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Stability-Indicating HPLC-DAD Method for the Determination of Granisetron Hydrochloride in Its Pharmaceutical Preparations

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ABSTRACT

A selective and reproducible high performance liquid chromatographic method has been developed for the determination of granisetron hydrochloride in presence of its hydrolytic, oxidation, photodecomposition and thermal degradation products. Successful separation of granisetron from its degradation products is achieved on X-Bridge C18 column using acetonitrile: 0.025 M KH_2PO_4 solution (20:80) adjusted to pH 2 as mobile phase. The method is selective for the determination of granisetron hydrochloride, benzyl alcohol (preservative) and sodium benzoate (preservative) in presence of benzaldehyde; the oxidation product of benzyl alcohol. The method is validated and validation acceptance criteria are met in all cases. Recovery experiments of granisetron hydrochloride, benzyl alcohol and sodium benzoate from a mixture of ICH stress-formed degradation products and benzaldehyde are between 99.5-100.5% with RSD% values less than 1.5%. The proposed validated stability-indicating method is applied to the determination of granisetron and the co-formulated preservatives in tablets, oral solution and injections.

INTRODUCTION

Granisetron (GRS), 1-Methyl-N-[(1R, 3r, 5S)- 9-methyl-9-azabicyclo (3.3.1) non-3-yl]-1H-indazole-3-carboxamide (Figure 1) is a selective 5-HT3 receptor antagonist used in prevention and treatment of cytotoxic chemotherapy-induced nausea and vomiting (Kirchner et al,1997; Cupissol *et al*, 1990; Carmichael *et al*, 1989). GRS.HCl is marketed as tablets, oral solutions that contain sodium benzoate (SBZ, Figure 1) as preservative and parenteral solutions, which are formulated as single dose-preservative free injections and multi-dose vials that contain benzyl alcohol (BZA, Figure 1) as bacteriostatic agent (Physicians Disk Reference, 57th edition, 2003). The literature reveals that several HPLC methods have been reported for the determination of GRS.HCl in biological fluids and pharmaceutical preparations. The HPLC methods used for determination of GRS.HCl in biological fluids included the use of fluorescence (Huang *et al*, 1998; Wada *et al*, 1998; McElvain *et al*, 1997; Pinguet *et al*, 1996; Huang *et al*, 1996; Boppana *et al*, 1995; Kudoh *et al*, 1999), UV (Capacio *et al*, 1993), mass spectrometric (Boppana *et al*, 1996; Jang *et al*, 2006; Dotsikes *et al*, 2006; Xiang *et al*, 2007; Wang *et al*, 2007; Bruijin *et al*, 2009) and electrochemical (Boppana *et al*, 1995) detectors. The HPLC methods used for the determination of GRS.HCl in pharmaceutical preparations included tablets (Xia *et al*, 2000) and intravenous infusion containing doxorubicin (Zhang *et al*, 1998).

The photo-stability of GRS.HCl in dextrose and saline solutions and their compatibility towards glass and polypropylene containers has been studied using an HPLC method (Hourcode *et al*, 1997). All of the above reported HPLC methods are non-stability indicating.

However, we have recently disclosed two HPLC-DAD methods under publication for the determination of granisetron hydrochloride, its major degradation product;1-methyl-1H-indazole-3-carboxylic acid (GRSD) and preservatives (benzyl alcohol, sodium benzoate, methylparaben and propylparaben) (Hewala *et al*, 2010; Hewala *et al*, 2011). The BP (British Pharmacopoeia, 2012) describes HPLC methods for determination of GRS.HCl and its related substances in raw material, meanwhile its pharmaceutical preparations are not official in BP (British Pharmacopoeia, 2012).

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USP (The United States Pharmacopoeia 35, 2012) monographs describe HPLC methods for the determination of the related substances and assay of bulk pharmaceuticals or preparations. The drug stability testing guideline of the International Conference on Harmonization (ICH Q1A (R2), 2003) requires that analysis of stability samples should be performed using validated stability-indicating analytical methods. It also recommends carrying out stress testing on the drug substance to elucidate its inherent stability characteristics and hence supporting the suitability of the proposed analytical procedure. The ICH recommended stress testing conditions encompasses the effect of heat, humidity, light and susceptibility of the substance towards oxidation and hydrolysis over a wide range of pH (Bakshi *et al*, 2002). To our knowledge, no article related to the HPLC stability-indicating method for determination of GRS.HCl in presence of ICH-formed degradation has ever been mentioned in the literature. Therefore, the aim of the present study is to develop and validate purposely a stability-indicating HPLC method to separate GRS.HCl from its ICH stress-formed degradation products and co-formulated adjuvants i.e. preservatives commonly added to GRS pharmaceutical preparations. The proposed HPLC method is validated according to USP analytical method validation parameters (United States Pharmacopoeia 35, 2012) and ICH guidelines (ICH Q2 (R1, 2005). Thereafter, the method has been successfully applied to the determination of GRS.HCl and the co-existing preservatives in GRS pharmaceutical preparations.

Materials and Methods

GRS.HCl, pharmaceutical grade (Carbo Mer, Inc, SanDigo, USA) was used and certified to contain 99.96%. The hydrolytic degradation product of GRS (GRSD, Figure 1) was prepared, purified and identified according to the described procedure (Hewala *et al*, 2010). The other hydrolytic degradation product; (1R, 3r, 5S)-9-methyl-9-azabicyclo [3.3.1] nonan-3-amine (GRSE, Figure 1); was obtained as USP reference standard, lot number FOF 287. The pharmaceutical grade of benzyl alcohol (BZA) and sodium benzoate (SBZ) were used and certified to contain 99.95% and 99.99%, respectively. Acetonitrile (HPLC grade) was obtained from BDH (Poole, UK). Orthophosphoric acid solution (85%) and potassium dihydrogen phosphate (HPLC grade) were obtained from Merck (Darmstadt, Germany). Sodium hydroxide pellets and hydrochloric acid (33%) were of analytical grade (Merck, Darmstadt, Germany). HPLC water was generated in-house using a Millipore, Milli-Q reverse osmosis plus system (Millipore, Bedford, MA, USA). Grantyl® tablets (labelled to contain 1.12mg GRS.HCl equivalent to 1 mg GRS), Grantyl® vials (labelled to contain 1.12 mg of GRS.HCl and 10 mg of BZA per 1 ml) and oral solution (labeled to contain 2.24 mg of GRS.HCl and 10 mg of SBZ per 10 ml) manufactured by Alexandria Company for Pharmaceuticals were used throughout the study.

Degradation experiments in acid, alkaline and neutral conditions were performed using a water bath (model DB28120-26, Thermolyne, Iowa, USA). Dry air oven (Postfach 102, GmbH Binder, Tuttlingen, Germany) was used to study the effect of dry heat. Metrohm pH meter model 744 (Metrohm Ltd. CH-9101 Herisau, Switzerland) was used to adjust the pH of the mobile phase and the stress-degraded sample solutions before analysis. A stability cabinet (Climacell, GmbH) was used for stability studies.

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 using Empower® 2 software (All Waters, Milford, MA, USA). The chromatographic separation was achieved on X-Bridge (Waters, Milford, MA, USA) C18 column (150 mm x 4.6 mm i.d), 3.5 µm particle size, 135°A pore diameter and 185 m^2g^{-1} surface area. The mobile phase consisted of acetonitrile: 0.025M KH_2PO_4 solution (20:80 v/v) adjusted to pH 2.0 using orthophosphoric acid. The mobile phase was filtered through 0.45 µm membrane filter and degassed ultrasonically before use. The detector was set at wavelength range of 200-350 nm with sampling rate at 10 points per second and the spectral resolution was 1.2 nm. The purity parameters included 100% active peak region and auto-threshold with non-purity pass at level 1. DAD library search was set at threshold degree 10.0 and level 3 in depth. A search threshold criterion of the noise due to instrument and solvent was at angle 1.0. The retention time research was at ±5%. The wavelength research was at ±1 nm.

