Interday (day 2)	0.10	0.82	0.57	0.30	0.66	0.38
Ruggedness	0.72	1.04	0.61	0.29	0.69	0.36
			50 % level accuracy[$]			
Amount added (µg/mL)	150	0.15	0.15	0.15	0.15	0.15
Recovered (µg/mL)	149.20	0.15	0.15	0.15	0.15	0.15
% Recovery	99.47	99.83	100.45	99.75	100.02	99.09
% RSD	0.85	0.67	0.60	0.46	0.54	1.52
			100 % level accuracy[$]			
Amount added (µg/mL)	200	0.20	0.20	0.20	0.20	0.20
Recovered (µg/mL)	199.91	0.20	0.20	0.20	0.20	0.20
% Recovery	99.95	99.64	100.07	99.82	99.84	99.96
% RSD	0.30	0.55	0.21	0.49	0.40	0.13
			150 % level accuracy[$]			
Amount added (µg/mL)	250	0.25	0.25	0.25	0.25	0.25
Recovered (µg/mL)	250.08	0.25	0.25	0.25	0.25	0.25
% Recovery	100.03	99.38	99.70	99.83	100.00	99.73
% RSD	0.19	0.83	0.46	0.38	0.13	0.29

n=3 ($) and n=6 ($$)

the determination of the % recovery. Notably, the mean % recovery values across all three levels, derived from the three replicates, consistently fell within the acceptable range of 98 % to 102 %, as stipulated by regulatory guidelines. These findings serve to confirm the method's accuracy, demonstrating its consistent ability to provide results that closely match the expected analyte concentrations. Detailed results are presented in *Table* 3 for reference.

To assess the method reliability for analyzing impurities of loteprednol, several key parameters were investigated. These parameters included variations in the pH, composition of the mobile phase, and detector wavelength. The % change, shifts in tR, and system suitability were closely monitored as responses to deliberate changes in method parameters. On mobile phase composition, variations were made within ± 5%, ± 0.1 % variation in pH and ± 5 nm variation in wavelength of specified method condition was performed. The comprehensive results of this study demonstrate that deliberate changes made within the specified parameter ranges do not affect elution, retention time, or system suitability. The mean % change remained stable across all conditions, exhibiting only slight variability well within acceptable limits (*Table* 4). These findings underscore the robustness of the proposed method, affirming its reliability under variations within the specified parameter ranges.

In accordance with the ICH stability guidelines, a variety of forced conditions, namely thermal, basic, acidic, oxidative, and photolytic were employed to conduct degradation studies using the pharmaceutical product loteprednol. These studies led to the identification and characterization of five distinct degradation products, designated as DP1 - DP5, using HPLC/MS analysis. The outcomes of these investigations have furnished valuable insights into the conditions that render the drug susceptible to degradation, thus facilitating the implementation of appropriate precautionary measures during the formulation process.

In the context of thermal degradation conditions, no significant degradation was observed, with an assay

Table 4. Robustness results

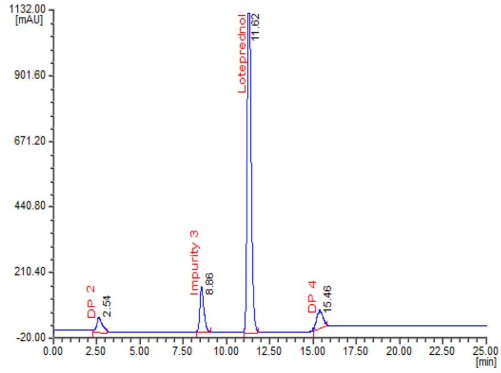
S No	Changed condition	Parameter	Results observed					
			Loteprednol	Impurity 1	Impurity 2	Impurity 3	Impurity 4	Impurity 5
1	MP 1	% change	1.44	1.44	0.62	1.44	1.44	0.24
		t_R	11.61	14.31	6.41	8.83	16.62	3.56
		N	8205	9748	5610	6837	1135	3501
2	MP 2	% change	0.12	0.78	1.3	0.78	1.06	0.91
		t_R	11.67	14.38	6.48	8.86	16.63	3.59
		N	8147	9581	5694	6925	1081	3617
3	pH 1	% change	0.22	0.88	0.88	0.88	0.51	0.88
		t_R	11.65	14.33	6.47	8.86	16.69	3.51
		N	8069	9632	5748	6625	1273	3366
4	pH 2	% change	0.14	0.06	0.03	0.47	0.8	0.46
		t_R	11.63	14.32	6.42	8.85	16.63	3.53
		N	8265	9749	5328	6518	1134	3478
5	WL 1	% change	0.02	1.05	0.67	0.02	0.61	0.5
		t_R	11.62	14.38	6.47	8.81	16.6	3.55
		N	8174	9880	5109	6309	1099	3590
6	WL 2	% change	0.47	0.19	0.19	0.19	0.19	0.66
		t_R	11.66	14.39	6.44	8.83	16.64	3.57
		N	8055	9591	5022	6704	1143	3308

