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Identification and characterization of degradation products of Nateglinide

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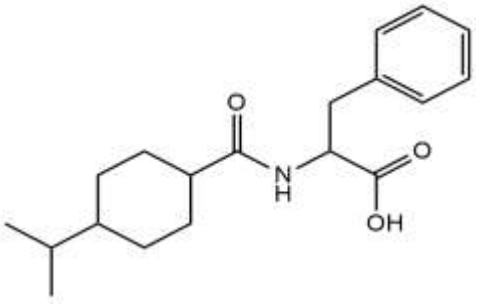
***Abstract*---**Nateglinide (NTG) was evaluated under a variety of ICH-recommended forced degradation settings and found to be degraded under acid and alkali hydrolysis but stable under other forced degradation conditions. Under acid hydrolytic conditions, three degradation impurities (I-III) were produced. All three contaminants were separated using a C-18 column with ammonium acetate buffer (20 mM), methanol, and acetonitrile as mobile phases provided in isocratic mode, and characterisation was done utilising +ESI-MSn and LC-MS-QTOF investigations. All three degrading impurities (I-III) were discovered to be novel. The most likely pathways of deterioration were presented and debated.

***Keywords*---**Nateglinide, LC-MS-QTOF, forced degradation, mass fragmentation, impurities.

Introduction

Nateglinide (NTG) is a new amino acid derivative, (Fig. 1) in the presence of glucose, this compound blocks ATP-sensitive K+ channels in pancreatic beta-cells, leading in an increase in insulin production. [1]. It is used to treat noninsulin-dependent diabetic mellitus and is chemically formulated as (N-trans-4-isopropyl cyclo hexyl carbonyl)-D-phenyl alanine [2,3]. In humans and experimental animals, NTG minimises meal-time glucose increase and lowers the post-absorptive propensity for hypoglycemia because of its early start and brief

duration of insulinotropic I action. [3]. It works by boosting the secretion of insulin from the pancreas, which reduces blood glucose levels. It achieves this by inhibiting potassium channels in the beta cell membrane that are ATP-dependent. The beta cells become depolarized, allowing voltage-gated calcium channels to open. Insulin-containing vesicles fuse with the cell membrane when calcium enters the cell, resulting in insulin production. [4].



Nateglinide (NTG)

Fig. 1: Chemical structure of Nateglinide

Forced degradation study is a convenient approach to generate all possible degradation impurities of a drug substance. According to the ICH Q1A(R2) recommendations, a drug substance must undergo a forced degradation study under circumstances of hydrolysis, oxidation, dry heat, and photolysis. [5]. There are currently just a few analytical approaches for NTG analysis that have been published in the literature. Some of these techniques are UV spectrophotometry [6, 7], VIS spectrophotometry [8, 9], HPLC [8-10], Liquid chromatography–mass spectrometry [11], and micellar electro kinetic chromatography technology [3]. However, in acidic and neutral media, no attempt has been made to define its breakdown products under acid and neutral hydrolytic conditions, oxidative conditions, and/or photolytic conditions. As a result, the goals of this study are to (i) conduct a forced degradation study on NTG in accordance with ICH Q1(R2) guidelines to identify all possible degradation products arising from various stress conditions such as hydrolysis and photolysis in acidic, alkaline, and neutral media, as well as oxidation; (ii) establish an HPLC method for the separation of degradation products (DP) and NTG; (iii) classify degradation impurities through MSn and LC-MS-QTOF studies; (iv) and determine the NTG degradation pathways.

Experimental

Chemicals and drugs

Venus Remedies Ltd. (Panchkula, India) provided NTG as a nice gift sample. Loba chemical Pvt. Ltd. provided sodium hydroxide (NaOH), hydrochloric acid (HCl), hydrogen peroxide (H2O2, 30%), and ammonium acetate (Mumbai, India). Merck Specialist Pvt. Ltd. supplied methanol, acetic acid, and acetonitrile (HPLC grade) (Mumbai, India). The HPLC grade water was acquired from the laboratory's Direct

Ultra water purification system (Bio-Age Equipment and Services, SAS Nagar, India).

