IJPSR (2017), Volume 8, Issue 11 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 25 March, 2017; received in revised form, 29 May, 2017; accepted, 25 June, 2017; published 01 November, 2017

DEVELOPMENT OF NOVEL STABILITY INDICATING HPLC METHOD AND CHARACTERIZATION OF OXIDATIVE DEGRADATION IMPURITY IN GRANISETRON DRUG SUBSTANCE

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Keywords:

Granisetron hydrochloride, Forced degradation, Impurity characterization, Method validation, HPLC

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ABSTRACT: The objective of the present study is to identification of the oxidative degradation impurity in the Granisetron hydrochloride active ingredient and also the novel method developed based on the forced degradation studies performed on Granisetron Hydrochloride to well resolve the impurities from the API. Forced degradation studies were conducted as per ICH technical requirements prescribed stress conditions using acid, base, oxidative, thermal stress, and photolytic degradation to show the stability indicating power of the method. Significant degradation was observed during basic hydrolysis and in oxidative studies and no degradation was observed in other stress conditions. Characterization of oxidative degradation impurity was conceded with a combination of analytical and preparative LC isolation followed by LC-MS/MS, IR and ^{1}H and ^{13}C NMR and 2D NMR spectral data. The HPLC chromatographic conditions were chosen to achieve satisfactory resolution between the impurities and API with shortest analysis time. Granisetron hydrochloride and its impurities were separated on Xbridge phenyl column (150mm×4.6 mm, 3.5 microns particle size). The mobile phase A composed of 10mM of Ammonium acetate in water (pH 8.5) and mobile phase B is Acetonitrile: Methanol (50:50, v/v) pumped at flow rate of 1mL/min with gradient. The column temperature was set at 40 °C and the detection wavelength at 305nm using UV detector. The developed HPLC method is suitable for LC–MS analysis which was further used to characterize the degradation products. Validation of the new developed HPLC-UV method was carried out in accordance with ICH guideline and met all required acceptance criteria.

INTRODUCTION: Chemotherapy induced nausea and vomiting (CINV) is one of the most common and distressed side effects reported by cancer patients [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(11).4591-03

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (11).4591-03

On increasing severity of nausea and vomiting can result in to medical complications such as dehydration, malnutrition, metabolic disturbances and aspiration pneumonia all which become life threatening [2]. CINV mechanism entails a complex interaction between neurotransmitters and receptors in both the central nervous system (CNS) and the peripheral nervous system initiated by the stimulation of dopamine, opiate, histamine, acetylcholine, neurokinin-1 (NK1) or serotonin (5-HT) receptors [2].

The 5-HT_3 receptors are located peripherally on vagal nerve terminals and centrally in the chemo receptor trigger zone (CTZ) of the area postrema. When a patient has CINV, 5HT_3 receptors are stimulated by serotonin, which is released from the mucosal enterochromaffin cells. In binding to 5-HT_3 receptors, Granisetron blocks serotonin stimulation and subsequent vomiting after emetogenic stimuli [3].

Granisetron hydrochloride is a selective 5-HT_3 receptor antagonist chemically termed as endo-1-methyl-*N*-[9-methyl-9-azabicyclo (3.3.1) non-3-yl]-^{1}H-indazole-3-carboxamidehydrochloride is an antinauseant and antiemetic agent [4]. It has a fewer side effects and has lower risk of drug interactions than other 5-HT_3 receptor antagonists. Various administrations such as oral and intravenous of Granisetron Hydrochloride formulations were now in the market. Granisetron is effective as an oral prophylactic in abolishing the retching and vomiting evoked by cytostatic therapy.

Granisetron hydrochloride official monograph is published in pharmacopeias [8, 9] describes the assay and related substances method. The European Pharmacopoeia monograph method for Granisetron hydrochloride and its related compounds (monitored for six impurities) is isocratic reverse phase HPLC method can resolve related compounds of Granisetron, is of total run time around 60 min and does not discuss about the stability indicating.

A thorough literature review revealed that several analytical methods for determination of Granisetron in biological fluids and dosage forms by various detectors like Fluorescence detector [10-16], UV-Vis [17-19], Mass detector [20-24] and Ampherometry detector were used. According to our research, none of the currently available analytical methods, including the monograph method, can separate and quantify all the known related compounds and degradation impurities Granisetron hydrochloride API. The Objective of current study is to develop novel well resolved short HPLC method for the separation of known impurities and identification of degradation products for Granisetron hydrochloride. It is, therefore, necessary to develop a new stability-indicating method for the determination and quantitative estimation of Granisetron hydrochloride and its related substances. This method was successfully validated according to the ICH guidelines [25]. Stress testing study of a drug substance was conducted to identify the likely degradation products and provides significant information on drug's inherent stability. Consecutively, it can be a fundamental contribution to develop and validate the stability indicating analytical method which can be used in testing for the quality and release of pharmaceutical products.

Any information regarding degradation products of a given drug substance is commonly obtained by extended stability studies carried out under different conditions, such as exposure to acid or base, oxidation, hydrolysis, thermal and photolytic conditions [5]. As per regulatory guidelines, the requirement of identification and characterizing of degradation products in pharmaceuticals are extremely essential in the wake of stringent purity requirements. Hence, structural elucidation of unknown impurities is an analytical challenge that requires both selective and sensitive methods [6, 7].

MATERIALS AND METHODS: Granisetron Hydrochloride standards and impurities were supplied by Dr. Reddy's Laboratories Limited, Hyderabad, India. Solvents such as Acetonitrile - Gradient grade is purchased from Merck Specialties (P) ltd. Methanol HPLC-grade is bought from RFCL limited, Faridabad, India. Ammonium acetate and Hydrogen peroxide (30% w/v) was purchased from Thermo Fischer scientific India (P) limited. Conc. Hydrochloric acid is purchased from RFCL Ltd, Mumbai, India. Aqueous ammonia solution 25% extra pure grade is bought from Sd Fine chemicals limited, Mumbai, India. Water for mobile phase and diluent was prepared in-house by using a Millipore Milli-Q Plus water purification system (Millipore Corporate Headquarters, Billerica, MA).

Chromatographic Conditions and Equipment: High pressure liquid chromatography was carried out on Agilent 1100 series instrument with a photodiode array detector (manufactured by Agilent Technologies, Waldbronn, Germany). The output signal was monitored and processed using chemstation software. The chromatographic column used Xbridge phenyl 150-mm, 4.6 mm, and

3.5 microns particle size. Separation was achieved using a gradient method. Mobile phase A contained a mixture of 10mM ammonium acetate buffer (pH adjusted to 8.5 using diluted aqueous ammonia). Mobile phase B contained a mixture of water and acetonitrile in a ratio of 50:50 (v/v) respectively. The flow rate of the mobile phase was 1.0 mL/min. The HPLC gradient program (T/%B) was set as 0.01/10, 10.0/35, 15.0/45, 16.0/10 and 20.0/10. The column temperature was maintained at 40 °C, and the detection was monitored at a wavelength of 305 nm. The injection volume was 10 microlitre.

