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# Development and application of a validated stability-indicating HPLC method for simultaneous determination of granisetron hydrochloride, benzyl alcohol and their main degradation products in parenteral dosage forms

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ABSTRACT

A simple, rapid and sensitive reversed phase high performance liquid chromatographic method using photodiode array detection was developed and validated for the simultaneous determination of granisetron hydrochloride, benzyl alcohol, 1-methyl-1H-indazole-3-carboxylic acid (the main degradation product of granisetron) and benzaldehyde (the main degradation product of benzyl alcohol) in granisetron injections. The separation was achieved on Hypersil BDS C8 (250 mm × 4.6 mm i.d., 5 μm particle diameter) column using a mobile phase consisted of acetonitrile:0.05 M $KH_2PO_4$:triethylamine (22:100:0.15) adjusted to pH 4.8. The column was maintained at 25 °C and 20 μL of solutions was injected. Photodiode array detector was used to test the peak purity and the chromatograms were extracted at 210 nm. Naphazoline hydrochloride was used as internal standard. The method was validated with respect to specificity, linearity, accuracy, precision, limit of quantitation and limit of detection. The validation acceptance criteria were met in all cases. Identification of the pure peaks was carried out using library match programmer and wavelengths of derivative optima of the spectrograms of the peaks. The method was successfully applied to the determination of the investigated drugs and their degradation products in different batches of granisetron injections. The method was proved to be sensitive for the determination down to 0.03 and 0.01% of granisetron degradation product and benzaldehyde, respectively, which are far below the compendia limits for testing these degradation products in their corresponding intact drugs.


## 1. Introduction

Intravenous chemotherapy in patients suffering cancer sometimes requires the co-administration of several medications to prevent the sever side effects that are enough to deter patients from continuing therapy [1]. Granisetron hydrochloride (GRS) – 1-methyl-N-[(1R, 3r, 5S)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-1H-indazole-3-carboxamide (Fig. 1) – is a selective 5-$HT_3$ receptor antagonist that has proved to be effective in the prevention and treatment of chemotherapy-induced nausea and vomiting following intravenous administration [2]. GRS is available as single dose-preservative free injections and multi-dose vials that contain benzyl alcohol (BZA, Fig. 1) as preservative [3].

Several HPLC methods have been reported for the determination of GRS in pharmaceutical preparations and biological fluids. These include the determination of GRS in tablets [4] and in intravenous infusion containing doxorubicin [5] using UV detector. The HPLC methods used for the determination of GRS in human plasma included the use of fluorescence [6–12], UV [13], mass spectrometric [14–19] and electrochemical [11] detectors. The BP [20] describes an HPLC method for the determination of GRS in bulk i.e. raw material but its pharmaceutical preparations are not official in both BP [20] and USP [21].

Being an amide, solutions containing GRS may undergo hydrolytic degradation reaction (Fig. 2) to give 1-methyl-1H-indazole-3-carboxylic acid (GRSD, I) which is a UV-absorbing compound and (1R, 3r, 5S)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine (II). The reaction is catalyzed by both acids and bases. Benzyl alcohol (BZA) preservative undergoes oxidative degradation to benzaldehyde (BZD). The British pharmacopoeia monograph for benzyl alcohol stated that benzyl alcohol intended for use in the formulations of parenteral dosage form contains not more than 0.05% of benzaldehyde [20]. The limit of GRSD in GRS raw material must not exceed 0.1% [20].

The aim of the present study is to develop and validate a simple and sensitive HPLC method for the simultaneous determination of GRS, BZA and their corresponding main degradation products i.e. GRSD and BZD. Thereafter, the method has been successfully

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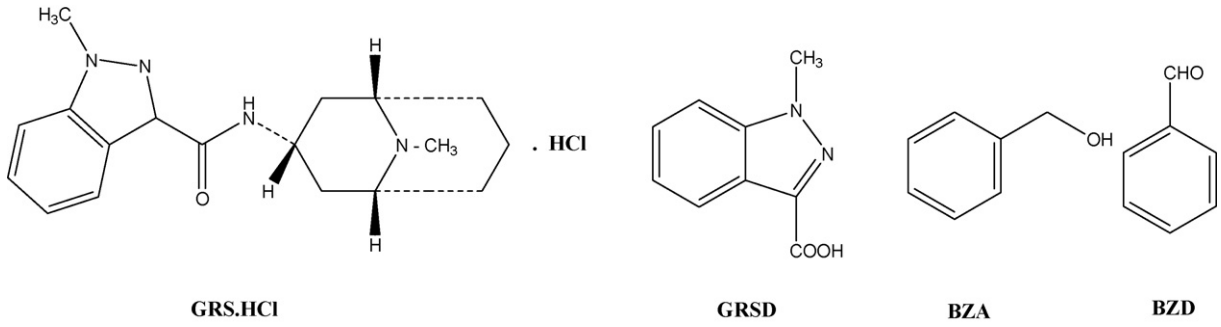


**Fig. 1.** Structural formulae of the investigated compounds.

applied to the determination of the fore-mentioned drugs and degradation products in vials containing GRS and BZA.

## 2. Experimental

### 2.1. Materials and reagents

Granisetron hydrochloride (GRS), pharmaceutical grade (Carbo Mer, Inc., San Diego, USA) was used and certified to contain 99.96%. Naphazoline hydrochloride (NF) was obtained form Sigma–Aldrich Co. (USA) and certified to contain 99.94%. Benzyl alcohol (BZA) and benzaldehyde (BZD) were of Lichrosolv grade (Merck, Darmstadt, Germany) and certified to contain 99.96 and 99.97%, respectively. Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Triethylamine, orthophosphoric acid (85%, w/v) and potassium dihydrogen phosphate were of analytical grade (Merck, Darmstadt, Germany). HPLC water was generated in-house by reverse osmosis using a Milli-Q plus system (Millipore, Bedford, MA, USA). Granisetron degradation product: 1-methyl-1H-indazole-3-carboxylic acid (GRSD) was prepared and its structure was elucidated in our laboratories. Different brands of GRS single dose, preservative free injections (labeled to contain 1.12 mg of GRS.HCl per mL) and multi-dose vials (labeled to contain 1.12 mg of GRS.HCl and 10 mg of BZA per mL) were obtained from local market.

### 2.2. Instrumentation

Waters Alliance HPLC system consisted of a solvent management system 2695, photodiode array detector (DAD) 2998, thermostatically controlled column apartment and auto-sampler with a 250 μL loop. The control of HPLC system and data processing were performed by Empower®2 software (All Waters, Milford, MA, USA).

The infrared spectra were recorded using a Shimadzu 408 infrared spectrometer operated in transmission mode with a nominal resolution of 10 $cm^{-1}$. The samples were prepared as KBr discs.

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 200 MHz NMR spectrometer. The sample was dissolved in deuterated DMSO and the $^1H$ NMR spectra were recorded using tetramethylsilane as internal standard.

The mass spectra were performed on Shimadzu GC-MS model VG-TRO 1000 operated on EI mode at 70 eV.

The pH measurements were carried out using Metrohm pH meter 744 (Metrohm Ltd., Switzerland).

### 2.3. Preparation of granisetron degradation product

200 mg of GRS.HCl was mixed with 25 mL of 0.5 M NaOH and the mixture was refluxed for 24 h where complete hydrolytic degradation of GRS was achieved (disappearance of the peak of GRS on the HPLC chromatogram of reaction mixture). The solution was extracted with diethylether (2× 50 mL) and the ether extract was rejected. The aqueous solution was acidified using 2 M HCl solution and extracted with diethylether (3× 50 mL). The ether extract was washed with water and dried using anhydrous sodium sulphate. The ether was distilled and the residue was dissolved in hot ethanol (25 mL), filtered into a clean crystallization flask, concentrated to a small volume and left for crystallization. The crystals were separated, dried under vacuum, its purity was confirmed using either HPLC-DAD or TLC and then its structure was elucidated using IR, NMR and MS.