Instruments and Equipments

Water bath and hot air oven with digital temperature control for hydrolytic and thermal deterioration control in a temperature range of 1 °C and 2 °C (Narang Scientific Works, New Delhi, India) were used. The drug samples were photodegraded using a photostability chamber (WTB Binder, Tuttlingen, Germany, KBF 240) equipped with temperature set to 25(2) °C with a relative humidity of 80% (RH) set at 55(±5%). For illumination the Option 2 of the ICH guideline Q1B [10] required that the chamber have a light source. An integrated Shimadzu HPLC LC 2010 CHT series system that includes a quaternary constant flow pump, auto injector, and UV detector was used for the analysis of NTG and its degraded samples. The data was processed on LC Solution Version 1.22 SP1 software. A reverse The chromatographic separations were performed on a phase C18 column (250 mm 4.6 mm i.d., 5m) (XTerra®, Waters, Miliford, USA). Transonic sonicator bath (570/H ELMA, Germany) was used to degass the mobile phase. LTQ-XL ion trap quadrupole mass spectrometer was used for the +ESI-MSn experiments (Thermo Electron Corporation, San Jose, USA). The LC-MS-QTOF experiments were performed on a micrOTOF-Q mass spectrometer (Bruker Daltonics GmbH, Germany) using microTOF control software version 2.0 in positive mode of electrospray ionisation (+ESI). Agilent 1100 series LC system (Agilent Technologies Inc., CA, USA) and Hystar (Ver. 3.1) software were used to control the LC components.

Preparation of Solutions and Reagents

0.1, 1 and 2 M (approximately) solutions of NaOH and HCl were prepared in HPLC grade water. The diluent was a 40 percent (v/v) DMSO solution in water. The diluent was used to make a stock solution of NTG (1 mg ml-1).

Forced Degradation Study

In separate volumetric flasks, around 0.1 g of NTG was combined with 100 ml of water, 0.1 M HCl, and NaOH solutions for hydrolytic breakdown. For 8 hours, each flask was held in a high-precision water bath at 85 °C. For oxidative degradation, approximately 0.1 g of NTG was dispersed in 100 ml of 30% H2O2 and stored at room temperature for 24 hours in the dark. Solid NTG in amber colour vials was thermally degraded for 30 days at 50 degrees Celsius. 2 ml of a 0.1 percent w/v solution of NTG in acetonitrile was combined with 3 ml of each stressor separately (i.e., 0.1 M HCl, 0.1 M NaOH, and H2O) in transparent glass vials for photolytic destruction. These vials, as well as the solid medication, were exposed to light in the photostability chamber as a thin layer in a petri-dish. The samples were put 9" away from the light source for 14 days, exposing them to around 200 Wh m-2 of UV and 1.2 million lux h of white light, respectively. To serve as a dark control, a separate set of solid drugs and drug solutions were stored in the dark for the same amount of time under similar conditions. Until the degraded samples were analysed, they were kept in the refrigerator.

Methodology of HPLC and Sample Preparation

On a reverse phase C18 column (250 mm X 4.6 mm i.d., 5m) held at ambient temperature (30 °C), the NTG and its degradation products were resolved optimally. The mobile phase was 70% (v/v) acetonitrile and 30% (v/v) ammonium acetate buffer, 20 mM (pH 4.0 adjusted with glacial acetic acid) was used to achieve the optimum separation. The 20 µl of the samples were injected on to the HPLC system through the auto injector and detection wavelength was fixed at 220 nm. Prior to analysis 10 times dilution of each degraded drug solution was done with diluent. Before diluting the hydrolyzed acid and alkali solutions, they were neutralised. Prior to dilution, solid drug samples were transformed into solutions (1 mg ml-1) in DMSO after being subjected to heat and photolytic conditions. Before analysis, each diluted sample was filtered through 0.45 nylon memberane.

