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Research Article

# Characterization of Degradation Products of Guanfacine Hydrochloride API: Development and Validation of a Stability-indicating Reversed Phase UHPLC Method

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ABSTRACT

Guanfacine hydrochloride API was subjected to forced degradation studies under various conditions of hydrolysis (acidic, alkaline, and neutral/water), oxidation, photolysis, and thermal. A short, simple, reverse phase UHPLC method was developed on a Shimadzu, Shimpack GIST, C18, (100 × 2.1) mm, 3.0 µm column. The gradient method consisted of 0.1% orthophosphoric acid as mobile phase A and acetonitrile as mobile phase B. The flow rate of the mobile phase was 0.3 mL/min. The method was validated using ICH guidelines considering the parameters solution stability, specificity, DL/QL, linearity, accuracy, precision and robustness. The drug was found highly sensitive to alkaline conditions and showed significant degradation. The drug was found sensitive to acidic degradation conditions. Slight degradation was observed in oxidative and water conditions. The drug was found to be stable in thermal and photolytic conditions. The mass compatible UHPLC method was prepared by simply substituting the orthophosphoric acid with formic acid in the mobile phase. Characterization of two major degradation products (DPs) was done. DP1 was characterized with LC–Q-TOF-MS/MS in combination with accurate mass measurements. DP2 was isolated and characterized with NMR, IR and HRMS spectroscopic techniques. The mechanisms of the formation of DPs were proposed.

## Introduction

Guanfacine hydrochloride (GFN) is an α-2-adrenergic ($\alpha_{2A}$) agonist and has been used to treat hypertension over the past 30 years. Its immediate-release formulation (Tenex™, Promius Pharma, LLC, NJ, USA) was approved by the US FDA in 1986.[1] GFN stimulates the $\alpha_{2A}$ receptors in the brain stem and reduces sympathetic nerve impulses from the vasomotor center to the heart and blood vessels. This decreases the peripheral vascular resistance and results in reduction of heart rate.[2] Besides hypertension, GFN is an effective drug for treating children with attention deficit hyperactivity disorder (ADHD) in children and adolescents aged 6 to 17 years. Its extended-release formulation (Intuniv®, Shire LLC, KY, USA) was approved in US in 2009 by US FDA, in Europe in 2015 by EMA and was approved for the treatment of pediatric ADHD in Japan in 2017 by PMDA.[3] The drug acts on the $\alpha_{2A}$ receptors located in the brain's prefrontal cortex, which controls the behavioural inhibition, attention regulation, distractibility, impulsivity and frustration tolerance.[1,4] ADHD affects approximately 8.7% of children between the ages of 6 and 17 years; therefore, the availability and use of guanfacine has increased.[5]

GFN is a derivative of the nucleic acid guanidine. Chemically it is described as *N*-Amidino-2(2,6-dichlorophenyl)

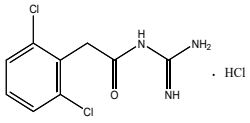


**Fig. 1:** Chemical structure of GFN.

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acetamide monohydrochloride. The chemical structure of GFN is depicted in Fig. 1.

Its molecular formula is $C_9H_9Cl_2N_3O.HCl$ and molecular weight is 282.55. The CAS No. of GFN is [29110-48-3]. Its description is white to off-white powder. It is sparingly soluble in water, methanol and ethanol. It is slightly soluble in acetone.[6] The solubility of GFN is 0.420 mg/mL in 0.1 N HCl, 1.265 mg/mL in acetate buffer (pH 4.5), and 1.302 mg/mL in phosphate buffer (pH 6.8).[7] Its melting point is 225 to 227°C. GFN has low solubility and high permeability and has been placed in the BCS Class II category.[8] It has a partition coefficient of 0.10 and a dissociation constant of 7.69.[9]