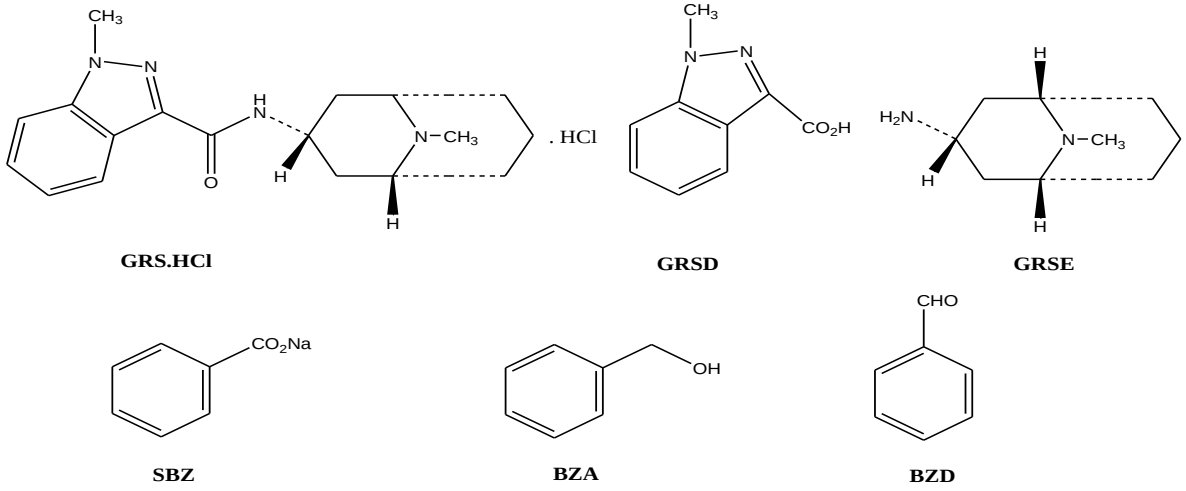


Fig. 1: Structural formulae of the investigated compounds.

Forced degradation studies

The forced degradation reactions were carried out using drug concentration of 1.3 mg mL^{-1}.

Acid-induced hydrolysis, alkaline-induced hydrolysis and neutral hydrolysis:

Hydrolytic degradations were carried out by preparing the drug solutions in 1M HCl, 1M NaOH and water, respectively. All the solutions were protected from light. The prepared solutions were refluxed for 8 h at 90°C.The solutions were neutralized to pH 5 and diluted with mobile phase to contain 130 μg mL^{-1}.

Hydrogen peroxide induced degradation

The stability of the drug toward oxidation was tested by preparing solutions in 0.3% and 3% H_2O_2, stored in dark at room temperature for 3 h and 12 h and then heated in boiling water bath at 80 °C for 10 min to remove the excess H_2O_2. The solutions were diluted with mobile phase to contain 130 μg mL^{-1}.

Photochemical degradation

The photochemical stability of the drug was studied by exposing the drug solutions (1.3 mg mL^{-1}); prepared in water, 0.1 M HCl and 0.1 M NaOH; to direct sunlight for 8 h. The solutions were neutralized and diluted with mobile phase to obtain final concentration of 130 μg mL^{-1}. Another study was carried out where two portions of GRS.HCl powder (200 mg each) were spread as thin films in two separate Petri-dishes. The fist portion was exposed to UV lamp at 254 nm for 8 h. The second portion was exposed to fluorescent lamp at 365 nm for 8 h. From each dish, a quantity of the stressed sample was dissolved in mobile phase and diluted to contain 130 μg mL^{-1}.

Dry heat degradation

The thermal stability of the drug was tested by subjecting it to dry heat at 90°C for 8 h. A quantity of the stressed sample was dissolved in the mobile phase and diluted to get a final concentration of 130 μg mL^{-1}.

All the prepared diluted solutions were filtered through 0.45 μm PVDF membrane filter and then 10 μl aliquots were injected into the HPLC system.

Optimization of the stability-indicating HPLC method

HPLC studies were carried out at first on individual stress-degraded samples of GRS and then on mixtures of solutions in which the decomposition was observed. Finally, resolution of the peaks due to GRS, BZA and SBZ from the GRS stress-formed degradation products and benzaldehyde; the oxidation product of BZA i.e. BZD (Figure 1) was optimized. Preliminary chromatographic trials were carried out on individual stress-degraded samples using a mobile phase composed of acetonitrile: 0.025 M KH_2PO_4 solution (30:70) adjusted to pH 2. However, as no good chromatographic separation of the mixture of GRS stress-degraded samples was achieved. The ratio of acetonitrile was decreased from 30% downwards until satisfactory resolution was obtained. The finally optimized conditions included the use of a mobile phase composed of acetonitrile: 0.025 M KH_2PO_4 solution (20:80) adjusted to pH 2, pumped at 1.5 mL min^{-1}, at ambient temperature. The injection volume was 10 μL and the detector wavelength was 240 nm.

Analysis of pharmaceutical preparations

Preparation of standard solution

Stock standard solutions of GRS.HCl and BZA were prepared in methanol to contain 1.12 mg mL^{-1} and 10 mg mL^{-1}, respectively. Stock standard solution of SBZ was prepared to contain 5 mg mL^{-1} in water. The stock standard solutions were suitably diluted with the mobile phase to obtain a standard mixture containing 33.6, 300 and 150 μg mL^{-1} of GRS.HCl, BZA and SBZ, respectively. The solution was filtered through 0.45 μm PVDF membrane filter and 10 μl aliquots were injected automatically in triplicate into the HPLC system.

Preparation of tablets test solution

A quantity of the powdered tablets (labeled to contain 1.12 mg per tablet) equivalent to 3.36 mg of GRS.HCl was transferred into a 100 ml volumetric flask and mixed with 50 ml of the mobile phase. The contents were stirred for 5 min, diluted to volume with the mobile phase, mixed well, filtered through 0.45 μm PVDF membrane filter and 10 μL aliquots were injected automatically in triplicate into the HPLC system.

Preparation of parenteral solution and oral solution test solutions

Aliquots of the GRS vial (labeled to contain 1.12 mg mL^{-1} of GRS.HCl and 10 mg mL^{-1} of BZA) and the GRS oral solution (labeled to contain 2.24 mg of GRS.HCl and 10 mg of SBZ per 10 ml) equivalent to 3.36 mg of GRS.HCl were transferred separately into 100 mL volumetric flasks and diluted to volume with the mobile phase. The solutions were filtered through 0.45 μm PVDF membrane filter and 10 μL aliquots were injected automatically in triplicate into the HPLC system.

Validation procedure

Specificity

The specificity of the proposed method was evaluated through chromatography of (a) solutions of standard analytes, (b) solutions of matrices i.e. placebo, (c) solutions of stress-degraded GRS, and (d) test solutions. In addition, DAD also supports the specificity through the assessment of the purity and spectral homogeneity of the HPLC peaks of GRS, BZA and SBZ in a mixture containing GRS stress-degraded samples, BZA, SBZ, BZD and the co-formulated adjuvant. The spectral homogeneity and purity of the peaks of GRS, BZA and SBZ were also checked using the methods of relative absorption spectra (RA) and log A versus the wavelength plots (Hewala *et al*, 2012; Hewala *et al*, 2010). The UV absorption spectra (spectrograms

extracted at different time intervals across the elution of the peaks of GRS.HCl, BZA and SBZ) were used to construct their RA spectra and log A versus λ plots(Hewala *et al*, 2012; Hewala *et al*, 2010). Identification of the active pharmaceutical ingredient i.e. GRS and its main degradation product (GRSD) in their mixtures was carried out using; in addition to the retention time of its peak; peak purity, spectral homogeneity and library match programme.

Linearity

The linearity of the detector response with the concentrations of the investigated drugs was evaluated. Stock standard solutions of GRS.HCl, BZA and SBZ were prepared at strength of 1.12 mg mL^{-1}, 10 mg mL^{-1} and 5 mg mL^{-1}, respectively. Dilution with the mobile phase was carried out to obtain solutions containing concentrations ranged from 10-60, 150-450 and 75-225 μg mL^{-1} of GRS.HCl, BZA and SBZ, respectively.

The solutions were injected in triplicate into the HPLC system. Peak areas were plotted versus the corresponding concentration to obtain calibration graphs. Regression data analysis was performed using least squares linear regression statistical analysis.

Accuracy

The accuracy of the method was evaluated by spiking a mixture of stress-degraded samples with GRS.HCl, BZA and SBZ at three different concentrations. The percentage recoveries of BZA and SBZ were calculated from the peak areas of their standards while the percentage recovery of GRS.HCl was calculated from the difference between the peak areas of fortified and unfortified solutions. Also recovery studies were carried out by applying the standard addition method to GRS tablets, GRS vials containing BZA and GRS oral solution containing SBZ.

Precision

The precision of the proposed HPLC method was verified by repeatability and intermediate precision studies. For determination of repeatability, ten samples solutions were prepared at 100% level of the analytical method concentration and the results were expressed as RSD% for the ten determinations. The intra-day precision studies were performed by analysis of three different concentrations; 80, 100 and 120% of the analytical concentration; of the drug in triplicate (n=3) on the same day. The inter-day precision studies were done by repeating the studies on three consecutive days.

Robustness

The robustness the method was tested through measurement of its capacity to remain unaffected by small variation in the % of the organic modifier and pH of the mobile phase. The robustness of the proposed method was evaluated in terms of system suitability parameters of drug peak in a mixture of stress-degraded samples and preservatives towards the small-intended aforementioned variations.

