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Characterization of forced degradation products of Netarsudil: Optimization and validation of stability-indicating RP-HPLC method for simultaneous quantification of process related impurities of Netarsudil

Short Title: LCMS Charcaterization of Netarsudil Degradation Products

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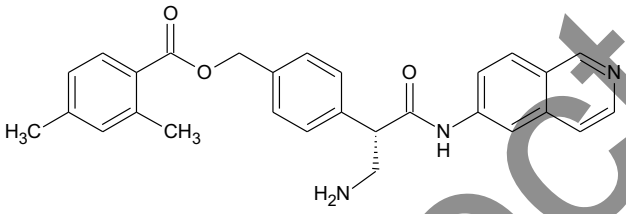
Abstract:
Objective: This study reports the resolution, identification and characterization of forced degradation products of netarsudil by liquid chromatography-tandem mass spectrometry by validating a simple and sensitive HPLC method for resolution, identification and quantification of two process related impurities in netarsudil.
Materials and Methods: The chromatographic separation was accomplished on an ZORBAX Eclipse XDB C18 (250×4.6 mm; 5 µ id) column at room temperature as stationary phase and 257 nm as 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 reports very sensitive detection limit of 0.008 µg/mL for impurity 1 and 0.003 µg/mL for impurity 1. The method produces 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, thermal and UV light to investigate the stabilityindicating ability of the method. Considerable degradation was observed in stress studies and the degradation products were well resolved from its process related impurities. The characterization of degradation products was performed based on 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 regular analysis of netarsudil and its process related impurities in bulk drug and pharmaceutical dosage forms and can also applicable for evaluation of stress degradation mechanism of netarsudil.
Key words: Netarsudil, Process related impurities, HPLC analysis, forced degradation studies, Characterization of degradation compounds

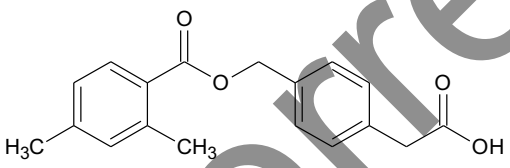
Introduction:

The pharmaceutical industry is rising day by day with an aim to investigate novel drugs that are isolated from natural products or synthesized chemically. The main challenge for that always remains is that the compound should be pure and purity was treated as significant factor for ensuring the drug quality [1]. In the process of synthesis of pure drug, there is a possibility of some unwanted compounds that remains in the pure drug and these unwanted compounds were considered as impurities. The presence of these impurities even at very low quantity may influence the quality, efficacy and safety of the drug product. Hence, identification and quantification of impurities was considered as very essential for producing the safe drug and HPLC was considered as simple and convenient procedure for identifying and quantifying the impurities in any source [2]. Netarsudil is a Rho kinase inhibitor and norepinephrine transporter inhibitor drug approved for decreasing the elevated intraocular pressure in people with open-angle glaucoma or ocular hypertension [3]. The key difference of netarsudil from other Rho kinase inhibitor is, it not only minimize the intraocular pressure by reducing outflow resistance but also minimize the aqueous humor production and episcleral venous pressure [4]. The side effects possible during the usage of netarsudil include eye pain upon instilling, eye or eye lid redness changes in vision, discoloration of the eye and teary eyes [5]. Its molecular structure was shown in figure 1 with IUPAC name of *[4-[(2S)-3-Amino-1-(isoquinolin-6-ylamino)-1-oxopropan-2-yl]phenyl]methyl 2,4-dimethylbenzoate* with molecular formula of $C_{28}H_{27}N_3O_3$ and mass of 453.542 g/mol.