The information about the chemical stability of a drug is very important as it affects both safety and efficacy. The stability studies generate evidence about the consistency in drug quality with time under the influence of various environmental factors such as temperature, humidity, and light.[10] A stability indicating analytical method must be capable of measuring the active ingredients in the presence of degradation products (DPs), process impurities, excipients, or other potential impurities.[11] It must be able to detect the relevant changes in quality attributes of drug substance or drug product during storage. ICH has recommended the forced degradation studies to identify the DPs, predict the molecule's intrinsic stability and establish degradation pathways, and validate the stability indicating procedures used.[9,12-14] The process and degradation impurities present in drugs should be identified. The identification threshold depends on maximum daily dose and the potency of impurities.[15] The spectroscopic techniques are extensively utilized for the characterization of DPs formed under various forced degradation conditions.[16-21]

Various analytical and bioanalytical methods have been developed for the determination of GFN. The initial methods were based on the derivatization of GFN molecule and subsequent analysis by electron capture GLC[22] and UV techniques.[23-24] The literature includes the LCMS method for the determination of GFN in urine,[25] LC-MS/MS methods in blood,[26-27] LC-MS/MS methods in plasma,[28] and LC-MS/MS methods in rat plasma.[29] The liquid chromatographic methods include the UPLC method for the estimation of GFN in tablets[30] and a stability indicating isocratic HPLC-UV method for the bulk drug and tablets.[31] In this reported stability indicating method, the peak of major degradant eluted at the void volume and there was no description about the degradation impurities. Despite the presence of GFN in the generic market for long period of time, inadequate information exists in the literature on the identification and characterization of its DPs. The drug is listed in the USP but the organic impurities (procedure 1 and 2) methods mentioned in the monograph are non-specific thin layer chromatography (TLC) methods.[6] Mobile phase of the assay by HPLC method involves the phosphate buffer and triethylamine (5mL/litre). USP is modernizing the GFN monograph and the recent proposal published in Pharmacopeial Forum (PF 48, Vol. 1, Jan. 2022) contains a specific HPLC-UV method for the determination of organic impurities.[32] The run time of the method is 65 minutes. The proposal contains 5 impurities however these have not been classified as DPs. Therefore, it becomes essential to know about complete degradation profile of the drug under stress conditions.

The present work describes the development and validation of a shorter and simpler stability indicating UHPLC method for the GFN API. Forced degradation studies were performed under various conditions of hydrolysis (acidic, alkaline, and neutral/water), oxidation, photolysis, and thermal. The MS compatible UHPLC method was prepared by simply substituting ortho phosphoric acid with the formic acid in the mobile phase. Two major degradation products (DPs) formed during forced degradation were characterized using spectroscopic techniques. The probable mechanisms for the formation of DPs have been proposed.

## Materials and Methods

### Chemicals and Reagents

GFN API was obtained from M/S Agnitio Pharma as a gift sample. Chromatographic purity of the sample was greater than 99%. Acetonitrile, orthophosphoric acid, formic acid, AR grade NaOH and 30% $H_2O_2$ solution were procured from M/S Merck. AR grade HCl and HPLC grade water were procured from M/S Rankem.

### Instruments

The chromatographic analysis was performed on a Shimadzu, N-Series-XS UHPLC system (Shimadzu Corporation, Kyoto, Japan). It consisted of sample manager, solvent manager with PDA detector. The software employed to run the experiments and data processing was LabSolutions Version 6.110. Water bath was employed for performing degradation experiments. Photo stability experiments were performed using M/S Thermolab's photostability chamber. Thermal degradation stability experiments were employed in dry hot air oven. The LC-MS studies were performed on Shimadzu Nexera X3 UHPLC system attached to a quadrupole time-of-flight (Q-TOF) mass spectrometer (LCMS-9030) and Waters ACQUITY UPLC H-class system attached to SYNAPT XS HDMS quadrupole time-of-flight (Q-ToF) mass spectrometer. In Shimadzu LCMS-9030, heated ESI interface was used for the study with following conditions: interface temperature: 300°C; desolvation line temperature: 250°C; heat block temperature: 400°C; heating gas flow: 10 L/min; nebulizing gas flow: 3 L/min; drying gas flow: 10 L/min. The collision energy spread for MS/MS was 18–52 V. Mass data was acquired using LabSolutions software.