### 2.4. Chromatographic conditions

The chromatographic separation was performed on a Hypersil C8 BDS column (250 mm × 4.6 mm, 5 μm; Thermo Electron Corporation, USA). The mobile phase consisted of acetonitrile–0.05 M $KH_2PO_4$–triethylamine (22:100:0.15) adjusted to pH 4.8 using diluted solution of $H_3PO_4$. The mobile phase was filtered through 0.45 μm nylon membrane filter, degassed ultrasonically before use and pumped in isocratic mode at a flow rate of 2 mL $min^{-1}$. The

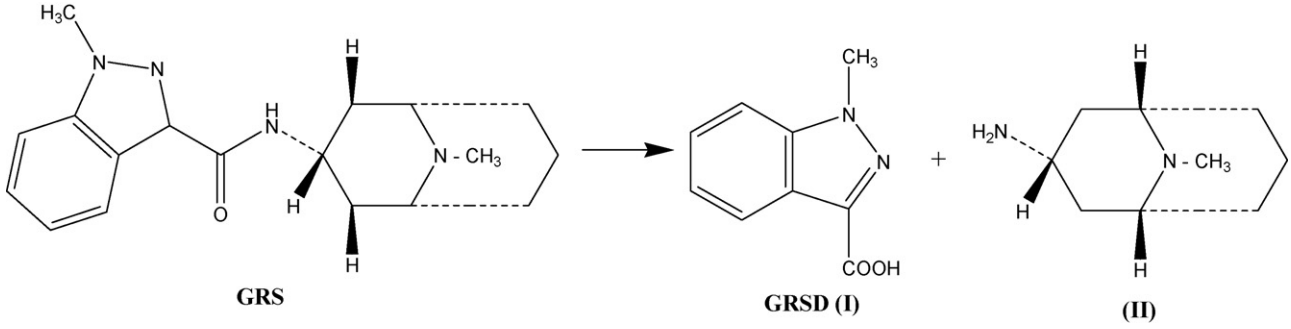


**Fig. 2.** Scheme of hydrolytic degradation of granisetron.

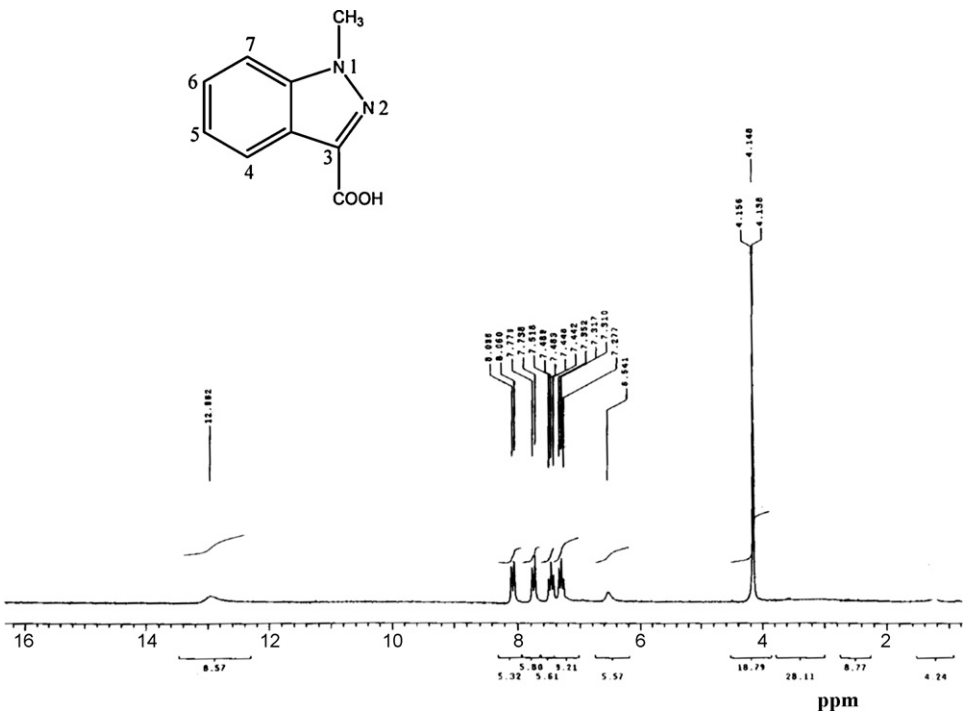


**Fig. 3.** PMR spectrum of 1-methyl-1H-indazole-3-carboxylic acid.

column was operated at ambient temperature. The injection volume was 20 μL using auto-sampler and the chromatographic run time was 15 min per sample. The chromatograms were extracted at 210 nm. The PDA detector was set at wavelength range 190–330 nm and sampling rate at 10 points/sec with spectral resolution 1.2 nm. The UV spectra i.e. spectrograms were smoothed at level 8 and their derivative spectra at level 20. The 3D plot of the chromatogram was adjusted at 15 angle and 40-degree height to view all peaks. The purity parameters included 100% active peak region and auto-threshold with purity pass at level 1. PDA library search was set at threshold degree 5 and level 2 in depth. A search threshold criterion of the noise due to instrument and solvent was at angle 1. The retention time search was at ±5% ($t_r$ window %). The wavelength search (window λ) was at ±2 nm.

### 2.5. Preparation of standard solutions

A mixture of acetonitrile–water (1:1, v/v) was used as a solvent for the preparation of the stock standard solutions of the investigated compounds. GRS.HCl and BZA stock standard solutions were prepared by accurately weighing 56 and 1000 mg separately into 50 mL volumetric flasks then dissolving and diluting to volume with the solvent. GRSD, BZD and NF stock standard solutions were prepared by accurately weighing 25 mg of each separately into 25 mL volumetric flasks, dissolving and then diluting to volume with the solvent.

A standard mixture solution for the method development and optimization was prepared by the appropriate dilution of the forementioned stock standard solutions with the mobile phase as diluent to yield a solution containing 0.66 μg mL$^{-1}$ of GRS.HCl, 40 μg mL$^{-1}$ of BZA and 10 μg mL$^{-1}$ of each of GRSD, BZD and NF.

A working standard solution for the determination of intact drugs was prepared by appropriate dilution of the stock standard solutions with the mobile phase as diluent to yield a solution containing 22.4 μg mL$^{-1}$ of GRS.HCl, 200 μg mL$^{-1}$ of BZA, 4.0 μg mL$^{-1}$ of GRSD and 10 μg mL$^{-1}$ of each of BZD and NF.

A working standard solution for the determination of degradation products was prepared by the appropriate dilution of the stock standard with the mobile phase as diluent to yield a solution containing 560, 0.40, 5000 and 1.6 μg mL$^{-1}$ of GRS, GRSD, BZA and BZD, respectively. The % (w/w) of GRSD relative to intact GRS was 0.07 while the % (w/w) of BZD relative to BZA was 0.032.

Out of the prepared standard solutions, portions were filtered through PVDF membrane filters (0.45 μm). The filtrates were placed in HPLC vials and 20 μL aliquots were injected automatically into HPLC system.

### 2.6. Assay of pharmaceutical preparations

For the determination of the intact drugs i.e. GRS and BZA, A 2 mL aliquot of the injections (labeled to contain 1.12 mg of GRS.HCl and 10 mg of BZA per mL) was transferred into a 100 mL volumetric flask. 1 mL of stock standard NF solution (1 mg mL$^{-1}$) was added and the contents were diluted to volume with the mobile phase.

For the determination of the degradation products i.e. GRSD and BZD, an aliquot of the injection solution equivalent to 5.6 mg of GRS.HCl was transferred into a 10 mL volumetric flask and diluted to volume with the mobile phase.

Out of the prepared solutions, portions were filtered through PVDF membrane filters (0.45 μm). The filtered solutions were placed in HPLC vials and 20 μL aliquots were injected automatically into HPLC system concurrently with the appropriate standard solutions. The peak area ratio (PAR) was used for the determination of intact drugs while the peak area was used for the determination of degradation products.