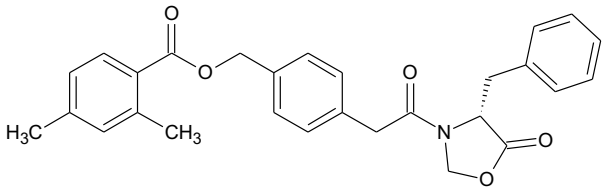
The literature survey was conducted for determining the available analytical method for quantification of netarsudil using various analytical techniques. In literature, it was observed that few analytical methods reported for quantification of netarsudil in combination with latanoprost using HPLC [6-10] and UPLC [11]. One UPLC/MS method reported for quantification of netarsudil in combination with timolol and latanoprost [12]. One LC-Q-TOF-MS/MS method reported for identification and characterization of netarsudil and its hydrolytic degradation products [13]. The literature review suggests that there is no method available for resolution, identification and quantification of process related impurities of netarsudil. Hence this study intended to fulfill the gaps identified in literature. The process related impurity 1 and 2 were available for the study and hence were selected. The molecular structure of netarsudil and its process related impurities was presented in figure 1.



Netarsudil



Impurity 1



Impurity 2

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

The origin of process related impurities in netarsudil synthetic drug was evaluated by observing the synthesis route of netarsudil [14] and was presented in figure 2. In the process of synthesis of netarsudil, the starting product of reaction i.e *(4-{[(2,4-dimethylbenzoyl)oxy]methyl}phenyl)acetic acid* (**4**) and the intermediated product (**6**) remains in the final product and these compounds were designated as impurity 1 and 2 respectively.

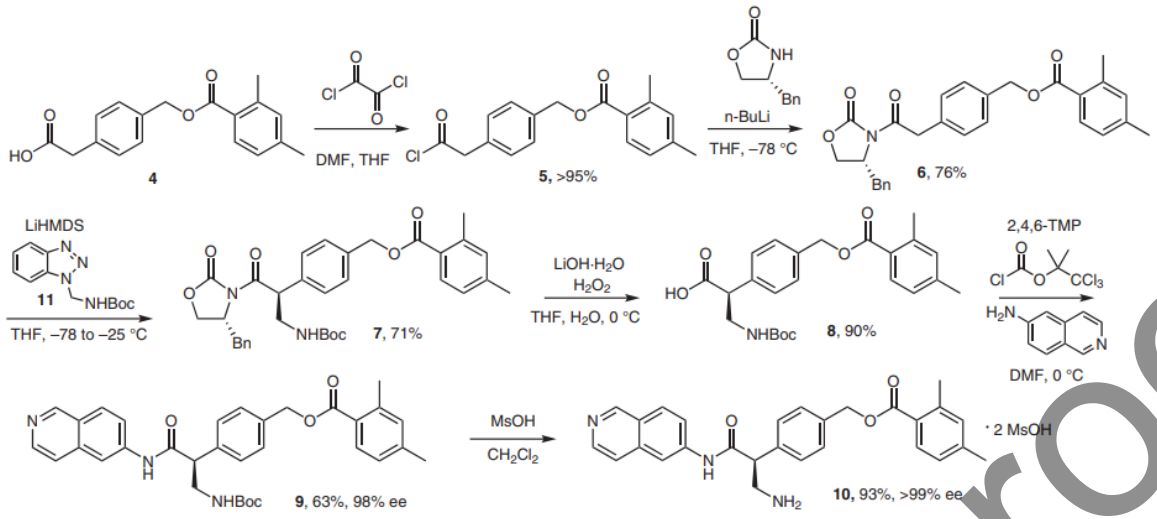


Figure 2: Netarsudil synthesis route

Materials and Methods:

Chemicals and reagents:

The analytical standard compound netarsudil with purity of 98.17%, its impurity 1 and 2 were procured from Ajanta Pharma Limited, Hyderabad, Telangana. The eye drop formulation containing 0.02% w/v of netarsudil with brand Netapride® was obtained from local pharmacy. The HPLC grade methanol, acetonitrile, Milli-Q® water were obtained from Merck chemicals, Mumbai. The 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:

The HPLC analysis was performed on Agilent 1100 (USA) instrument that coupled with quaternary pump (G1311 A) for solvent delivery. The analytes were injected through temperature adjustable auto sampler (G 1329A) having injection capacity of 0.1–1500 µL. The column eluents were detected using programmable ultraviolet (UV) detector (G 1314 A) and the chromatographic integrations was carried using Agilent chem-station software. The LCMS analysis was performed on Waters LCMS (Japan) equipped with triple quadruple mass detector. During the mass spectral analysis, splitter was placed in between the column and detector that can allow 40 % of the chromatographic eluents were entered in to the 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). The nebulization (40 Psi) and drying (300 ºC, 9 L/h) was 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 solvent using an ultrasonic bath sonicator. Then the analytes were filtered through 0.2 µ membrane filter and the final volume was made up to the mark using same solvent to obtain 1000 µg/mL concentration of netarsudil and its impurities separately. During the analysis, selected volume of required concentration of individual analyte was mixed separately.