Semi Preparative Apparatus and Conditions: A semi preparative Xbridge phenyl OBD column (150mm×19mm, 5µm; Waters, USA) was used for preparative purposes using a mobile phase consisting of aqueous solution of 10mM ammonium acetate and acetonitrile (70:30, v/v). Agilent 1200 Preparative HPLC instrument (manufactured by Agilent Technologies, Waldbronn, Germany) is used for separation and fraction collection at the detector wavelength if 305 nm. The flow rate 20 ml/min was employed throughout the preparation. The volume of solution injected into the semi preparative column was 1000 µL.

NMR Spectroscopy: The ^{1}H, ^{13}C NMR, DEPT and 2D-NMR data for Granisetron Hydrochloride and oxidative degradation impurity were recorded on Varian Unity INOVA500MHz FT NMR Spectrometer (Varian, Germany) using CD_3OD as solvent (Cambridge Isotopic Labs, USA). The ^{1}H and ^{13}CNMR chemical shift values were reported on the δ scale in ppm, relative to solvent signal 3.35 ppm in ^{1}H and δ = 49.5 ppm in ^{13}C NMR spectrum as internal standards.

LCMS/MS and HR-MS Spectroscopy: Agilent 1200 series HPLC coupled with 6410 QQQ mass detector (manufactured by Agilent Technologies, Waldbronn, Germany) is used to get the molecular mass and MS/MS fragmentation information with Electrospray ionization mode. Capillary voltage maintained at 4000 V, Fragmentor voltage 135, Nebulizer gas 50L/min (Nitrogen) and MS/MS collision energy is 20 eV. High resolution mass spectral data was analyzed on the Micro-mass LCT Premier XE mass spectrometer equipped with an ESILock spray source for accurate mass values (Water Corporation, Milford, MA, USA). Leucine enkephalin was used as an internal reference compound, which was introduced *via* the Lockspray channel. Data were acquired using the positive mode.

FT-IR Spectroscopy: The infra-red spectrum for the Granisetron Hydrochloride and oxidative degradation product is obtained from 4000 cm^{-1} to 450cm^{-1} in the Perkin Elmer FT-IR instrument connected with Spectrum v.2 software using dried potassium bromide pellet as medium. The instrument is calibrated using polystyrene film 0.038µm.

Preparation of Standard Solutions: For the analysis purpose, a working solution of 1 mg/mL of Granisetron Hydrochloride in diluent was prepared for related substances determination analysis. An individual impurity stock solution for all the impurities and degradation product were prepared at concentration 0.01mg/mL was also prepared in same diluent.

Validation of New Developed Analytical Test Procedure: The simple gradient HPLC conditions developed for determination of related substances in Granisetron Hydrochloride was intended to be applicable to the quantification of impurities. Critical validation parameters such as specificity in presence of degradants, repeatability, limit of detection and quantification, linearity, recovery and robustness of the method were evaluated as per ICH guidelines [25] to demonstrate the stability indicate capability of the new developed method is discussed in this article. The forced degradation study of the drug substance (Granisetron Hydrochloride) was performed by exposing to acidic, alkaline, oxidative, photolytic and thermal stress conditions. The HPLC analysis was carried out by with a PDA detector and peak purity of the main peak is established for every stress condition. 10µL of each forced degradation samples were injected at regular intervals and the final stress conditions were established in such a way that 10-20% degradation of drug substance.

Acid Degradation: 50mg of Granisetron Hydrochloride sample was taken into a 50mL volumetric flask, 10ml of 5N hydrochloric acid solution was added, contents were mixed well and

kept for constant stirring for 2 days at 70 °C and the mixture was neutralized. 1.0mL of this solution was diluted to 10 1.0mL with diluent.

Base Degradation: 50mg of Granisetron Hydrochloride sample was taken into a 50mL volumetric flask and 10mL of 0.5N aqueous sodium hydroxide solution was added and the contents were mixed well Kept for 8 days in a water bath and also heated at 70 °C for 12 hours. The sample is neutralized and tested for impurity profile as per method of analysis.

Oxidative Degradation: 50mg of Granisetron Hydrochloride sample was taken into a 50mL volumetric flask, 10mL of 10% H_2O_2 solution was added and contents were mixed well and kept for constant stirring for 3 hr at room temperature. 1.0 mL of this solution was diluted to 10mL with diluent.

Thermal Degradation: Granisetron Hydrochloride sample was exposed to heat by keeping in a convention oven at 105 °C temperature 8 days. Aliquot of Sample is withdrawn at every day interval to check the degradation profile as per the chromatographic conditions captured.

Photolytic Degradation: Granisetron Hydrochloride sample was placed in a UV cabinet (Vision lab equipments, India) and exposed to light in an overall illumination of ≥ 200 Watt hours/ square meter at 25 °C with UV radiation and 1.2 million lux hours visible light. Control samples which were protected from light with aluminium foil were also placed in the light cabinet and exposed concurrently. These samples were analyzed as per the chromatographic conditions.

RESULTS AND DISCUSSION:

Development and Optimization of LC Method: Related substance by HPLC method is available in Pharmacopeia which can able to separate the listed impurities and its degradation products. However, the mobile phase used for the separation is not volatile buffer and not well resolved the oxidative degradation product. During the specificity a study, an oxidative degradation impurity is observed while the sample was oxidative stress study. This impurity is eluted at about 5.5 min *i.e.*, with a relative retention time (RRT) of 0.8 for impurity with respect to Granisetron Hydrochloride. Hence the orthogonal method development approach is handled in this article to separate the degradation product and other known impurities listed in the pharmacopeia. Granisetron hydrochloride drug substance has reported pKa value: 9.5.

The main target of the chromatographic method is to achieve the separation of impurities, degradation products and the main component. The new method developed for the determination of related substances is volatile and LCMS compatible. Xbridge phenyl column of dimensions 150mm length and 4.6mm ID with 3.5 microns particle size is used in the finalized conditions. The optimum chromatographic resolution of Granisetron and its impurities and degradation products was influenced by the pH of the aqueous phase (mobile phase A), ratio of the organic modifier(mobile phase B) and the flow rate of the mobile phase to get the base line separation of Granisetron with other impurities. Slight change in the pH of the buffer has greater impact on resolution of new method is thoroughly studied in this article.