Experimental study of +ESI-MS^n and LC-MS-QTOF

The LC-MS-QTOF investigation used the same chromatographic conditions as the HPLC approach. The MSn and LC-MS-QTOF experiments were performed in +ESI mode. Dry heat temperature of 200 oC, capillary voltage of 4.5 KV, end plate offset voltage of -500 V, collision cell RF of 400.0 vpp, and nebulizer set at 1.2 bar were the working parameters for mass spectrometric detection. 6.2 L min-1 was selected as the dry gas flow rate. The data in the LC-MS investigation was obtained at 10 and 15 V ionisation potential, whereas the spectra in the MSn study were recorded at 18.0 to 22.0 V ionisation potential. Each analyte peak found in the total ion chromatogram (TIC) in the LC-MS study had its mass scans recorded in the m/z 50-1000 range.

Results

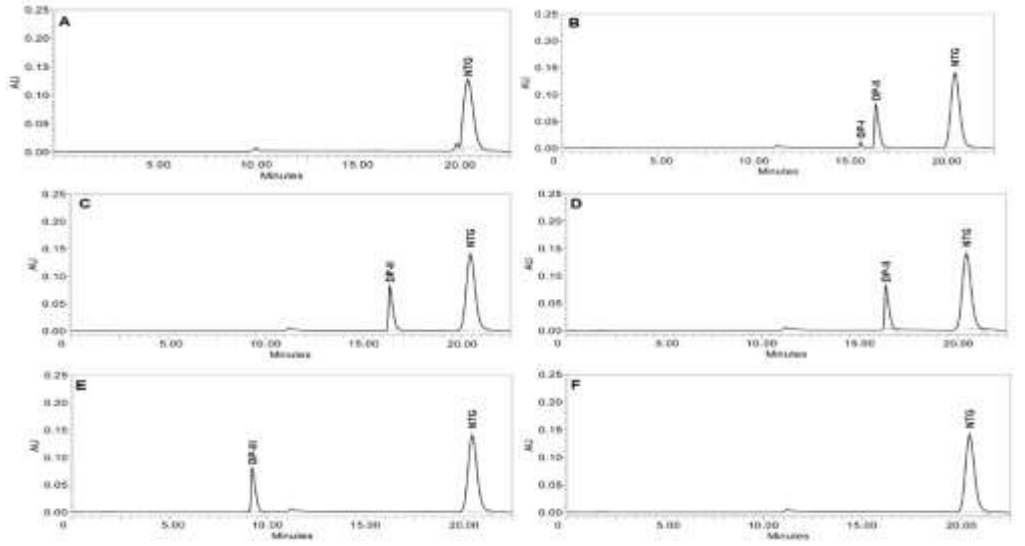


Fig. 2: Chromatograms of standard-solution of nateglinide (NTG) (A) and of drug-solutions subjected to 1.0 M NaOH (B), 1.0 M HCl (C), neutral conditions (D), oxidation (E) and thermal degradation (F)

