Research Article

Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Estimation of Guaifenesin and Dextromethorphan Impurities in Pharmaceutical Formulations

Thummala V. Raghava Raju,^{1,2} Noru Anil Kumar,¹ Seshadri Raja Kumar,¹
Annarapu Malleswara Reddy,¹ Nittala Someswara Rao,² and Ivaturi Mrutyunjaya Rao²

¹ Analytical Research and Development, Integrated Product Development, Dr. Reddy's Laboratories Limited, Bachupally, Hyderabad-500072, India

² School of Chemistry, Andhra University, Visakhapatnam, Andhra Pradesh 530003, India

Correspondence should be addressed to Thummala V. Raghava Raju; raghavartv@drreddys.com

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A sensitive, stability-indicating gradient RP-HPLC method has been developed for the simultaneous estimation of impurities of Guaifenesin and Dextromethorphan in pharmaceutical formulations. Efficient chromatographic separation was achieved on a Sunfire C18, 250 × 4.6 mm, 5 μ m column with mobile phase containing a gradient mixture of solvents A and B. The flow rate of the mobile phase was 0.8 mL min⁻¹ with column temperature of 50°C and detection wavelength at 224 nm. Regression analysis showed an *r* value (correlation coefficient) greater than 0.999 for Guaifenesin, Dextromethorphan, and their impurities. Guaifenesin and Dextromethorphan formulation sample was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal, and photolytic degradation. Guaifenesin was found stable and Dextromethorphan was found to degrade significantly in peroxide stress condition. The degradation products were well resolved from Guaifenesin, Dextromethorphan, and their impurities. The peak purity test results confirmed that the Guaifenesin and Dextromethorphan peak was homogenous and pure in all stress samples and the mass balance was found to be more than 98%, thus proving the stability-indicating power of the method. The developed method was validated according to ICH guidelines with respect to specificity, linearity, limits of detection and quantification, accuracy, precision, and robustness.

1. Introduction

Guaifenesin (GN), (+)-3-(2-methoxyphenoxy)-propane-1,2-diol, is a widely used expectorant, useful for the symptomatic relief of respiratory conditions. Its empirical formula is C₁₀H₁₄O₄, which corresponds to a molecular weight of 198.21. It is a white or slightly gray crystalline substance with a slightly bitter aromatic taste. Its solid oral dosage form is available as extended release tablets for oral administration [1].

Dextromethorphan (DN) [2–4] is the dextrorotatory enantiomer of the methyl ether of levorphanol and stereoisomer of levomethorphan. DN is an antitussive (cough suppressant) drug and used for pain relief and psychological

applications [5–7]. Its empirical formula is C₈H₂₅NO, which corresponds to a molecular weight of 271.4. It is a white powder. The combination of GN and DN is used to treat cough and chest congestion caused by the common cold, infections, or allergies. The chemical structures of GN and DN are shown in Table 1.

Impurity profiling of active pharmaceutical ingredients (API) in both bulk material and finalized formulations is one of the most challenging tasks for pharmaceutical analytical chemists under industrial environment [8]. The presence of unwanted or in certain cases unknown chemicals, even in small amounts, may influence not only the therapeutic efficacy but also the safety of the pharmaceutical products [9]. For these reasons, all major international pharmacopoeias

have established maximum allowed limits for related compounds for both bulk and formulated APIs. As per the requirements of various regulatory authorities, the impurity profile study of drug substances and drug products has to be carried out using a suitable analytical method in the final product [10, 11].

GN and DN are official in the United States pharmacopeia and European pharmacopeia, but its combination is not official in any of the pharmacopeias. In the literature survey, there were several LC assay methods that have been reported for the determination of GN and DN in pharmaceutical preparation either individually or in combination with other drugs [12–20] and in human plasma by LC-MS [21, 22]. Few methods were available for the determination of impurities individually for GN and DN [27–29].

There is no single method reported for the simultaneous determination of the impurities in pharmaceutical formulations of GN and DN. It is felt to develop a stability-indicating method for simultaneous determination of GN and DN related impurities in pharmaceutical formulation.