MP (mobile phase) 1: 1 % phosphoric acid, methanol, and acetonitrile 45:20:35; MP 2: 1 % phosphoric acid, methanol, and acetonitrile 45:30:25; WL (wavelength) 1: 248 nm; WL 2: 238 nm; pH 1: 5.8; pH 2: 5.6

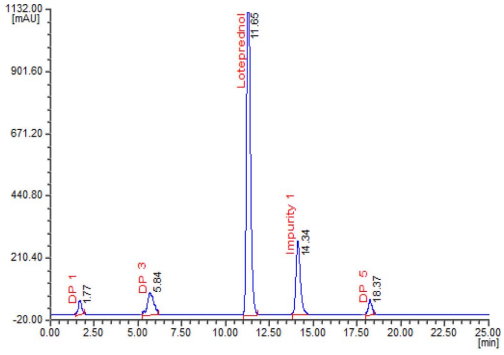
Table 5. Stress degradation results

Stress	% degradation[#] of loteprednol	% assay[#] of loteprednol	% Mass balance[$]	Remark
Acidic	7.18	92.82	98.25	DP 2 and 4 were noticed
Basic	9.14	90.86	99.63	DP 1, 3, and 5 were noticed
Peroxide	5.69	94.31	97.35	DP 2 was noticed
UV light	4.15	95.85	98.04	No degradation was identified
Thermal	2.11	97.89	98.74	DP 3 was noticed

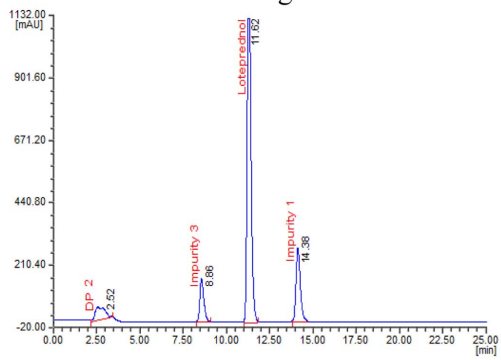
[#]n=3; [$]sum of loteprednol, impurities, and DPs



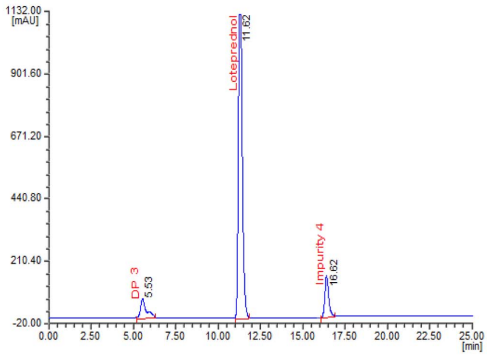
A) DP 2 and 4 visualized in acid degradation chromatogram



B) DP 1, 3, and 5 visualized in basic study



C) DP 2 visualized under peroxide conditions



DP 3 visualized in UV light degradation chromatogram of loteprednol

Fig. 3. Forced degradation chromatograms of loteprednol.