### 2.7. Validation procedure

The proposed method was validated according to USP requirements [21] and ICH guidelines [22].

The specificity and selectivity of the HPLC method was tested by the comparison of standard mixture, sample solution and placebo chromatograms. DAD also supports the selectivity of the method through peak purity assessment using purity plots and match plots for each compound in both sample and standard solutions. In addition, the spectral homogeneity of the resolved peaks was

further checked by the comparison of the UV absorption spectra extracted at different time intervals across the eluted peak i.e. spectrograms and their corresponding derivative (1st, 2nd, 3rd and 4th) spectra. Another test for the selectivity of the HPLC-DAD method was the comparison of the mathematically transformed data of the spectrograms of the test compound with the corresponding spectrochromatogram of standard compound. The mathematical transformation included the construction of log *A* versus the wavelength plots [23].

The linearity of the HPLC detector response with the concentrations of the intact drugs was evaluated using 6 concentration levels between 50 and 150% of the working concentration of the method. Stock standard solutions of GRS and BZA were diluted with the mobile phase to concentration ranges of 11.2–33.6 and 100–300 $\mu g\,mL^{-1}$, respectively. Each of the prepared solutions contained 10 $\mu g\,mL^{-1}$ of NF as internal standard. For the degradation products i.e. GRSD and BZD, the linearity was tested by the dilution of their stock standard solutions with the mobile phase to concentration ranges of 0.03–2.0 and 0.01–10.0 $\mu g\,mL^{-1}$, respectively. Triplicate injections were made for each concentration. Peak area ratios (PAR) for intact drugs and peak area for the degradation products were plotted versus the concentration for construction of calibration curves and regression analysis.

The accuracy of the method for the determination of intact drugs i.e. GRS.HCl and BZA was calculated as the percent recoveries of known added amounts of their standards to the placebo solution. The accuracy was evaluated in triplicate using three concentration levels: 80, 100 and 120 of the analytical method concentration. To assess the accuracy for the determination of degradation products, laboratory prepared mixtures containing 560 $\mu g\,mL^{-1}$ of GRS.HCl and 5000 $\mu g\,mL^{-1}$ of BZA were spiked with different concentrations of GRSD and BZD and their percent recoveries were calculated.

To measure the repeatability, 10 solutions were prepared at 100% level of the analytical method concentration and the results were expressed as RSD% for the 10 determinations. The intra-day precision was evaluated by the analysis of three different concentrations: 80, 100 and 120% of the analytical concentration, in