Hence, an attempt has been made to develop an accurate, rapid, specific, and reproducible method for the determination of GN and DN impurities (Tables 2(a) and 2(b)) in pharmaceutical dosage forms along with method validation as per ICH norms [23, 24]. The stability tests were also performed on both drug substances and drug products as per ICH norms [25, 26].

2. Experimental

2.1. Chemicals and Reagents. GN + DN tablets were received from the formulation research and development laboratory of Dr. Reddy's Laboratories Ltd., IPDO, Hyderabad, India. GN API and impurities were procured from Synthochem Lab., India. DN API and impurities were procured from Wochardt Laboratories Ltd., India. Sodium dihydrogen phosphate monohydrate, 1-octane sulfonic acid sodium salt monohydrate, HPLC grade acetonitrile, methanol, and orthophosphoric acid were purchased from Merck, Germany, Regis Technologies Inc, USA, and highly pure water was prepared by using Millipore MilliQ plus purification system.

2.2. Equipment. The LC system used for method development and method validation was Waters with a diode array detector (model: 2998 detector and e2695 separation module). The output signal was monitored and processed using Waters Empower software. Weighing was performed with a Mettler XS 205 Dual Range (Mettler-Toledo GmbH, Greifensee, Switzerland). Photo stability studies were carried out in a photo stability chamber (SUN TEST XLS+, Atlas, USA). Thermal stability studies were performed in a dry air oven (Merck Pharmatech, Hyderabad, India).

2.3. Chromatographic Conditions. HPLC measurements were carried out using a reversed phase Sunfire, C18, 250 × 4.6 mm, 5 μ m particle size column (Waters India Pvt. Ltd.) operated at 50°C with gradient elution at 0.8 mL min⁻¹ using a mobile phase buffer as a mixture of 0.01 M sodium dihydrogen

phosphate monohydrate and 0.0046 M 1-octane sulfonic acid sodium salt monohydrate of pH 3.0 (pH adjusted with diluted orthophosphoric acid), UV absorbance at 224 nm, injection volume 20 μ L. The mobile phase A consisted of pH 3.0 buffer and acetonitrile (90 : 10 v/v); mobile phase B consisted of pH 3.0 buffer, acetonitrile, and methanol (10 : 10 : 80 v/v/v). The LC gradient program was set as and time (min)/% mobile phase B: 0.01/15, 15/5, 20/30, 30/50, 60/85, 65/15, and 75/15. Pressure Range was 1700 psi to 2200 psi. Mixture of water, acetonitrile, and methanol (60 : 20 : 20 v/v/v) was used as diluent for sample preparation.

2.4. Preparation of Standard Solution and System Suitability Solution. We prepared individual stock solutions for GN, DN, and their impurities (each 500 μ g/mL). Working solution was prepared from the above stock solutions for related substances determination (48 μ g/mL of GN and 2.4 μ g/mL of DN). A mixture of all impurities (48 μ g/mL of GN impurities and 2.4 μ g/mL of DN impurities) along with GN and DN (24000 μ g/mL of GN and 1200 μ g/mL of DN) was also prepared in diluent. Stock solutions were used for method development and method validation.

2.5. Preparation of Test Solution. Twenty tablets (1200 mg of GN + 60 mg of DN) were weighed and the average weight was calculated. The tablets were crushed into fine powder, and powder equivalent to 6000 mg of GN (or equivalent to 300 mg of DN) was transferred into a 250 mL volumetric flask. Approximately 170 mL of diluent was added, shaken to disperse the material, and sonicated for 20 minutes with intermediate shaking. The solution was then diluted to 250 mL and centrifuged at 3000 rpm for 10 min. The supernatant (24000 μ g/mL of GN and 1200 μ g/mL of DN) was collected and filtered through a 0.45 μ m pore size nylon 66-membrane filter (make: Rankem). The filtrate was used as sample solution.

3. Method Validation

The proposed method was validated as per ICH guidelines [24].