percentage of 97.89 %. Among the various degradation conditions, the most pronounced degradation noticed in base-induced degradation study, where the base-induced degradation reached 9.14 %. *Fig*. 4A depicts the chromatogram from this study, revealing well-separated degradation products with t_R of 1.7, 5.8, and 18.3 min, denoted as DP 1, DP 3, and DP 5, respectively. In the acid-induced degradation study, as depicted in *Fig*. 4B, two distinct degradation products were clearly resolved at retention times of 2.54 min, and 15.46 min, designated as DP 2 and DP 4, respectively. The percentage degradation under acidic degradation conditions was measured at 7.18 %. In addition, the chromatogram revealed impurity 3 at a retention time of 8.8 min. In the peroxide degradation study, the assay percentage for loteprednol

was calculated to be 5.69 %. The chromatogram for this study clearly delineated a single degradation product with a retention time of 2.5 min, labelled DP 2. The outcomes of peak purity, conducted using a PDA detector, provided robust validation of the purity and consistency of the loteprednol peak across stress studies under examination. The mass balance was assessed in the performed stress studied, and more than 98 % results were noticed. These consistent results from the peak purity tests unequivocally affirmed the uniformity and purity of loteprednol in stress studies. Notably, the loteprednol assay showed remarkable stability, further attesting to the specificity and efficacy of the developed method in detecting stability. *Table* 5 presents the recovery results and *Fig*. 3 visualize the stress study chromatograms.

3.1. LC-MS/MS characterization of the DPs

The stress-induced DPs of loteprednol were characterized via LC-MS/MS. The optimized LC conditions remained unchanged, and the mass operating conditions were fine-tuned to maximize the detection of mass fragments with significantly less noise. The collision-induced dissociation spectra of each DP along with its accurate mass measurements were noted to evaluate the structure of each DP formed in the stress study.

Fig. 4 illustrates the fragmentation mechanism of DP1, with the ESI spectrum (*Fig*. 9A) revealing that the most intense [M+H] ion at m/z 395 represents a mass of DP 1 of 394.88 g/mol. The MS/MS spectrum of DP1 exhibited highly intense product ion peaks at m/z-162 (indicating the loss of $C_{13}H_{19}ClO_4$), 286 (resulting from the loss of $C_2H_2ClO_3$ from m/z 395), and m/z 121 (loss of $C_{13}H_{19}ClO_4$ from m/z 395), and accurate mass measurements suggest the molecular composition of these fragments. DP 1 was identified as-*(6-(6-((6-hydroxypyridin-3-yl)methyl)-3,6-diazabicyclo [3.1.1] heptan-3-yl) pyridin-3-yl)-6-methoxypyrazolo[1,5-a]pyridine-3-carbonitrile* with formula of $C_{24}H_{35}N_7O_3$.

Fig. 5 illustrates the fragmentation mechanism of DP 2 of loteprednol. The fragmentation spectrum (*Fig*. 9B) of DP 2 shows the parent ion with an m/z of 419 (m+1). In the fragmentation spectra, there were notable