***Formulation solution*:**

The eye drop formulation containing 0.02% w/v of netarsudil with brand Netapride® was utilized for preparing formulation solution. An accurately measured Netapride® solution (25 mL) was taken in a 50 mL calibrated flask having 10 mL methanol. The flask was sonicated to dissolve the formulation completely in the solvent and then the volume make up to mark with same solvent. The solution was filtered through 0.2 µ membrane filter to obtain 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 suitable wavelength for the detection of netarsudil and its impurities using UV detector. 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 the iso-absorption wavelength that was suitable for the detection of netarsudil and its impurities.

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

Method Validation:

The method optimized for evaluation of netarsudil and its impurities was validated as per methodology reported literature [15-18] as well as ICH guidelines [19].

System suitability:

The 100 % concentration level solution was analysed six times in the proposed method for establishing the system suitability of the developed method. The chromatographic response of the resultant chromatograms in each study was summarized for evaluating the system suitability of the developed method. The 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 detection of impurities was evaluated by assessing 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 impurities of netarsudil was analysed in the developed method and the chromatographic response (signal) along with 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 contains 0.1 % of impurity. Various levels of netarsudil standard solution spiked with 0.1 % of studied impurities was analysed in the developed method. The chromatographic response of each analyte was tabulated and calibration curve was plotted individually by considering obtained peak area response on y-axis and its prepared concentration on x-axis. The best fitted calibration range for each analyte was considered as 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 for evaluating the repeatability and reproducibility of the developed method. The solution was prepared and injected six times in one day (intraday), in three days (interday) and by different analysts in the same day (ruggedness). The peak area response of each analyte in each study was tabulated and the % RSD was calculated for each analyte in each study. The % 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 composition of mobile phase, ±5 nm variation in wavelength of detector and ±0.1 factor variation in mobile phase pH was made intentionally and the 100 % concentration of netarsudil containing 0.1 % of each impurity was injected in each changed method condition. The chromatographic response and the system suitability of the obtained chromatograms in each condition were summarized. The % change in the peak area response of each analyte was calculated by comparing it corresponding regression equation and a % change of less than 2 was treated as acceptable.

Recovery:

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

Force degradation studies:

The method applicability for the separation and analysis of stress degradation compounds generated during the stress exposer of netarsudil was confirmed by performing forced degradation studies. In this, 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 study respectively. The stressed samples were incubated for 24 hours to induce degradation in netarsudil drug. Then the solution was neutralized, diluted to 100 % concentration level and then analysed. The standard netarsudil was taken in a petri dish and exposed to 60 ^{0}C for 24 h in an air oven for thermal degradation and exposed to UV light at 254 nm for 24 h for photolytic degradation study. Then the stressed sample was diluted to 100 % concentration level and the dilute solution was analysed in the developed method. The chromatograms observed for each stress sample analysis was observed for evaluation of method efficiency for the separation and analysis of stress degradation compounds. The % degradation of netarsudil was calculated by comparing the peak area response of stressed sample with the un-stressed sample of the same concentration level. The peaks correspond to degradation products was characterized using mass spectral analysis.

