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ORIGINAL ARTICLE

Characterization of Forced Degradation Products of Netarsudil: Optimization and Validation of a Stability-Indicating RP-HPLC Method for Simultaneous Quantification of Process-Related Impurities

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ABSTRACT

Objectives: The aim of this study is to examine resolution, identification, and characterization of forced degradation products of netarsudil by liquid chromatography-tandem mass spectrometry by validating a simple and sensitive high-performance liquid chromatography method for the resolution, identification, and quantification of two process-related impurities in netarsudil.

Materials and Methods: Chromatographic separation was accomplished on a ZORBAX Eclipse XDB C18 (250 x 4.6 mm; 5 µ id) column at room temperature as the stationary phase and 257 nm as the detector wavelength with the mobile phase consisting of acetonitrile, methanol, and pH 4.6 phosphate buffer in 45:35:20 (*v/v*) at 1.0 mL/min flow rate in isocratic elution.

Results: The method reported very sensitive detection limits of 0.008 µg/mL for impurity 1 and 0.003 µg/mL for impurity 1. The method produces a calibration curve linear in the concentration level of 25-200 for netarsudil and 0.025-0.2 µg/mL for impurities. The proposed method gives acceptable results for other validation parameters such as accuracy, precision, ruggedness, and robustness. The drug was subjected to various stress conditions such as acid, base, peroxide, and thermal and ultraviolet light to investigate the stability-indicating ability of the method. Considerable degradation was observed in stress studies, and the degradation products were well resolved from process-related impurities. The characterization of degradation products was performed on the basis of collision-induced dissociation mass spectral data, and the possible structures of the six degradation compounds of netarsudil were proposed.

Conclusion: The outcomes of other validation studies were likewise satisfactory and proven adequate for the regular analysis of netarsudil and its process-related impurities in bulk drug and pharmaceutical dosage forms and can also be applied for the evaluation of the stress degradation mechanism of netarsudil.

Keywords: Netarsudil, process related impurities, HPLC analysis, forced degradation studies, characterization of degradation compounds

INTRODUCTION

The pharmaceutical industry is rising day by day with the aim of investigating novel drugs that are isolated from natural products or synthesized chemically. The main challenge that always remains is that the compound should be pure, and purity was treated as a significant factor for ensuring drug quality.[1] In the process of synthesis of a pure drug, there is a possibility of some unwanted compounds remaining in the pure drug, and these unwanted compounds are considered as impurities. The presence of these impurities even in very low quantities may influence the quality, efficacy, and safety of the drug

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product. Hence, identification and quantification of impurities are considered essential for producing safe drugs, and high-performance liquid chromatography (HPLC) is considered a simple and convenient procedure for identifying and quantifying impurities from any source.[2]

Netarsudil is a Rho kinase inhibitor and norepinephrine transporter inhibitor drug approved for decreasing elevated intraocular pressure in patients with open-angle glaucoma or ocular hypertension.[3] The key difference between netarsudil and other Rho kinase inhibitors is that it not only minimizes intraocular pressure by reducing outflow resistance but also minimizes aqueous humor production and episcleral venous pressure.[4] The side effects possible during the usage of netarsudil include eye pain upon instillation, eye or eyelid redness changes in vision, discoloration of the eye, and teary eyes.[5] Its molecular structure is shown in Figure 1 with the International Union of Pure and Applied Chemistry name of *[4-[(2S)-3-Amino-1-(isoquinolin-6-ylamino)-1-oxopropan-2-yl] phenyl]methyl 2,4-dimethylbenzoate* with a molecular formula of $C_{28}H_{27}N_3O_3$ and a mass of 453.542 g/mol.

A literature survey was conducted to determine the available analytical method for quantification of netarsudil using various analytical techniques. In the literature, it was observed that few analytical methods have been reported for the quantification of netarsudil along with latanoprost using HPLC[6-10] and Ultra Performance Liquid Chromatography.[11] One Ultra Performance Liquid Chromatography/Mass Spectrometry method has been reported for the quantification of netarsudil along with timolol and latanoprost.[12] One Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry/Tandem Mass Spectrometry method has been reported for the identification and characterization of netarsudil and its hydrolytic degradation products.[13] The literature review suggests that no method is available for the resolution, identification, and quantification of process-related impurities of netarsudil. Hence, this study intended to fill the gaps identified in the literature. Process-related impurities 1 and 2 were available for the study and hence were selected. The molecular structure of netarsudil and its process related impurities is presented in Figure 1.

Netarsudil

The origin of process-related impurities in the netarsudil synthetic drug was evaluated by observing the synthesis route of netarsudil[14] and is presented in Figure 2. In the process of synthesis of netarsudil, the starting product of the reaction, *i.e.*, (4-[(2,4-dimethylbenzoyl)oxy]methylphenyl)acetic acid (4), and the intermediate product (6) remain in the final product, and these compounds are designated as impurities 1 and 2, respectively.

MATERIALS AND METHODS

Chemicals and reagents

The analytical standard compound netarsudil with purity of 98.17% and impurities 1 and 2 were procured from Ajanta Pharma Limited, Hyderabad, Salangana. The eye drop formulation containing 0.02% *w/v* netarsudil was obtained from a local pharmacy. HPLC grade methanol, acetonitrile, and Milli-Q® water were obtained from Merck Chemicals, Mumbai. Reagent grade chemicals such as acetic acid, sodium acetate, hydrochloric acid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide were purchased from Fisher Scientific, Mumbai.