RESULTS AND DISCUSSION

Development and optimization of the stability-indicating method

Stress-degraded samples were analysed by HPLC using X-Bridge C18 column (150 mm x 4.6 mm i.d., 3.5 μm particle size) and a mobile phase initially, composed of methanol: 0.025 M KH_2PO_4 solution (50:50).

As the separation and the shape of the peaks were not good, the % of the organic modifier was changed from 50% to 25% but no improvement was observed. Subsequent attempts were made through replacement of methanol by acetonitrile and lowering the pH to be around 2.5. There was an improvement in the resolution of the closely eluted peaks. Ultimately a mobile phase composed of acetonitrile : 0.025 M KH_2PO_4 solution (20:80) adjusted to pH 2 flowing at 1.5 mL min^{-1}, at ambient temperature was found to resolve GRS, BZA, SBZ and BZD from the ICH stress-formed GRS degradation products.

The system suitability test parameters of the optimized method were in agreement with the requirements of good HPLC practice. The capacity factors of the compounds to be determined i.e. GRS.HCl, BZA and SBZ were higher than 1.4. The resolution between the peaks of the compounds to be determined and the closest peak were higher than 2. The plate count of all peaks of the compounds to be determined was more than 2000 and their symmetry factors were in between 0.92 and 1.25.

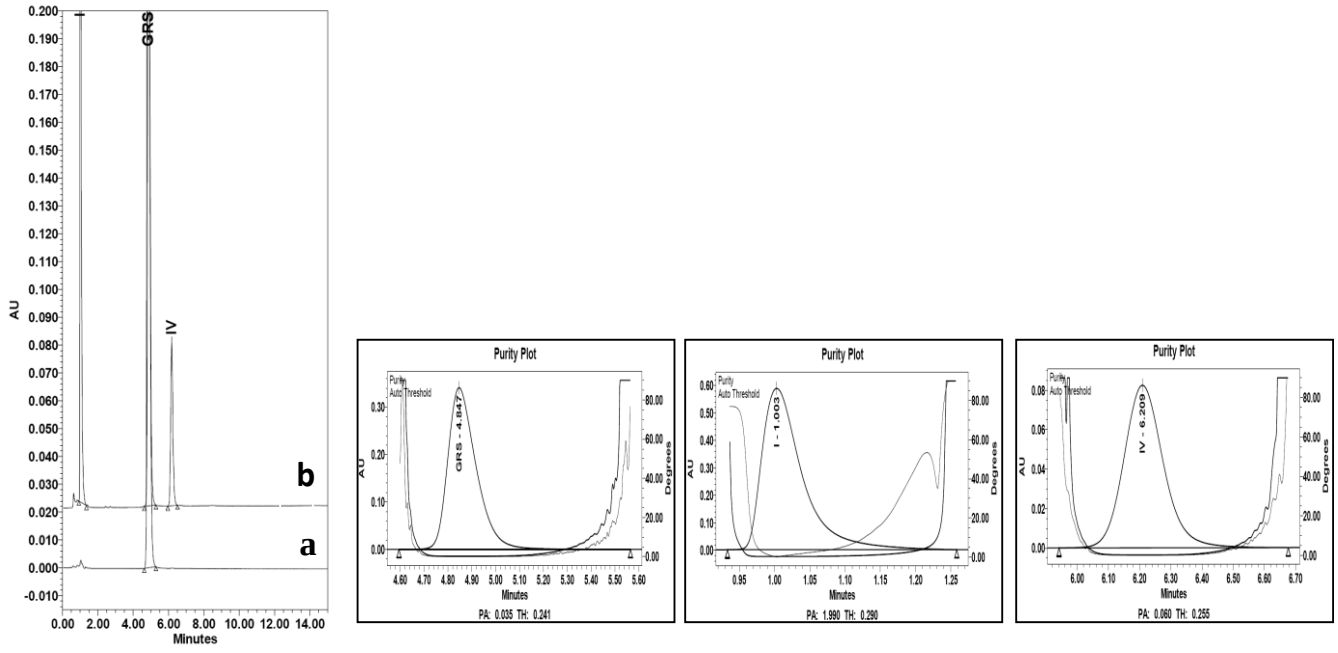
Stability of granisetron hydrochloride

GRS.HCl undergoes degradation to more than one product in most of the stress conditions except neutral hydrolysis and thermal stress conditions. The relative retention times of the degradation products; which carry the symbols I, II, III, IV and V; to the intact GRS were 0.16, 0.53, 0.7, 1.27 and 1.95, respectively. The HPLC studies of the stress-degraded samples showed the following behaviour:

Hydrolytic degradation studies

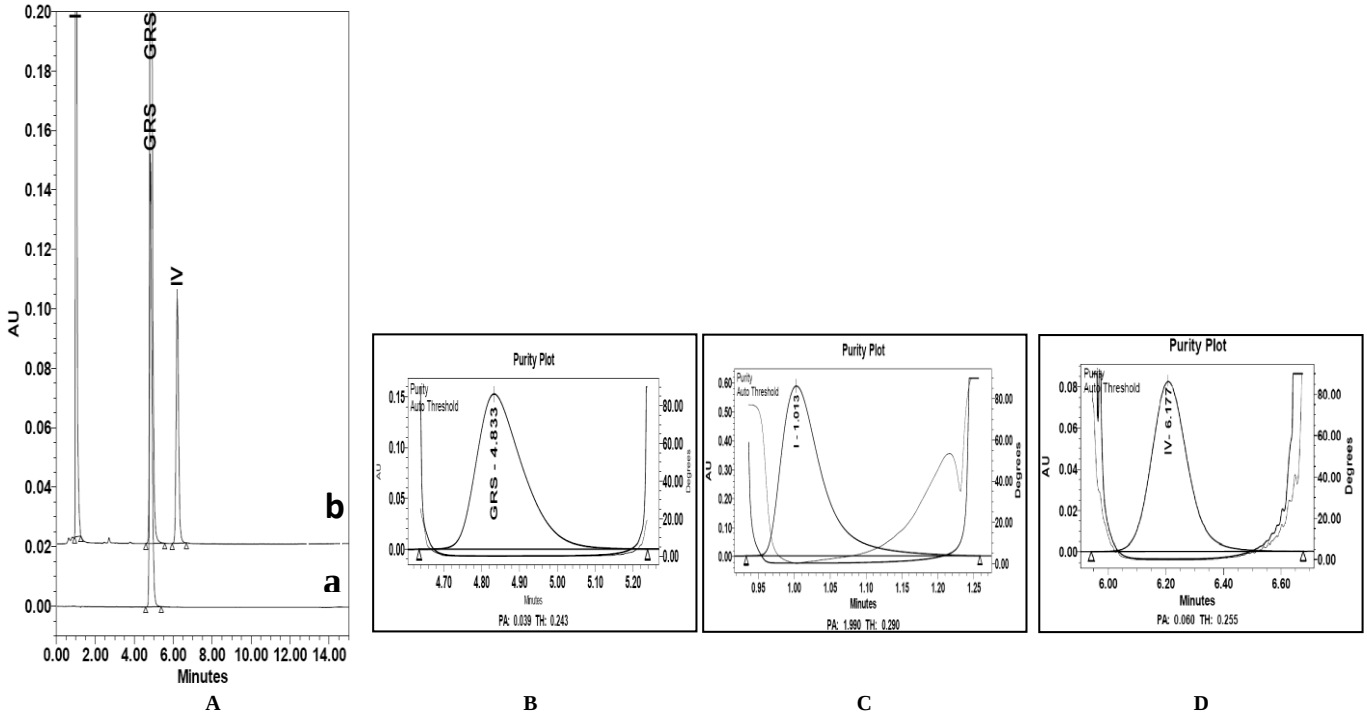
The drug showed ≈5% degradation in 1.0 M HCl and ≈ 12% degradation in 1.0 M NaOH when refluxed for 8 h at 90°C. However, the drug was found to be stable towards hydrolysis in neutral solution. The chromatograms of the acid-induced degradation (Figure 2A) and the alkaline-induced degradation (Figure 3A) showed two degradation products with RRTs of 0.16 and 1.27.

The purity plots indicated that the peak with RRT of 0.16 was spectrally impure (Figures 2C & 3C) while that whose RRT 1.27 was spectrally pure (Figures 2D &3D). The peak due to the non-reacted GRS is spectrally pure (Figures 2B &3B). These findings proved that the acid-and alkaline- induced degradation products would not interfere with the peak due to non-reacted GRS using the proposed HPLC method.



A **B** **C** **D**

Fig. 2: Overlay of chromatograms (A) of (a) standard GRS and (b) its partial degradation in IM HCl and the purity plots of the non-reacted GRS (B), the degradation product I (C) and the degradation product IV (D) of the acid-degraded sample.