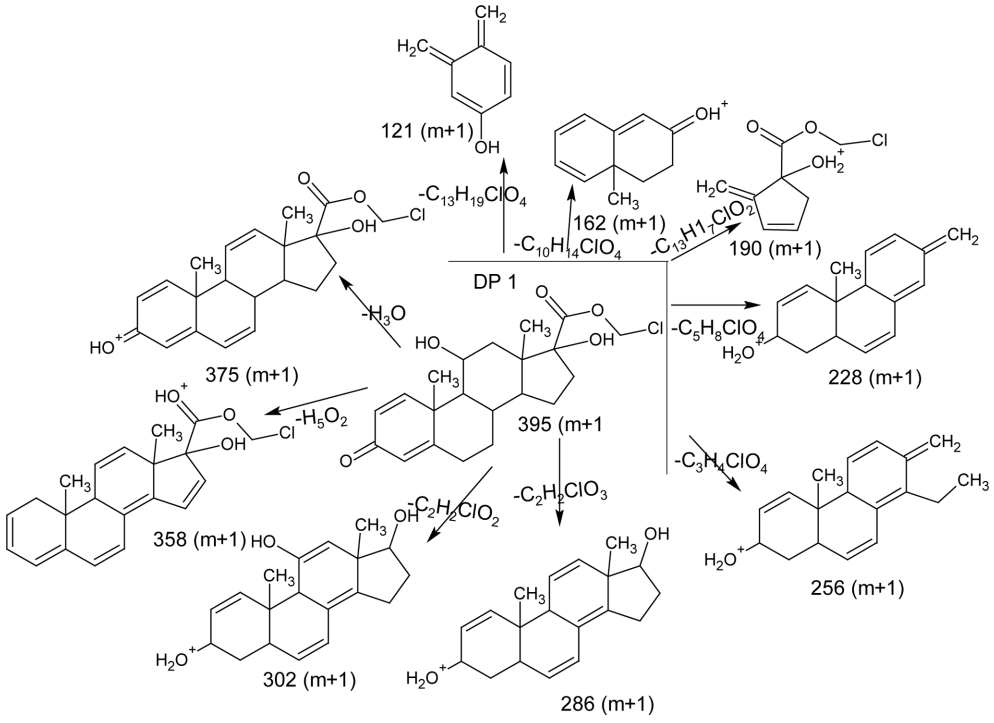


Fig. 4. Fragmentation mechanism proposed for DP 1.

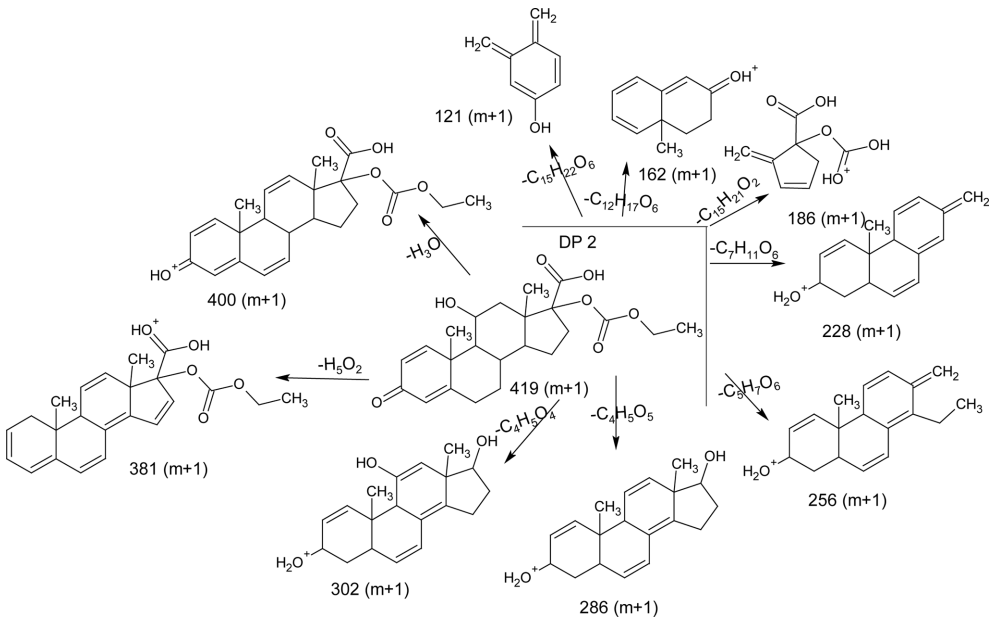


Fig. 5. Fragmentation mechanism proposed for DP 2.