3.1. System Suitability. System suitability parameters were evaluated to verify the system performance. System precision was determined on six replicate injections of standard preparations. All the important characteristics, including the relative standard deviation, peak tailing, and theoretical plate number, were measured. Resolution between impurities was measured by injecting system suitability solution. All these system suitability parameters covered the system, method, and column performance.

3.2. Specificity. Stress studies were performed at an initial concentration of 24000 μ g mL⁻¹ of GN and 1200 μ g mL⁻¹ of DN in active pharmaceutical ingredients (API) and formulated sample to provide the stability-indicating property and specificity of the proposed method. Intentional degradation

TABLE 1: Structures of Guaifenesin and Dextromethorphan.

Molecule name	Chemical name	Chemical structure
Guaifenesin	(+)-3-(2-Methoxyphenoxy)-propane-1,2-diol
Dextromethorphan	Ent-3-methoxy-17-methylmorphinan

was attempted by the stress conditions of exposure to photolytic stress (1.2 million lux hours followed by 200 Watt hours m^{-2}), heat (exposed at 105°C for 15 h), acid (0.5 N HCl for 2 hours at 60°C), base (0.5 N NaOH for 2 hours at 60°C), oxidation (10% peroxide for 30 min at 60°C), water (refluxed for 12 hours at 60°C), and humidity (exposed to 90% RH for 7 days).

3.3. Precision. The precision of the determination of the impurities was checked by injecting six individual preparations of (24000 $\mu g/mL$ of GN and 1200 $\mu g/mL$ of DN) test preparation spiked with 48 $\mu g mL^{-1}$ of GN impurities and 2.4 $\mu g mL^{-1}$ of DN impurities (0.2% of the target test concentration) and calculating the % RSD of % impurity for each compound. The intermediate precision of the method was also evaluated using different analysts and a different instrument in the same laboratory.

3.4. Limits of Detection (LOD) and Quantification (LOQ). LOD and LOQ for all impurities including GN and DN were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at LOQ level by injecting six individual preparations of impurities and % RSD was calculated.

3.5. Linearity. Linearity test solutions for the method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to the target test concentration. The peak area versus concentration in $\mu g mL^{-1}$ data was subjected to least-squares linear regression analysis.

3.6. Accuracy. Accuracy of the method was evaluated by using concentration levels LOQ, 0.1%, 0.2%, 0.4%, 0.8%, and 1.0% on GN + DN tablets. Six preparations were performed at LOQ and 1.0% level and three preparations were performed at different levels. Standard addition and recovery experiments were conducted on real sample to determine

accuracy of the related substance method. The percentages of recoveries for all impurities, GN, and DN were calculated.

3.7. Robustness. To determine the robustness of the developed method, experimental conditions were deliberately changed and the resolution between GN, DN, and their impurities, tailing factor, and theoretical plates of GN and DN peaks were evaluated. Also relative retention times for all the impurities and column pressure throughout the run were monitored.

To study the effect of the flow rate on the developed method, it was changed from 0.8 $mL min^{-1}$ to 0.6 and 1.0 $mL min^{-1}$. The effect of column temperature on the developed method was studied at 45 and 55°C (instead of 50°C). The effect of pH was studied by varying ± 0.2 pH units (i.e., 2.8 and 3.2) and the mobile phase composition was changed $\pm 10\%$ from the initial composition. In all the above varied conditions, the component of the mobile phase was held constant.

3.8. Stability in Solution and in the Mobile Phase. GN and DN spiked samples (impurities spiked at 0.2% of the target test concentration, i.e., 48 $\mu g mL^{-1}$ of GN impurities and 2.4 $\mu g mL^{-1}$ of DN impurities) were prepared in the diluent leaving the test solutions at room temperature. The spiked samples were injected at 0, 24, and 48 hrs time intervals. The impurity content was calculated, and the consistency in the % area of the principal peak at each interval was checked. The prepared mobile phase was kept constant during the study period. The mobile phase study was demonstrated by injecting the freshly prepared sample solution at different time intervals (0–2 days).

4. Results and Discussion

4.1. Optimization of Chromatographic Conditions. The main criterion was developing an RP-HPLC method for the simultaneous determination of impurities in GN and DN pharmaceutical dosage form in a single run, with emphasis

TABLE 2: (a) Structures of Guaifenesin impurities. (b) Structures of Dextromethorphan impurities.