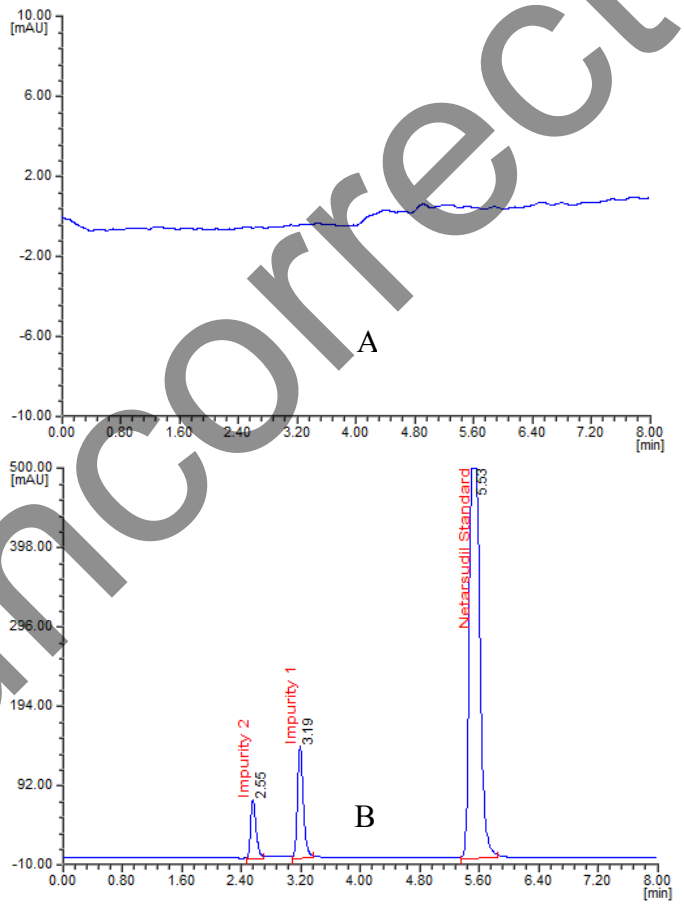
Sample analysis:

The Netapride® formulation solution was analysed in 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 and Discussions:

The literature survey for the available analytical methods proved that there is no method reported for resolution and quantification of process related impurities of netarsudil in synthetic drug as well as in pharmaceutical formulations. In view of the above, this study was intended to develop a simple and sensitive HPLC method for quantification of 2 process related impurities namely impurity 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 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 (250×4.6 mm; 5 µ id) column at room temperature as stationary phase and 257 nm as detector wavelength. In this proposed method conditions, the chromatogram of blank (Figure 3A) don't 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 corresponds to analytes in the study (Figure 3B). This result proved that the method was specific for the detection of netarsudil and its impurities in the study.



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

Figure 3: Specificity chromatograms observed in the proposed method

The tail factor of less than 1.5, plate count of more than 2500 and resolution of more than 2 was noticed for netarsudil and its impurities suggest that the method passes system suitability and having good selectivity. The s/n method was utilized for evaluating method sensitivity and results were expressed in terms of LOD and LOQ. The detection limit 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 impurity 1 and 2 respectively. The results obtained for both I impurities indicating the higher sensitivity of the method.

The higher quantification limit concentration of impurities i.e 0.025 µg/mL was taken as initial concentration for constructing the calibration curve for both impurities. The netarsudil standard solution was prepared such that the solution contains 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 in the study.

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

Parameter	**Results**		
	Netarsudil	**Impurity 1**	**Impurity 2**
System suitability$			
t_R (min)	5.53	3.19	2.55
RRT	--	0.58	0.46
RRF	--	0.088	0.067
R_S	9.14	3.97	--
$K^!$	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

t_R (min) = retention time; RRT = relative retention time; RRF = relative response factor; R_S = resolution; $K^!$ = retention factor; A_S = tail factor; N = No. of theoretical plates; r^2 = slope; $average of three determinations; $$average of six determinations

Table 1: Summary results of method validation study

Spiked recovery at three spiked levels was performed to evaluate the accuracy of the method proposed for evaluating netarsudil and its impurities. The % recovery in each analysis as well as the % RSD in each studied levels was noticed to be with in the acceptable level for netarsudil and its impurities studied. The acceptable % recovery and % RSD was observed suggest the method was accurate. Results in recovery study were presented in table 2.

Accuracy level	Results		
	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

Table 2: Recovery results in the study

In all deliberately altered chromatographic conditions such as mobile phase composition, pH and detector wavelength, all analytes were resolved and order of elution was unchanged. Very nominal % variation of less than 1 was noticed for netarsudil and its studied impurities. The variability in the estimation of netarsudil and impurities was within the acceptable level of less than 2 indicating the robustness of the method. Table 3 presents the robustness study results obtained in the developed method.