Instrumentation

HPLC analysis was performed on an Agilent 1100 (USA) instrument coupled with a quaternary pump (G1311 A) for solvent delivery. The analytes were injected through a temperature-adjustable autosampler (G1329A) with an injection capacity of 0.1-1500 µL. The column eluents were detected using a programable ultraviolet (UV) detector (G1314A), and the chromatographic integration was performed using Agilent chem-station software. Liquid Chromatography-Mass Spectrometry (LCMS) analysis was performed on a Waters LCMS (Japan) equipped with a triple quadruple mass detector. During the mass spectral analysis, a splitter was placed between the column and detector to allow 40% of the chromatographic eluents to be entered into the electrospray Ionization (ESI) source. The mass detector was operated in positive ESI mode with suitable fragmentor voltage (70 V), capillary voltage (3200-3600 V), and skimmer voltage (60 V). Nebulization (40

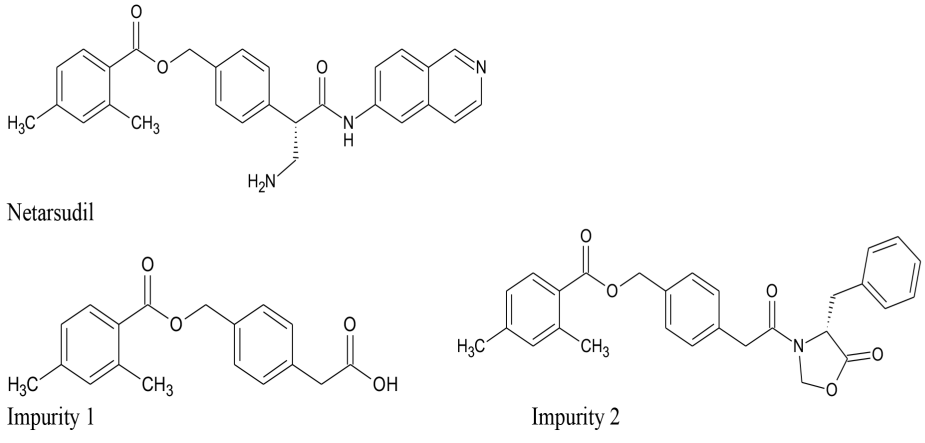

Figure 1. Molecular structure of netarsudil and its impurities in the study

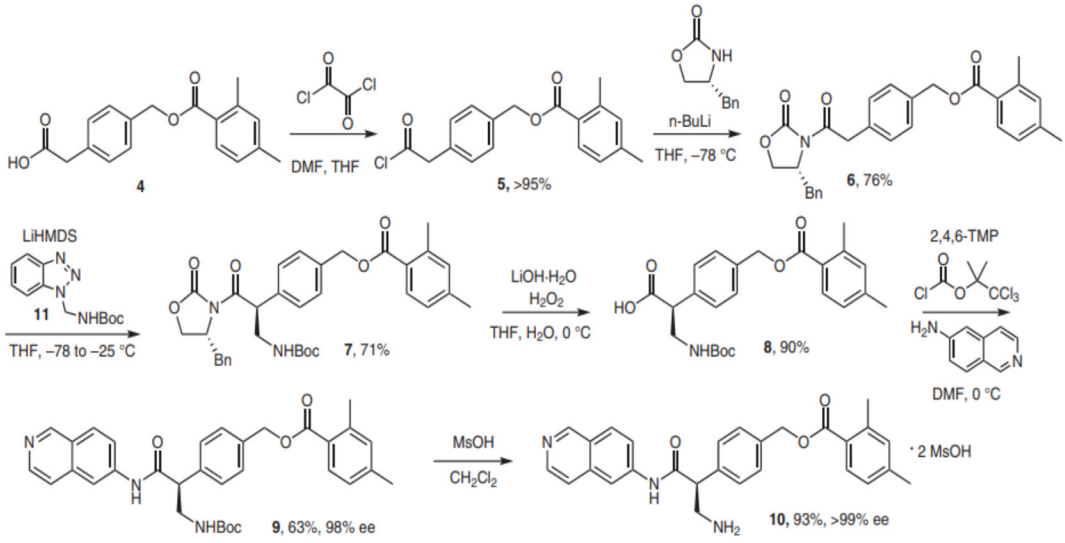

Figure 2. Netarsudil synthesis route

Psi) and drying (300 °C, 9 L/h) were performed using nitrogen gas. The spectra throughout the analysis were recorded under similar experimental conditions and 20-30 average scans were conducted.

Preparation of solutions

Netarsudil and impurity solutions

The standard netarsudil and its impurities at 1 mg/mL (1000 µg/mL) were prepared separately by accurately weighing 25 mg of analyte in 25 mL of volumetric flask containing 15 mL of methanol. The analytes were dissolved in a solvent using an ultrasonic bath sonicator. Then, the analytes were filtered through a 0.2 µ membrane filter, and the final volume was made up to the mark using the same solvent to obtain a 1000 µg/mL concentration of netarsudil and its impurities separately. During the analysis, the selected volume of the required concentration of individual analytes was mixed separately.

Formulation solution

The eye drop formulation containing 0.02% *w/v* of netarsudil was used for preparing the formulation solution. An accurately measured netarsudil solution (25 mL) was taken in a 50 mL calibrated flask containing 10 mL methanol. The flask was sonicated to dissolve the formulation completely in the solvent and then the volume was made up to mark with the same solvent. The solution was filtered through a 0.2 µ membrane filter to obtain the formulation solution at a concentration of 100 µg/mL, and the same solution was used for formulation analysis.

Method development

The method development was initiated by identifying a suitable wavelength for the detection of netarsudil and its impurities using a UV detector. The UV-visible spectrophotometer was used for the identification of suitable wavelength for the detection of netarsudil and its impurities. The standard solution at 10 µg/mL concentration of netarsudil and its impurities was scanned individually at a scan range of 400-200 nm. The overlay UV absorption spectra of netarsudil and its impurities confirm that the isoabsorption wavelength is suitable for detecting netarsudil and its impurities.