Mass fragmentation pattern of NTG

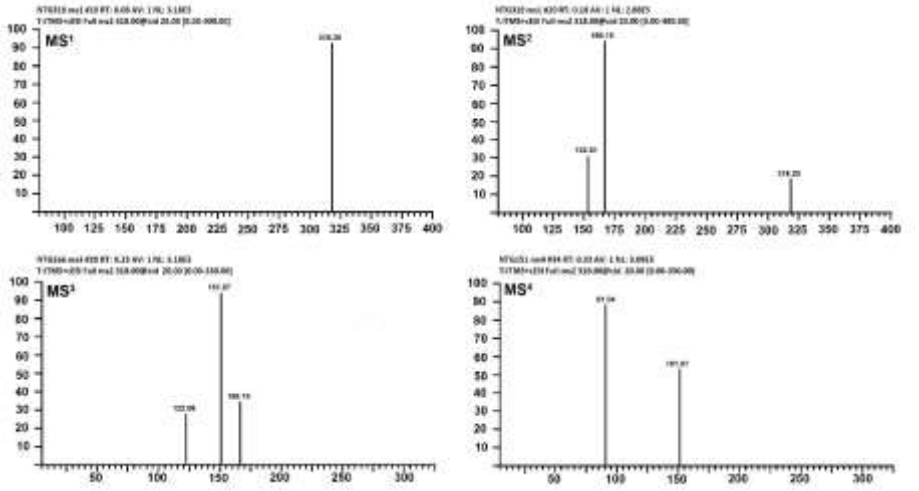


Fig. 3: Four stage mass fragmentation spectra of NTG

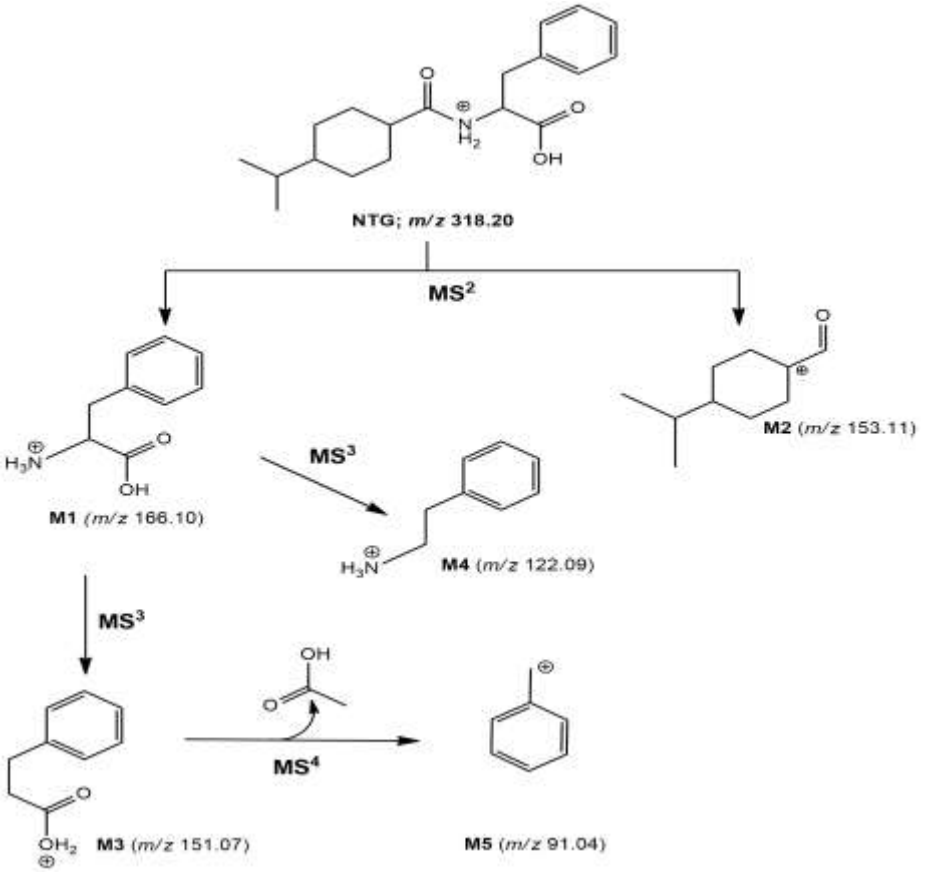


Fig. 4: Postulated pattern of mass fragmentation of NTG

Characterization of degradation impurities