Chromatographic conditions	t_R			N			% change in peak area		
	NTD	Imp 1	Imp 2	NTD	Imp 1	Imp 2	NTD	Imp 1	Imp 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

NTD = Netarsudil; Imp = Impurity; $average of three determinations

Table 3: results observed in robustness study

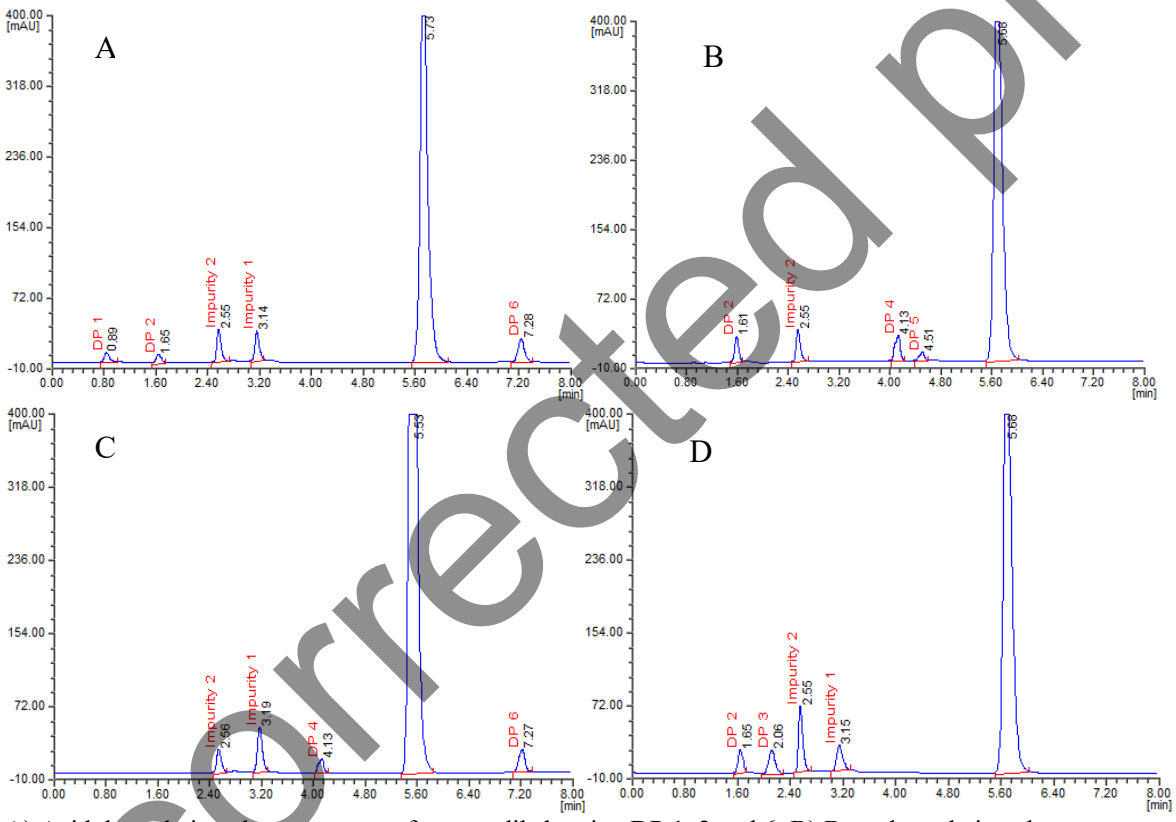
No considerable degradation of netarsudil drug substance was observed under thermolytic stress conditions. Significant degradation of netarsudil drug substance was observed in other degradation conditions studied. The assay of netarsudil for three determinations in acid degradation was calculated to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

The purity of netarsudil in each stress study was evaluated using PDA detector and 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 results suggest that the method was specificity and stability-indicating. Table 4 presents the results and figure 4 shows the representative chromatograms observed in forced degradation study.