(a)		
Impurity name	Chemical name	Chemical structure
Impurity A (degradant)	2-Methoxyphenol	
Impurity B (degradant)	2-(2-Methoxyphenoxy)propane-1,3-diol	
Impurity C (degradant)	1,1'-Oxybis[3-(2-methoxyphenoxy)propan-2-ol]	
Impurity D (process related impurity)	1,3-Bis(2-methoxyphenoxy)propan-2-ol	
(b)		
Impurity name	Chemical name	Chemical structure
Impurity A (degradant)	Ent-3-methoxymorphinan	
Impurity B (degradant)	Ent-17-methylmorphinan-3-ol	
Impurity C (degradant)	Ent-3-methoxy-17-methylmorphinan-10-one	
N-Oxide (degradant)	3-Methoxy-N-methylmorphinan N-oxide	
N-Formyl Morphine (NFM) (process related impurity)	3-Methoxy-6,7,8,8a,9,10-hexahydro-5H-9,4b-(epiminoethano)phenanthrene-11-carbaldehyde	
N-Formyl octabase (NFO) (process related impurity)	(S)-1-(4-Methoxybenzyl)-3,4,5,6,7,8-hexahydroisoquinoline-2(1H)-carbaldehyde

FIGURE 1: Spectra of GN, DN, and their impurities.

on the method being accurate, reproducible, robust, stability-indicating, linear, free of interference from other formulation excipients, and convenient enough for routine use in quality control laboratories.

Individual stock solutions of GN, DN, and their impurities were injected and the spectra were checked of each component (Figure 1). From the spectra all the impurities were having absorbance maximum at about 224. Hence 224 nm was selected for the estimation of GN and DN impurities.

A spiked solution of impurities ($48 \mu\text{g mL}^{-1}$ of GN impurities and $2.4 \mu\text{g mL}^{-1}$ of DN impurities), GN + DN ($24000 \mu\text{g mL}^{-1} + 1200 \mu\text{g mL}^{-1}$), and placebo peaks were subjected to separation by RP-HPLC. Initially, the separation was tried with the existing methods (USP, Pharma Europe and the literature method) [27–29]. It was observed that placebo peaks and GN impurity peaks were merging with

each other and two DN known impurities (NFM and NFO) were not eluting in DN API Pharma Europe method. In USP and Pharma Europe GN API method, DN impurities were not separated from DN peak and two DN known impurities were eluting at longer retention with broad peak shapes. In case of the literature method [29] DN impurities were not separated from DN peak and two DN known impurities were eluting at longer retention times with better peak shapes.

Method development was initiated by changing different gradient programmes, different pH values of the mobile phase buffer, different phosphate buffers, and different columns with the literature method [29]. Sharp peak shapes were observed with Sunfire column and sodium dihydrogen phosphate monohydrate buffer at pH 3.0, but separation was not up to the mark for DN, DN-N-oxide, and DN-impurity C. Sharp peak shapes were due to the properties of the Sunfire column (high mass loading capability, excellent low pH

FIGURE 2: Overlaid chromatogram of blank and system suitability preparation.

FIGURE 4: Typical chromatogram of peroxide stressed sample.

FIGURE 3: Overlaid chromatogram of placebo and spiked test preparation.

stability, superior peak shapes, and high efficiency). Since we were using a very high concentration of GN ($24000 \mu\text{g mL}^{-1}$), it was decided to use Sunfire column for further development trails by using ion pair reagent in the mobile phase for better separation between DN, DN-Impurity A, and DN-Impurity B. Separation was achieved between all the pairs of peaks but peak shapes of DN-NFM Impurity and DN-NFO Impurity are not sharp. Acetonitrile was added in the mobile phase in addition to methanol to get sharp peaks.