The suitable stationary phase for the separation of netarsudil and its impurities in the study was selected based on the resolution and chromatographic responses of the analytes in each column studied. Various C18 columns such as zodiac, zorbax XDB, phenomenex luna, and prontoSIL ODS columns with 250- and 100-mm column lengths were studied. The solvents like methanol and acetonitrile were selected as organic modifiers, and various strengths of acetate and phosphate buffer were selected as pH modifiers. Various compositions of these solvents were pumped at a flow range of 0.5 to 1.5 mL/min was studied for the best resolution of netarsudil and its impurities. In the method development conditions performed, the 100% standard solution containing 0.01% of impurities 1 and 2 was analyzed. The condition that gives the best resolution of netarsudil and its impurities with acceptable system suitability was considered suitable for the study.

Method validation

The method optimized for the evaluation of netarsudil and its impurities was validated as per methodology reported in literature[15-18] as well as International Conference on Harmonization (ICH) guidelines.[19]

System suitability

The 100% concentration level solution was analyzed six times in the proposed method to establish the system suitability of the developed method. The chromatographic response of the resultant chromatograms in each study was summarized to evaluate the system suitability of the developed method. Chromatographic parameters such as retention time (RT), asymmetric factor (tail factor), plate count (number of theoretical plates), and resolution factor were used to evaluate method system suitability.

Sensitivity

The method sensitivity of the proposed method for the detection of impurities was evaluated by assessing the detection limit (LOD) and quantification limit (LOQ). The signal (s) to noise (n) ratio method was adopted for evaluating the sensitivity levels of impurities in the developed method. The minimal concentration of netarsudil impurities was analyzed using the developed method, and the chromatographic response (signal) along with the baseline (noise) response was summarized. The signal-to-noise ratio of 3 and 10 was considered as LOD and LOQ, respectively.

Linearity and range

The calibration concentrations were prepared such that the netarsudil solution contained 0.1% impurity. Various levels of netarsudil standard solution spiked with 0.1% of the studied impurities were analyzed in the developed method. The chromatographic response of each analyte was tabulated, and the calibration curve was plotted individually by considering the obtained peak area response on the y-axis and its prepared concentration on the x-axis. The best-fit calibration range for each analyte was considered to be a suitable range of analysis in the developed method.

Precision

The 100% concentration level in the linearity level spiked with 0.1% of each impurity was used to evaluate the repeatability and reproducibility of the developed method. The solution was prepared and injected six times in one day (intraday), three days (interday), and by different analysts on the same day (ruggedness). The peak area response of each analyte in each study was tabulated, and the percentage relative standard deviation (RSD) was calculated for each analyte in each study. A RSD% of less than 2 was acceptable in each study as per the guidelines.

Robustness

The influence of minor variations in the developed method conditions for the separation and quantification of netarsudil and its impurities was evaluated in robustness. The ± 5 mL variation in the composition of the mobile phase, ± 5 nm variation in the wavelength of the detector and ± 0.1 factor variation in the mobile phase pH were intentionally made, and a 100% concentration of netarsudil containing 0.1% of each impurity was injected in each changed method condition. The chromatographic response and system suitability of the obtained chromatograms under each condition are summarized. The percentage change in the peak area response of each analyte was calculated by comparing it with the corresponding regression equation, and a percentage change of less than 2 was treated as acceptable.

Recovery

The 50, 100 and 150% levels to a known concentration (100%) in the linearity range were used for evaluating the method's accuracy. The percentage recovery in each analysis was calculated by correlating the recovery results with the calibration results. The percentage RSD in every studied spiked level was calculated for netarsudil and its impurities. The percentage recovery in the range of 98-102 and percentage RSD of < 2 in each level was considered acceptable.

Force degradation studies

The applicability of this method for the separation and analysis of stress degradation compounds generated during the stress exposure of netarsudil was confirmed by performing forced degradation studies. The standard netarsudil at a quantity of 50 mg was separately mixed with 50 mL of 0.1 N HCl, 0.1 N NaOH, and 3% peroxide solution for acid, base, and peroxide degradation studies, respectively. The stressed samples were incubated for 24 hours to induce degradation of the netarsudil drug. Then, the solution was neutralized, diluted to a 100% concentration level, and analyzed. The standard netarsudil was taken in a Petri dish and exposed to 60 °C for 24 hours in an air oven for thermal degradation and exposed to UV light at 254 nm for 24 hours for photolytic degradation study. The stressed sample was then diluted to a 100% concentration level, and the dilute solution was analyzed using the developed method. The chromatograms observed for each stress sample analysis were used to evaluate the efficiency of the method for the separation and analysis of stress degradation compounds. The percentage degradation of netarsudil was calculated by comparing the peak area response of the stressed sample with that of the un-stressed sample of the same concentration level. The peaks corresponding to degradation products were characterized using mass spectral analysis.

Sample analysis

The netarsudil solution was analyzed using the developed method. The formulation solution spiked with known and concentration of the impurities was also analysed. Based on results noticed during formulation analysis, the % assay of netarsudil and its impurities was calculated using its corresponding calibration equation.

RESULTS

The literature survey for the available analytical methods proved that there is no method reported for the resolution and quantification of process-related impurities of netarsudil in synthetic drugs or pharmaceutical formulations. In view of the above, this study was intended to develop a simple and sensitive HPLC method for quantification of two process-related impurities, namely impurities 1 and 2, along with netarsudil in formulations.