A **B** **C** **D**

Fig. 3: Overlay of chromatograms (A) of (a) standard GRS and (b) its partial degradation in IM NaOH and the purity plots of the non-reacted GRS (B), the degradation product I (C) and the degradation product IV (D) of the base-degraded sample.

Oxidative degradation studies

GRS.HCl was found to be relatively stable when kept in 0.3% H_2O_2 solution for 4 h at room temperature. However, the drug showed slight oxidative degradation when prepared in 3.0% H2O2 solution and kept for 8 h at room temperature. The chromatogram of the oxidative degradation (Figure 4A) showed three impure peaks with RRTs of 0.16, 0.53 and 0.70 and a pure peak with RRT of 1.27. The peak of non-reacted GRS was spectrally pure (Figure 4B) indicating that the oxidative degradation products would not interfere with the determination of GRS.

Photolytic degradation studies

Exposure of GRS.HCl either in its solid form to radiation at 254 nm and 365 nm for 4 h or in its solution in 0.1 M HCl or 0.1 M NaOH to sunlight for 6 h resulted into slight degradation ≈ 1%. The chromatograms of GRS subjected to photolytic studies were similar and showed a small peak with RRT of 1.95 (Figure 5A). The peak of GRS after subjection to radiation was spectrally pure as indicated by its purity plot (Figure 5B). This finding indicated that photo-degradation product(s) would not interfere with the peak due to GRS.HCl.

Thermal degradation studies

The chromatogram of GRS.HCl exposed to dry heat at 90 ^{o}C for 8 h was identical to that of standard GRS. This was considered as evidence that the drug is stable toward thermal degradation under the prescribed conditions.

Identification of GRSD as major degradation product of GRS

The chromatogram of a mixture of the ICH stress formed-degradation products of GRS.HCl (Figure 6A) showed five resolved peaks due to the degradation products alongside the peak of the non-degraded GRS.HCl. The peak of GRS was spectrally pure as indicated by its purity plot (Figure 6B) and library match plot (Figure 6D). The degradation products of GRS.HCl are spectrally impure except the product IV that was spectrally pure as indicated by its purity plot (Figure 6C) and library match plot (Figure 6E). Degradation product (IV) was formed during hydrolytic degradation in both acid (Figure 2) and alkaline (Figure 3) media. GRS.HCl; an amide; is liable to undergo acid-and alkaline-induced hydrolytic degradation to give GRSE and GRSD (Figure 7). Spiking the mixture of stress- formed degraded solutions with either GRSE or GRSD resulted into enrichment of the peaks with RRT 0.16 or 1.26, respectively which may considered as evidence for their presence in the degraded mixture. Identification of the peaks of degradation product IV and the non-degraded GRS in the stress-degraded mixture was carried out through allocation of wavelengths of derivative (D_1, D_2, D_3 and D_4) optima (Fig. 8, Table 1) of the spectrograms extracted at different time intervals throughout the elution of the peaks and their corresponding standards(Hewala *et al,* 2012; Hewala *et al,* 2010). The similarity of absorption and derivative optima (Table 1, Figure 8) of the spectrograms of degradation product IV and GRS peaks of the degradation reaction mixture to those of the corresponding reference standards confirmed their identification and that the degradation product IV is GRSD.

Validation of the method

The optimized RP-HPLC stability-indicating method was validated according to USP analytical method validation parameters (USP 35, 2012) and ICH guidelines (ICH Q2(R1) concerning specificity, accuracy, linearity, range, precision and robustness. System suitability test parameters were assessed.

Specificity

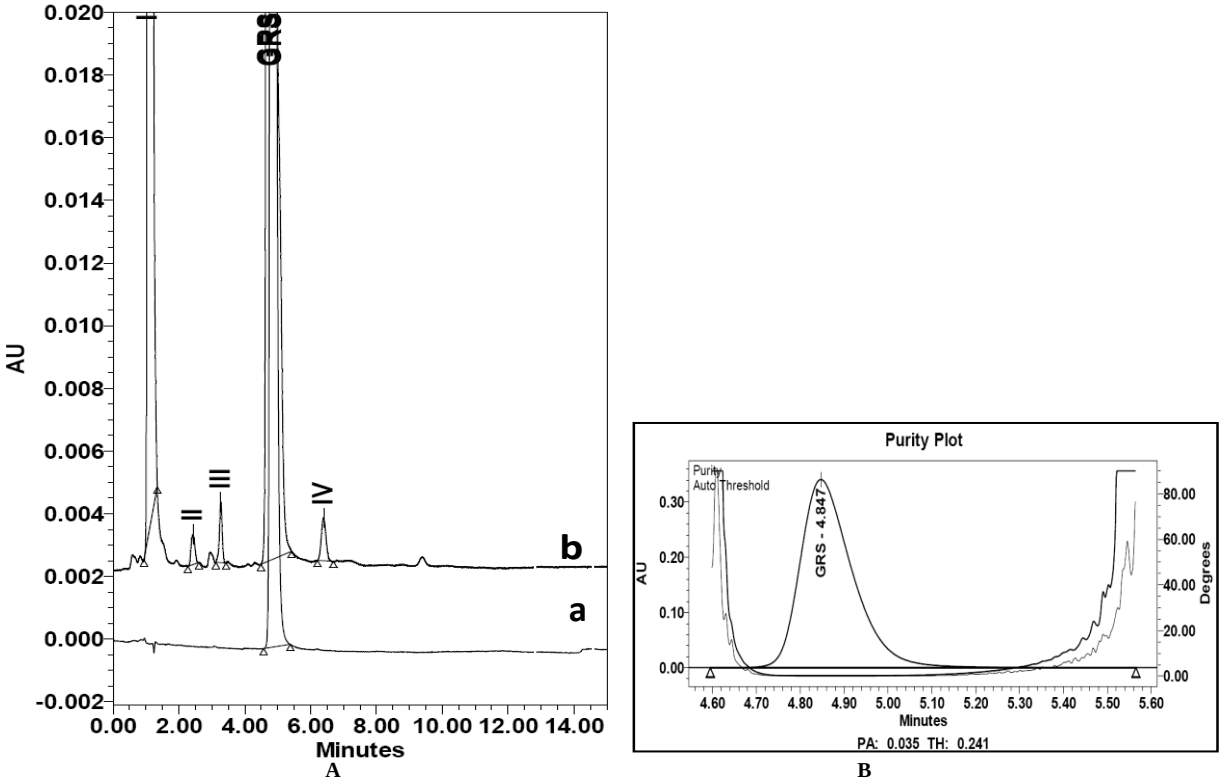
The specificity of the proposed HPLC method for determination of GRS.HCl, BZA and SBZ in the investigated pharmaceutical preparations in presence of the ICH stress-formed degradation products was proved by comparison of retention times for such peaks on the chromatograms of recovery experiments to that of standard solutions. The absence of peaks on the chromatograms of placebo solution indicated the absence of interference from co-formulated recipients.

Purity plots for GRS.HCl, BZA and SBZ (Figure 9) in a mixture of stress-degraded samples containing BZD proved that the peaks are pure and the method is specific for determination of the investigated drugs as their purity angles were less then threshold angles (Table 2).

The chemical purity and identity of the three investigated drugs were also assessed using library matching. The library match plots (Figure 9) and the lower values of match angles compared to threshold angles (Table 2) confirmed that the peaks eluted at 4.94, 3.71 and 8.71 min are pure and due to GRS.HCl, BZA and SBZ, respectively. Furthermore, the spectral homogeneity and purity of the peaks were confirmed using relative absorption (RA) spectra and log A versus the wavelength plots constructed from the data extracted from the spectrograms of each peak. The superimpose of the relative absorption spectra (Figure 10) and the traces of log A versus the wavelengths plots (Figure 10) with those of corresponding standard for each peak proved the absence of interference and hence specificity of the method for determination of the investigated compounds (Figure 11).