The chromatographic separation was achieved by a reversed phase Sunfire, C18, $250 \times 4.6 \text{ mm}$, $5 \mu\text{m}$ particle size column operated at 50°C with gradient elution at

0.8 mL min^{-1} using a mobile phase buffer as a mixture of 0.01 M sodium dihydrogen phosphate monohydrate and 0.0046 M 1-octane sulfonic acid sodium salt monohydrate of pH 3.0 (pH adjusted with diluted orthophosphoric acid), UV absorbance at 224 nm , and injection volume $20 \mu\text{L}$. The mobile phase A consisted of pH 3.0 buffer and acetonitrile (90:10 v/v); mobile phase B consisted of pH 3.0 buffer, acetonitrile, and methanol (10:10:80 v/v/v). The LC gradient program was set as: time (min)/% mobile phase B: 0.01/15, 15/5, 20/30, 30/50, 60/85, 65/15, and 75/15. All the impurities were well separated with a resolution greater than 2. No chromatographic interference due to the blank (diluent) and other excipients (placebo) at the retention time of GN, DN, and their impurities was observed. The typical overlay chromatogram of blank and system suitability solution and spiked test is shown in Figures 2 and 3.

4.2. Method Validation. After the development of the method it was subject to method validation as per ICH guidelines [24]. The method was validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics (system suitability, specificity, accuracy, precision, linearity, robustness, ruggedness, solution stability, LOD, and LOQ and stability-indicating capability).

4.2.1. System Suitability. The percentage relative standard deviation (RSD) of area from six replicate injections was below 5.0% (diluted standard solution, $48 \mu\text{g mL}^{-1}$ of GN and $2.4 \mu\text{g mL}^{-1}$ of DN). Low values of RSD for replicate injections indicate that the system is precise. The results of other system suitability parameters such as resolution, peak tailing, and theoretical plates are presented in Table 3. As seen from this data, the acceptable system suitability parameters would be as follows: the relative standard deviation of

FIGURE 5: (a) Linearity graph of Guaifenesin. (b) Linearity graph of Guaifenesin impurity A. (c) Linearity graph of Guaifenesin impurity B. (d) Linearity graph of Guaifenesin impurity C. (e) Linearity graph of Guaifenesin impurity D. (f) Linearity graph of Dextromethorphan. (g) Linearity graph of Dextromethorphan impurity A. (h) Linearity graph of Dextromethorphan impurity B. (i) Linearity graph of Dextromethorphan impurity C. (j) Linearity graph of Dextromethorphan N-oxide. (k) Linearity graph of Dextromethorphan impurity NFM. (l) Linearity graph of Dextromethorphan impurity NFO.

TABLE 3: System suitability results.

Parameter	% RSD* of standard		Theoretical plates*		Tailing factor*		Resolution 1	Resolution 2
	GN	DN	GN	DN	GN	DN		
As such method	0.5	0.7	45320	498659	1.0	1.0	2.7	2.3
At 0.6 mL/min flow rate	1.2	0.6	41091	495497	1.0	1.0	2.9	2.5
At 1.0 mL/min flow rate	0.9	0.4	45828	515551	1.0	1.0	2.5	2.2
At 50°C column temperature	1.5	0.8	45321	508966	1.0	1.0	2.6	2.3
At 60°C column temperature	2.1	2.3	40929	515807	1.0	1.0	2.9	2.6
At pH 2.8 (buffer pH)	1.1	0.5	43970	463987	1.0	1.0	2.4	2.3
At pH 3.2 (buffer pH)	0.7	1.5	44619	532891	1.0	1.0	2.6	2.7

* Average of six replicate standard injections.

TABLE 4: (a) Forced degradation data for Guaifenesin. (b) Forced degradation data for Dextromethorphan.