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 min), 2 (1.65 min) and 6 (7.28 min) were identified
Base	6.32	93.68	99.78	DP 2 (1.61 min), 4 (4.13 min) and 5 (4.51 min) were identified
Peroxide	4.01	95.99	99.63	DP 4 (4.13 min) and 6 (7.27 min) 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 min) and 3 (2.06 min) were identified

[$]average of three replicate experiments

Table 4: Summary of netarsudil forced degradation results 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 4 and 6; D) UV light degradation chromatogram of netarsudil showing DP 2 and 3

Figure 4: Chromatogram observed in forced degradation study of netarsudil in the proposed method

Characterization of DPs by LC-MS/MS:

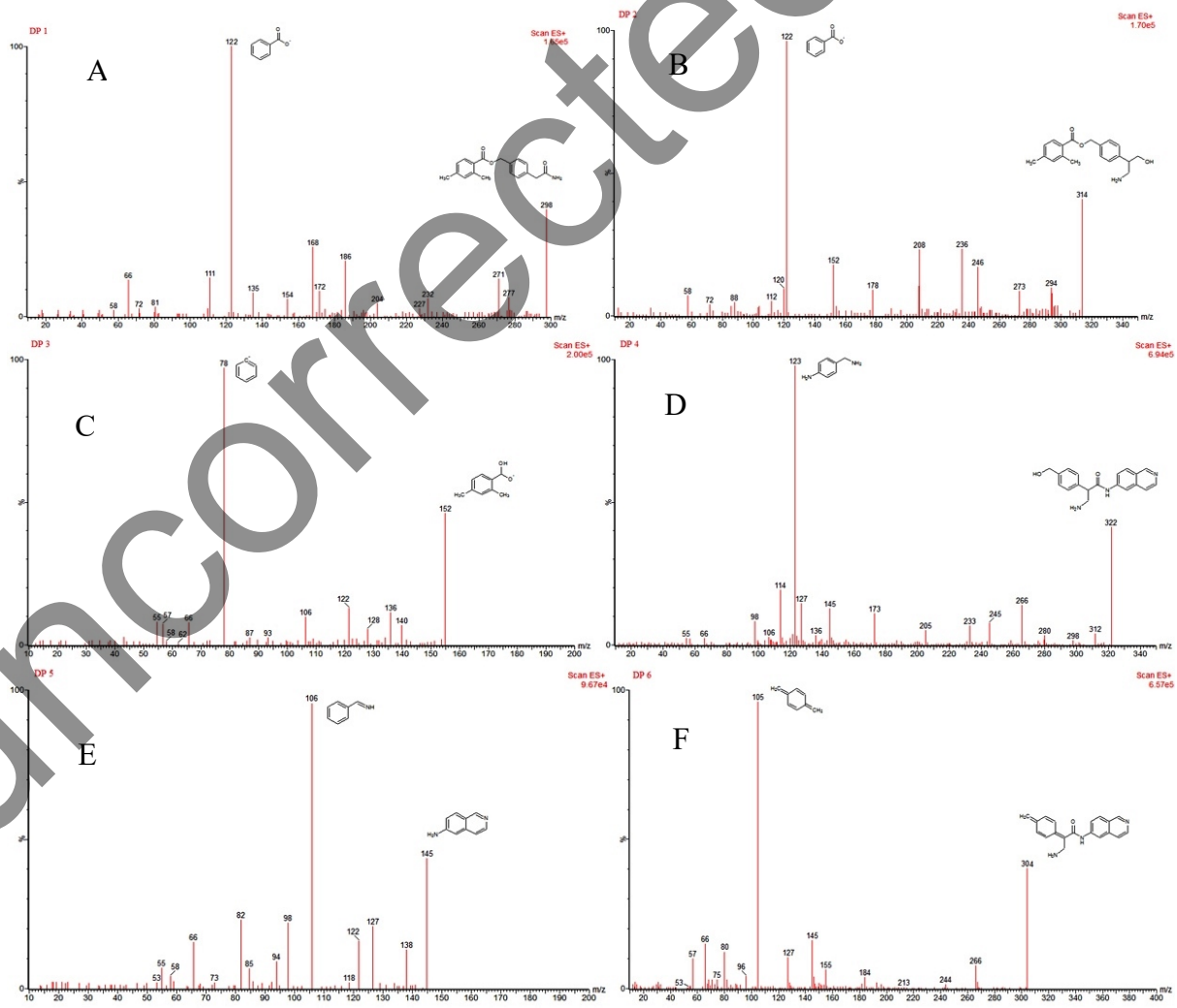
Netarsudil and its DPs (DP 1 to DP 6) were well resolved by LC and were identified at its specified retention time. All the DPs along with standard netarsudil exhibited abundant protonated molecular ions ($[M+H]^+$) in positive ionization mode. The structural confirmation of DPs was performed using collision 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 show abundant parent ion at 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 low abundant product ions at m/z of 122 (m+1) corresponds to benzoate ion with molecular formula of $C_7H_6O_2$ by losing $C_{11}H_{14}NO$. The proposed structure of DP 1 was presented in figure 7 and its fragmentation spectra were shown in figure 5A.