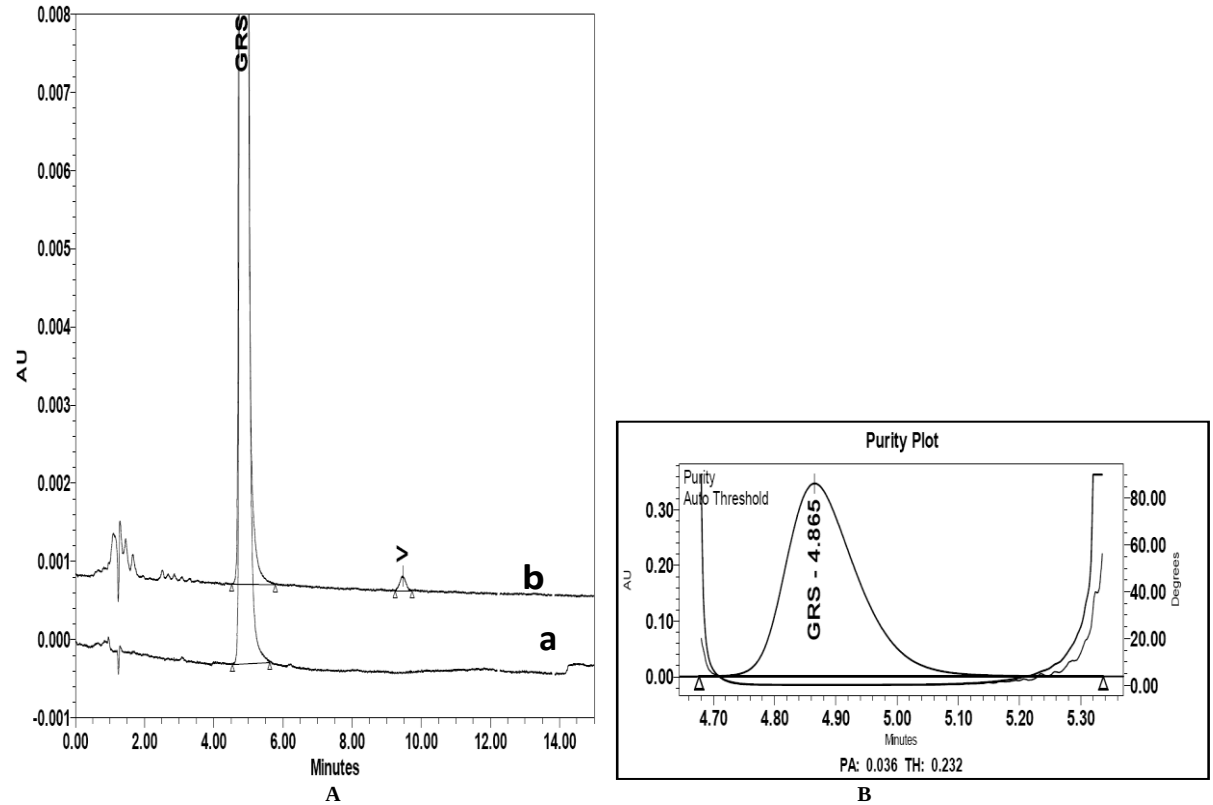
Linearity

Calibration curves with six points were constructed by injecting a series of standard mixtures covering 50% to 150% of SBZ and BZA and 30% to 180% of GRS.HCl. The wider concentration range of GRS was designed to cover the analysis of mixture of stress-degraded samples fortified with intact GRS. The equations were obtained by least squares linear regression analysis of the peak area versus the concentration. The values of correlation coefficient were close to unity indicating good linearity. The regression data, values of correlation coefficient (r) and other statistical parameters are listed (Table 3).



A B

Fig. 4: Overlay of chromatograms (A) of (a) standard GRS and (b) GRS partially oxidized sample and (B) the purity plot of the non-reacted GRS in the oxidized sample.



A B

Fig. 5: Overlay of chromatograms (A) of (a) standard GRS and (b) GRS partially photo-decomposed sample and (B) the purity plot of the non-reacted GRS in the photo-decomposed sample.

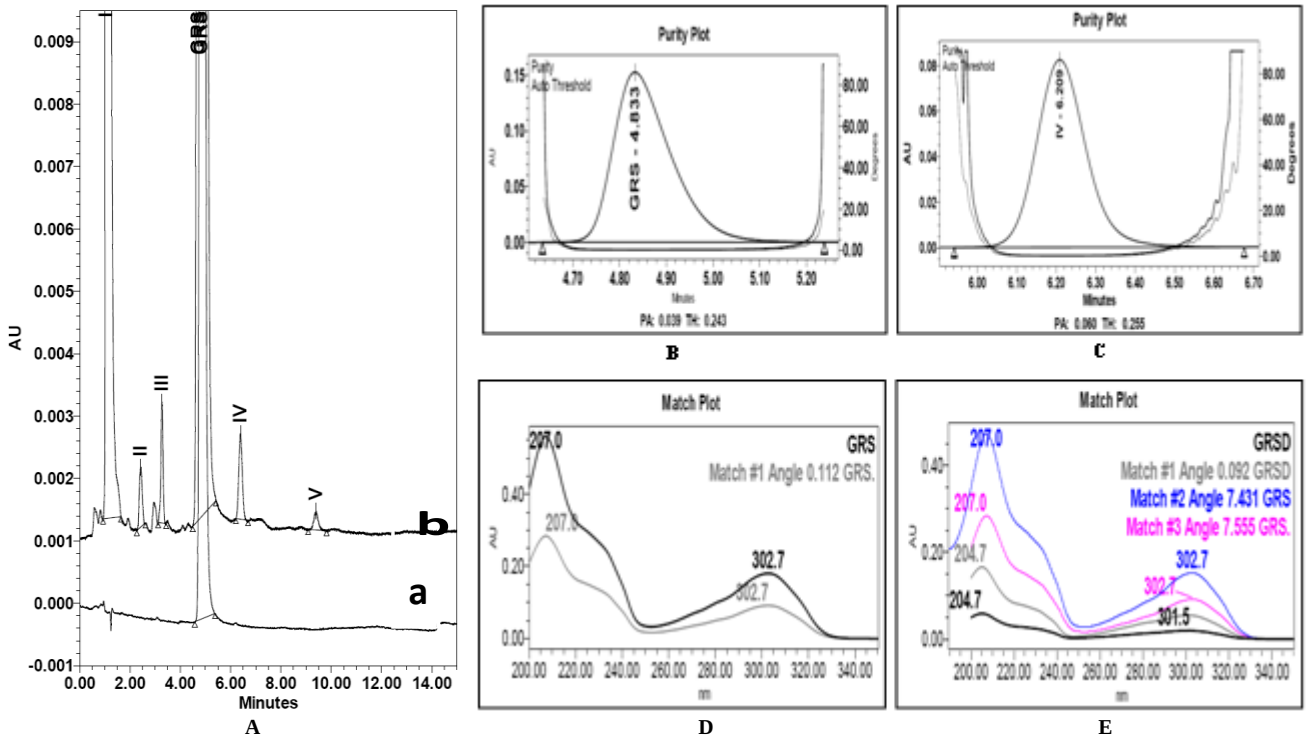


Fig. 6: Overlay of chromatograms (A) of (a) standard GRS and (b) standard GRS in a mixture of stress-degraded samples, the purity plots of non-reacted GRS (B) and the degradation product IV (C) and the library match plots of the non-reacted GRS against GRS reference standard (D) and degradation product IV against standard GRSD (E).

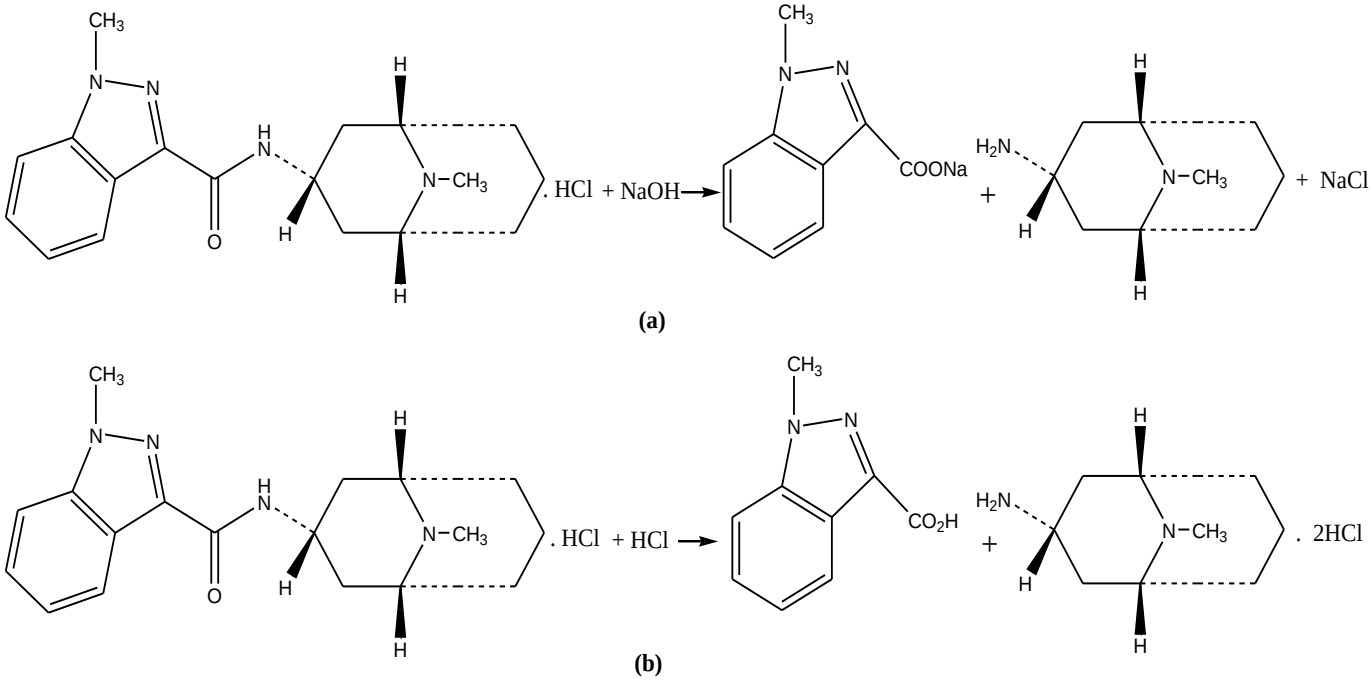


Fig. 7: Hydrolytic degradation of GRS.HCl in alkaline (a) and acid (b) media.