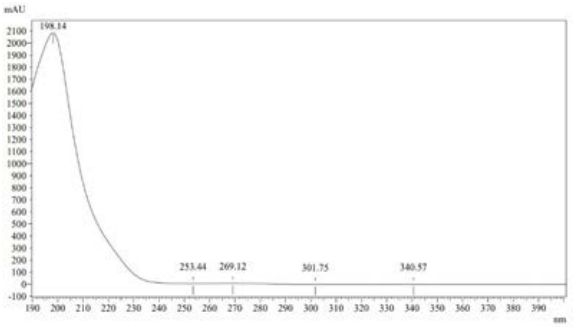


**Fig. 2:** UV-spectrum of Guanfacine hydrochloride

In Waters SYNAPT XS HDMS, the conditions were: Ionisation mode: ESI; desolvation gas: 950 L/Hr; cone gas: 50 L/Hr; desolvation temperature: 550°C; source temperature: 120°C; capillary voltage: 3.22 keV; cone voltage: 50V; collision energy: 4 eV. The NMR data was recorded on Avance-II Bruker 500 MHz (Bruker, Fallanden, Switzerland) instrument. The infrared spectroscopy data were recorded on a PerkinElmer Spectrum Two FTIR spectrophotometer (PerkinElmer Inc. Massachusetts, United States).

## Forced Degradation (FD) Studies

FD studies were performed according to the regulatory guideline, ICH Q1A (R2).[10] The purpose of FD studies was to establish the specificity and the stability indicating nature of the method. All solutions for force degradation studies were prepared at initial concentration of 0.50 mg/mL. Solution state stressed conditions included acid hydrolysis (1-mL of 1 N HCl, 80°C, 3 hours), alkaline hydrolysis (1-mL of 1 N NaOH, 80°C, 2 hours), water degradation (5 mL of water, room temperature, 5 hours) and oxidative degradation (1-mL of 30% $H_2O_2$, room temperature, 5 hours). For all solution state degradation, 1-mL of diluent was also added along with degradation reagent solutions, to solubilize the samples. Acidic and alkaline degradation samples were neutralized with NaOH and HCl before diluting with diluent. Solid state degradation involved photolytic stress (1.2 million lux h visible light and 200 wh/square $m^2$ UV light) and heat stress (80°, 24 hours).

## UHPLC Method Development

The solubility of GFN was studied and a mixture of water and acetonitrile in the ratio of 30:70 v/v was selected as diluent. The UV absorption spectra (refer Fig. 2) of GFN was generated with the help of PDA detector.

Based on UV spectrum, a wavelength of 210 nm was selected for further studies. The UHPLC column used in the study was Shimadzu, Shimpack GIST, C18, (100 x 2.1) mm, 3.0 µm. GFN molecule contains the basic amine functional group and its pKa value 7.69.[9] Therefore, to avoid the peak tailing due to silanol effect, method development trials were started with acidic pH. A solution of 0.1% orthophosphoric acid in water was considered as buffer solution and acetonitrile as organic component. Base degradation sample was considered for the method development purpose. Trials were taken with different flow rates and different composition of the gradient to obtain good peak shapes, adequate resolution and retention times for the GFN peak and major degradation products (DP1 and DP2). Sample concentration was also optimized to achieve the adequate DL/QL values.

Optimum conditions were achieved using the gradient program set as follows: $T_{time}$/mobile phase-A: B (%): $T_0$/90:10, $T_5$/90:10; $T_{15}$/50:50; $T_{18}$/;50:50 $T_{18.01}$/90:10; $T_{24}$/90:10 with a flow rate of 0.5 mL/min. Injection volume used was 5 µL and kept the column temperature and sample cooler temperature at 25°C. A concentration of 500 µg/mL was finalized for sample solution. The standard solution was prepared at a concentration of 0.1% with respect to sample solution *i.e.,* 0.5 µg/mL. A sensitivity solution of 0.25 µg/mL concentration was prepared.