In the process of method development, various method conditions were optimized by comparing the results attained in each studied condition. The method optimization was concluded by achieving the best resolution of analytes with acceptable system suitability. The mobile phase composition of acetonitrile, methanol and pH 4.6 phosphate buffer in 45:35:20 (*v/v*) as mobile phase at 1.0 mL/min flow rate, ZORBAX Eclipse XDB C18 (250 x 4.6 mm; 5 µ id) column at room temperature as stationary phase and 257 nm as detector wavelength. Under the proposed method conditions, the chromatogram of the blank (Figure 3A) did not show any chromatographic detection

throughout the run time, whereas 100% solution of netarsudil spiked with 0.1% impurities was noticed to be well resolved and retained peaks corresponded to analytes in the study (Figure 3B). This result proved that the method was specific for detecting netarsudil and its impurities.

A) Chromatogram obtained for bank analysis in the developed method; B) standard chromatogram observed for analysing netarsudil standard solution at 100 µg/mL concentration spiked with 0.1% impurities

A tail factor of less than 1.5, plate count of more than 2500, and resolution of more than 2 were noticed for netarsudil, and its impurities suggest that the method passes system suitability and has good selectivity. The s/n method was used for evaluating method sensitivity, and the results were expressed in terms of LOD and LOQ. The LOD was observed to be 0.008 µg/mL and 0.003 µg/mL, whereas the LOQ was identified as 0.025 µg/mL and 0.010 µg/mL for impurities 1 and 2, respectively. The results obtained for both I impurities indicate the higher sensitivity of the method.

The higher LOQ concentration of impurities, *i.e.* 0.025 µg/mL) was taken as the initial concentration for constructing the calibration curve for both impurities. The netarsudil standard solution was prepared such that the solution contained 0.1% of each impurity, and an accurate fit calibration curve was obtained in the concentration level of 25-200 for netarsudil and 0.025-0.2 µg/mL for impurities.

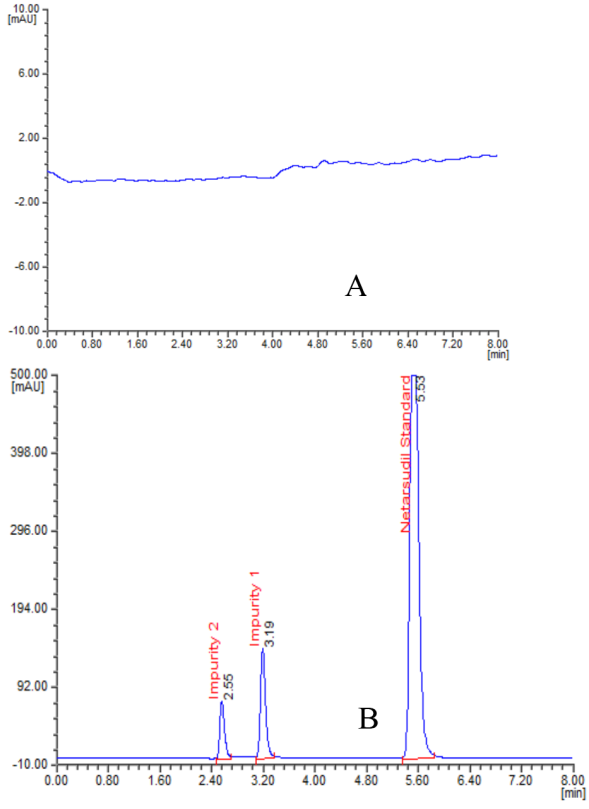

Figure 3. Specificity chromatograms observed in the proposed method, A) chromatogram obtained for bank analysis in the developed method; B) standard chromatogram observed for analysing netarsudil standard solution at 100 µg/mL concentration spiked with 0.1% impurities

The RSD% values of peak areas obtained were below 2 for impurities and netarsudil in intraday, interday precision, precision at the LOQ level, and ruggedness study, indicating good precision of the method. The summary results observed in the system suitability, linearity, precision, accuracy, and sensitivity study in the proposed method are presented in Table 1.

Spiked recovery at three spiked levels was performed to evaluate the accuracy of the proposed method for evaluating netarsudil and its impurities. The percentage recovery in each analysis as well as the percentage RSD in each studied level was noticed to be within the acceptable level for netarsudil and its impurities studied. The acceptable percent recovery and percentage RSD were observed, suggesting that the method was accurate. Results in the recovery study are presented in Table 2.

In all deliberately altered chromatographic conditions, such as mobile phase composition, pH, and detector wavelength, all analytes were resolved and the order of elution was unchanged. A very nominal percentage variation of less than 1 was noticed

Table 1. Summary results of method validation study

Parameter	Drugs		
	Netarsudil	Impurity 1	Impurity 2
System suitability[$]			
t_R (minute)	5.53	3.19	2.55
RRT	-	0.58	0.46
RRF	-	0.088	0.067
R_S	9.14	3.97	-
K^I	1.81	0.62	0.29
A_S	0.98	1.04	1.07
N	6525	7908	12410
Linearity			
Range in µg/mL	25-200	0.025-0.2	0.025-0.2
Slope	7108.2	627533	479773
Intercept	19692	-809.74	-258.73
r^2	0.9995	0.9992	0.9994
Precision[$$]			
Intraday	0.22	0.23	0.85
Interday (day 1)	1.31	0.89	1.30
Interday (day 2)	0.94	0.72	0.34
LOQ level	-	0.83	1.02
Sensitivity			
LOD (µg/mL)	-	0.008	0.003
LOQ (µg/mL)	-	0.025	0.010

[$]Average of three determinations, [$$]average of six determinations, t_R (minute): Retention time, RRT: Relative retention time, RRF: Relative response factor, R_S: Resolution, K^I: Retention factor, A_S: Tail factor, N: No of theoretical plates, r^2: Slope

for netarsudil and its studied impurities. The variability in the estimation of netarsudil and impurities was within the acceptable level of 2, indicating the robustness of the method. Table 3 presents the robustness study results obtained using the developed method.