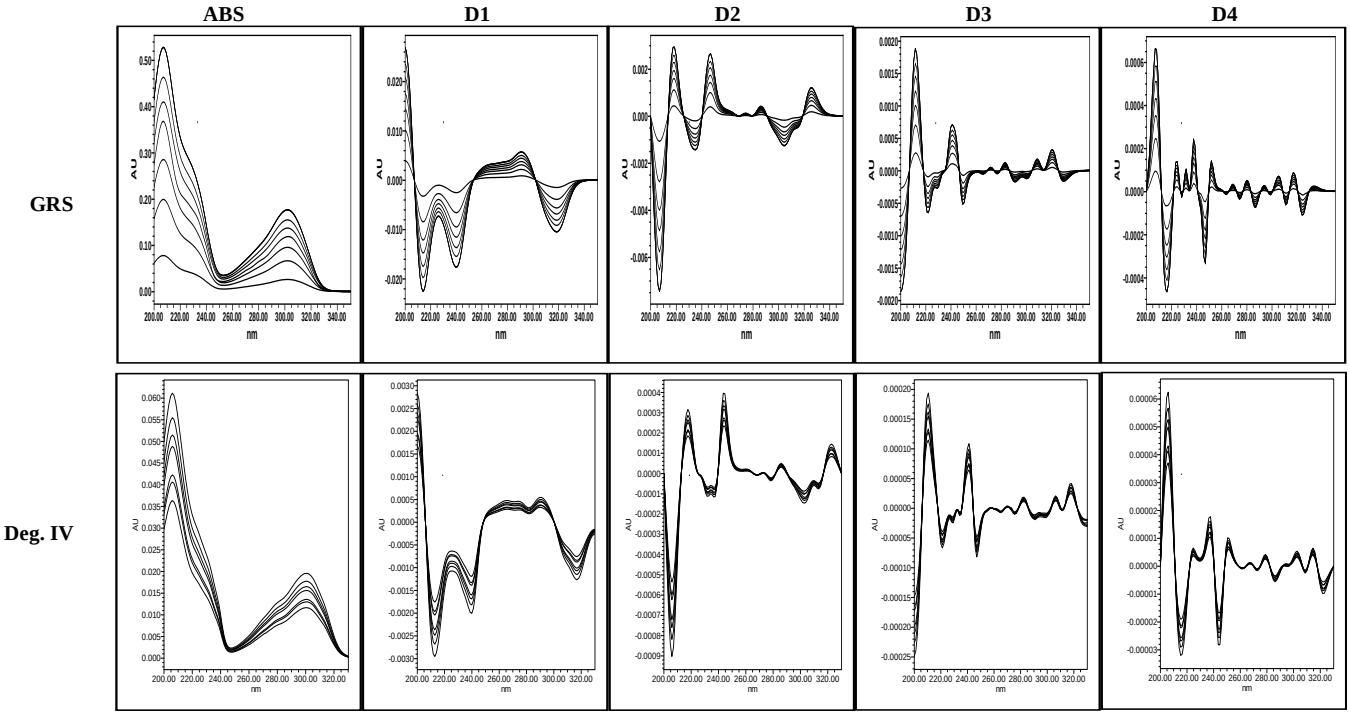


Fig. 8: The spectrograms (ABS) of the peaks of GRS and degradation product IV and their first (D1), second (D2), third (D3) and fourth (D4) derivative spectra.

Table. 1: Wavelengths of absorption (A) and derivative (first D_1, second D_2, third D_3 and fourth D_4) optima for the spectrograms extracted at different time intervals for GRS and degradation product IV HPLC peaks and their corresponding standards.

Solution	Compound	Wavelength of optima				
		A	**D_1**	**D_2**	**D_3**	**D_4**
Standard mixture	GRS	207	- 215	- 205	+213	+213
		250	- 227	+220	- 224	- 224
		302.7	- 246	- 232	+240	+240
			+289	+247	+320	+320
			- 315	- 302	- 330	- 330
				+326		
	GRSD	204.7	- 211	- 205	+210	+205
		247	- 225	+218	- 221	+234
		300.3	+271	- 230	+239	- 245
			+290	+246	- 247	+253
			- 315	+287	+318	+301
				- 305	- 333	- 325
				+325		
Stress-degraded solution	GRS	207	- 216	- 206	+213	+214
		250	- 226	+221	- 225	- 224
		302.5	- 246	- 232	+240	+240
			+290	+246	+321	+319
			- 315	- 302	- 329	- 331
				+326		
	Degradation product (IV)	204.2	- 212	- 205	+210	+205
		247	- 225	+217	- 220	+235
		300.3	+270	- 230	+239	- 245
			+290	+245	- 247	+253
			- 315	+287	+318	+301
				- 305	- 334	- 324
				+325		

\+ : Optimum on the positive side of the spectrum.
\- : Optimum on the negative side of the spectrum.

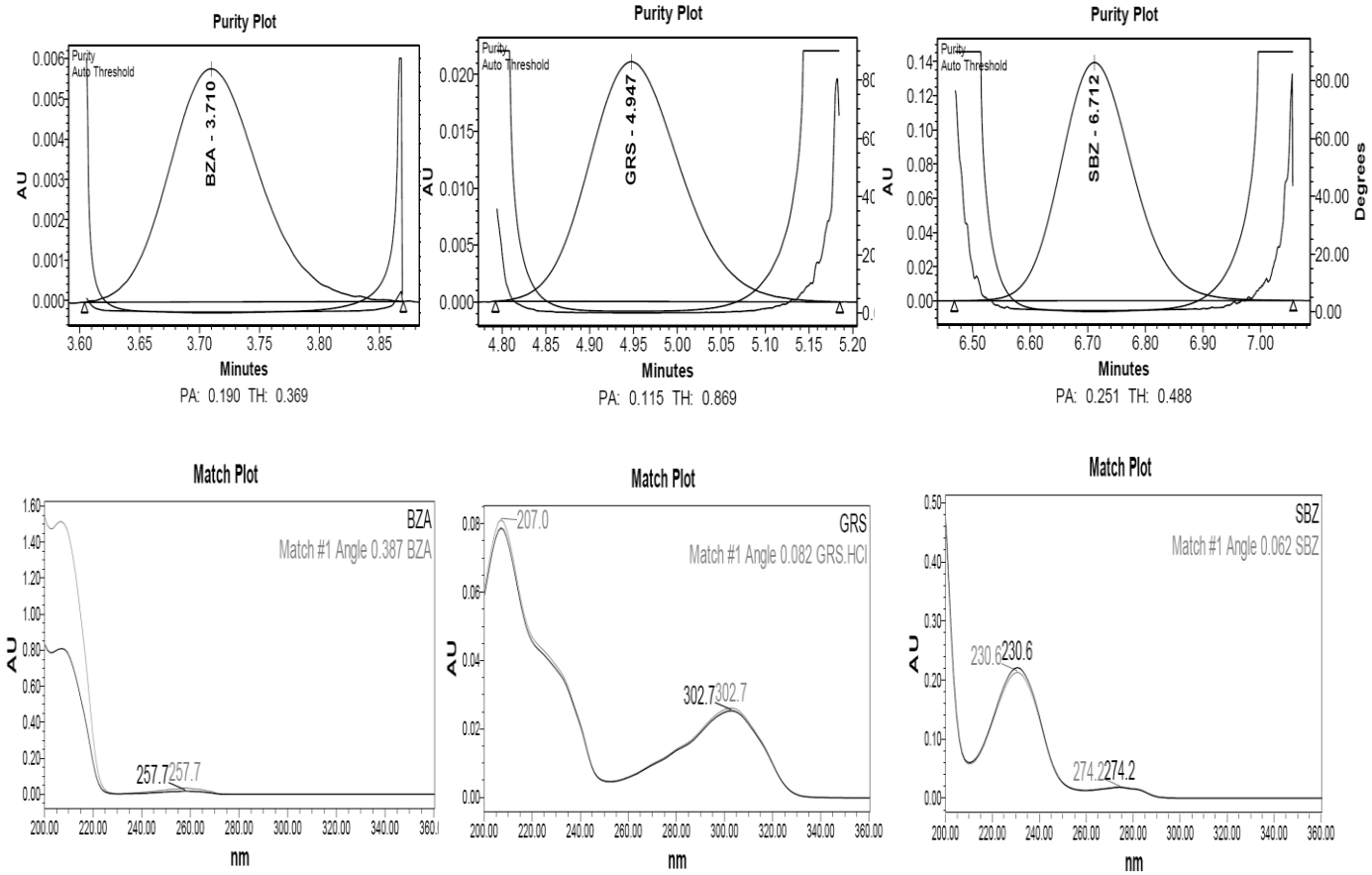


Fig. 9: Purity plots and library match plots of the peaks of BZA, GRS and SBZ obtained from chromatography of a mixture containing degraded samples, BZA, GRS, BZD and SBZ.