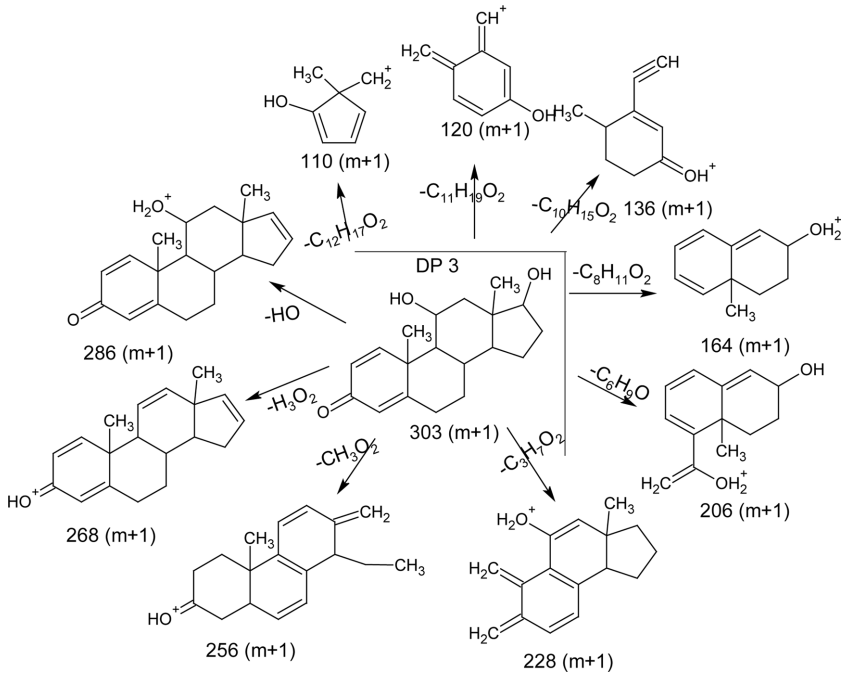


Fig. 6. Fragmentation mechanism proposed for DP 3.

product ions at m/z 162 (indicative of the loss of $C_{12}H_{17}O6$), 256 (resulting from the loss of $C_5H_7O_6$), 121 (stemming from the loss of $C_{15}H_{22}O_6$ from parent ion), and m/z 228 (indicative of the loss of $C_7H_{11}O_6$ from parent ion). The MS/MS results, along with accurate mass assessments, provide strong support for the proposed fragmentation scheme. DP 2 was identified as (6-methoxy-4-(6-(6-((6-methoxypyridin-3-

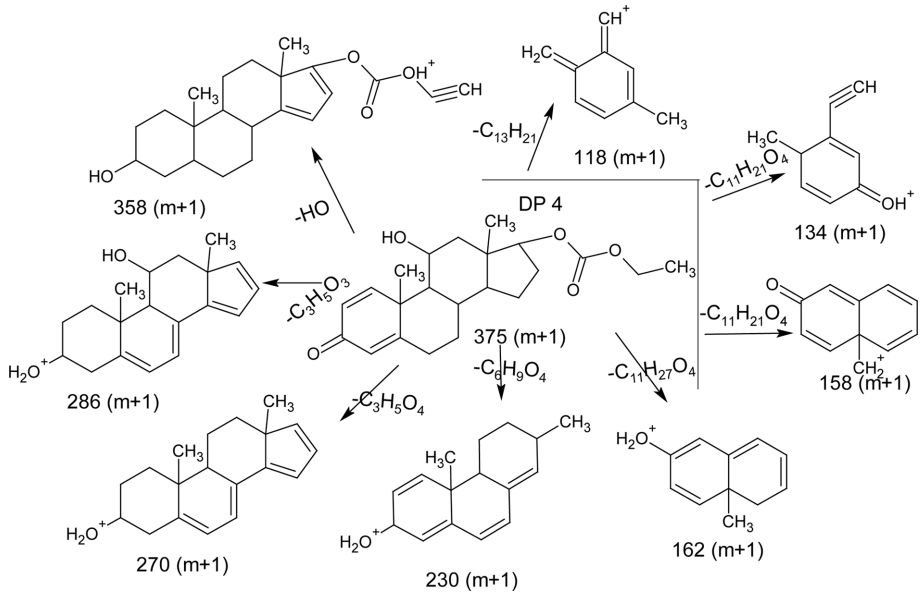


Fig. 7. Fragmentation mechanism proposed for DP 4.