## Development of MS Compatible UHPLC Method

The MS compatible method was prepared by simply substituting the non-volatile orthophosphoric acid with volatile formic acid. The remaining chromatographic conditions were kept same. The peak elution pattern obtained in the MS compatible method matched with the peak elution pattern obtained in the stability indicating method for the different degradation samples (acid, alkaline and oxidative).

## Method Validation

Analytical method for the estimation of degradation products and other related impurities was validated to demonstrate that the method is suitable for its intended purpose. Method was validated as per ICH guideline Q2 (R1).[13]

### *System Suitability*

To ensure the proper functioning of chromatographic system during routine analysis, one parameter each from detector, injector and column was selected for assessing the performance. A signal to noise (s/n) ratio criteria of more than 10 for GFN peak from sensitivity solution was considered. The % RSD of area of standard solution from six injections of standard solutions should be less than 5.0%. The average tailing factor of GFN peak from six injections of standard solutions should be less than 2.0.

### *Solution Stability*

The stability of sample solution of Guanfacine was evaluated by injecting it at different intervals 0, 12, 18 and 24 hours.

### *Detection Limit and Quantitation Limit*

The detection limit (DL) and quantitation limit (QL) were established based on visual evaluation as well as s/n ratio method. A series of samples of different concentrations (0.05, 0.1, 0.15, 0.2, 0.25 µg/mL or 0.01, 0.02, 0.03, 0.04,

0.05% with respect to test concentration of 0.5 mg/mL) were prepared and injected. As per visual evaluation approach given in ICH Q2 (R1) guideline,[13] the signal should be detected reliably at DL level and as per s/n ratio criteria, the typical s/n value given is 3 or 2. The signal should be quantifiable at QL level with acceptable accuracy and precision, and the typical s/n value given is 10. To verify the precision at QL level, six replicates of QL solution were injected and the value of %RSD is calculated.

### *Linearity*

To verify the linearity of the method, a series of Guanfacine concentrations were prepared from QL level to 150% of specification level. The concentrations of these solutions were 0.15, 0.375, 0.5, 0.625 and 0.75 ug/mL (or 0.03, 0.05, 0.10, 0.125 and 0.15% with respect to the sample concentration of 0.5 mg/mL. Concentration (%) was plotted against the average Guanfacine peak area and the slope, intercept and correlation coefficient values were calculated.

### *Specificity*

To demonstrate the specificity of the method, retention time of Guanfacine and major degradation products in the chromatograms of sample solutions were compared with the blank chromatogram.

### *Accuracy*

Accuracy of the method was evaluated at three levels (QL, 100 and 150%). Test solutions of GFN were prepared in triplicate at 0.03, 0.10 and 0.15% levels. An injected single injection of all nine preparations. Calculated the amount added, amount recovered and %recovery at each level. Also, the %RSD at each recovery level and cumulative % RSD of %recovery were calculated.

### *Precision*

Six replicates of standard solution were injected to evaluate the system precision, and the %RSD of retention time and area of Guanfacine peak was calculated. The average tailing factor was also calculated. Six different preparations of sample solution were made and injected to demonstrate the repeatability of the method.

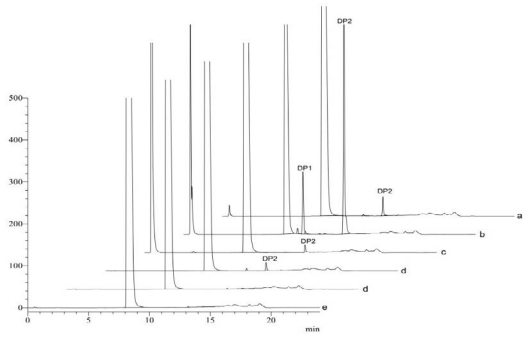


**Fig. 3:** Forced degradation chromatograms: (a) Acid hydrolysis; (b) alkaline hydrolysis; (c) Oxidative degradation; (d) water degradation; (e) Thermal degradation; (f) Photolytic degradation.