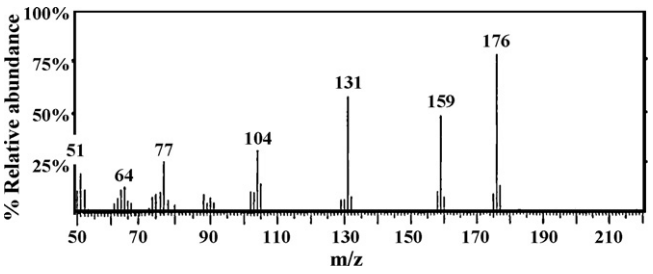


**Fig. 4.** Mass spectrum of 1-methyl-1H-indazole-3-carboxylic acid.

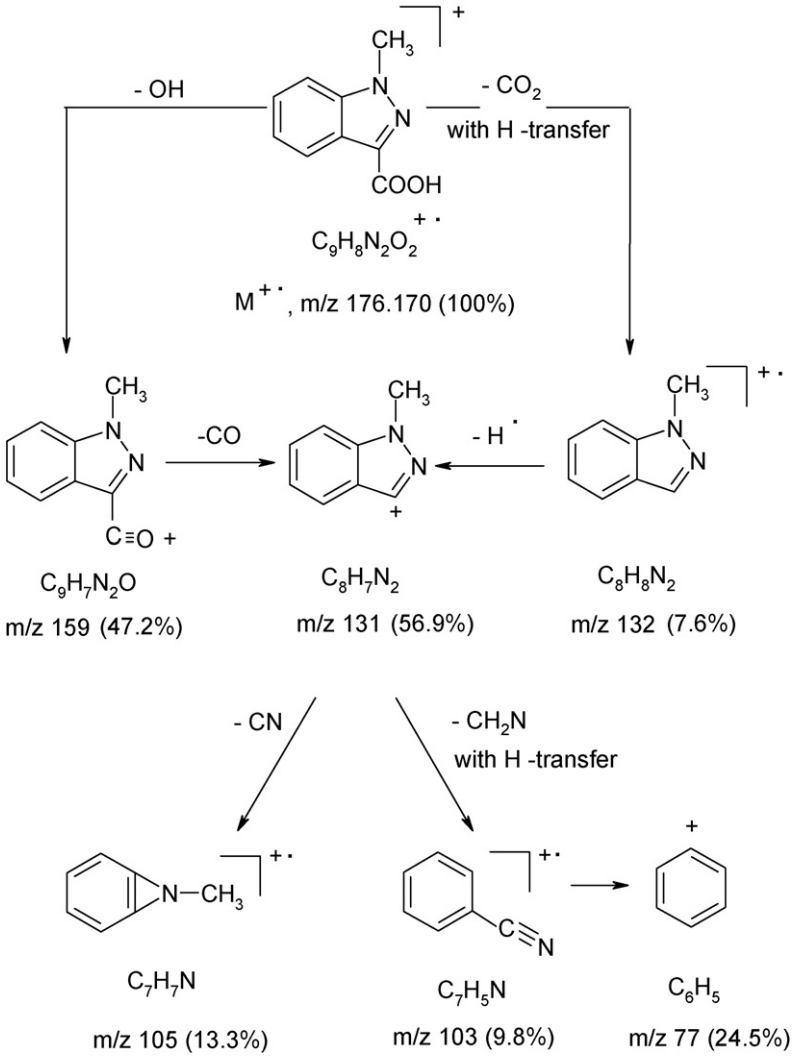


**Fig. 5.** Fragmentation pattern of 1-methyl-1H-indazole-3-carboxylic acid.

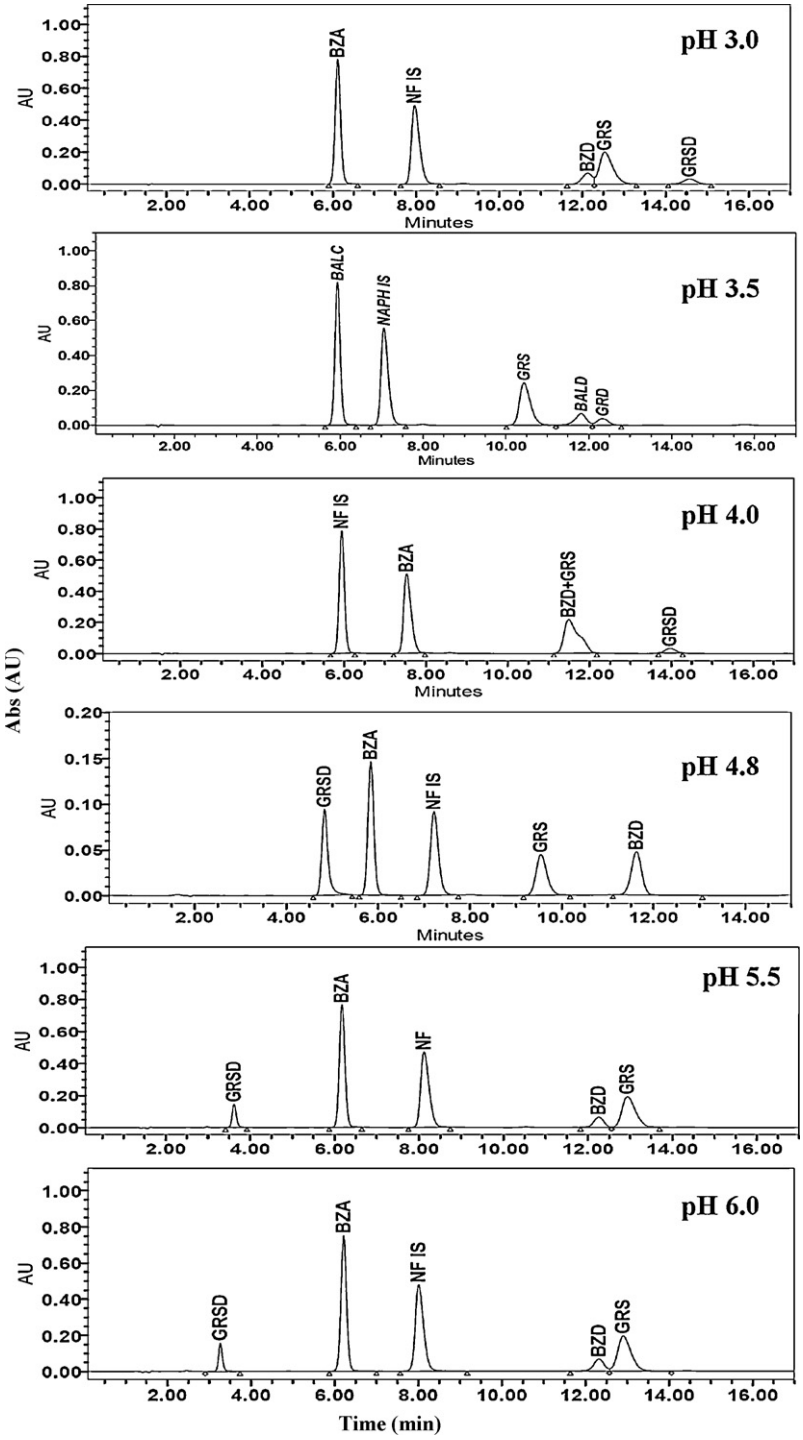


**Fig. 6.** Representative chromatograms of the effect of pH of the mobile phase on chromatographic behaviour of the investigated compounds.

triplicate on the same day. The inter-day precision studies were performed by repeating the studies on three consecutive days. The data of intermediate precision were evaluated as RSD% for the determinations.

The robustness of the method was performed by testing its capacity to be unaffected by a slight change of the percent of organic modifier (±2% absolute) and pH (±0.1 unit) of the mobile phase. The results were evaluated in terms of capacity factor and resolution.

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the residual standard deviation of the regression line ($\delta$) of the calibration curve and its slope ($S$) in accordance to the following equations: LOD = 3.3($\delta$/$S$) and LOQ = 10($\delta$/$S$).

### 2.8. System suitability

System suitability test (SST) parameters were performed during the development and optimization of method as well as through the validation procedure. In addition, SST parameters must be checked to ensure that the system is working correctly during the analysis. The test was performed by injecting the standard mixture in triplicate and the parameters were calculated using Empower®2

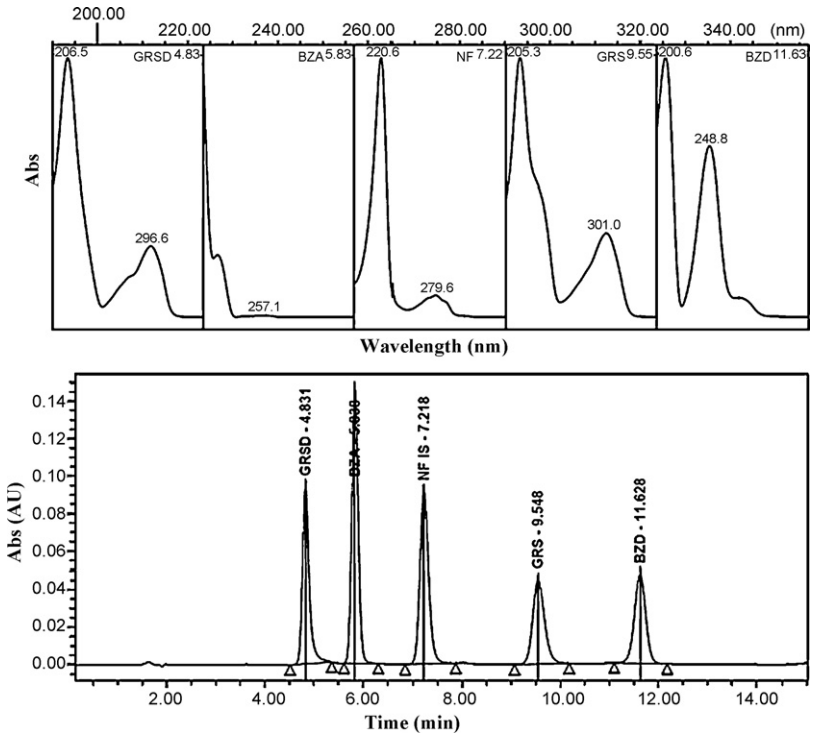


**Fig. 7.** Spectrum index plot obtained from chromatography of a mixture containing GRS.HCl (0.66 μg mL$^{-1}$), BZA (40 μg mL$^{-1}$), GRSD (10 μg mL$^{-1}$), BZD (10 μg mL$^{-1}$) and NF.HCl (10 μg mL$^{-1}$) internal standard.

software as reported by United States pharmacopeia and ICH guidelines. SST parameters include capacity factor ($k'$), selectivity factor ($\alpha$), resolution (Rs), column efficiency (number of theoretical plates, $N$) and tailing factor.

## 3. Results and discussion

### 3.1. Structure assignment of the main degradation product of granisetron hydrochloride

The assignment of the degradation product as 1-methyl-1H-indazole-3-carboxylic acid was based on its IR, PMR and mass spectral data of a purified specimen, separated from the degradation reaction.

The IR (KBr) of the degradation product was different from that of the parent drug as it showed a broad band at 3250–2000 cm$^{-1}$ corresponding to the carboxylic –OH and a strong band at 1695 cm$^{-1}$ due to the carboxylic –C=O, in addition to the mixed absorptions of C=N, C=C and aromatics between 1530 and 1320 cm$^{-1}$. Whereas, the intact drug showed the amide –NH as strong absorption band with multiple splits between 3500 and 2000 cm$^{-1}$ and the carbonyl-amide as a sharp strong band at 1650 cm$^{-1}$.

The PMR (DMSO-$d_6$) (Fig. 3) displayed the $CH_3$ a singlet at $\delta$ 4.148 ppm, the four aromatic protons as two triplets at $\delta$ 7.313 ppm for C-5-H and at $\delta$ 7.483 for C-6-H, and two doublets at $\delta$ 7.6 ppm for C-7-H and at $\delta$ 7.8 ppm for C-4-H. The spectrum also showed the carboxylic –OH proton as singlet at $\delta$ 12.96 ppm.

The mass spectrum (Fig. 4) revealed the molecular ion peak ($M^{+\bullet}$) at $m/z$ 176 (100%) which constitutes the base peak of the spectrum. The relative intensities % of M+1 ($m/z$ 177) and M−1 ($m/z$ 175) peaks were 13.1 and 9.1, respectively. In addition, the spectrum showed characteristic fragmentation signals, which are indicative of the assigned structure. The predominant fragmentation pattern is illustrated in Fig. 5.