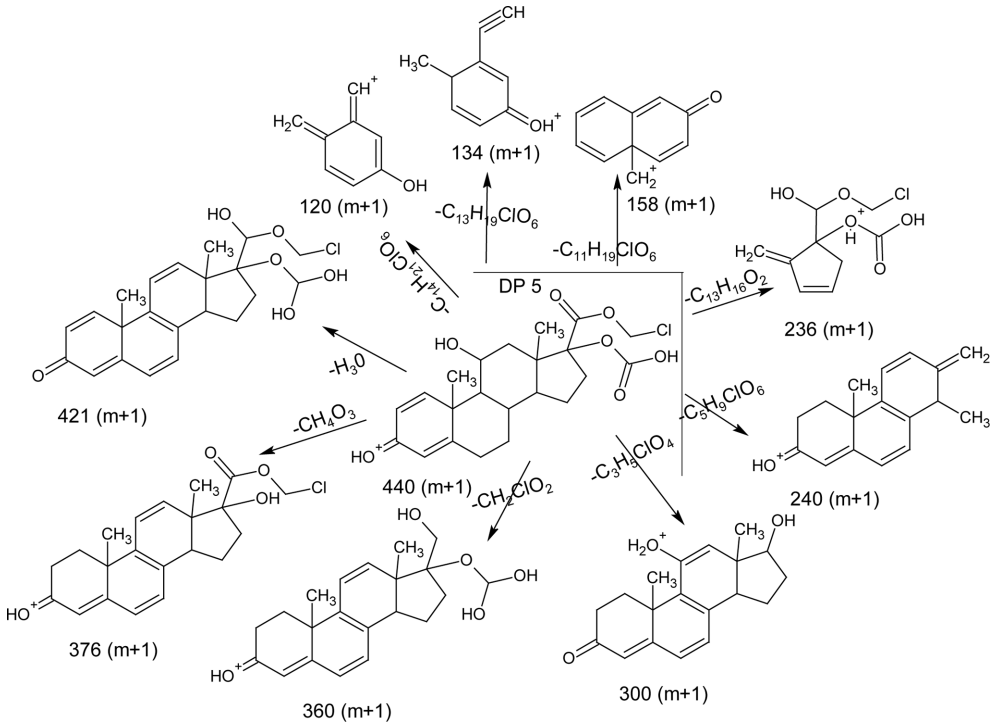


Fig. 8. Fragmentation mechanism proposed for DP 5.

yl)methyl)-3,6-diazabicyclo[3.1.1]heptan-3-yl)pyridin-3-yl)pyrazolo[1,5-a] pyridin-3-yl)methanamine with formula of $C_{23}H_{30}O_7$.

Fig. 6 illustrates the fragmentation mechanism of DP3 of loteprednol. The fragmentation spectra of DP 3 (*Fig.* 9C) visualize parent ion with an m/z of 303 (m+1). In the fragmentation spectra, there were notable product ions at m/z 110 (generated by losing