The maximum absorption wavelength of the reference drug solution, process bi-products and forcibly degraded drug solutions is 305 nm, which is the intersecting value obtained from the UV absorption spectra; hence, 305 nm was selected as the detection wavelength for LC analysis. During the initial development, ammonium acetate buffer at pH 6.5 was tried to separate all the known impurities and the degradants. It was found that the impurities A, C, F were merged with main peak. Development continued by increasing the pH of the buffer to 7.5 and also 8.0, impurity A has resolved from the main peak however, other two impurities F and C was still merged with the main component. At pH 8.5, all the known impurities and the degradants were well separated.

To elute these non-polar impurities and better separations, mixtures of different ratios of acetonitrile and methanol have been used and finally 50:50 (v/v) acetonitrile and methanol was fixed. Sharp peaks with good resolution were attained using 1.0mL/min. A successful gradient run (mentioned in the experimental section) was employed to resolve all the known impurities and the degradation products which were formed during degradation. The blended solution containing

50µg/mL of Granisetron Hydrochloride and 1µg/mL of each of the seven impurities were prepared in the diluent.

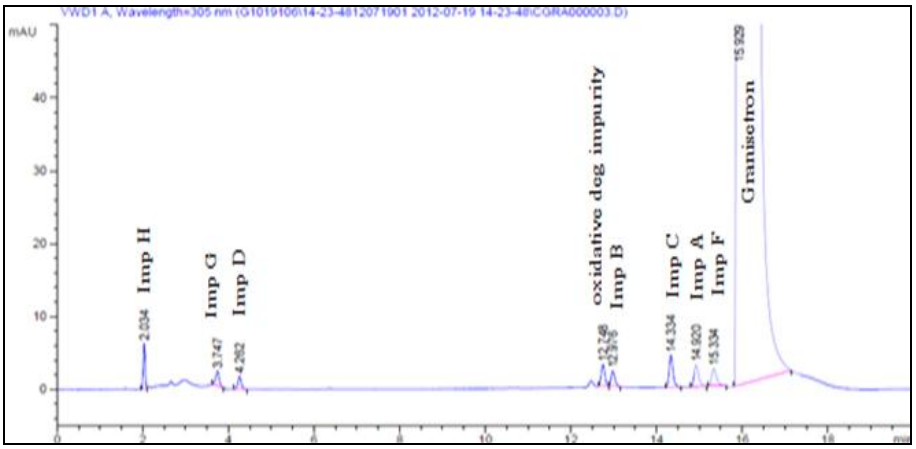

FIG. 1: GRANISETRON, ITS PROCESS AND N-OXIDE IMPURITY CHROMATOGRAM

Isolation of Impurity by Preparative HPLC: Oxidative test sample was subjected to preparative HPLC as per the conditions mentioned above. The preparative LC fractions were initially analyzed by analytical HPLC-UV method and then pooled together. The fractions were concentrated on rota vapor and then freeze-dried to remove organic solvent and water, respectively. The purity of isolated impurity is found to be 90% (area normalization method).

Spectral Characterization of Oxidative Degradation Impurity:

IR Spectrum Data Analysis: The IR spectrum recorded in the KBr pellet for the oxidative degradation product was comparable with that of the parent drug as it showed a strong band at 1646 cm^{-1} and 1552 cm^{-1} corresponding to the carbonyl-amide as a sharp strong band and NH bending respectively. Additionally the degradation product has a strong band at 1494 cm^{-1} due to the formation of N-O bond in the structure. Mixed absorptions of C-C aromatics between were observed at 1474cm^{-1} and 1300cm^{-1}.

ESI-MS and HRMS Spectral Data Analysis: The mass spectrum revealed the molecular ion peak for the parent at m/z 313 [M+H] and the oxidative degradation impurity at m/z 329 [M+H] which constitutes the base peak of the spectrum. In addition, the fragmentation analysis was carried out for both parent as well as impurity. MS/MS fragmentation showed for Granisetron at m/z 138.1 and for impurity at 154.1. The difference of 16 mass units is observed between parent and the impurity in both molecular ion and fragment. The HR-MS analysis also performed to determine the elemental composition using instrumental parameters mentioned. The elemental composition obtained is $C_{17}H_{22}N_4O_2$.

NMR Spectral Data Analysis: Identification and structure confirmation of unknown oxidative impurity was confirmed by NMR study of the isolated impurity. The assignment of NMR signals was performed for Oxidative impurity and the proposed structure was unequivocally confirmed. The ^1H NMR spectral data of the oxidative degradation impurity recorded in CD_3OD is close to the assignment of Granisetron Hydrochloride data. The isolated compound showed very similar correlations with Granisetron Hydrochloride analogue in the 2D-NMR experiments, suggesting the close structural comparison. Total number of protons of aliphatic region and aromatic region is found to be same.

The major shift in ^1H NMR spectrum was observed for an aliphatic methyl proton is observed at δ 2.94 ppm for 22nd carbonin parent. In the oxidative impurity spectrum the chemical shift value is shifted to downwards and observed at δ 3.60 ppm. This could be due to the formation of N-O bond formation in the impurity leads to the shift towards downfield region. Similarly, the ^{13}C NMR experiments have confirmed the chemical shift values precisely matches to the Granisetron hydrochloride drug substance. However, Methyl

exhibited a chemical shift at δ 38.8 ppm has shifted to δ 67.4 ppm for the oxidative impurity. The NMR signals observed in ^{1}H and ^{13}C spectrum is correlated by 2D-NMR experiments. In gHSQC experiment the correlation for proton signal at 2.94 ppm is observed at δ 38.8 ppm and in for oxidative impurity correlation for δ 3.60 ppm is observed at 67.4 ppm for parent and oxidative impurity respectively.