(a)

Degradation conditions	Guaifenesin			
	% degraded	Purity angle	Purity threshold	Mass balance (%)
Photo. stress (1.2 million lux hours followed by 200 Watt hours)	0.06	3.585	4.142	100.2
Exposed to humidity at 25°C and 90% RH for about 7 days	0.07	3.881	4.241	99.8
Refluxed with purified water for about 12 hours at 60°C	0.05	3.285	3.845	98.6
Refluxed with 0.5 N HCL solution for about 2 hours at 60°C	0.10	5.293	7.073	99.2
Refluxed with 0.5 N NaOH solution for about 2 hours at 60°C	0.06	3.067	3.489	98.9
Refluxed with 10% H ₂ O ₂ solution for about 30 min at 60°C	0.13	2.755	3.607	98.3
Exposed to dry heat for about 15 hours at 105°C	0.07	3.346	4.331	99.4

(b)

Degradation conditions	Dextromethorphan			
	% degraded	Purity angle	Purity threshold	Mass balance (%)
Photo. stress (1.2 Million lux hours followed by 200 Watt hours)	0.01	0.115	0.242	99.8
Exposed to humidity at 25°C and 90% RH for about 7 days	0.01	0.124	0.261	99.5
Refluxed with purified water for about 12 hours at 60°C	0.01	0.101	0.261	98.9
Refluxed with 0.5 N HCL solution for about 2 hours at 60°C	0.02	0.154	0.262	98.1
Refluxed with 0.5 N NaOH solution for about 2 hours at 60°C	0.01	0.103	0.256	99.2
Refluxed with 10% H ₂ O ₂ solution for about 30 min at 60°C	1.05	0.102	0.250	99.7
Exposed to dry heat for about 15 hours at 105°C	0.01	0.099	0.261	98.7

TABLE 5: Results of precision.

Compound	Prep-1	Prep-1	Prep-3	Prep-4	Prep-5	Prep-6	Avg	% RSD
GN-Impurity A	0.198	0.201	0.189	0.213	0.205	0.209	0.203	4.2
GN-Impurity B	0.209	0.195	0.192	0.201	0.206	0.194	0.200	3.5
GN-Impurity C	0.189	0.195	0.186	0.192	0.198	0.184	0.191	2.8
GN-Impurity D	0.208	0.190	0.192	0.193	0.188	0.186	0.193	4.1
DN-Impurity A	0.215	0.214	0.215	0.209	0.212	0.205	0.212	1.9
DN-Impurity B	0.204	0.200	0.202	0.203	0.205	0.191	0.201	2.5
DN-Impurity C	0.195	0.186	0.198	0.199	0.184	0.194	0.193	3.2
DN-N-oxide	0.216	0.214	0.218	0.219	0.211	0.210	0.215	1.7
DN-NFM Impurity	0.216	0.218	0.215	0.215	0.216	0.212	0.215	0.9
DN-NFO Impurity	0.198	0.195	0.213	0.192	0.214	0.215	0.205	5.2
GN	0.203	0.212	0.204	0.206	0.205	0.210	0.207	1.7
DN	0.182	0.189	0.194	0.188	0.187	0.201	0.190	3.4

TABLE 6: Results of intermediate precision.

Compound	Prep-1	Prep-1	Prep-3	Prep-4	Prep-5	Prep-6	Avg.	% RSD
GN-Impurity A	0.191	0.204	0.201	0.199	0.210	0.212	0.203	3.8
GN-Impurity B	0.212	0.204	0.210	0.209	0.211	0.204	0.208	1.7
GN-Impurity C	0.192	0.198	0.191	0.194	0.202	0.209	0.198	3.5
GN-Impurity D	0.185	0.192	0.191	0.194	0.193	0.192	0.192	1.5
DN-Impurity A	0.202	0.202	0.203	0.202	0.202	0.204	0.203	0.4
DN-Impurity B	0.191	0.188	0.204	0.209	0.189	0.202	0.197	4.5
DN-Impurity C	0.183	0.189	0.191	0.186	0.205	0.206	0.193	5.1
DN-N-oxide	0.197	0.210	0.213	0.202	0.198	0.212	0.205	3.5
DN-NFM Impurity	0.183	0.187	0.179	0.184	0.185	0.188	0.184	1.7
DN-NFO Impurity	0.206	0.197	0.209	0.212	0.194	0.208	0.204	3.5
GN	0.199	0.205	0.201	0.204	0.207	0.208	0.204	1.7
DN	0.187	0.185	0.192	0.186	0.194	0.191	0.189	1.9

TABLE 7: LOD, LOQ, and precision data.