Table. 2: Library match angles and purity angles of the investigated compounds.

Solution	Composition	PDA match 1 angle	PDA match1 threshold	Purity1 angle	Purity 1 threshold
Standard mixture	BZA	0.387	0.467	0.190	0.369
	GRS	0.082	1.367	0.115	0.869
	SBZ	0.062	1.124	0.251	0.488
Standard mixture & GRS stress-degraded solution	BZA	0.433	0.556	0.240	0.399
	GRS	0.152	1.597	0.155	0.911
	SBZ	0.087	1.564	0.279	0.499

Table 3: Linearity data.

Parameters	GRS	BZA	SBZ
Calibration range (μgmL^{-1})	10-60	150-450	75-225
Regression equation (Y)[a]			
Slope (b)	9758.76	196.7	15843.3
Standard deviation of the slope (Sb)	123.37	2.817	54.153
Relative standard deviation of the slope (%)	1.26	1.432	0.342
Confidence limit of the slope [b]	9366.15-10151.36	187.75-205.69	15670.86-16015.54
Intercept (a)	-1910.84	-870.48	1152.32
Correlation coefficient (r)	0.999760	0.999692	0.99982

[a] Y=a+ bC; where C is the concentration in μgmL^{-1} and Y is the peak area.
[b] 95% confidence limit.

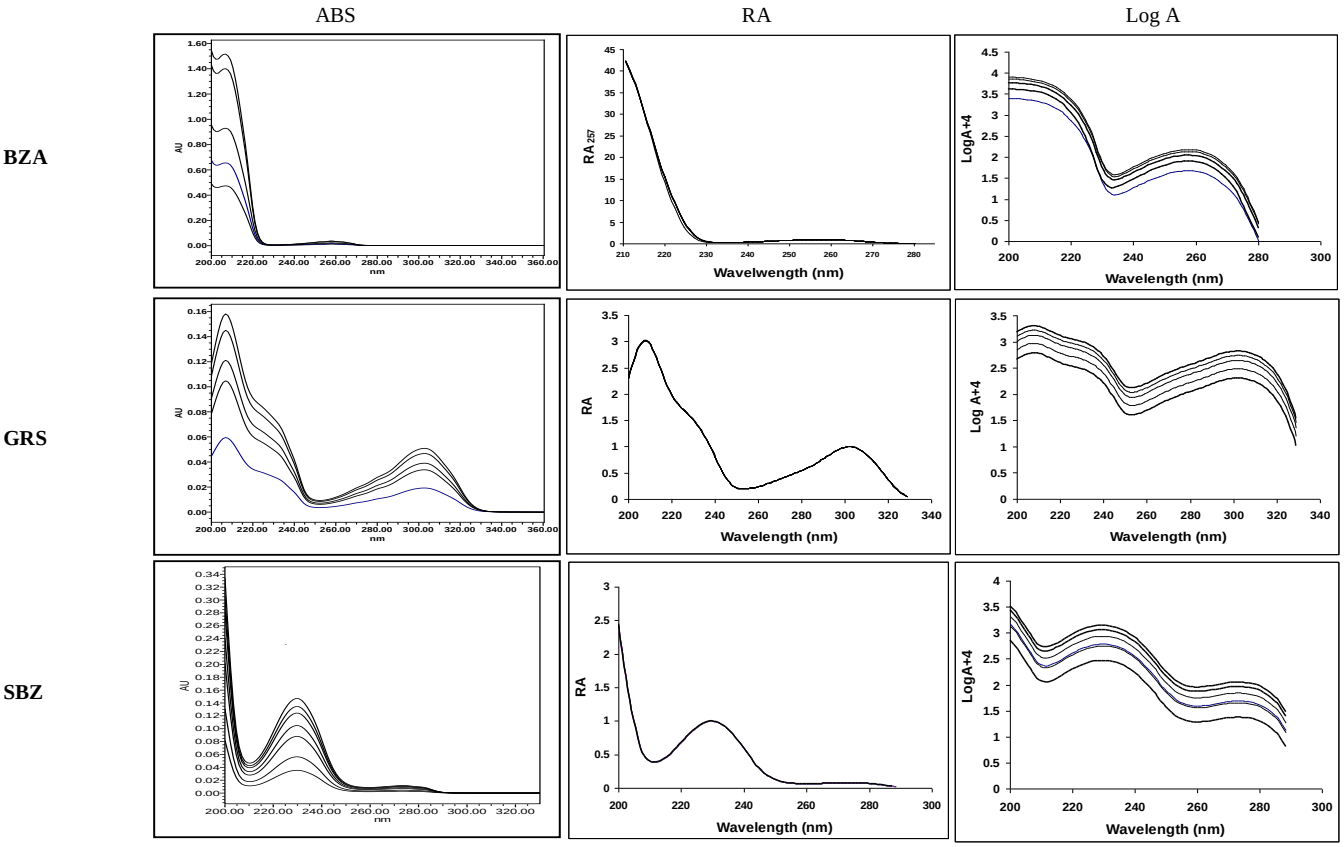


Fig. 10: The spectrograms (ABS) of the peaks of BZA, GRS and SBZ, their relative absorption (RA) spectra and their (log A) versus the wavelength plots.

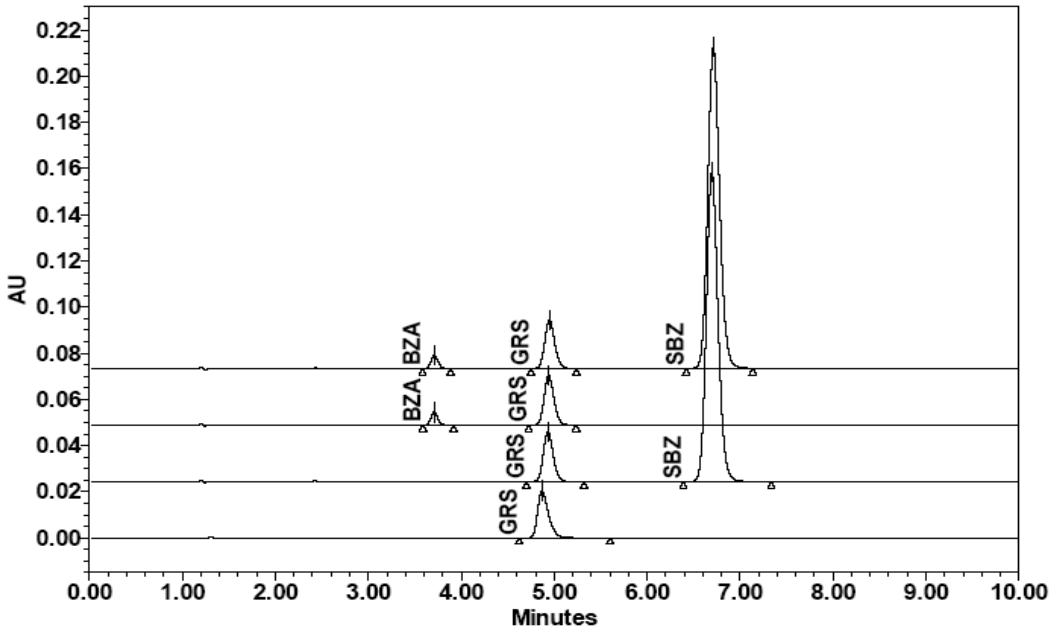


Fig. 11: Overlay of chromatograms of (a) standard mixture containing GRS.HCl (33.5 µg mL-1), BZA (311 µg mL-1) and SBZ (148 µg mL-1), (b) GRS injection test solution labeled to contain 33.6 µg mL-1 of GRS.HCl and 300 µg mL-1 of BZA, (c) GRS oral solution test solution labeled to contain 33.6 µg mL-1 of GRS and 150 µg mL-1 of SBZ and (d) GRS tablets test solution labeled to contain 33.6 µg mL-1 of GRS.HCl.