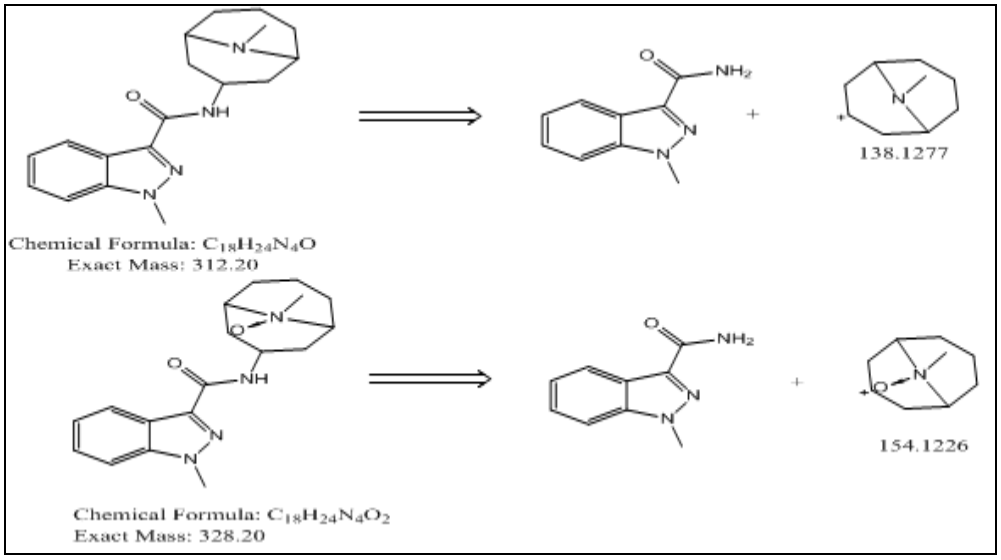

FIG. 2: LCMS AND MS/MS FRAGMENTATION PATTERN

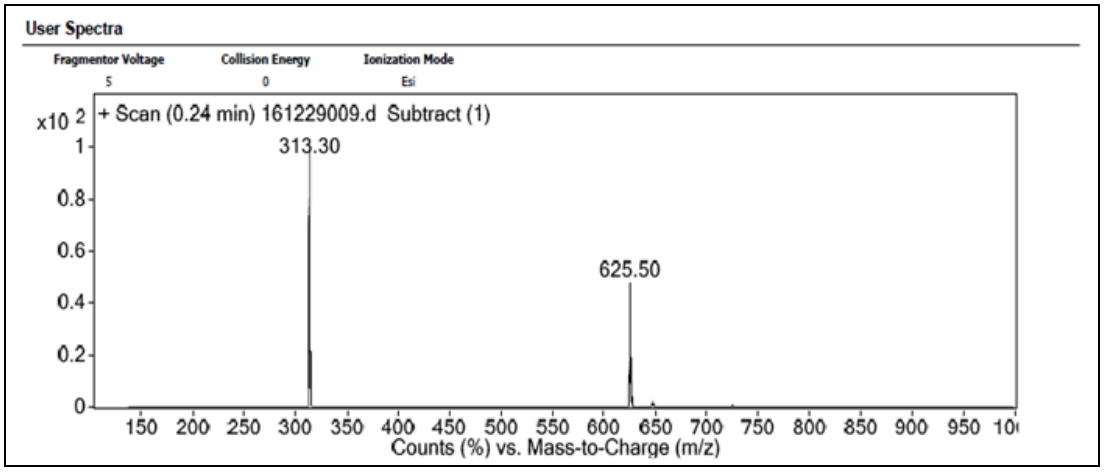

FIG. 3: MASS SPECTRUM OF GRANISETRON

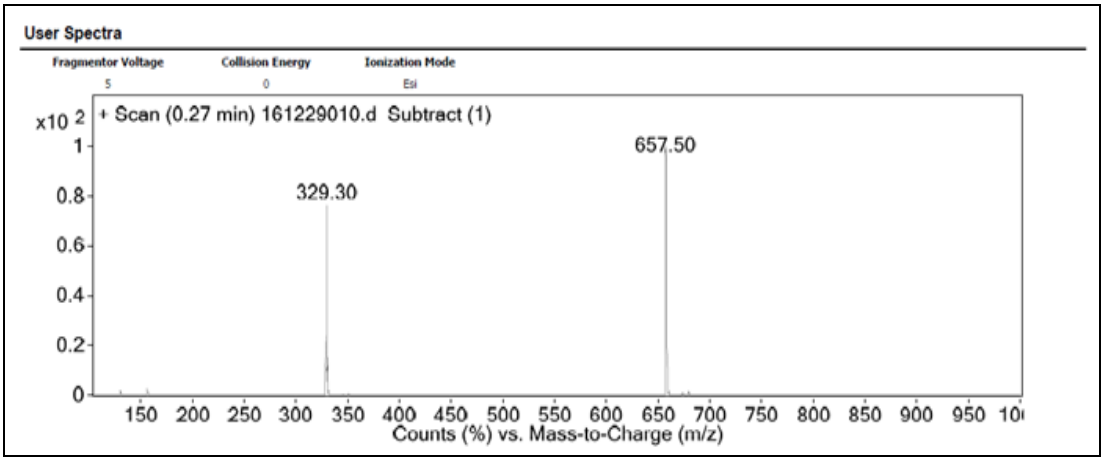

FIG. 4: MASS SPECTRUM OF GRANISETRON N-OXIDE IMPURITY

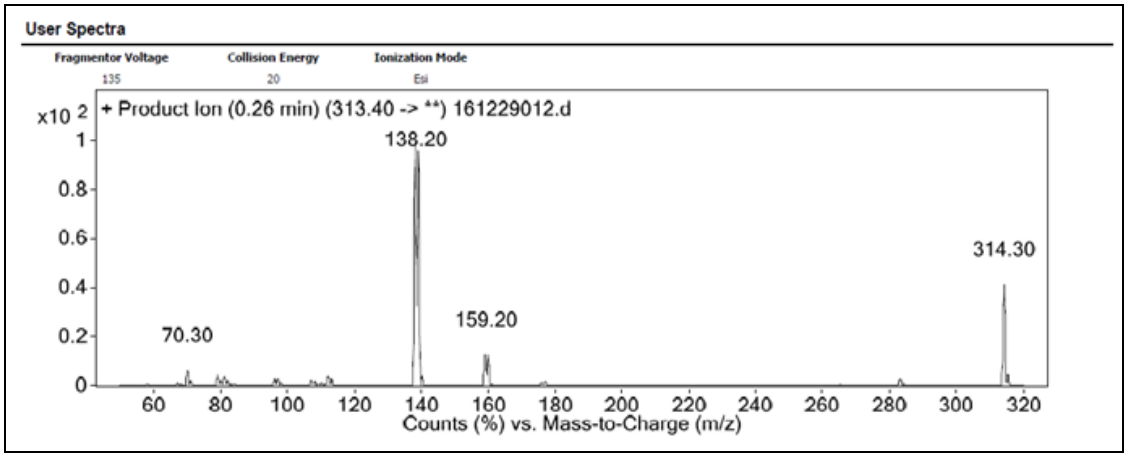

FIG. 5: MS/MS SPECTRUM OF GRANISETRON AT 20 eV COLLISION ENERGY

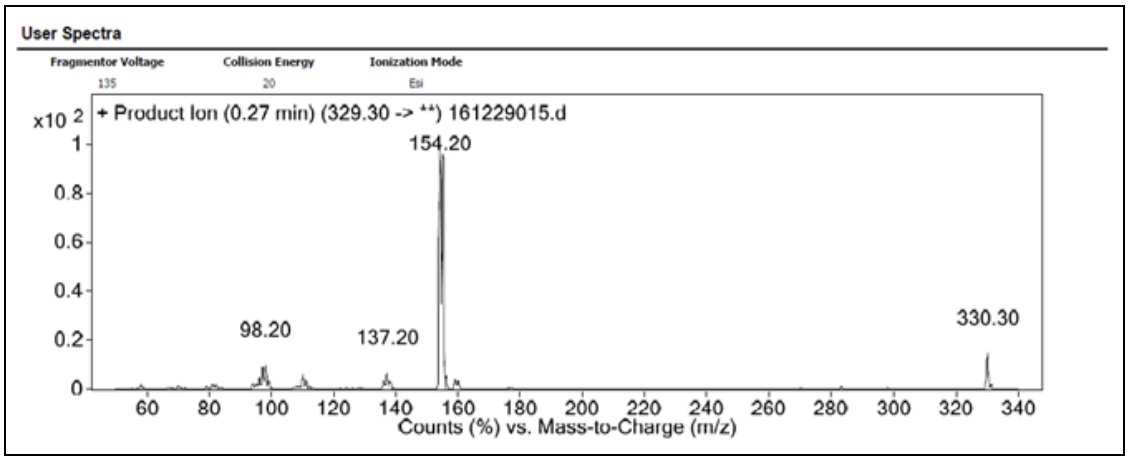

FIG. 6: MS/MS SPECTRUM OF GRANISETRON N-OXIDE IMPURITY AT 20 eV COLLISION ENERGY

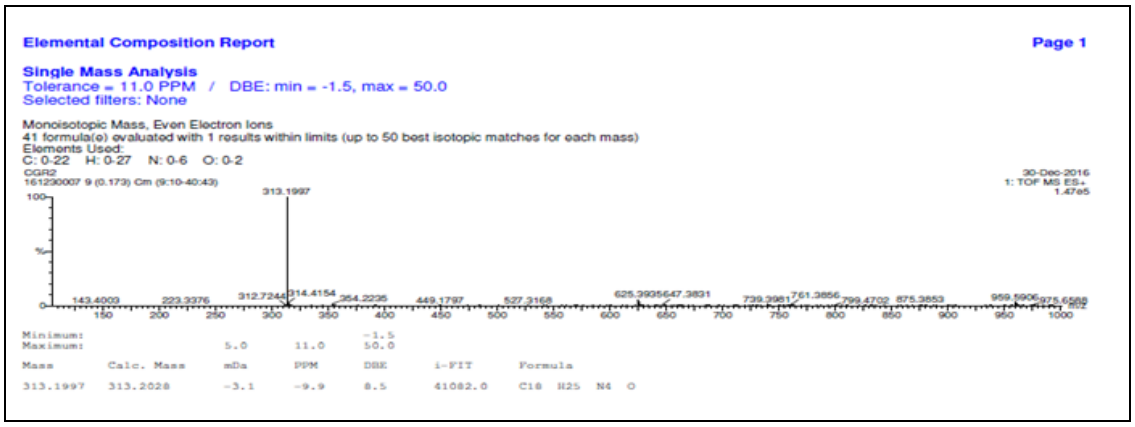

FIG. 7: HR-MS DATA OF GRANISETRON IMPURITY FOR ELEMENTAL COMPOSITION

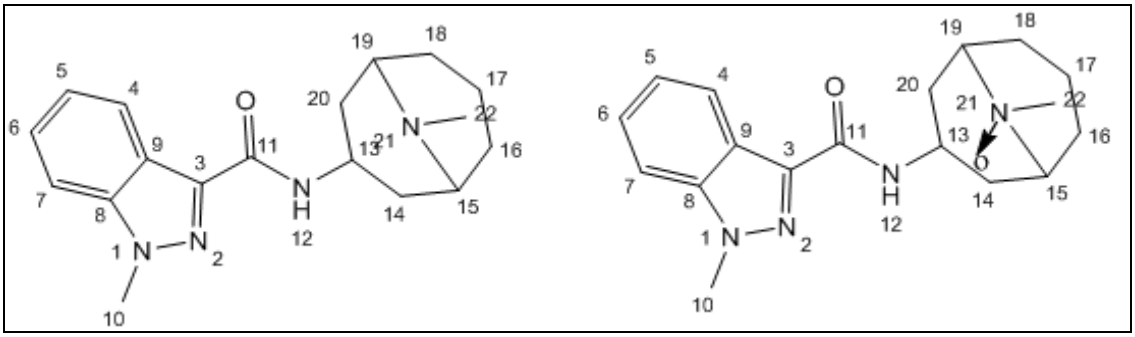

FIG. 8: NMR STRUCTURAL NUMBERING OF GRANISETRON AND N-OXIDE IMPURITY

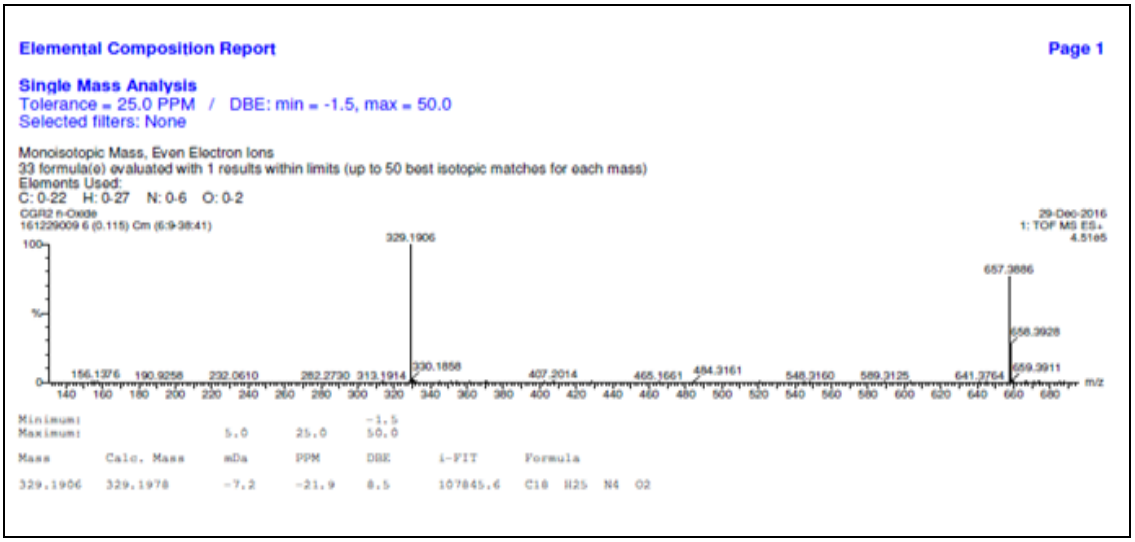

FIG. 9: HR-MS DATA OF GRANISETRON N-OXIDE IMPURITY FOR ELEMENTAL COMPOSITION

TABLE 1: NMR ASSIGNMENTS OF GRANISETRON HYDROCHLORIDE

Position [a]	^{1}H	δ (ppm), J Hz	gDQCOSY	^{13}C, δ (ppm)	DEPT	gHSQC
3	-	-	-	138.16	-	-
4	^{1}H	8.20, d(8.0)	5H, 7.29	124.44	CH	4H, 8.20
5	^{1}H	7.29, d(7.2)	4H, 8.20 6H, 7.47	124.28	CH	5H, 7.29
6	^{1}H	7.47, d(7.2)	5H, 7.29 7H, 7.57	128.50	CH	6H, 7.47
7	^{1}H	7.57, d(8.4)	6H, 7.47	111.45	CH	7H, 7.57
8	-	-	-	143.23	-	-
9	-	-	-	123.32	-	-
10	^{3}H	4.14, s	-	36.84	CH_3	10H, 4.14
11	-	-	-	164.99	=C=	-
13	^{1}H	4.62, m	14,20 Ha, 1.98 14,20 Hb, 2.59	39.65	CH	13H, 4.62
14, 20	^{2}Ha	1.98, m	13 H, 4.62 14,20 Hb, 2.59 15,19 H, 3.73 16, 18 Ha, 1.59 16,18 Hb 2.22	32.64	CH_2	14,20Ha 2.59
	^{2}Hb	2.59, m	13 H, 4.62 14,20 Ha, 1.98 15,19 H, 3.73			14,20Hb 1.98