No considerable degradation of netarsudil was observed under thermolytic stress conditions. Significant degradation of the netarsudil drug substance was observed in other degradation conditions studied. The assay of netarsudil for three determinations in acid degradation was calculated to be 91.09%, whereas, in the presence of impurities and degradation products, it was 99.73%. The chromatogram clearly resolves three degrdation products (DPs) identified at RTs of 0.89, 1.65, and 7.28 minutes, and these impurities are marked as DP 1, DP 2, and DP 6, respectively. Three degradation products were identified in the base degradation study with a percentage degradation of 6.32%. The percentage degradation of 4.01 and 9.85 was noticed in the peroxide and UV light degradation studies. Based on the t_R of the degradation products identified, it was confirmed that 6 DPs were observed in the stress degradation study of netarsudil.

Table 2. Recovery results in the study

Accuracy level	Drugs		
	Netarsudil	Impurity 1	Impurity 2
50%[$]			
Amount added (µg/mL)	50	0.05	0.05
Amount recovered (µg/mL)	49.478	0.04939	0.04940
Recovery %	98.96	98.78	98.7900
RSD %	0.79	0.90	0.76
100%[$]			
Amount added (µg/mL)	100	0.10	0.10
Amount recovered (µg/mL)	99.05	0.09879	0.09891
Recovery %	99.05	98.79	98.91
RSD %	0.49	0.99	0.78
150%[$]			
Amount added (µg/mL)	150	0.15	0.15
Amount recovered (µg/mL)	147.86	0.15086	0.15081
Recovery %	98.57	100.57	100.54
RSD %	0.47	0.37	0.60

[$]Average of three determinations, RSD: Relative standard deviation

The purity of netarsudil in each stress study was evaluated using a photodiode array detector, and the results proved that the peak was homogeneous and pure. A very high mass balance in the level of 99.02-99.84% was noticed in the stress study, and the results suggest that the method was specific and stable. Table 4 presents the results and Figure 4 shows the representative chromatograms observed in the forced degradation study.

A) Acid degradation chromatogram of netarsudil showing DP 1, 2, and 6; B) base degradation chromatogram of netarsudil showing DP 2, 4, and 5; C) peroxide degradation chromatogram of netarsudil showing DP 4 and 6; D) UV light degradation chromatogram of netarsudil showing DP 2 and 3.

Characterization of DPs by LC-MS/MS

Netarsudil and its DPs (DP 1 to DP 6) were well resolved by LC and identified at the specified RT. All DPs, along with standard netarsudil, exhibited abundant protonated molecular ions ($[M+H]^+$) in the positive ionization mode. Structural confirmation of DPs was performed using collision-induced dissociation (CID) spectra of the molecular ions of netarsudil. The ESI MS spectrum of DP 1 identified at t_R of 0.8 min showed abundant parent ions at an m/z of 298 (m+1), which might be due to *4-(2-amino-2-oxoethyl)benzyl 2,4-dimethylbenzoate* (loss of $C_{10}H_8N_2$ from $C_{28}H_{27}N_3O_3$ of netarsudil). In addition, the spectrum also showed a low abundance of product ions at m/z of 122 (m+1), which corresponds to benzoate ions with the molecular formula of $C_7H_6O_2$ by losing $C_{11}H_{14}NO$. The fragmentation spectra are shown in Figure 5A.

Table 3. Results observed in robustness study

Chromatographic conditions	t_R			Number of theorietical plates			Change in peak area %		
	NTD	Impurity 1	Impurity 2	NTD	Impurity 1	Impurity 2	NTD	Impurity 1	Impurity 2
Mobile phase composition (*v/v* of acetonitrile, methanol and buffer)[$]									
40:40:20	5.51	3.19	2.57	6904	8015	12328	0.42	0.47	0.76
50:30:20	5.53	3.20	2.54	6858	8146	12507	0.26	0.99	0.98
Detector wavelength[$]									
252 nm	5.53	3.08	2.55	6562	7858	12269	0.51	0.88	0.57
262 nm	5.50	3.10	2.53	6749	7940	12351	0.96	0.74	0.13
Mobile phase pH[$]									
4.5	5.55	3.11	2.54	6631	8414	12499	0.28	0.52	0.61
4.7	5.52	3.10	2.56	6503	8329	12407	0.69	0.95	0.31

[$]Average of three determinations (n= 3), NTD: Netarsudil

The mass fragmentation spectra of DP 2 (Figure 5B) show an abundant parent ion at an m/z of 314 (m+1) under the negative ionization mode. The spectrum also shows fragment ions at m/z of product ions at m/z of 122 (m+1), which corresponds to benzoate ions with a molecular formula of $C_7H_6O_2$. Based on the achieved date, DP 2 was confirmed to be *4-(1-amino-3-hydroxypropan-2-yl)benzyl 2,4-dimethylbenzoate* with a molecular formula of $C_{19}H_{23}NO_3$.

The ESI-MS spectrum identified at a RT of 2.0 min shows a parent ion at m/z= 152 corresponding to the $[M+H]^+$ of DP 3 formed under acidic stress (Figure 5C). The spectrum showed abundant product ions at m/z 78, and the production fragments correlated well with the fragmentation pattern of benzene. The purity test and CID studies of DP 3 suggest that it is one of the degradation products of DP-2 observed in the study. All these product ions and patent ions confirm DP 3 as *(2,4-dimethylphenyl)(hydroxy)methanolate* with the molecular formula $C_9H_{11}O_2$.