### 3.2. Method development and optimization

During the method development, different parameters were manipulated to obtain an acceptable resolution between the components and to satisfy the HPLC system suitability. These parameters included the use of different types of C8 columns, ratio of organic modifier/phosphate buffer and the pH of the mobile phase. The preliminary work was conducted using three different brands of reversed phase C8 columns i.e. Zorbax C8 (250 mm × 4.6 mm, 10 μm), Symmetry C8 (150 mm × 3.9 mm, 5 μm) and Hypersil BDS C8 (250 mm × 4.6 mm, 5 μm) using a mobile phase consisted of acetonitrile–phosphate buffer in different ratios. Of the stationary phases experienced, Hypersil BDS C8 gave results, in terms of peak shape, resolution and analysis time better than the other two columns. The combined effects of organic modifier fraction and the pH of the mobile phase on chromatographic performance of GRS, BZA, GRSD and BZD were investigated. The effect of percent acetonitrile (ranged from 12 to 24% in the mobile phase) on the retention parameters, utilizing Hypersil C8 BDS stationary phase, was investigated. It was concluded that 18% acetonitrile in the mobile phase resulted into suitable retention and optimal resolution between the four investigated components (Table 1). Higher percent acetonitrile disturbed the resolution between BZA and GRS while lower percent acetonitrile decreased their resolution to unacceptable values. The effect of pH of the mobile phase on the chromatographic performance revealed that pH 4.8 was the most suitable (Fig. 6). Adjusting the pH to lower or higher values resulted into bad resolution between GRS and BZD. Triethylamine (0.12%) was added to minimize the peak tailing. Consequently, a mobile phase composed of acetonitrile–$KH_2PO_4$–TEA (22:100:0.15) adjusted to pH 4.8 flowing at 2 mL min$^{-1}$ was used for the successful separation of GRS, BZA, GRSD and BZD in their mixtures using Hypersil BDS C8 column (250 mm × 4.6 mm, 5 μm) as stationary phase. NF was used as internal standard as its peak eluted symmetrically between BZA and GRS with acceptable resolution. The spectrum index of the chromatogram was plotted (Fig. 7).

**Table 1**
Effect of the % acetonitrile in the mobile phase on chromatographic behaviour of a mixture containing GRS, BZA, GRSD and BZD.

| % ACN | Chromatographic parameter | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Capacity factor ($k'$) | | | | Number of theoretical plates ($N$) | | | | Tailing factor ($T$) | | | | Resolution (Rs) | | | Selectivity ($\alpha$) | | |
| | GRSD | BZA | GRS | BZD | GRSD | BZA | GRS | BZD | GRSD | BZA | GRS | BZD | GRSD and BZA | BZA and GRS | GRS and BZD | GRSD and BZA | BZA and GRS | GRS and BZD |
| 12 | 3.14 | 4.03 | 9.28 | 10.58 | 8558 | 12,159 | 13,041 | 7185 | 0.99 | 1.07 | 1.63 | 1.65 | 4.96 | 1.95 | 2.94 | 1.28 | 2.30 | 1.14 |
| 16 | 2.56 | 3.18 | 6.69 | 7.54 | 8324 | 11,867 | 6907 | 13,076 | 1.02 | 1.06 | 1.63 | 0.967 | 4.08 | 13.86 | 2.59 | 1.24 | 2.12 | 1.13 |
| 18[a] | 2.22 | 2.90 | 5.36 | 6.75 | 7668 | 11,223 | 5866 | 13,349 | 1.24 | 1.16 | 1.14 | 1.00 | 5.21 | 11.09 | 5.26 | 1.35 | 1.89 | 1.29 |
| 20 | 1.23 | 2.04 | 2.76 | 5.11 | 7479 | 11,055 | 6551 | 12,814 | 1.25 | 1.16 | 1.56 | 1.04 | 7.55 | 4.94 | 11.88 | 1.66 | 1.36 | 1.85 |
| 24 | 0.72 | 1.40 | 1.48 | 3.64 | 7564 | 8541 | 3820 | 12,965 | 1.15 | – | – | 1.03 | 8.92 | – | 13.58 | 1.94 | – | 2.46 |

(Rs) and ($\alpha$) are the resolution and selectivity, respectively, between two consecutive peaks.
[a] HPLC method conditions.

**Table 2**
System suitability test results.

| Name | Selectivity[a] ($\alpha$) | Capacity factor ($k'$) | Resolution[a] (Rs) | EP plate count ($N$) | Symmetry factor ($T$) |
|---|---|---|---|---|---|
| GRSD | – | 2.22 | – | 7668 | 1.24 |
| BZA | 1.30 | 2.89 | 5.21 | 11,223 | 1.16 |
| NF.IS | 1.32 | 3.81 | 5.00 | 8925 | 1.14 |
| GRS | 1.41 | 5.37 | 6.20 | 5866 | 1.16 |
| BZD | 1.26 | 6.75 | 5.26 | 12,349 | 0.998 |

[a] (Rs) and ($\alpha$) are the resolution and selectivity, respectively, between two consecutive peaks.

**Table 3**
Library match angles and purity angles of the investigated compounds.

| Solution | Composition | PDA match 1 angle | PDA match 1 threshold | Purity 1 angle | Purity 1 threshold |
|---|---|---|---|---|---|
| Standard mixture | GRS | 0.138 | 1.568 | 0.415 | 1.389 |
| | BZA | 0.936 | 1.948 | 1.556 | 2.605 |
| | GRSD | 0.129 | 1.402 | 0.535 | 1.292 |
| | BZD | 0.384 | 1.634 | 0.314 | 1.410 |
| Test solution[a] | GRS | 0.111 | 1.222 | 0.399 | 1.125 |
| | BZA | 0.985 | 1.844 | 1.221 | 2.211 |
| | GRSD | 0.141 | 1.351 | 0.510 | 1.195 |
| | BZD | 0.322 | 1.457 | 0.333 | 1.233 |

[a] Test solution spiked with GRSD and BZD.

### 3.3. Method validation

#### 3.3.1. System suitability test

SST results (Table 2) confirmed that the chromatographic system was adequate for the analyses planned to be done. The capacity factor values were found to be within the range of $2.2 < k' < 6.8$, the resolution between two adjacent peaks was greater than 4.0, tailing factors were <1.25 and the column's efficiency, calculated as number of theoretical plates, was >5800. The calculated SST parameters were within the acceptance criteria for good HPLC practice. The RSD% values for the calculated SST parameters for replicate injections were less than 2%.

#### 3.3.2. Specificity and selectivity

The specificity of the proposed HPLC method was proven as the retention times for each compound in recovery experiments were identical to those of standard solution. In addition, no peak was eluted in the chromatogram of placebo solution indicating the

**Table 4**
Linearity and regression analysis data.

| Parameters | GRS[a] | BZA[a] | GRSD[b] | BZD[a] |
|---|---|---|---|---|
| Calibration range ($\mu g\,mL^{-1}$) | 11.2–33.6 | 100–300 | 0.03–2.0 | 0.01–10 |
| Detection limit ($\mu g\,mL^{-1}$) | 0.2855 | 0.888 | 0.007 | 0.0029 |
| Quantitation limit ($\mu g\,mL^{-1}$) | 0.8563 | 2.693 | 0.023 | 0.0087 |
| Regression equation ($Y$)[c] | | | | |
| Slope ($b$) | 0.1051 | 0.0525 | 65,705 | 460,652 |
| Standard deviation of slope ($S_b$) | 0.0014 | 0.0007 | 204.63 | 1869.9 |
| Relative standard deviation for slope % | 1.3333 | 1.340 | 0.311 | 0.41 |
| Confidence limit of slope[d] | 0.1016–0.1086 | 0.050397–0.054652 | 65,205.2–66,206.6 | 456,233.4–465,072.5 |
| Intercept ($a$) | −0.0586 | 0.2118 | 19,832 | 5658.95 |
| Correlation coefficient ($r$) | 0.99958 | 0.999757 | 0.999942 | 0.999981 |

[a] Peak area ratio using naphazoline hydrochloride as internal standard.
[b] Peak area.
[c] $Y = a + bC$, where $C$ is the concentration in $\mu g\,mL^{-1}$ and $Y$ is the peak area or peak area ratio.
[d] 95% confidence limit.