15, 19	^{2}H	3.73, m	14,20 Ha, 1.98 14,20 Hb, 2.59 16,18Ha, 1.59 16,18Ha, 2.22	55.73	CH	15, 19H 3.73
16, 18	^{2}Ha	1.59, m	15,19 H, 3.73 16,18Hb, 2.22 14,20 Ha, 1.98 17Ha, 1.95 17Hb, 1.59	24.60	CH_2	16,18H 1.69
	^{2}Hb	2.22, m	15,19 H, 3.73 14,20 Ha, 1.98 16,18Ha, 1.59 17Ha, 1.94 17Hb, 1.59			16,18H 2.22
17	Ha	1.95, m	16,18Ha, 1.59 16,18Hb, 2.22 17Hb, 1.59	13.08	CH_2	17Ha 1.95

	Hb	1.59, m	16,18Ha, 1.59 16,18Hb, 2.22 17Ha, 1.95			17Hb 1.59
22	^{2}H	2.94, s	-	38.86	CH_3	22H 2.94

s, singlet; d, doublet; t, triplet; m, multiplet; dd, double doublet, Refer structures (**Fig. 8**) for numbering.

TABLE 2: NMR ASSIGNMENTS OF OXIDATIVE DEGRADATION IMPURITY

Position [a]	^{1}H	δ (ppm), J Hz	gDQCOSY	^{13}C, δ (ppm)	DEPT	gHSQC
3	-	-	-	138.13	-	-
4	^{1}H	8.16, d(8.5)	5H, 7.26	124.37	CH	4H, 8.16
5	^{1}H	7.26, t(8.5)	4H, 8.16 6H, 7.44	124.31	CH	5H, 7.26
6	^{1}H	7.44, t(7.0)	5H, 7.26 7H, 7.57	128.54	CH	6H, 7.44
7	^{1}H	7.57, d(8.0)	6H, 7.44	111.44	CH	7H, 7.57
8	-	-	-	143.16	-	-
9	-	-	-	123.28	-	-
10	^{3}H	4.12, s	-	36.85	CH_3	10H, 4.12
11	-	-	-	165.34	=C=	-
13	^{1}H	4.39, t	14,20 Ha, 2.37 14,20 Hb, 2.96	38.37	CH	13H, 4.39
14,20	^{2}Ha	2.37, m	13 H, 4.39 14,20 Hb, 2.96 15,19 H, 3.29 16, 18 Ha, 1.89 16,18 Hb 2.60	32.90	CH_2	14,20Ha 2.37
	^{2}Hb	2.96, m	13 H, 4.39 14,20 Ha, 2.37 15,19 H, 3.29			14,20Hb 2.96
15,19	^{2}H	3.29, t	14,20 Ha, 2.37 14,20 Hb, 2.96 16,18Ha, 1.89 16,18Ha, 2.60	55.17	CH	15, 19H 4.40
16,18	^{2}Ha	1.89, dd (14.5,5.5)	15,19 H, 3.29 16,18Hb, 2.60 14,20 Ha, 2.37 17Ha, 2.34 17Hb, 1.65	26.61	CH_2	16,18H 1.89