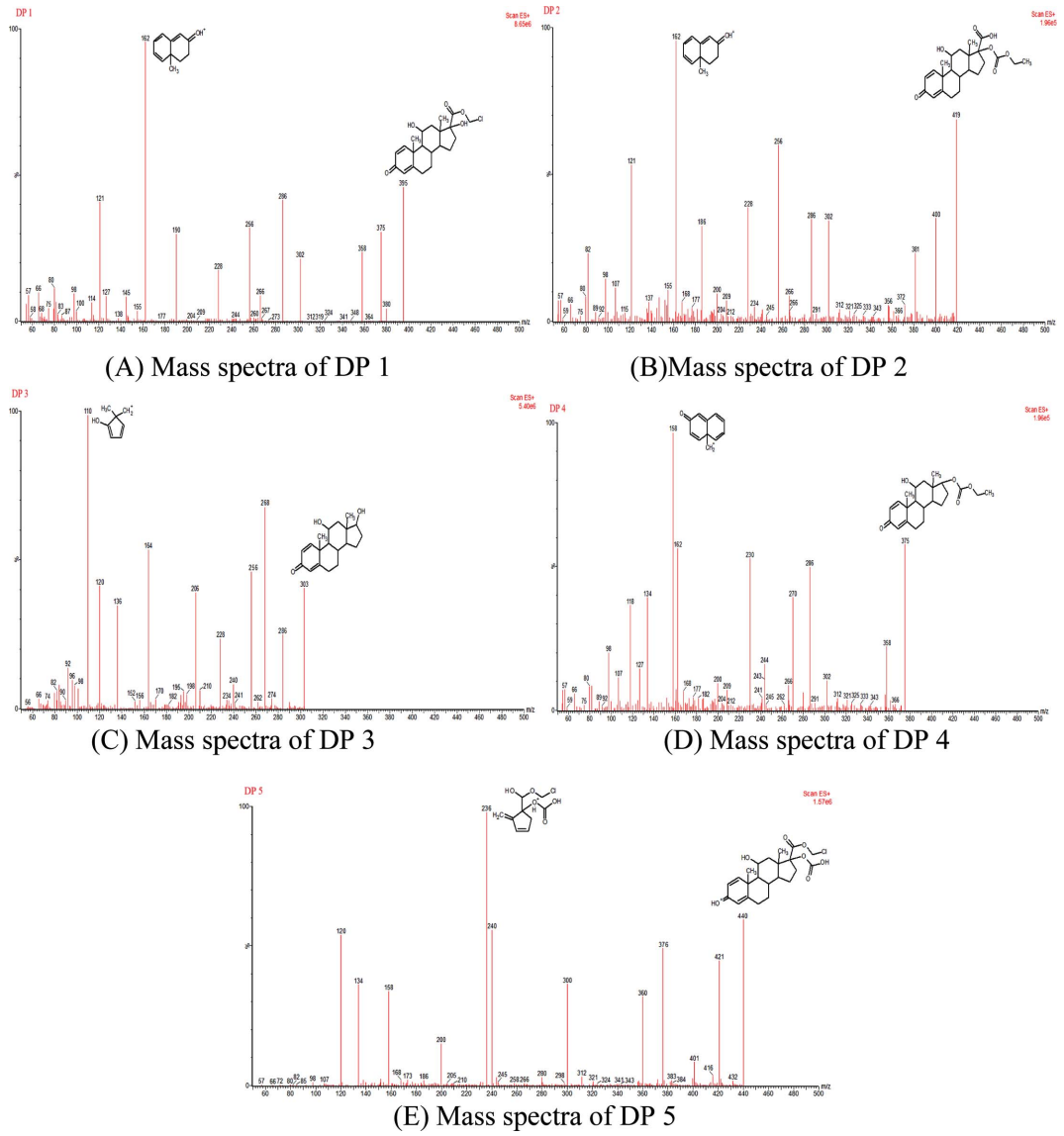
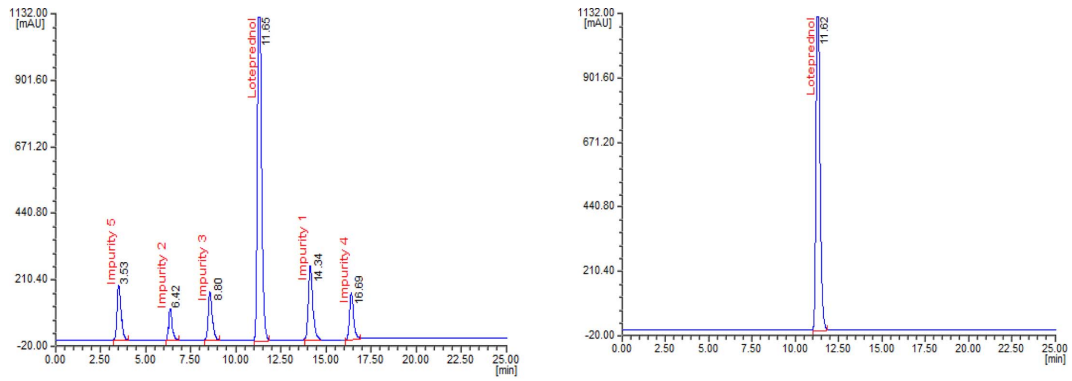


Fig. 9. Mass spectra of DPs observed in the forced degradation study.

-$C_{12}H_{17}O_2$) and m/z 268 (generated by losing H_3O_2 from parent ion). DP 3 was identified as *4-(6-aminopyridin-3-yl)-6-methoxypyrazolo[1,5-a]pyridine-3-carbonitrile* with the formula $C_{19}H_{26}O_3$.