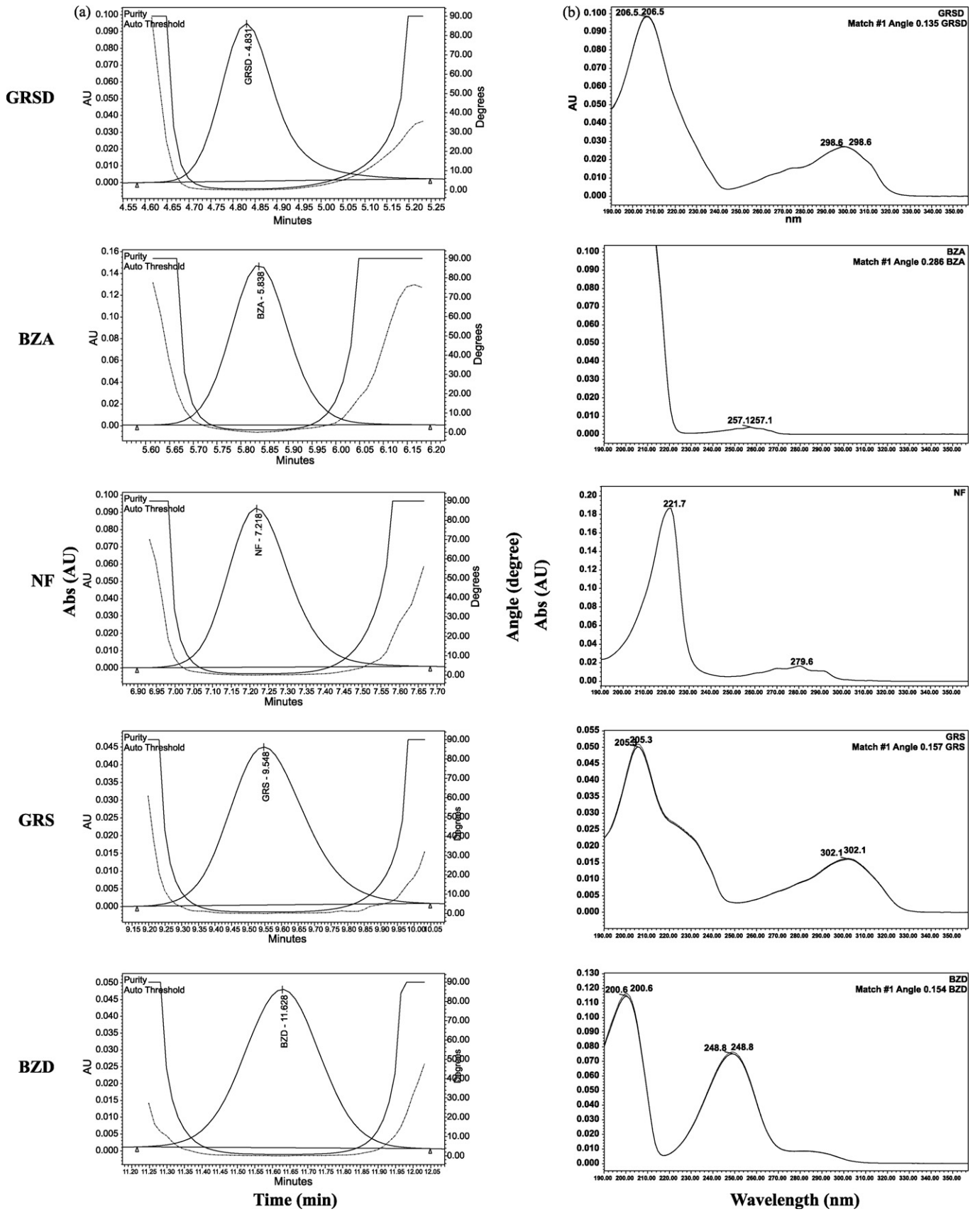


**Fig. 8.** The purity plots (a) and library match plots (b) of the peaks obtained from HPLC of a mixture containing GRS.HCl (0.66 μg mL$^{-1}$), BZA (40 μg mL$^{-1}$), GRSD (10 μg mL$^{-1}$), BZD (10 μg mL$^{-1}$) and NF.HCl (10 μg mL$^{-1}$) prepared in formulation matrix.

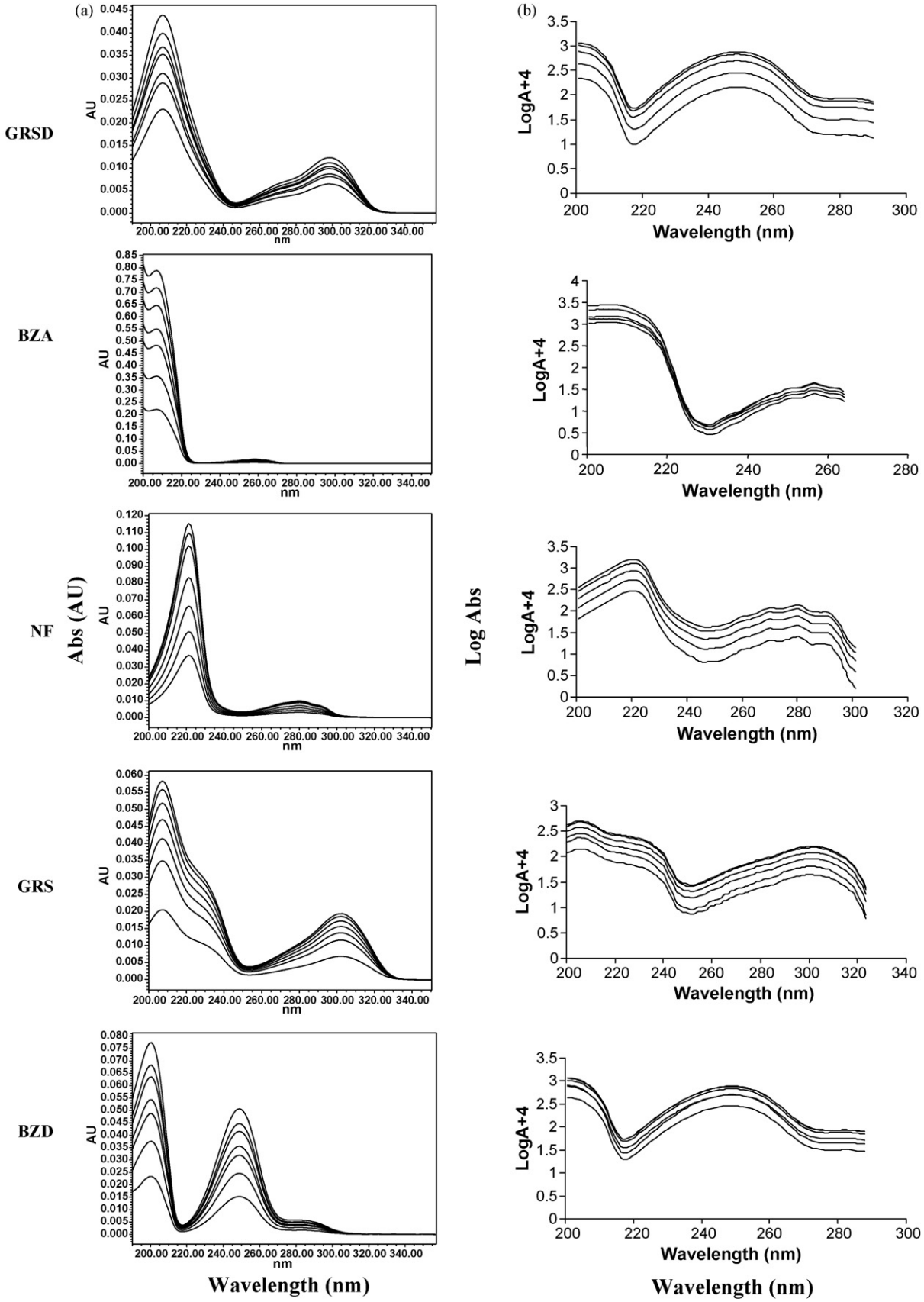


**Fig. 9.** The spectrograms (a) and their log *A* versus the wavelength plots (b) of the peaks obtained from HPLC of a mixture containing GRS.HCl (0.66 μg mL$^{-1}$), BZA (40 μg mL$^{-1}$), GRSD (10 μg mL$^{-1}$), BZD (10 μg mL$^{-1}$) and NF.HCl (10 μg mL$^{-1}$) prepared in formulation matrix.