### *Robustness*

To evaluate the robustness of the method, deliberate variations were made in few chromatographic parameters and system suitability criteria were evaluated. Also, the base degradation sample was injected in each robustness condition to check any impact on degradation products also. The parameters considered in the study were flow rate (± 10%), column temperature (± 10°C) and mobile phase composition (± 10% w.r.t organic part).

## Results and Discussion

### FD Studies

From the forced degradation studies, GFN molecule was considered highly sensitive to the alkaline degradation. The degradation product, denoted as DP1, was formed about 2.23%. The major degradation product, denoted as DP2 was formed about 11.11%. GFN molecule was found sensitive to the acidic degradation. The major degradation product *i.e.,* DP2 was formed about 1.06%. Slight degradation was observed in oxidative and water degradation conditions and major degradation product i.e., DP2 was formed about 0.36 and 0.42%, respectively. No degradation was observed in thermal and photolytic conditions (refer Fig. 3).

Force degradation studies demonstrated that the method of analysis is specific, as no interference was observed with GFN peak as well as DP1 and DP2 peaks. The peaks of GFN, DP1 and DP2 were regarded as pure, as the minimum peak purity index values obtained from the LabSolutions software were ≥ 0. Hence, the method is considered as stability indicating.

### LC–ESI-Q-TOF-MS/MS Study of GFN and its DPs

The LC–ESI-MS/MS analysis of GFN, DP1 and DP2 were performed. The m/z values of different ions and their MS/MS data were obtained. Based on the accurate mass measurements, structures were assigned and fragmentation pathways were proposed for the m/z values. The mass accuracy values were less than 5 ppm (refer Table 1) which satisfied the acceptance criteria for the conformation of identity.[33]

### MS/MS of GFN

The fragmentation pathway of protonated GFN was established using LC–ESI-MS/MS combined with accurate mass measurements. The ESI-MS/MS spectrum (refer Fig. 4) showed the product ions at m/z 203.9984 (loss of $CH_2N_2$ from m/z 246.0185) and m/z 158.9769 (loss of CO and $NH_3$ molecules from m/z 203.9984).

The peak at m/z 158.9769 was observed as base peak which was explained due to the formation of stable dichlorinated tropylium ion (refer Fig. 5).

**Table 1:** Mass accuracy data of the GFN, DP1 and product ions

| *Drug/DPs* | *Elemental composition [M+H]⁺* | *Observed (m/z)* | *Theoretical (m/z)* | *Mass accuracy (ppm)* |
|---|---|---|---|---|
| GFN | $C_9H_{10}Cl_2N_3O^+$ | 246.0185 | 246.0195 | 4.06 |
| Product ion of GFN | $C_8H_8Cl_2NO_+$ | 203.9984 | 203.9977 | -3.43 |
| Product ion of GFN | $C_7H_5Cl_2^+$ | 158.9769 | 158.9763 | -3.77 |
| DP1 | $C_8H_8Cl_2NO^+$ | 203.9973 | 203.9977 | 1.96 |
| Product ion of DP1 | $C_7H_5Cl_2^+$ | 158.9761 | 158.9763 | 1.26 |
| Product ion of DP1 | $C_7H_4Cl^+$ | 122.9992 | 122.9996 | 3.25 |
| Product ion of DP1 | $C_7H_5^+$ | 89.0382 | 89.0386 | 4.49 |