Table . 4: Recovery data.

Matrix	Added (μgmL^{-1})			Mean* % recovery and (RSD%)		
	GRS	**BZA**	**SBZ**	**GRS**	**BZA**	SBZ
Mixture of stress- degraded samples & 0.5μgmL^{-1} BZD	15.6	240	120	99.82 (0.78%)	101.34 (0.82%)	99.98 (1.14%)
	20.2	300	150	99.39 (0.91%)	100.77 (0.98%)	100.95 (1.03%)
	25.5	360	180	100.11 (0.59%)	99.88 (1.06%)	100.58 (0.98%)
Grantyl®vial placebo & 0.5μgmL^{-1} BZD	26.8	240	(—)	99.87 (0.94%)	101.77 (1.05%)	(—)
	33.6	300	(—)	100.90 (1.11%)	100.92 (1.00%)	(—)
	40.32	360	(—)	99.66 (0.92%)	100.47 (0.99%)	(—)
Grantyl® oral solution Placebo	26.10	(—)	120	101.23 (0.89%)	(—)	99.76 (1.09%)
	33.60	(—)	150	101.11 (1.02%)	(—)	98.87 (1.01%)
	41.08	(—)	180	100.96 (1.16%)	(—)	99.77 (0.98%)
Grantyl® tablets Placebo	27.2	(—)	(—)	99.96 (0.88%)	(—)	(—)
	33.60	(—)	(—)	99.97 (0.99%)	(—)	(—)
	41.31	(—)	(—)	99.72 (1.05%)	(—)	(—)

* Mean of three determinations.
(—) means it is not a component of formulation

Table . 5: Precision data.

Pharmaceutical preparation	Labeled concentration μgmL^{-1}			Found * concentration and (RSD%)					
				Intra-day			Inter-day		
	GRS	**BZA**	**SBZ**	**GRS**	**BZA**	**SBZ**	**GRS**	**BZA**	**SBZ**
Grantyl®vial	26.8	240	(—)	26.62 (0.92%)	244.5 (1.12%)	(—)	26.58 (0.98%)	243.44 (1.22%)	(—)
	33.6	300	(—)	33.45 (1.22%)	306.7 (1.22%)	(—)	33.51 (1.05%)	304.66 (1.21%)	(—)
	40.32	360	(—)	40.21 (1.35%)	363.3 (1.14%)	(—)	40.19 (1.09%)	357.11 (1.19%)	(—)
Grantyl® oral solution	26.10	(—)	120	26.23 (1.23%)	(—)	122.55 (1.11%)	26.13 (1.14%)	(—)	121.33 (0.97%)
	33.60	(—)	150	33.69 (1.11%)	(—)	149.22 (1.29%)	33.55 (1.25%)	(—)	149.90 (1.11%)
	41.08	(—)	180	41.26 (1.10%)	(—)	179.30 (1.16%)	41.22 (1.31%)	(—)	178.99 (1.02%)
Grantyl®tablets	27.2	(—)	(—)	27.34 (0.96%)	(—)	(—)	27.30 (1.01%)	(—)	(—)
	33.60	(—)	(—)	33.86 (1.03%)	(—)	(—)	33.71 (1.26%)	(—)	(—)
	41.31	(—)	(—)	41.46 (0.95%)	(—)	(—)	41.40 (1.00%)	(—)	(—)

* Mean of three determinations.
(—) means it is not a component of formulation.

Table . 6: Robustness data.

Mobile Phase	Mobile phase Composition		pH	k\						Rs				
	% Acetonitrile	% 0.025 M KH_2PO_4		III	BZA	GRS	(IV)	SBZ	BZD	III & BZA	BZA &GRS	GRS& (IV)	(IV) &SBZ	SBZ& BZD
A	19	80	2.0	1.44	2.66	3.93	5.26	5.71	7.06	6.25	6.65	5.95	1.95	5.11
B*	20	80	2.0	1.45	2.71	3.99	5.33	5.77	7.11	6.34	6.77	5.93	2.02	5.02
C	21	80	2.0	1.44	2.64	3.88	5.28	5.67	6.98	6.32	6.88	5.88	1.99	5.00
D	20	80	1.9	1.39	2.79	3.99	5.37	5.66	7.16	6.31	6.69	5.99	1.99	5.16
B*	20	80	2.0	1.45	2.71	3.99	5.33	5.77	7.11	6.34	6.77	5.93	2.02	5.02
E	20	80	2.1	1.51	2.67	4.11	5.41	5.55	6.98	6.39	6.81	5.89	1.97	5.09

*Mobile phase of the proposed method.
Rs: resolution between two adjacent peaks in the same order of elution

Table. 7: Assay results.

Pharmaceutical preparation	Batch Identity symbol	Mean % found (RSD%)*		
		GRS.HCl	BZA	SBZ
Grantyl® Tablets	BT I	99.58 (0.51)	(—)	(—)
	BT II	99.76 (0.62)	(—)	(—)
	BT III	99.81(0.46)	(—)	(—)
Grantyl® Vial	BV I	100.21 (0.32)	100.21 (0.88)	(—)
	BV II	100.04 (0.48)	101.10 (1.19)	(—)
	BV III	99.89 (0.69)	100.29 (0.59)	(—)
Grantyl® oral solution	BS I	98.96 (1.31)	(—)	100.93 (1.21)
	BS II	99.69 (0.91)	(—)	101.31 (0.42)
	BS III	99.33 (0.49)	(—)	100.24 (0.69)

* Mean and RSD % for three determinations.
Grantyl® tablets labeled to contain 1.12 mg of GRS.HCl per tablet.
Grantyl® vial labeled to contain 1.12 mg of GRS.HCl and 10 mg of BZA per 1mL.
Grantyl® oral solution labeled to contain 2.24 mg of GRS.HCl and 10 mg of SBZ per 10 mL.
(—) means it is not a component of formulation.

Accuracy

The results obtained for the accuracy studies in the various matrices are presented (Table 4). The method is accurate for determination of GRS.HCl, BZA and SBZ in presence of a mixture of GRS stress-formed degradation products, BZD (the oxidation product of BZA) and the co-formulated adjuvant of GRS oral solution and parenteral.

The mean percentage recoveries of the determined GRS.HCl, BZA and SBZ ranged from 98.9 to 100.5% and the RSD % values were less than 1.5%.

Precision

The results of the repeatability and intermediate precision experiments are presented (Table 5). The developed method was found to be precise as the RSD% values for repeatability and intermediate precision studies were less than 1.4%.

Range

The mean percentage found of GRS.HCl, BZA and SBZ from three sets of GRS tablets, GRS oral solution and GRS vials containing 90%, 100% and 110% of the method concentration were between 98.9 and 100.4% of the label claimed contents. The RSD% values for these determinations were less than 1.5%. Consequently, the method fulfils to the compendia specifications for the ranges for determination of intact drugs in tablets, oral solutions and parenteral.

Robustness

The results of robustness studies (Table 6) proved that slight but deliberate changes of the optimized chromatographic parameters would neither affect the retention of the compounds; as indicated by their capacity factors (k`); nor the resolution between any two consecutive peaks i.e. the method is robust.

Applications to pharmaceutical preparations

The proposed validated stability-indicating HPLC method was applied to the determination of GRS.HCl in Grantyl® tablets, GRS.HCl and SBZ in Grantyl® oral solution and GRS.HCl and BZA in Grantyl® vials (Figure 11). The mean percentage drug found and the RSD% values (Table 7) indicated that the proposed validated stability-indicating HPLC method could be adopted for the selective determination of the investigated drugs in their pharmaceutical preparations without interference from either GRS degradation products formed under ICH-recommended stress conditions, co-formulated adjuvant or the oxidation product of benzyl alcohol.

CONCLUSION

A validated stability-indicating HPLC-DAD method has been developed for determination of GRS.HCl, BZA and SBZ in their pharmaceutical preparations. The method has been proved to be selective for determination of GRS.HCl and the preservatives (BZA & SBZ) in presence of GRS degradation products formed under ICH-recommended stress testing conditions and BZD (the oxidation product of BZA).

The developed robust method is simple, selective, specific, accurate and precise for determination of the investigated drug and preservatives without interference from co-formulated adjuvant of the tested pharmaceutical preparations. The method has been applied successfully for determination of GRS.HCl in tablets, GRS.HCl and SBZ in oral solution as well as GRS.HCl and BZA in parenteral. The method has been adopted for stability studies of GRS.HCl and preservatives in their pharmaceutical preparations stored under normal (25oC and 65% RH) and accelerated (40°C and 65% RH) stability studies conditions.

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