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

The ESI-MS spectrum identified at a retention time of 2.0 min show parent ion at m/z of 152 corresponding to the $[M+H]^+$ of DP 3 formed in acidic stress (Figure 5C). The spectrum showed abundant product ions at m/z 78 and the product ion fragments were well correlate with the fragmentation pattern of benzenide. The purity test and CID studies of DP 3 suggest it as one of the degradation product of DP-2 observed in the study. All these product ions and patent ion confirms DP 3 as *(2,4-dimethylphenyl)(hydroxy)methanolate* with molecular formula $C_9H_{11}O_2$ and its structure was presented in figure 7.

The ESI-MS spectrum of DP 4 (Figure 5D) identified at retention time of 4.1 min show parent ion at m/z of 322 (m+1) with parent ion at m/z of 123 (m+1). The parent ion shows molecular formula of $C_{19}H_{19}N_3O_2$ that show fragment ion with molecular formula of $C_7H_{10}N_2$ by losing $C_{12}H_9NO_2$. The DP 4 was identified as *3-amino-2-[4-(hydroxymethyl)phenyl]-N-(isoquinolin-6-yl)propanamide* with molecular mass of 322 (m+1) and its proposed structure was given in figure 7. The DP 5 (Figure 5E) observed at a retention time of 4.5 min was identified as *isoquinolin-6-amine* with molecular formula of $C_9H_8N_2$ and molecular mass of 145 (m+1).

The characterization of DP 6 (Figure 5F) was carried based on its ESI MS spectrum $[M+H]^+$ that showed abundant product ions at m/z 304 (m+1). The product ion 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 it as one of the degradation product of DP 4 observed in the study. Based on the studies, the DP 6 was identified as *3-amino-N-(isoquinolin-6-yl)-2-(4-methylidenecyclohexa-2,5-dien-1-ylidene)propanamide* with 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 7.



Mass spectra identified at t_R 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 5: Mass spectra of DPs observed in forced degradation study