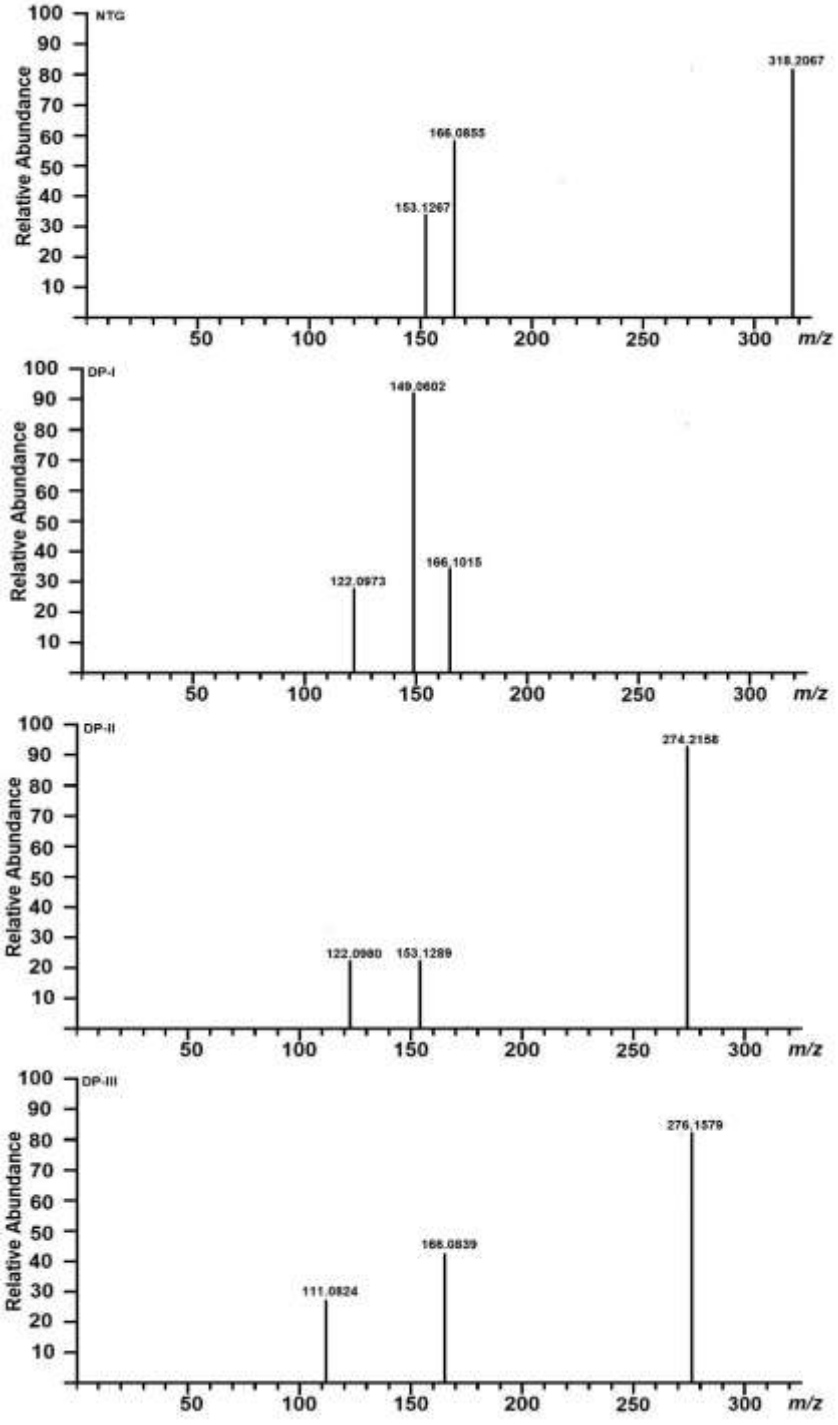


Fig. 5: LC-MS-QTOF spectra of NTG and its degradation impurities

Table 1: Molecular formulae corresponding to the measured mass of varied peaks in MS-QTOF spectra of NTG and degradation products