	^{2}Hb	2.60, m	15,19 H, 3.29 14,20 Ha, 2.37 16,18Ha, 1.89 17Ha, 2.34 17Hb, 1.65			16,18H 2.60
17	Ha	2.34, m	16,18Ha, 1.89 16,18Hb, 2.60 17Hb, 1.65	13.78	CH_2	17Ha 2.34
	Hb	1.65, m	16,18Ha, 1.89 16,18Hb, 2.60 17Ha, 2.34			17Hb 1.65
22	^{2}H	3.62, s	-	67.41	CH_3	22H 3.62

s, singlet; d, doublet; t, triplet; m, multiplet; dd, double doublet, Refer structures (**Fig. 8**) for numbering.

Validation of Analytical Method:

Specificity: Specificity of the analytical method is termed as an ability to measure the analyte response in the presence of its potential impurities [25]. Specificity and stability indicating capability of the new developed method is demonstrated by forced degradation studies and also no interference at the retention time of Granisetron and all known impurities. Significant degradation was observed when Granisetron hydrochloride was subjected to oxidation and acid whereas no degradation was observed when subjected to base, thermal and UV conditions. The peak homogeneity of main peak is passed in each stressed sample was examined

individually by peak purity testing using PDA detector [26]. Additionally the LCMS analysis was performed for all the stressed samples and mass spectra was scanned for main peak under positive Electrospray ionization (ESI +ve mode) and no significant change in mass spectra was found across main peak. The degradation products obtained during the stress study were observed to be well separated from each other and from the main peak which proved that the adopted method is specific.