Compound	LOD (%)	LOQ (%)	S/N ratio		% RSD at LOQ Level precision
			LOD	LOQ	
GN-Impurity A	0.004	0.001	2.7	9.5	4.2
GN-Impurity B	0.008	0.002	2.9	9.8	2.5
GN-Impurity C	0.005	0.002	3.0	9.8	3.1
GN-Impurity D	0.003	0.001	2.9	9.7	2.5
DN-Impurity A	0.014	0.004	2.8	10.1	1.1
DN-Impurity B	0.021	0.006	2.7	10.4	3.4
DN-Impurity C	0.023	0.007	2.8	9.9	4.9
DN-N-oxide	0.016	0.005	3.1	9.8	2.2
DN-NFM Impurity	0.016	0.005	2.7	10.1	1.5
DN-NFO Impurity	0.041	0.012	2.9	10.6	4.2
GN	0.006	0.002	3.1	9.8	2.1
DN	0.015	0.005	2.8	9.6	2.8

replicate injections is not more than 5.0%, resolution between impurities 2.0, the tailing factor for GN and DN is not more than 1.5, and the theoretical plates are not less than 5000.

4.2.2. Specificity. All forced degradation samples were analyzed with the aforementioned HPLC conditions using a PDA detector to monitor the homogeneity and purity of the GN, DN, and their related impurities. Individual impurities, placebo, GN, and DN were verified and proved to be noninterfering with each other thus proving the specificity of the method.

Figure 3 shows that there is no interference at the RT (retention time) of GN, DN, and all known impurities from the other excipients. Degradation was not observed in photolytic stress, humidity, acid hydrolysis, base hydrolysis, water hydrolysis, and thermal stress studies. Significant degradation was observed in oxidative conditions. The typical oxidative stressed chromatogram was shown in Figure 4. It was interesting to note that all the peaks due to degradation were well

resolved from the peaks of GN, DN, and their impurities. Further the peak purity of GN, DN, and their impurities was found to be homogeneous based on the evaluation parameters such as purity angle and purity threshold using Waters Empower Networking Software. The verification of peak purity indicates that there is no interference from degradants, facilitating error-free quantification of GN and DN impurities. Also the mass balance of stressed samples was found to be more than 98%. Thus, the method is considered to be “stability-indicating.” The specificity results were shown in Tables 4(a) and 4(b).

4.2.3. Precision. The % RSD for the individual % of all impurities in impurities method precision study was within 3.4%. The results obtained in the intermediated precision study for the % RSD of the individual % of all impurities were well within 4.1%, conforming high precision of the method. The results are shown in Tables 5 and 6.

TABLE 8: Accuracy of the method.

Compound	% Recovery at each level ^a					
	LOQ	0.1%	0.2%	0.4%	0.8%	1.0%
GN-Impurity A	98.5	99.5	101.2	96.7	99.9	103.5
GN-Impurity B	105.6	102.7	99.7	105.1	98.5	97.5
GN-Impurity C	102.6	105.6	101.9	106.1	103.5	102.1
GN-Impurity D	95.6	97.2	96.5	95.8	96.5	98.5
DN-Impurity A	93.5	96.5	95.2	94.2	98.5	96.7
DN-Impurity B	98.5	96.7	99.9	98.9	97.5	96.5
DN-Impurity C	101.1	102.3	102.5	103.7	102.5	101.8
DN-N-oxide	105.6	106.5	105.1	104.5	102.9	103.7
DN-NFM Impurity	101.5	98.5	102.5	101.2	99.5	98.6
DN-NFO Impurity	107.1	105.6	106.9	105.8	106.5	105.8
GN	98.5	96.5	97.2	96.8	97.4	98.7
DN	99.2	98.5	97.1	99.5	100.5	101.2

^a Average of six determinations at LOQ level & 1.0% level and three determinations at remaining levels.

TABLE 9: Regression statistics.