**Table 5**
Accuracy results.

| Compound | % of Targeting concentration | Matrix for injections solution | | | |
|---|---|---|---|---|---|
| | | Quantity ($\mu g\,mL^{-1}$) | | | |
| | | Added | Found[a] | % Recovery ± SD | RSD% |
| GRS[b] | 80 | 17.92 | 17.85 | 99.61 ± 1.44 | 1.45 |
| | 100 | 22.40 | 22.25 | 99.33 ± 1.35 | 1.36 |
| | 120 | 26.88 | 26.57 | 99.85 ± 1.21 | 1.22 |
| Average ± SD (RSD%) | 99.60 ± 0.404 (0.41%) | | | | |
| BZA[b] | 80 | 160 | 162.55 | 101.59 ± 1.66 | 1.63 |
| | 100 | 200 | 202.33 | 101.12 ± 1.22 | 1.22 |
| | 120 | 240 | 237.81 | 99.09 ± 1.77 | 1.79 |
| Average ± SD (RSD%) | 100.61 ± 1.094 (1.09%) | | | | |
| GRSD[c] | 0.03 | 0.168 | 0.170 | 101.19 ± 1.77 | 1.75 |
| | 0.04 | 0.224 | 0.222 | 99.11 ± 1.39 | 1.40 |
| | 0.05 | 0.280 | 0.279 | 99.64 ± 1.43 | 1.43 |
| | 0.06 | 0.336 | 0.337 | 100.30 ± 1.37 | 1.37 |
| | 0.08 | 0.448 | 0.449 | 100.22 ± 1.12 | 1.12 |
| Average ± SD (RSD%) | 100.10 ± 0.76 (1.84%) | | | | |
| BZD[d] | 0.01 | 0.500 | 0.511 | 102.20 ± 1.33 | 1.30 |
| | 0.02 | 1.000 | 1.010 | 101.00 ± 1.11 | 1.10 |
| | 0.03 | 1.500 | 1.530 | 102.00 ± 1.24 | 1.22 |
| | 0.04 | 2.000 | 2.020 | 101.01 ± 1.05 | 1.04 |
| | 0.05 | 2.500 | 2.530 | 101.20 ± 1.06 | 1.05 |
| Average ± SD (RSD%) | 101.48 ± 0582 (0.57%) | | | | |

[a] Mean of three determinations for each concentration.
[b] % of targeting concentration of intact drug presented as % of the method concentration.
[c] % of targeting concentration is presented as % (w/w) relative to intact GRS.HCl (560 $\mu g\,mL^{-1}$).
[d] % of targeting concentration is presented as % (w/w) relative to BZA (5000 $\mu g\,mL^{-1}$).

**Table 6**
Precision data.

| Compound | Theoretical concentration ($\mu g\,mL^{-1}$) | Intra-day measured concentration ($\mu g\,mL^{-1}$) | | Inter-day measured concentration ($\mu g\,mL^{-1}$) | |
|---|---|---|---|---|---|
| | | Mean[a] | RSD% | Mean[a] | RSD% |
| GRS | 17.92 | 17.89 | 1.52 | 17.88 | 1.42 |
| | 22.40 | 22.36 | 1.32 | 22.34 | 1.66 |
| | 26.88 | 26.83 | 1.29 | 26.82 | 1.33 |
| BZA | 160.0 | 162.5 | 1.63 | 161.8 | 1.71 |
| | 200.0 | 201.3 | 1.24 | 201.2 | 1.33 |
| | 240.0 | 239.1 | 1.46 | 238.9 | 1.55 |

[a] Mean and RSD% of three determinations.

absence of interference from excipients used in such formulations. DAD also supported the selectivity of the method and provided evidences for the identification and spectral homogeneity of the eluted peaks through purity plots and library match plots for GRS, BZA and their degradation products in standard mixture as well as samples used in recovery experiments (Fig. 8a and b). The library match angles and the purity angles for the peaks in the investigated mixtures were less than their corresponding threshold angles (Table 3) confirmed the identity and spectral purity of the resolved peaks. In addition, the observation that the wavelengths of derivative optima

**Table 7**
Robustness data.

| Mobile Phase | Composition | | pH | $k'$ | | | | | Rs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acetonitrile | 0.05 M $KH_2PO_4$ | | GRSD | BZA | NF | GRS | BZD | GRSD and BZA | BZA and NF | NF and GRS | GRS and BZD |
| A | 21 | 100 | 4.8 | 2.33 | 2.95 | 3.95 | 5.44 | 6.52 | 5.18 | 4.99 | 5.99 | 5.28 |
| B[a] | 22 | 100 | 4.8 | 2.22 | 2.90 | 3.82 | 5.36 | 6.75 | 5.21 | 5.01 | 6.01 | 5.26 |
| C | 23 | 100 | 4.8 | 2.35 | 3.01 | 3.81 | 5.31 | 6.99 | 5.22 | 5.11 | 6.02 | 4.87 |
| D | 22 | 100 | 4.7 | 2.41 | 2.94 | 3.99 | 5.41 | 6.50 | 5.22 | 4.94 | 5.88 | 5.23 |
| B[a] | 22 | 100 | 4.8 | 2.22 | 2.90 | 3.82 | 5.36 | 6.75 | 5.21 | 5.01 | 6.01 | 5.26 |
| E | 22 | 100 | 4.9 | 2.30 | 3.11 | 3.83 | 5.33 | 6.88 | 5.28 | 5.11 | 6.22 | 5.27 |

$k'$ is the capacity factor; Rs is the resolution between two consecutive peaks.
[a] HPLC method conditions.

(first, second, third and fourth) of the spectrograms of the peaks (Fig. 9a) of the test solution were identical to those of corresponding standard was considered as evidence confirming the identity of the investigated compounds [23]. Moreover, the spectral homogeneity and purity of the peaks were confirmed using the log *A* versus the wavelength plots constructed from the data obtained from the spectrograms (Fig. 9a) of the peaks [23]. The superimposed traces of log *A* versus the wavelengths with each other and with those of the corresponding standard for each peak (Fig. 9b) proved further the purity of HPLC peaks.

### 3.3.3. Linearity

Under optimum chromatographic conditions, linear relationship existed between the peak area ratios and the concentration of each of GRS and BZA. Also linear relationship existed between the peak areas and the corresponding concentration of GRSD and BZD. The values of correlation coefficient were close to unity indicating good linearity. The characteristic parameters for the regression equations obtained by least square treatment of the results are presented (Table 4).

### 3.3.4. Accuracy

The results obtained for the accuracy study from 9 samples ($n = 3$ for each concentration level) are presented (Table 5) for the determination of GRS, BZA, GRSD and BZD in matrix of injections solutions. The results indicated that the proposed HPLC-DAD method is accurate for the determination of the investigated drugs and their degradation products, if existed, in their pharmaceutical preparation i.e. injections.

### 3.3.5. Precision

The RSD% values for PAR of GRS and BZA were less than 1.25% for 10 consecutive injections of the working standard mixture indicated good precision of the system. The RSD% for 10 determinations of GRS and BZA in sample solution was less than 2% indicating good precision of the method. The results of intra-day and inter-day precision confirmed good precision of the proposed HPLC-DAD method (Table 6).

### 3.3.6. Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated from the linearity data for each compound (Table 4). The results indicated that the method is sensitive for the determination of very small concentrations of the degradation products.