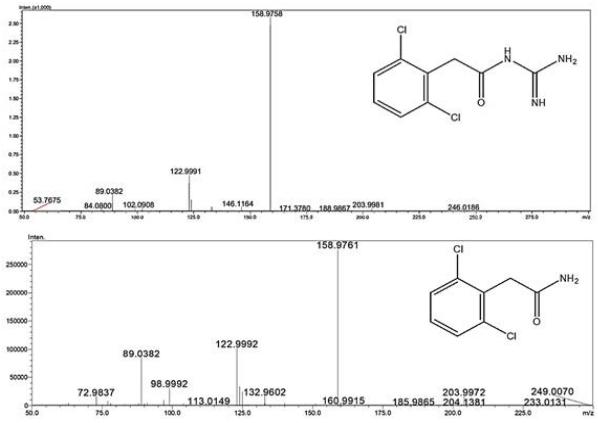


**Fig. 4:** LC-ESI-MS/MS spectrum of GFN (top) and DP1 (bottom).

## LC–ESI-Q-TOF-MS/MS Study of DP1

The LC-ESI-MS and LC-ESI-MS/MS spectrum of DP1 were recorded in positive mode. The MS/MS spectrum protonated DP1 (m/z 203.9973, Rt = 13.18 min.) showed the product ions at m/z 158.9761, 122.9992 and 89.0382 (refer Fig. 4). Similar to GFN, the dichlorinated tropylium ion peak (m/z 158.9761) appeared as base peak in both LC-ESI-MS and LC-ESI-MS/MS data of DP1. The proposed fragmentation pathway for protonated DP1 is demonstrated in Fig. 5. Based on the characteristic fragment ions in combination with accurate mass data, the structure 2-(2,6-dichlorophenyl)acetamide was assigned for DP1.

## LC–ESI-Q-TOF-MS/MS Study of DP2

The LC–ESI-MS and LC-ESI-MS/MS analysis of DP2 were performed in positive mode. In both the spectrum, DP2 showed base peak corresponding to stable dichlorinated tropylium ion (m/z 158.9763). To further explore, the LC–ESI-MS and LC-ESI-MS/MS analysis of DP2 was performed in negative mode. The base peak corresponding to dichlorinated benzyl anion (m/z 158.9774) was observed in both the spectrum in negative mode. This indicated that the concerned degradation product is quite labile and easily prone to fragmentation in MS conditions and the cleaved fragment could be very stable. The structure of DP2 could not be assigned based on MS analysis only. Hence, it was decided to enrich the DP2 by using the harsher alkaline degradation conditions and isolate it for further characterization.

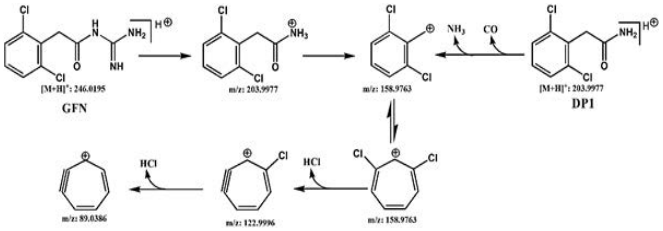


**Fig. 5:** Proposed fragmentation pathways of GFN and DP1.

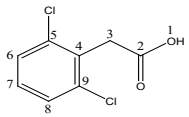

**Fig. 6:** Chemical structure of DP2.

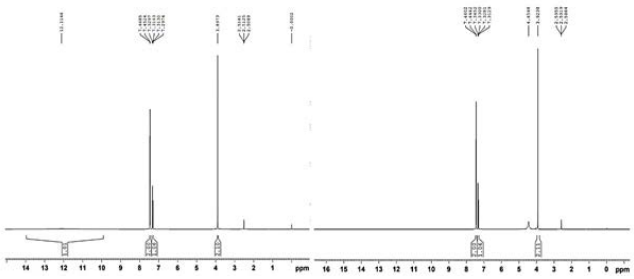

**Fig. 7:** $^1$H-NMR spectrum of DP2 (left) and $^1$H-NMR spectrum of DP2 (right) after $D_2O$ shake, solvent DMSO-d6.

## Enrichment and Isolation of DP2

A total of 5 g of GFN API sample was taken and 100 mL of 2N NaOH solution was added. Raised the temperature of the content and heated under reflux conditions for 8 hours. Cooled and adjusted the pH of the solution to 2.0 with 5N HCl solution. Filtered the solution. Washed the residue with 50 mL of 5N HCl solution and dried at 80°C under vacuum for 5 hours.