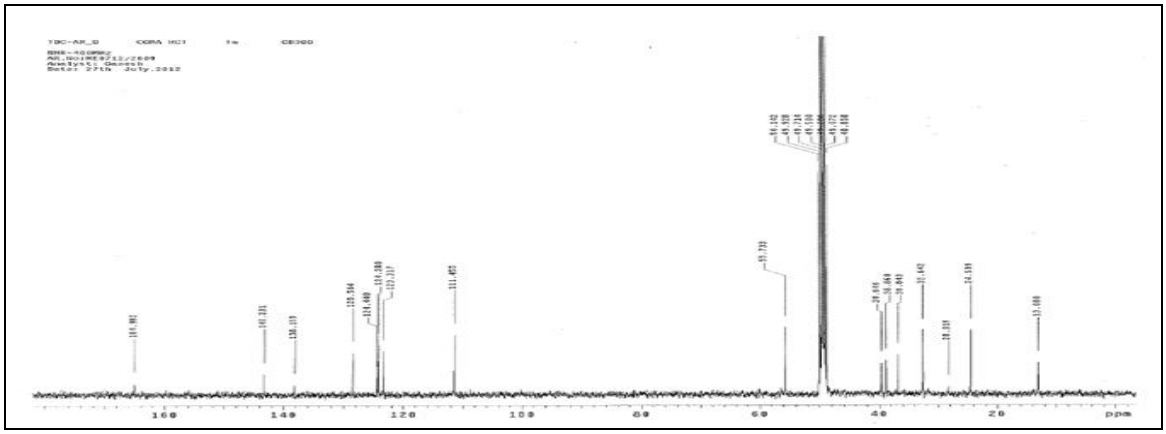

FIG. 10: C13 NMR SPECTRUM OF GRANISETRON HCL

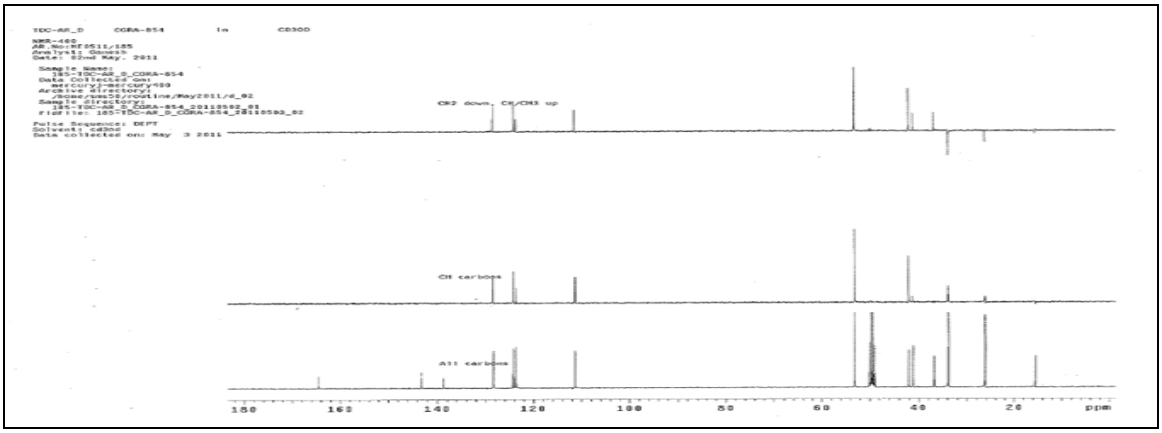

FIG. 11: DEPT NMR SPECTRUM OF GRANISETRON HCL

Repeatability: Repeatability of the test method was performed by injecting six individual preparations of Granisetron Hydrochloride (1.0 mg/ml) spiked with 0.15% of individual impurities along with oxidative degradation impurity with respect to analyte concentration. Method precision was obtained by determining the % R.S.D. of area response for each impurity was calculated and tabulated in **Table 2**. The intermediate precision results also tabulated in **Table 2** by doing the same exercise using different analyst, different instrument and different day in the same laboratory. The % R.S.D. of test results obtained for method precision and intermediate precision studies were within 10.0% shows appreciable precision of the method.

Limit of Detection and Limit of Quantification: The LOD and LOQ for all Granisetron Hydrochloride, related substances and oxidative deg impurity in the developed method was established by injecting a known concentration solutions prepared by series of dilutions to achieve the peak height criteria of signal-to-noise ratio of 3:1 and 10:1, respectively. The limit of detection and limit of quantification established concentration values were tabulated in **Table 3**. Precision at limit of quantification study was also carried at the LOQ level by injecting six independent preparations of all impurities and drug substance and the % R.S.D. of the area response of each peak was calculated and found to be less than 10.0 which complies with the ICH guidelines requirement [25].