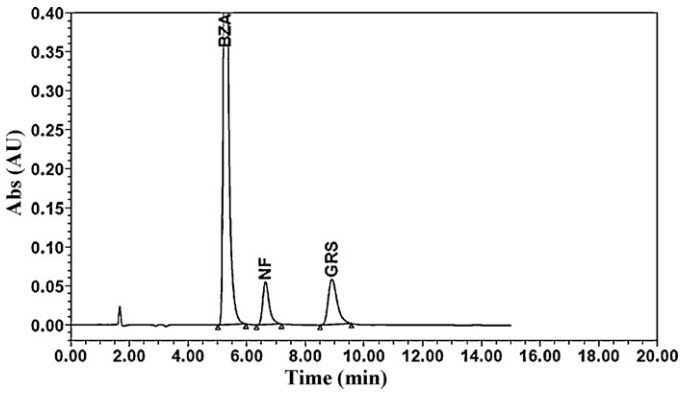


**Fig. 10.** Chromatogram of GRS vial test solution labeled to contain 22.4 μg mL$^{-1}$ of GRS.HCl and 200 μg mL$^{-1}$ of BZA using 10 μg mL$^{-1}$ of NF.HCl as internal standard.

**Table 8**
Determination of GRS and BZA in GRS-vial solutions.

| Batch number | % Found ± SD[a] | | RSD% | |
|---|---|---|---|---|
| | GRS | BZA | GRS | BZA |
| B1 | 101.22 ± 1.55 | 102.45 ± 1.54 | 1.53 | 1.50 |
| B2 | 100.58 ± 1.75 | 101.55 ± 1.33 | 1.74 | 1.31 |
| B3 | 99.21 ± 1.42 | 99.42 ± 1.16 | 1.43 | 1.16 |

B1, B2, B3 refer to three different batches.
[a] Mean of three determinations.

### 3.3.7. Robustness

SST parameters remained unaffected over deliberate small changes in the chromatographic conditions viz. slight change in the % acetonitrile in the mobile phase and slight change in the pH of the mobile phase (±0.1 pH units). The method was demonstrated to be robust over an acceptable working range of its HPLC operational parameters (Table 7).

## 3.4. Application to pharmaceutical preparations

### 3.4.1. Determination of intact drugs

The proposed validated HPLC method was applied to the determination of GRS and BZA in three different batches of GRS-vials. Fig. 10 shows representative chromatogram for such determination. Satisfactory results were obtained as the mean percentage found for GRS and BZA were in good agreement with the label claimed. The mean percentage found and the RSD% values (Table 8) indicated that the proposed method could

**Table 9**
Determination of GRSD and BZD in GRS-vial solutions.

| Batch number | Labeled intact drug (μg mL$^{-1}$) | | Mean[a] degradation found (μg mL$^{-1}$) ± SD | | % (w/w) of BZD[b] |
|---|---|---|---|---|---|
| | GRS.HCl | BZA | GRSD | BZD | BZD |
| 1 | 560 | 5000 | BQL | 0.800 ± 0.011 | 0.016 |
| 2 | 560 | 5000 | BQL | 1.445 ± 0.017 | 0.029 |
| 3 | 560 | 5000 | BQL | 1.168 ± 0.012 | 0.023 |

BQL, below quantitation limit.
[a] Mean of three determinations.
[b] Weight of BZD relative to weight of BZA.

**Table 10**
Determination of GRSD in GRS-vial solution spiked with GRSD.

| Sample number | Labeled GRS (μg mL$^{-1}$) | Spiked GRSD (μg mL$^{-1}$) | % (w/w) GRSD | Mean %[a] recovery | RSD% |
|---|---|---|---|---|---|
| 1 | 560 | 0.200 | 0.036 | 103.51 | 1.34 |
| 2 | 560 | 0.400 | 0.072 | 103.22 | 1.60 |
| 3 | 560 | 0.500 | 0.089 | 102.99 | 1.19 |

[a] Mean of three determinations.

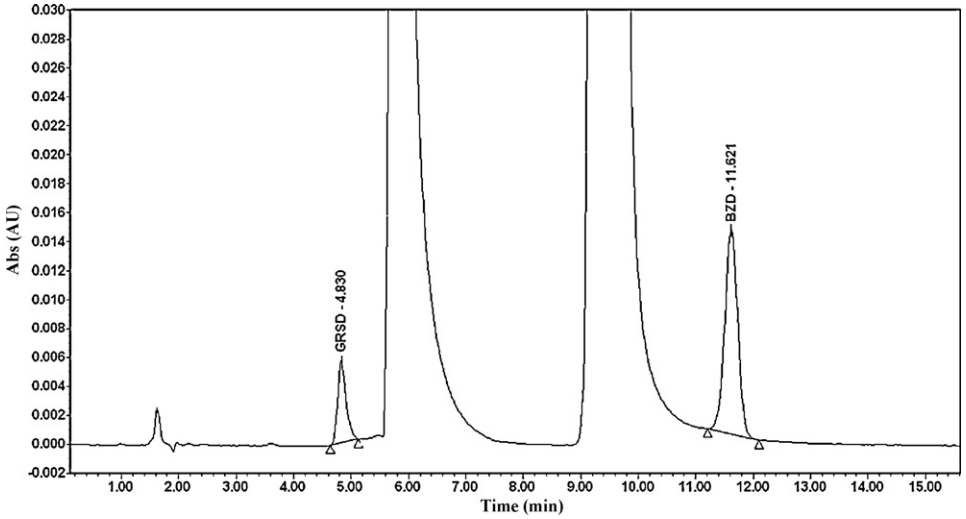


**Fig. 11.** Chromatogram of GRS vial test solution labeled to contain 560 μg mL$^{-1}$ of GRS.HCl and 5000 μg mL$^{-1}$ of BZA and spiked with 0.4 μg mL$^{-1}$ of GRSD.

be adopted for the determination of GRS and BZA in GRS-vials.

*3.4.2. Determination of degradation products*

The proposed validated HPLC method was applied to the determination of GRSD and BZD in GRS-vials. The results (Table 9) showed that GRSD was non-detected in the investigated GRS-vials. The contents of BZD in three different batches of GRS-vials were between 0.015 and 0.03% (w/w). The concentration of BZD in the vials is far below the BP limit of BZD in BZA raw material (0.05% w/w). The results (Table 9) indicated that the investigated GRS-vials were formulated using high quality GRS and BZA raw materials.

The applicability of the proposed HPLC method for the determination of very small concentrations of GRSD was verified by spiking the investigated vial solution with GRSD. Fig. 11 shows representative chromatogram of spiked samples. The high percentage recovery of GRSD (Table 10) could be due to the contribution of the original non-detected GRSD in GRS-vials. The results (Table 10) indicated that the proposed validated HPLC method could be used for the determination of GRSD in GRS-vials in concentrations far below the pharmacopoeia limit of GRSD in GRS raw material (0.1% w/w).

## 4. Conclusion

A simple, sensitive and stability-indicating HPLC-DAD method has been developed and validated for simultaneous identification and determination of granisetron hydrochloride, its main degradation product, benzyl alcohol and benzaldehyde (oxidation product of benzyl alcohol) in granisetron injections. The chromatographic method is accurate, precise and specific for the determination of the intact drugs and their main degradation products without interference from the co-formulated adjuvant. The method is sensitive to monitor down to 0.03% (w/w) of granisetron degradation product and 0.01% (w/w) of benzaldehyde which are far below the compendia limits for their detection in granisetron and benzyl alcohol, respectively. The chromatographic separation is accomplished in 12 min. Therefore, the chromatographic method is recommended for routine quality control analysis for the determination of granisetron hydrochloride, benzyl alcohol and their putative degradation product in granisetron injection. In addition, the procedure could be applied for the detection and determination of granisetron main degradation product and benzaldehyde in granisetron hydrochloride and benzyl alcohol bulk material, respectively.

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