## Characterization of DP2 by NMR, IR and HRMS

The $^1$H-NMR and $^{13}$C-NMR spectrum were recorded in DMSO-d6 solvent and tetramethylsilane (TMS) as internal standard. The $^1$H chemical shift values were reported on δ

**Table 2:** Interpretation of NMR and IR data of DP2

| *Position* | *δH (J/Hz)* | *δC (J/Hz)* | *IR absorption bands ($cm^{-1}$)* |
|---|---|---|---|
| 1 | 12.11 (1H, s) | - | 3005 (aromatic C-H stretch) |
| 2 | - | 173.14 | 2932 (aliphatic C-H stretch) |
| 3 | 3.89 (2H, s) | 39.42 | 1712 (C=O stretch) |
| 4 | - | 134.52 | 1244 (C-O stretch) |
| 5, 9 | - | 138.22 | 933 (aromatic C-H bend) |
| 6, 8 | 7.45 (2H, d, 8.05 Hz) | 131.05 | 778 (C-Cl stretch) |
| 7 | 7.31 (1H, m) | 132.39 | |

**Table 3a:** Robustness-system suitability

| *Robustness condition* | *System suitability parameter* | | |
|---|---|---|---|
| | *s/n ratio* | *%RSD* | *Average tailing factor* |
| Ideal | 21.63 | 0.49 | 1.14 |
| Flow decrease | 21.34 | 1.18 | 1.00 |
| Flow increase | 24.88 | 1.95 | 0.95 |
| Column temp. decrease | 20.53 | 0.73 | 1.14 |
| Column temp. increase | 22.56 | 1.65 | 1.40 |
| Composition decrease | 22.13 | 1.46 | 1.04 |
| Composition increase | 21.42 | 1.59 | 0.92 |

**Table 3b:** Robustness-retention time and relative retention times

| *Robustness condition* | *RT of GFN (min.)* | *RRT w.r.t GFN* | |
|---|---|---|---|
| | | *DP1* | *DP2* |
| Ideal | 8.35 | 1.18 | 1.58 |
| Flow decrease | 8.82 | 1.16 | 1.54 |
| Flow increase | 8.10 | 1.17 | 1.58 |
| Column temp. decrease | 8.62 | 1.16 | 1.55 |
| Column temp. increase | 8.10 | 1.19 | 1.61 |
| Composition decrease | 9.28 | 1.13 | 1.48 |
| Composition increase | 7.28 | 1.23 | 1.70 |

scale in ppm, relative to TMS (δ = 0.0 ppm) and in the $^{13}C$ chemical shift values were reported relative to DMSO-d6 (δ = 39.4 ppm). The IR spectrum was recorded in the solid state as KBr dispersion over the range of 4000–400 $cm^{-1}$. The spectrum was acquired by accumulation of 42 scans with 4 $cm^{-1}$ resolution. Similar to the observations of LC–ESI-MS, the DP2 molecule was also found labile in direct mass analysis. In positive ion mode, the base peak was observed at the m/z value 158.9770 (corresponding to dichlorinated tropylium ion) and in negative ion mode at m/z value 158.9780 (corresponding to dichlorinated benzyl anion). The interpretation of NMR and IR data is presented in Table 2 and Fig. 6.

In $^{1}H$-NMR spectrum, beside the aromatic and benzylic protons, a broad peak was observed at 12.11 ppm which could be due to carboxylic proton. The 1H-NMR spectrum was recorded again after D2O shake to confirm the same. The carboxylic proton (H-1) disappeared from the spectrum (refer Fig. 7).

The presence of carboxylic group was further confirmed from the presence of carbonyl carbon (C-2) in $^{13}C$-NMR spectrum and a peak corresponding to C=O stretching in IR spectrum. Thus, from the spectroscopic data, the structure 2-(2,6-dichlorophenyl)acetic acid is assigned for the DP2. The peak elution pattern i.e., late elution of DP2 (containing acidic functional group) as compared to DP1 (containing amide functional group) in the acidic mobile phase also supports the proposed structure of DP2.