The ESI-MS spectrum of DP 4 (Figure 5D) identified at a RT of 4.1 min shows a parent ion at m/z= 322 (m+1) with a parent ion at m/z= 123 (m+1). The parent ion shows a molecular formula of $C_{19}H_{19}N_3O_2$ and a fragment ion with a molecular formula of $C_7H_{10}N_2$ by losing $C_{12}H_9NO_2$. DP 4 was identified as *3-amino-2-[4-(hydroxymethyl)phenyl]-N-(isoquinolin-6-yl)propanamide* with a molecular mass of 322 (m+1), DP 5 (Figure 5E) observed at an RT of 4.5 min was identified as *isoquinolin-6-amine* with

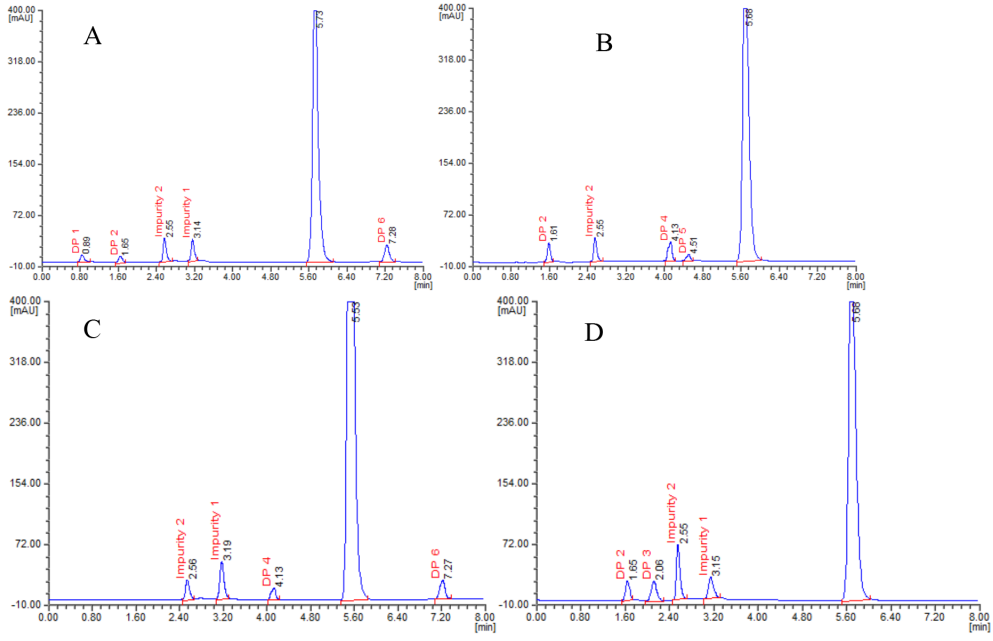

Figure 4. Chromatogram observed in forced degradation study of netarsudil in the proposed method, A) acid degradation chromatogram of netarsudil showing DP 1, 2 and 6; B) base degradation chromatogram of netarsudil showing DP 2, 4 and 5; C) peroxide degradation chromatogram of netarsudil showing DP 1, 4 and 6; D) UV light degradation chromatogram of netarsudil showing DP 2 and 3

UV: Ultraviolet, DP: Degrdation product

Table 4. Summary of netarsudil forced degradation results in the proposed method

Stress condition	% Degradation[$] of netarsudil	% Assay[$] of netarsudil	% Mass balance[$] (assay + total impurities)	Remark
Acid	8.91	91.09	99.47	DP 1 (0.89 minute), 2 (1.65 minute) and 6 (7.28 minute) were identified
Base	6.32	93.68	99.78	DP 2 (1.61 minute), 4 (4.13 minute) and 5 (4.51 minute) were identified
Peroxide	4.01	95.99	99.63	DP 4 (4.13 minute) and 6 (7.27 minute) were identified
Thermal	3.28	96.72	99.95	No degradation was identified
UV light	9.85	90.15	99.58	DP 2 (6.15 minute) and 3 (2.06 minute) were identified

[$]Average of three replicate experiments, UV: Ultraviolet, DP: Degrdation product

a molecular formula of $C_9H_8N_2$ and a molecular mass of 145 (m+1).

The characterization of DP 6 (Figure 5F) was carried out based on its ESI MS spectrum $[M+H]^+$ that showed abundant product ions at m/z 304 (m+1). The production at m/z 105 may be formed by the loss of $C_{11}H_9NO_3$ from m/z 304, resulting in a p-xylylene ion. The peak purity CID studies of DP 6 confirm that it is one of the degradation products of DP 4 observed in the study. Based on these studies, DP 6 was identified as *3-amino-N-(isoquinolin-6-yl)-2-(4-methylidenecyclohexa-2,5-dien-1-ylidene)propanamide* with a molecular mass of 303 g/mol and formula of $C_{19}H_{17}N_3O$. The DP 1 to 6 generated during the stress study of netarsudil was presented in Figure 6.

The developed HPLC method was applied to quantify process-related impurities of netarsudil in pharmaceutical formulations. The formulation sample was directly analyzed for the evaluation of impurities present in it, and the formulation sample spiked with impurities was analyzed to evaluate the effectiveness of the method for the resolution and quantification of impurities in the formulation. The chromatogram obtained for the impurity spiked formulation solution (Figure 7A) shows a clear identification of peaks corresponding to the impurities in the study. The chromatogram observed for the unspiked formulation solution (Figure 7B) shows peaks corresponding to impurity 1 only. Impurity 2 was not identified in the chromatogram, proving that the quantity of impurity in the sample was less than the LOD of impurity B. The peak area response of impurity 1 was substituted in its corresponding regression equation, and the percentage assay was calculated. The percentage assay of impurity 1 was estimated to be 0.02%. This confirms that the quantity of impurity in the sample was noticed to be under the permissible level, and the proposed method can be successfully applied for the quantification of process-related impurities in netarsudil.