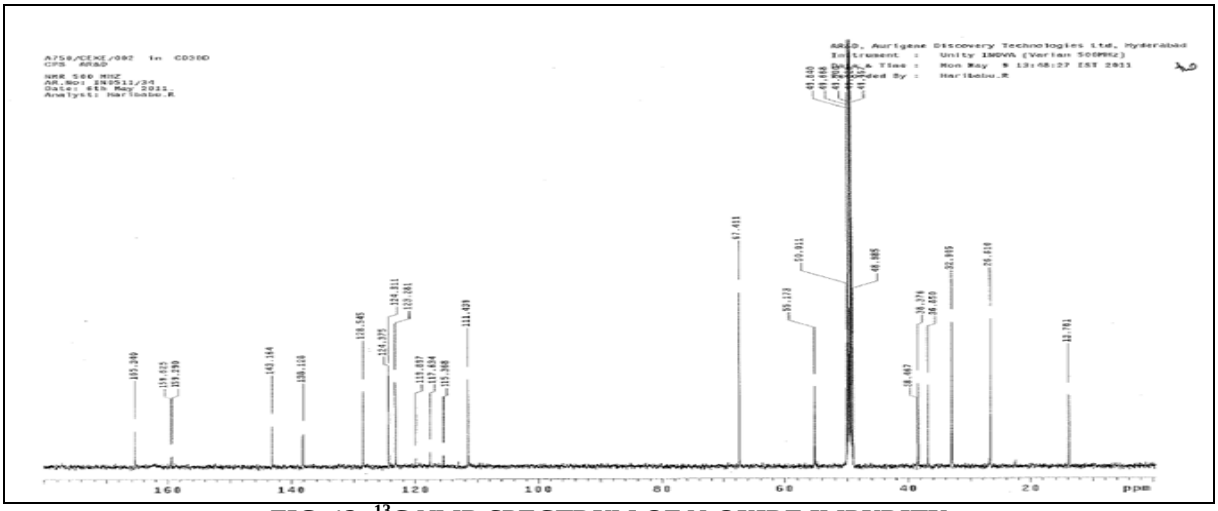

FIG. 12: ^{13}C NMR SPECTRUM OF N-OXIDE IMPURITY

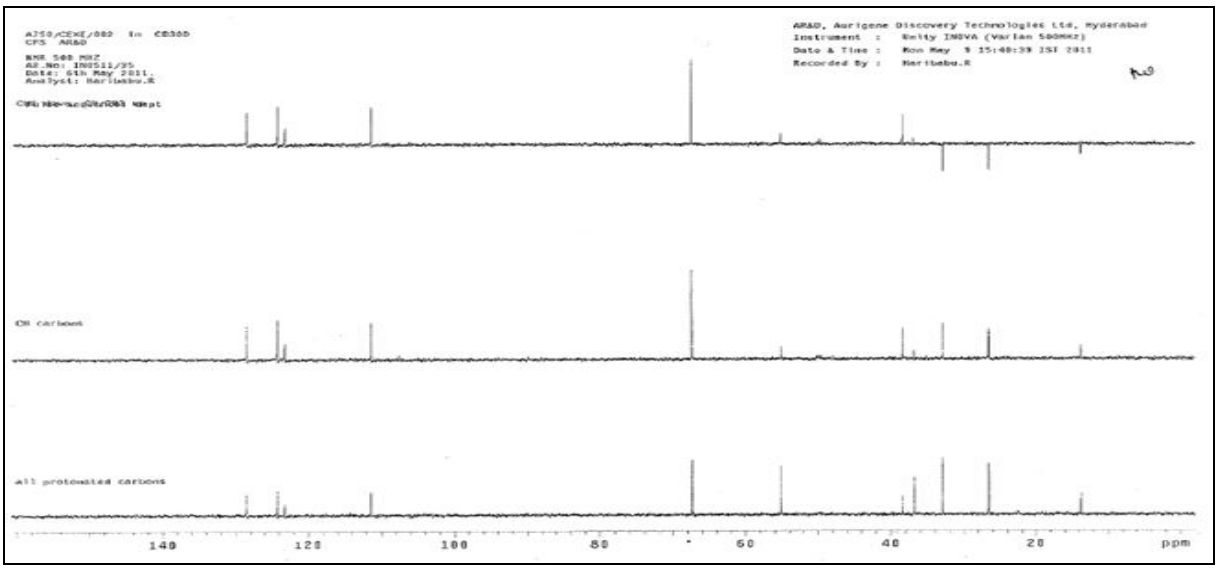

FIG.13: DEPT NMR SPECTRUM OF N-OXIDE IMPURITY

Linearity and Accuracy: The linearity of a developed analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in that sample within a given range. Linearity test solutions were prepared diluting the Impurity stock solution at five concentration levels from LOQ to 200% with respect to the impurities specification level of 0.15% (*i.e.* LOQ, 0.075% w/v, 0.10% w/v, 0.15% w/v, and 0.20% w/v) relative to sample concentration (1.0mg/mL) and injected in to the HPLC as per the chromatographic condition in Section 2. The calibration curve was plotted the peak areas of individual peak versus its corresponding concentration. The resulted slope, intercept and correlation coefficient of determination (r^2) of the plot are presented in **Table 3** shows the method is linear to the specified range. Recovery experiments were carried out to calculate the accuracy by spiking known amount of individual impurities at 0.075, 0.15 and 0.225% with Granisetron hydrochloride with respect to the sample concentration (1mg/mL). Each preparation was analyzed in triplicate (n = 3) and percent recovery was calculated from the slope and *Y*-intercept of the calibration curve.

The % recovery for the impurities were calculated obtained is tabulated in **Table 2**.

Robustness: No significant variation on system suitability parameters was observed such as RSD, tailing factor, or the theoretical plates of Granisetron hydrochloride when small but deliberate changes were made to chromatographic conditions such as change in flow rate, buffer

composition detector wavelength, column temperature and pH of the buffer. The results are presented in **Table 3**, along with the system suitability parameters of normal conditions. Thus, the method was found to be robust with respect to variability in applied conditions.

TABLE 3: METHOD VALIDATION DATA FOR GRA.HCL, OXIDATIVE DEG IMPURITY AND OTHER RELATED SUBSTANCES

Validation parameter Results									
Linearity	**Parent**	**Oxi deg impurity**	**Imp A**	**Imp B**	**Imp C**	**Imp E**	**Imp F**	**Imp G**	**Imp H**
Calibration Intervals (n=3)	5	5	5	5	5	5	5	5	5
Calibration range (%, w/w)	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30	0.05, 0.1, 0.15, 0.20, 0.30
Slope	188.445	98.476	180.451	106.701	103.571	59.2205	110.61	66.289	129.703
Intercept	1.10154	-0.3532	0.47106	-0.5489	-0.3596	-1.4966	-0.1639	0.109	1.34191
Correlation Coefficient (r2)	0.99959	0.99894	0.99921	0.99943	0.99951	0.99793	0.99972	1.000	0.99993
LOD and LOQ									
Detection limit (%, w/w)	0.012	0.015	0.015	0.013	0.012	0.025	0.009	0.007	0.005
Quantification limit (%, w/w)	0.037	0.047	0.045	0.039	0.036	0.075	0.027	0.020	0.014
Repeatability									
Precision at QL Concentration (%, w/w) (n = 6, % R.S.D.)	0.14	0.53	2.27	0.58	1.26	1.75	2.77	1.78	0.75
Standard precision* Concentration (%, w/w) (n = 6, % R.S.D.)	0.54	0.76	0.67	0.52	0.86	0.83	0.49	0.83	0.73
Intermediate precision# Concentration (%, w/w) (n = 6, % R.S.D.)	0.09	0.22	2.89	8.74	0.35	7.12	2.73	4.18	2.13
Overall (n = 12) % R.S.D	0.32	0.49	1.78	4.63	0.60	3.97	1.61	2.51	1.43
Accuracy 50%, 100%, 150%									
Average recovery (%)		95.5	95.8	88.3	107.6	98.8	110.0	85.6	86.2

* = Analyst 1, Column 1, HPLC 1, and Day 1. # = Analyst 2, Column 2, HPLC 2, and Day 2. R.S.D. = Relative standard deviation.

CONCLUSION: In this paper, the oxidative degradation impurity observed in stress study of Granisetron Hydrochloride by HPLC-UV method was identified by LC–MS, isolated by preparative LC and fully characterized by LC-MS, HR-MS and NMR spectral data. The quick and efficient and well-defined stability indicating HPLC method for the measurement of all the related impurities and patent has been described in the above section. The method has apparent advantages due to high separation efficiency and sensitivity over those previously reported such as pharmacopeial and other similar methods. Though the stress study shows the immediate formation of oxidative degradation impurity, several batches of Granisetron Hydrochloride manufactured was tested to check the presence of oxidative impurity and does not show the impurity even at detection limit.

ACKNOWLEDGEMENT: The authors wish to thank Dr. Vilas Dahanukar, Vice president, Dr. Reddys Laboratories limited and the management

of Dr. Reddys Laboratories limited for the constant support and encouragement. We would like to acknowledge the colleagues of our department for their co-operation in carrying out this work.

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

NOTE: DRL-IPDO communication No.: IPDO IPM-00500 has been allotted for this research article.

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How to cite this article:
Balakumaran K, Rajana N, Babu JM and Jayashree A: Development of novel stability indicating HPLC method and characterization of oxidative degradation impurity in Granisetron drug substance. Int J Pharm Sci Res 2017; 8(11): 4591-03.doi: 10.13040/IJPSR.0975-8232. 8(11).4591-03.

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