Analyte peak	Observed mass (Da)	Mass difference	Most probable composition (Theoretical mass; Tolerance)* For the mass difference	For the Observed mass
NTG	318.2067 [M+H$^+$]			$C_{19}H_{28}NO_3^+$ (318.2069; -0.22)
	166.0855	152.1212	$C_{10}H_{16}O$ (152.1201; 1.09)	$C_9H_{12}NO_2$ (166.0868; -1.30)
	153.1267	165.0800	$C_9H_{11}NO_2$ (165.0790; 1.02)	$C_{10}H_{17}O$ (153.1279; -1.24)
I	166.1065 [M+H$^+$]			$C_9H_{12}NO_2$ (166.1101; -3.54)
	149.0602	17.0463	NH_3 (17.0283; 7.75)	$C_9H_9O_2^+$ (149.0597; 0.5)
	122.1059	44.0006	CO_2 (43.9898; 9.75)	$C_8H_{12}N^+$ (122.0964; 7.6)
II	274.2158 [M+H$^+$]			$C_{18}H_{28}NO^+$ (274.2165; -0.7)
	153.1289	121.0902	$C_8H_{11}N$ (121.0891; 1.0)	$C_{10}H_{17}O^+$ (153.1274; -1.0)
	122.0980	152.1180	$C_{10}H_{16}O$ (152.1201; -2.1)	$C_8H_{12}N^+$ (122.0964; 1.6)
III	276.1579			$C_{16}H_{22}NO_3^+$ (276.1594; -1.5)
	166.0839	110.0695	$C_7H_{10}O$ (110.0740; -3.7)	$C_9H_{12}NO_2^+$ (166.0839; -2.3)
	111.0824	165.0804	$C_9H_{11}NO_2$ (165.0790; 1.4)	$C_7H_{11}O^+$ (111.0804; 2.0)

DP I and DP II

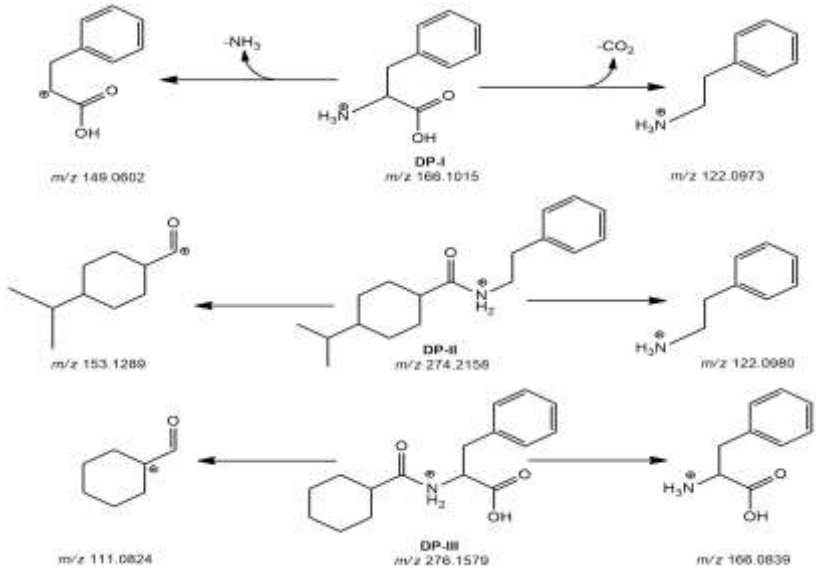


Fig. 6: Proposed mass fragmentation pattern of the degradation impurities.