DISCUSSION

The study addresses a critical gap in existing literature by developing a novel HPLC method for the precise quantification of process-related impurities (impurities 1 and 2) along with netarsudil in pharmaceutical formulations. Through syntematic method optimization including the optimization of mobile phase composition, column selection, and detector wavelength, optimal conditions were established to ensure effective resolution

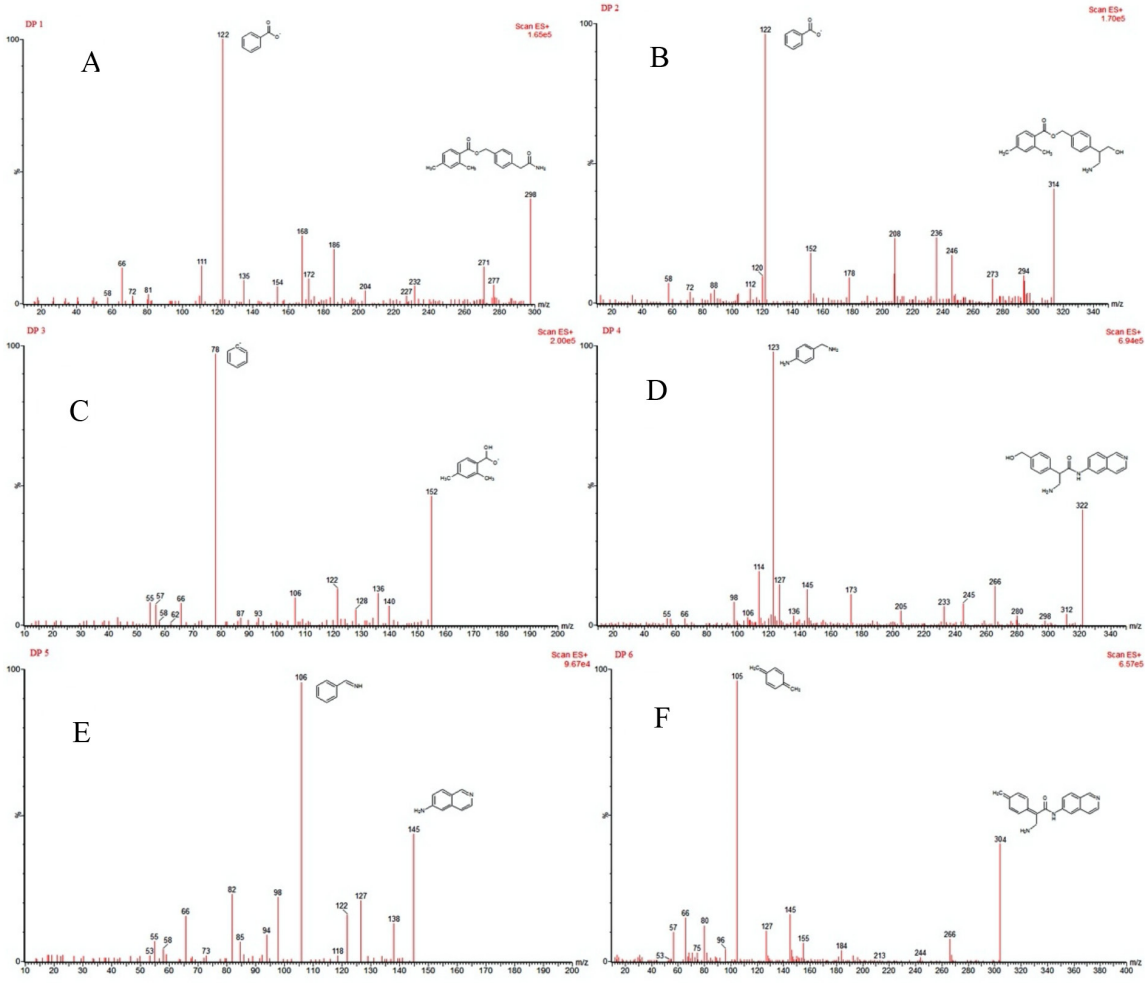

Figure 5. Mass spectra of DPs observed in forced degradation study, mass spectra identified at tR of 0.8 min for DP 1 (A), 1.6 min for DP 2 (B), 2.0 min for DP 3 (C), 4.1 min for DP 4 (D), 4.5 min for DP 5 (E) and 7.2 min for DP 6 (F)

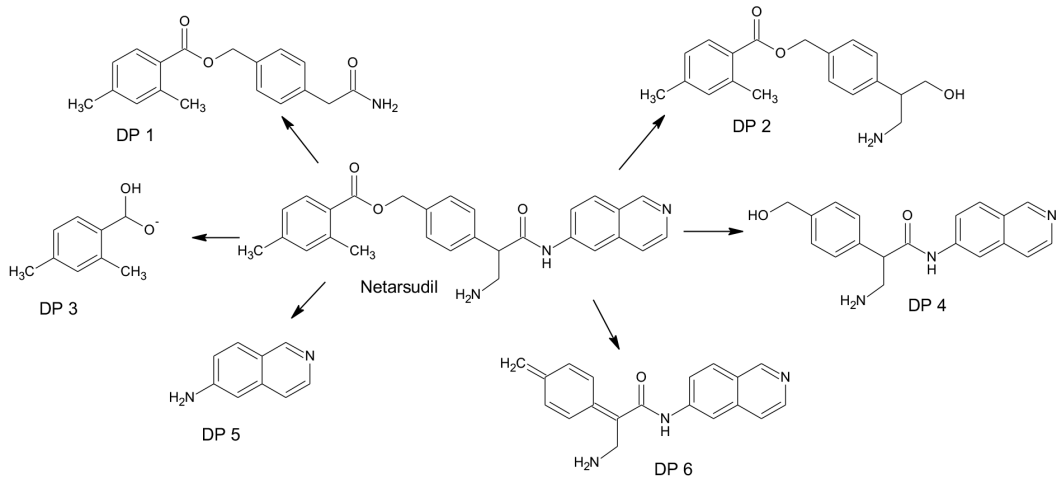

Figure 6. Degradation products formed during the forced degradation study of netarsudil