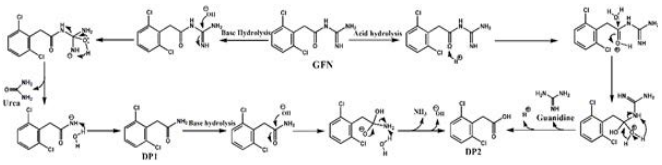


**Fig. 8:** Mechanism of formation of DP1 and DP2.

## Mechanisms of formation of DP1 and DP2

The mechanism of formation of DP1 under alkaline degradation conditions involves the loss of stable urea molecule. The DP1 was further hydrolyzed to DP2 *via* the loss of ammonia molecule. Under acidic degradation conditions, GFN molecule was hydrolyzed to DP2, generating the stable guanidine molecule (refer Fig. 8).

## Method Validation

### *System Suitability*

All the system suitability criteria were met successfully. The value of s/n ratio obtained for the GFN peak in sensitivity solution was 21.63, the value of % RSD of GFN peak area in standard solution was 0.49 and the value of average tailing factor of GFN peak in standard solution was 1.14.

### *Solution Stability*

No differences were observed in the levels of 'any other impurity' and % purity after 12, 18 and 24 hours. Hence the solution of GFN sample was considered stable for at least 24 hours at a temperature of 15°C.

### *Detection Limit and Quantitation Limit*

The signal of GFN was reliably detected and also the s/n ratio was more than 3 (12.24 and 13.26 for two injections) for the solution of concentration 0.05 µg/mL. Hence, the concentration of 0.05 µg/mL (or 0.01% w.r.t test concentration) was considered as detection limit. Also, the value of s/n ratio was greater than 10 (42.35 and 51.16 for two injections) for the solution of concentration 0.15 µg/mL. Hence, 0.15 µg/mL (or 0.03% w.r.t test concentration) was considered as quantitation limit. The %RSD value for six replicate injections of QL was 2.17 which was less than 10% acceptance criteria, hence the established QL was found to be precise.

### *Linearity*

The linearity plots were drawn by taking concentration on x-axis and peak area on y-axis. The linear regression equation was y = 269309x + 3801.9 with correlation coefficient 0.9993. It indicated that method has good linearity and obey Beer-Lamberts law.

### *Specificity*

There was no interference found in the blank at the retention time of GFN peak.

### *Accuracy*

The %recovery values observed were well within the acceptable range of 80 to 120%. The values of %RSD of %recoveries at QL, 100 and 150% levels were 1.70, 0.66 and 0.55, respectively. The cumulative %RSD of % recovery values was 2.43. As both the individual (at each recovery level) and cumulative (overall) %RSD of % recovery values were less than 10.0, the analytical method demonstrated acceptable accuracy at each level.

### *Precision*

The % RSD values of retention time and area of GFN peak in standard solution were 0.07 and 0.49, less than the acceptance criteria of 5.0%. The %RSD values of 'any other impurity' and '%Purity' were calculated from six different preparations of sample solution and were found (0 and 0.05, respectively) to be less than the acceptance criteria of less than 10%. Thus, both the system precision and repeatability were demonstrated.

### *Robustness*

The summary of system suitability data obtained in different robustness conditions and comparison of RT of GFN peak and RRT of DP1 and DP2 data is tabulated in Tables 3a-b. The data shows that the method is robust w.r.t changes in flow rate, column temperature and mobile phase composition.

## Conclusion

The intrinsic stability of GFN was determined using a specific validated stability indicating UHPLC method that was developed to study the degradation behaviour of the drug under hydrolysis (acid, base, and neutral), oxidation, photolysis, and thermal degradation conditions. Two DPs (DP1 and DP2) were formed under basic conditions and DP1 was formed under acidic, oxidative and water conditions. Thermal and photolytic degradation conditions revealed no degradation. LC-ESI-MS/MS experiments combined with accurate mass measurements were used to characterize the DP1. The DP2 was isolated and characterized using $^{1}H$ and $^{13}C$-NMR, HRMS and IR studies. The impurities 2-(2,6-dichlorophenyl)acetamide i.e., DP1 and 2-(2,6-dichlorophenyl)acetic acid i.e., DP2 were reported as degradants for the first time. The mechanisms of the formation of DP1 and DP2 were explained.

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