Drug degradation mechanisms of NTG

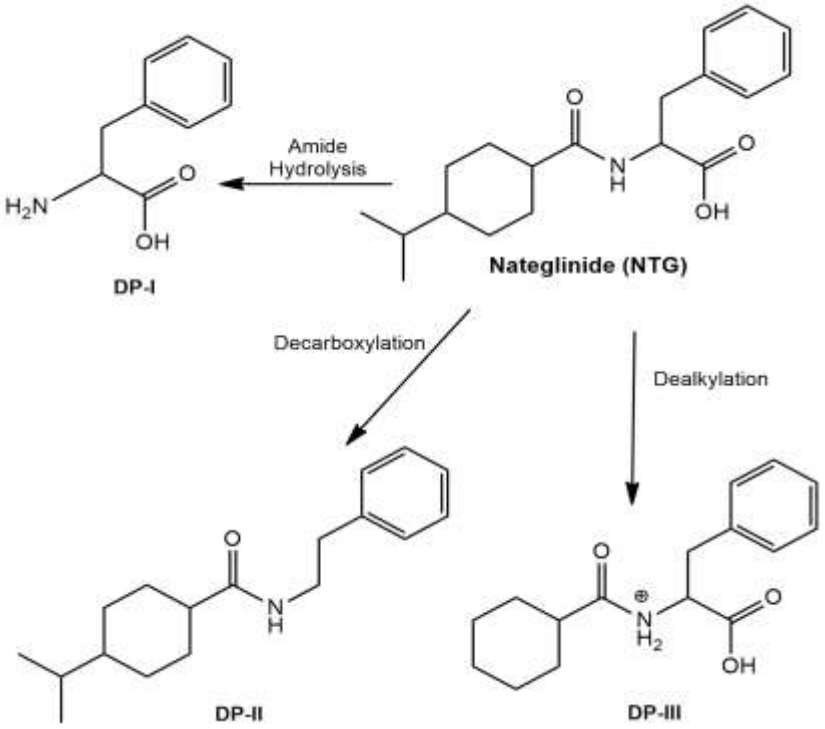


Fig. 7: Mechanisms of NTG degradation.

Discussion

Forced degradation behavior

HPLC methods for the quantification of NTG in the literature were evaluated, and chromatographic conditions reported by Pathare et al. [6] were used to design HPLC method for improved separation of NTG and its degradation products. The chromatographic settings were changed as needed, and the best resolution of NTG and all degradation products was obtained using the chromatographic conditions described in section 2.5. In the LC-UV chromatogram of the NTG standard solution, no impurities were found (Fig. 2A). The medicine degraded to two degradation products (I-II) in 1.0 M NaOH at 80 °C for 8 h (Fig. 2B). The drug degraded to two degradation products (I-II) in 1.0 M NaOH at 80 °C for 8 h (Fig. 2B). The degradation product II was also noted as the single major impurity in 1.0 M HCl (Fig. 2C) and neutral conditions (Fig. 2D) 8 hours at 80°C. Under oxidatively driven circumstances, one degradation product (III) was also discovered (Fig. 2E). These findings indicated that NTG was resistant to photolysis as well as thermal deterioration. As a result of these findings, NTG was discovered to be exclusively susceptible to acidic, alkaline hydrolysis, and oxidative stress conditions.

Mass fragmentation pattern of NTG

There are a few papers in the literature that use mass spectrometry to analyse NTG quantitatively [11], but none of them reveal or explain the mass fragmentation pattern of NTG. As a result, four stage mass spectra (MS4) of NTG were acquired in the current investigation (Fig. 3) to characterize its mass fragmentation pattern (Fig. 4), which aided in the characterization of its degradation products. In the MS1 spectrum (Fig. 2), NTG was discovered as parent ion [M+H+] at m/z 318.20, which corresponds to its molecular mass of 317 Da. In the MS2 spectrum, further fragmentation of NTG yielded fragments of m/z 166 and 153. (Fig. 2). The loss of cyclohexyl portion, corresponding to the other fragment in the MS2 (m/z 153) of NTG, was proposed to create the m/z 166 fragment, as shown by the mass difference of 152 Da between M-1 and the heaviest fragment in the MS2 spectrum. For DP-I, the m/z 166.1006 fragment was also found. The fragment m/z 166 was utilised as a precursor ion to record the MS3 spectrum, which revealed m/z 151 and 122 as product ions, based on the amount of fragment ions in the MS2 spectrum. The loss of an amine molecule might produce the m/z 151, which is 15 Da less than the precursor ion, whereas decarboxylation of the precursor ion could provide the m/z 122.