Substance	Linearity range ($\mu\text{g mL}^{-1}$)	Correlation coefficient (<i>R</i>)	Y-intercept bias in %
GN-Impurity A	0.93 to 239.61	0.999	0.9
GN-Impurity B	2.01 to 242.78	0.999	1.0
GN-Impurity C	1.26 to 241.92	1.000	0.7
GN-Impurity D	0.79 to 242.55	0.999	0.8
DN-Impurity A	0.17 to 14.10	1.000	0.4
DN-Impurity B	0.25 to 10.10	1.000	0.1
DN-Impurity C	0.27 to 12.23	1.000	0.8
DN-N-oxide	0.19 to 12.49	1.000	0.6
DN-NFM Impurity	0.19 to 12.19	0.998	1.5
DN-NFO Impurity	0.48 to 12.22	1.000	0.9
GN	1.53 to 244.78	0.999	1.6
DN	0.18 to 12.15	1.000	0.1

TABLE 10: Results of robustness study.

S. no.	Impurity name	As such method	RRT's of impurities					
			At 0.6 mL/min flow rate	At 1.0 mL/min flow rate	At 50°C column temperature	At 60°C column temperature	At pH 2.8 (buffer pH)	At pH 3.2 (buffer pH)
1	GN-Impurity A	0.74	0.76	0.73	0.72	0.71	0.74	0.73
2	GN-Impurity B	1.12	1.11	1.13	1.11	1.10	1.12	1.13
3	GN-Impurity C	2.04	2.06	2.05	2.05	2.02	2.04	2.05
4	GN-Impurity D	2.14	2.12	2.15	2.13	2.11	2.13	2.14
5	DN-Impurity B	0.83	0.82	0.81	0.82	0.82	0.83	0.82
6	DN-Impurity C	0.98	0.97	0.98	0.98	0.97	0.97	0.98
7	DN-N-oxide	1.01	1.02	1.03	1.02	1.03	1.02	1.04
8	DN-Impurity A	1.03	1.04	1.05	1.04	1.05	1.04	1.06
9	DN-NFM Impurity	1.28	1.31	1.30	1.31	1.29	1.28	1.19
10	DN-NFO Impurity	1.38	1.41	1.42	1.39	1.39	1.39	1.41

Note: GN known impurities RRTs were calculated against GN main peak, and DN known impurities RRTs were calculated against DN main peak.

4.2.4. Limit of Detection (LOD) and Limit of Quantification (LOQ). The determined limit of detection, limit of quantification, and precision at LOQ values for GN, DN, and their impurities were reported in Table 7. The RSD for peak areas of GN, DN, and their related impurities at limit of quantification level was within 10.0%.

4.2.5. Accuracy. The recovery of all the impurities from finished pharmaceutical dosage form ranged from 85.0% to 115.0%. The summary of % recovery for individual impurity was mentioned in Table 8.

4.2.6. Linearity. Linear calibration plot for the related substance method was obtained over the calibration ranges tested, that is, LOQ to 1.0% of the target test concentration. The correlation coefficient obtained was greater than 0.997 for all the components. The slope and y-intercept values were also provided in Table 9, which confirmed good linearity between peak areas and concentration. The linearity graphs were shown in Figure 5(a) to Figure 5(l).

4.2.7. Robustness. No significant effect was observed on system suitability parameters such as resolution, RSD, tailing factor, RRTs of impurities, or the theoretical plates of GN and DN when small but deliberate changes were made to chromatographic conditions. The results were presented in Tables 3 and 10, along with the system suitability parameters of normal conditions. Thus, the method was found to be robust with respect to variability in applied conditions.

4.2.8. Stability in Solution and in the Mobile Phase. No significant changes were observed in the content of impurities during solution stability and mobile phase stability experiments when performed using the impurities method. The solution stability and mobile phase stability experiment data confirms that the sample solution and mobile phases used during the impurity determination were stable for at least 48 h.

5. Conclusions

The gradient HPLC method developed for the simultaneous determination of GN and DN impurities in pharmaceutical dosage form was precise, accurate, and specific. The method is validated as per ICH guidelines and found to be specific, precise, linear, accurate, rugged, and robust. The developed method can be used for the stability analysis of GN and DN either individually or in their combination dosage forms.

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