DP 4 was noticed at t_R of 15.4 min (*Fig*. 9D) in acid-induced degradation chromatogram that exhibits the parent ion fragment at m/z 375. The fragmentation spectrum visualizes various fragment peaks at m/z 158 (m+1), 230 (m+1), 286 (m+1) and 162 (m+1). The spectral interpretation of fragment ions suggest that DP 4 as *5-((3-(5-(3-methylpyrazolo[1,5-a]pyridin-4-yl)pyridin-2-yl)-3,6-diazabicyclo[3.1.1]heptan-6-yl) methyl) pyridin-2-ol*, with a molecular formula of $C_{22}H_{30}O_5$. *Fig*. 7 visualizes the fragmentation pattern of DP 4.

In the base stress chromatogram, a peak at 18.3 minutes was prominently observed and designated as DP 5, which was not detected in other stress studies conducted. The fragmentation spectra of DP 5 revealed a prominent parent fragment at m/z 440 (m+1).



A) Spiked formulation B) Unspiked formulation

Fig. 10. Loteprednol formulation analysis chromatogram.

Furthermore, the spectrum exhibited fragment ions at m/z 236 (m+1), resulting from the loss of $C_{13}H_{16}O_2$. Based on acquired data (*Fig*. 8), DP 5 was conclusively identified as *4-(6-(6-((6-hydroxypyridin-3-yl)methyl)-3,6-diazabicyclo[3.1.1]heptan-3-yl)pyridin-3-yl)pyrazolo [1,5-a]pyridin-6-ol*, with $C_{22}H_{28}ClO_7$ as molecular formula.

The established HPLC technique was used for the quantification of the investigated impurities in an ophthalmic solution. The spiked sample analysis chromatogram visualizes well-retained peaks representing impurities along with loteprednol (*Fig*. 10A). However, the direct formulation chromatogram does not visualize any peak representing impurities (*Fig*. 10A) suggesting that the impurities not detected in the sample. This highlights that the method was adequate for analyzing loteprednol impurities.

4. Conclusions

In this investigation, a rapid, cost-effective, highly sensitive, and readily accessible HPLC procedure was optimized for the evaluation of loteprednol impurities in ophthalmic formulations. This method offers numerous advantages, including reduced analysis time, cost-effectiveness, accessibility, robustness, sensitivity, and reproducibility. Our study delved into the degradation behaviour loteprednol under various stress conditions, encompassing hydrolysis (acidic, basic), oxidation, thermal, and photolysis stress. Notably, our findings revealed that the drugs exhibited stability under thermal hydrolysis conditions but underwent degradation in acidic and alkaline photolysis environments. The identification of DPs was achieved through the observation of [M+H]+ ions, and the proposed structural elucidation was further corroborated by HPLC-MS/MS experiments, supported by accurate mass measurements. The DPs were characterized as *4-(6-(6-((6-hydroxypyridin-3-yl)methyl) -3,6-diazabicyclo[3.1.1]heptan-3-yl)pyridin-3-yl)-6-methoxypyrazolo[1,5-a]pyridine-3-carbonitrile* (DP 1), *5(6-methoxy-4-(6-(6-((6-methoxypyridin-3-yl)methyl) -3,6-diazabicyclo[3.1.1]heptan-3-yl)pyridin-3-yl) pyrazolo[1,5-a]pyridin-3-yl)methanamine* (DP 2), *4-(6-aminopyridin-3-yl)-6-methoxypyrazolo[1,5-a] pyridine-3-carbonitrile* (DP 3), *5-((3-(5-(3-methyl-pyrazolo[1,5-a]pyridin-4-yl)pyridin-2-yl)-3,6-diaza-bicyclo[3.1.1]heptan-6-yl) methyl) pyridin-2-ol* (DP 4), and *4-(6-(6-((6-hydroxypyridin-3-yl)methyl)-3,6-diazabicyclo[3.1.1]heptan-3-yl)pyridin-3-yl)pyrazolo [1,5-a]pyridin-6-ol* (DP 5). This study effectively elucidated the fragmentation pathways and characterized the degradation products of loteprednol. Consequently, this developed method can serve not only for assessing the process-related impurities of loteprednol but also for evaluating sstress-induced DPs.

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