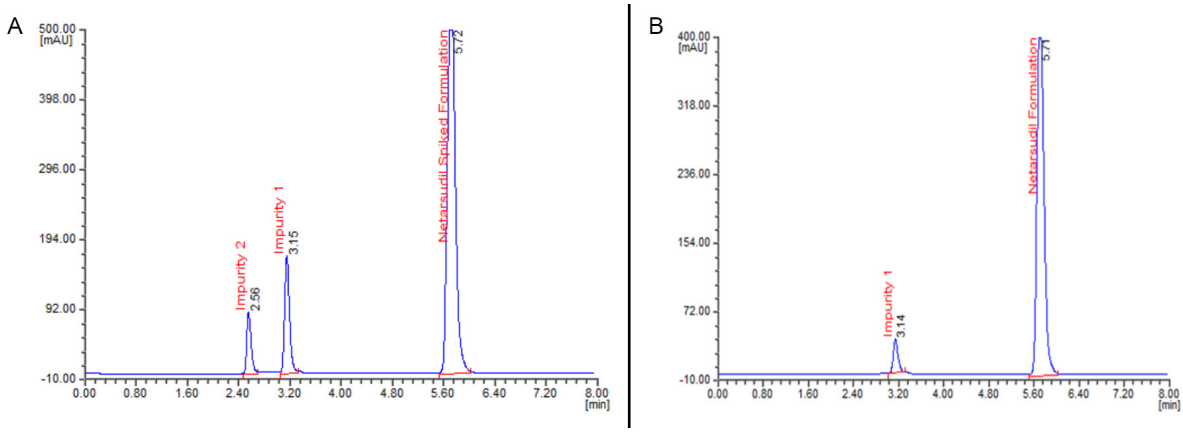

Figure 7. Formulation chromatogram of netarsudil in the developed method, A) chromatogram observed for analysing netarsudil solution spiked with 0.1% impurities; B) chromatogram observed for analysing netarsudil formulation solution spiked with no impurities

and system suitability. The finalized method exhibited robust chromatographic parameters, including tailing factors below 1.5, plate counts exceeding 2500, and resolution values greater than 2, confirming its specificity and reliability. Sensitivity assessments revealed low limits of detection (LOD) at 0.008 µg/mL and 0.003 µg/mL for impurities 1 and 2, respectively, underscoring the method's ability to detect trace impurities. Calibration curves constructed across a broad concentration range (25-200 µg/mL for netarsudil and 0.025-0.2 µg/mL for impurities) demonstrated excellent linearity, essential for accurate quantification. Precision studies demonstrated consistent results with RSD% values below 2, both within-day and between-day, reinforcing the method's reliability. Furthermore, robustness testing under varied chromatographic conditions showed minimal impact on quantification, affirming the method's stability and reproducibility. Application of the method to pharmaceutical formulations effectively identified and quantified impurities, with analysis of spiked and unspiked samples confirming its suitability for routine quality control. The study's comprehensive approach, including forced degradation studies and characterization of degradation products through LC-MS/MS, provided valuable insights into netarsudil's stability under stress conditions, enhancing its applicability in pharmaceutical analysis and ensuring compliance with regulatory standards.

CONCLUSION

A simple, sensitive isocratic reversed-phase HPLC method has been optimized and subsequently used for the evaluation of a stability-indicating assay of netarsudil and its two process-related impurities in bulk drugs and its dosage forms. The developed method was validated to be selective, sensitive, linear, accurate, precise, and sensitive and is applicable for evaluating process-related impurities and degradation products at trace levels in bulk drugs and formulations. The degradation behavior of netarsudil was assessed under different stress conditions as per the ICH prescribed guidelines. In total, six degradation products were formed and characterized by LCMS. The DPs were characterized as *4-(2-amino-2-oxoethyl)benzyl 2,4-dimethylbenzoate* (DP 1), *4-(1-amino-3-hydroxypropan-2-yl)benzyl 2,4-dimethylbenzoate* (DP 2), *(2,4-dimethylphenyl)(hydroxy)methanolate* (DP 3), *3-amino-2-[4-(hydroxymethyl)phenyl]-N-(isoquinolin-6-yl)propanamide* (DP 4), *isoquinolin-6-amine* (DP 5), and *3-amino-N-(isoquinolin-6-yl)-2-(4-*

methylidenecyclohexa-2,5-dien-1-ylidene) propanamide (DP 6). Thus, this method can be used for process development and quality assurance of netarsudil in bulk drugs.

Ethics

Ethics Committee Approval: This study does not involve the use of animals, and therefore ethical approval is not required to complete my research study.

Informed Consent: In this study, a no-consent model was employed to complete this research work.

Authorship Contributions

Concept: V.R.A., B.S.K., J.H., Design: V.R.A., B.K.T., T.E., Data Collection or Processing: V.R.A., B.S.K., J.H., Analysis or Interpretation: V.R.A., B.S.K., J.H., Literature Search: V.R.A., B.K.T., T.E., Writing: V.R.A., B.S.K., J.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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