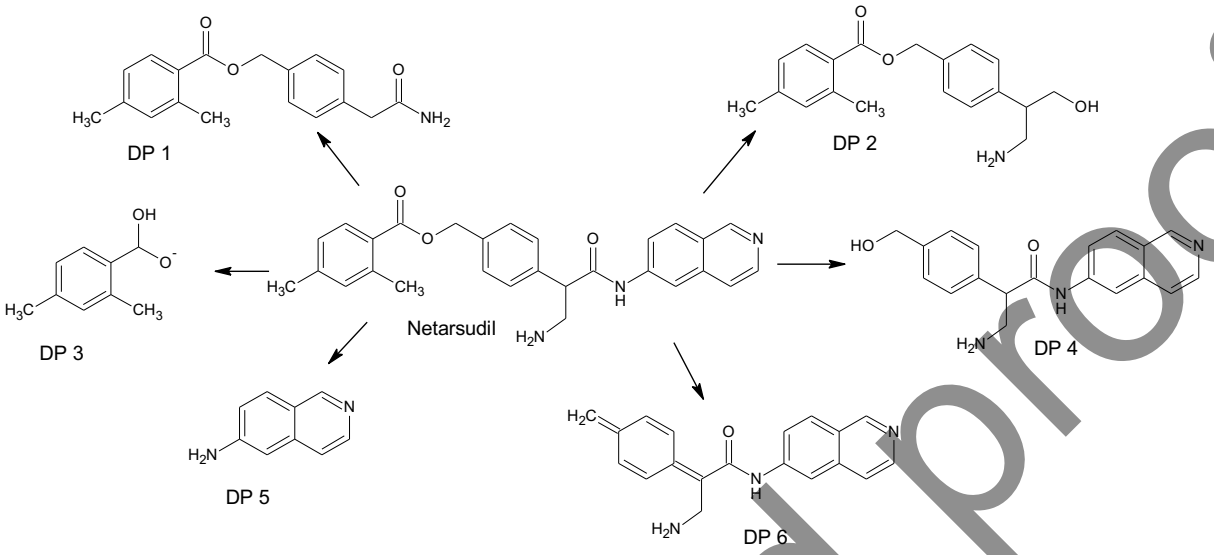
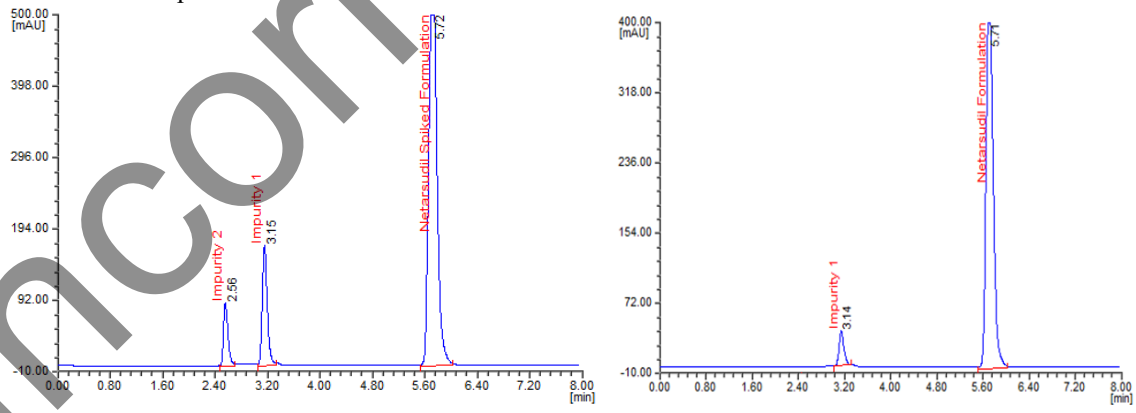


Figure 7: degradation products formed during the forced degradation study of netarsudil

The developed HPLC method was applied for the quantification of process related impurities of netarsudil in pharmaceutical formulation. The formulation sample directly analysed for the evaluation of impurities present in it and also the formulation sample spiked with impurities was analysed for evaluating the effectiveness of the method for the resolution and quantification of impurities in formulation. The chromatogram obtained for impurities spiked formulation solution (Figure 8A) show clear identification of peaks corresponds to impurities in the study. Whereas the chromatogram observed for unspiked formulation solution (Figure 8B) shows peaks corresponds to impurity 1 only. The impurity 2 was not identified in the chromatogram proved that the quantity of impurity in the sample was less than the detection limit of impurity B. The peak area response of impurity 1 was substituted in its corresponding regression equation and the % assay was calculated. The % 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 successfully applicable for the quantification of process related impurities in netarsudil.



A B

A) Chromatogram observed for analysing Netapride® formulation solution spiked with 0.1 % impurities; B) Chromatogram observed for analysing Netapride® formulation solution spiked with no impurities

Figure 8: Formulation chromatogram of netarsudil in the developed method

Conclusion:

A simple, sensitive isocratic reversed phase HPLC method has been optimized and subsequently for 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 behaviour of netarsudil was assessed under different stress conditions as per ICH prescribed guidelines. In total, six degradation products were formed and were characterized by LCMS. The DPs 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, the method can be used for process development as well as quality assurance of netarsudil in bulk drugs.

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