Characterization of degradation impurities

DP I

It was recognized as parent [$M+H^+$] ion at m/z 166.1015 with two other fragments at m/z 149.0702 and 122.0973 (Fig. 5). The [$M+H^+$] ion peak was also detected in the LC-MS and MS^n spectrum of NTG. So, on this basis, DP-I was suspected to be M_1 observed in the MS^n spectrum. The LC MS-QTOF spectrum of **I** revealed two fragments at m/z 149.0702 and m/z 122.0973 formed by the loss of 17.0463 and 44.0006 Da from [$M+H^+$] ion. Loss of these same masses from [$M+H^+$] ion was proposed to form M3 and M4, respectively in LC-MS as well as MS^n studies (Fig. 4 and 6). On the basis of this similarity in mass-fragmentation, fragments of m/z 149.0702 and m/z 122.0973 were proposed to form from M1 as shown in Fig. 4. Hence, **I** was categorised as 2-amino-3-phenylpropanoic acid and was possible to form due to hydrolysis of the amide linkage in NTG [2].

DP II

Its LC-MS-QTOF-spectrum revealed the most common as well as, bulkiest ion at *m/z* 274.2158 (Fig 5). Based on the mass difference of *m/z* 43.9909 between the two, the *m/z* 274.2158 NTG molecular ion peak (*m/z* 318.2067) the DP-II was suspected to be NTG without carboxylic acid. Two fragments at *m/z* 153.1289 and *m/z* 122.0980 detected in-case of LC-MS spectrum of DP-II. The fragment-component of *m/z* 153.1289 has also been observed as M2 in the MS^2 spectrum of NTG and have been proposed to form by breakage of amide linkage and loss of 2-phenylethanamine from DP-II (Fig. 4). Another fragment *i.e. m/z* 122.0980 was hypothesised as the result of the loss of 4-iso-propylcyclohexane carbaldehyde the other part of DP-II. On the basis of this assessment, DP- II was postulated as NTG without carboxylic acid part *i.e.* 4-isopropyl-N-phenethylcyclohexanecarboxamide (Fig. 1) which fragmented as shown in Fig. 6.

DP III

[M+H$^+$] ion was proposed at *m/z* 276.1579 in addition to, 2 fragments on *m/z* 166.0839 and *m/z* 111.0824 (Fig. 5). The component of *m/z* 166.0839 has also been seen as the molecular ion peak for DP-I also. The formation of this fragment indicated that the phenyl part of NTG is remained intact in DP-III and changes had been happened in the cyclohexyl part. This is further supported by the mass difference in the fragment formed in from DP-II and *m/z* 111.0824 is exactly the molecular weight of side propane chain. On the basis of this the DP-III was proposed to be NTG without propane side chain *i.e.* 2-(cyclohexanecarboxamido)-3-phenylpropanoic acid (Fig. 6).

Pathways of Drug Deterioration

Fig 7 depicts the most likely route for the generation of degrading impurities from NTG. The DPI possibly is formed via well-known hydrolysis of the amide linkage under acidic, alkaline and neutral medium. The DP II was possible to form from NTG by decarboxylation. The DP III characterized as propane less NTG, has been postulated to generate oxidative dealkylation of tertiary carbon.

Conclusions

NTG was subjected to ICH-mandated forced degradation tests, and it was discovered to be vulnerable towards acid, alkaline and neutral hydrolysis and oxidative conditions where three DPs were formed in total. An HPLC method was developed to separate DPs and NTG and validated for different validation parameters. LC-MS-QTOF tests were used to characterise all of the degrading impurities. The three degradation products were characterized as 2-amino-3-phenylpropanoic acid (DP I), 4-isopropyl-N-phenethylcyclohexanecarboxamide (DP II) and 2-(cyclohexanecarboxamido)-3-phenylpropanoic acid (DP III).

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