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Development and Validation of Analytical Method Using Gas Chromatography with Triple Quadrupole Mass Spectrometry for the Detection of Alkyl Halides as Potential Genotoxic Impurities in Posaconazole

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Abstract: Effective risk management and control methods for potentially genotoxic impurities (PGIs), including alkyl halides, are of significant importance in the medicinal (pharmaceutical) sector. The three alkyl halides in posaconazole are PGIs. The detection and assessment of genotoxic substances is a top priority for all regulatory organizations. Quantifying PGIs at trace levels using standard analytical techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), is challenging for the pharmaceutical manufacturing industry. Thus, the detection of trace quantities of PGIs in posaconazole is essential for developing sensitive analytical methodologies. The objective of this study was to establish an analytical technique for quantifying the three PGIs (alkyl halides) in posaconazole and its intermediate. These alkyl halides are 1-(2,4-difluorophenyl) ethan-1-one (PGI-1), (Z)-1-(1-bromoprop-1-en-2-yl)- 2,4-difluorobenzene (PGI-2), and 1-bromo-2-(2,4-difluorophenyl) propan-2-ol (PGI-3). To identify trace quantities (parts per million (ppm)) of these impurities, we employed a gas chromatography (GC-MS/MS) equipped with a triple quadrupole mass spectrometry detector. The GC column was a USP phase G43, which is a mid-polar 6% cyanopropyl; 94% polydimethylsiloxane, with a 60 m length, 0.32 mm inner diameter, and 1.8 µm film thickness. Helium (He) was used as the carrier gas, with a flow rate of 1.5 mL/min. A thermal gradient elution program was used for this procedure. The method was calibrated for the three PGIs with limits of detection (LOD) and quantification (LOQ) of 0.01 and 0.025 ppm, respectively. The linear range of concentrations (25–150%) was maintained with respect to the specification level. This method was validated according to the ICH regulations and was shown to be specific, rugged, robust, precise, sensitive, accurate, linear, and stable. Therefore, in this newly developed method, the combination of suitable analytical techniques, such as GC-MS/MS and proper chromatographic conditions and column selection with the lowest LOD and LOQ, have allowed the induction of excellent ionization. These conditions have successfully facilitated the identification of PGI-1, PGI-2, and PGI-3 in posaconazole and its intermediate during routine analysis.

Keywords: alkyl bromide; haloalkane; alkyl halide; posaconazole; potentially genotoxic impurities

Citation: Chittireddy, H.N.P.R.; Kumar, J.V.S.; Bhimireddy, A.; Shaik, M.R.; Khan, M.; Khan, M.; Oh, T.H.; Shaik, B. Development and Validation of Analytical Method Using Gas Chromatography with Triple Quadrupole Mass Spectrometry for the Detection of Alkyl Halides as Potential Genotoxic Impurities in Posaconazole. *Separations* **2023**, *10*, 295. https://doi.org/10.3390/separations10050295

Academic Editors: Miguel Ángel Rodríguez-Delgado and Bárbara Socas-Rodriguez

Received: 13 March 2023
Revised: 30 April 2023
Accepted: 3 May 2023
Published: 6 May 2023

1. Introduction

Alkanes and halogens react to form alkyl halides. Owing to their high reactivity, simplicity of use, low cost, and wide commercial availability, these compounds are mostly utilized in alkylation processes via nucleophilic substitution in the synthesis of active pharmaceutical ingredients (APIs). Alkyl halides are potential genotoxic impurities (PGIs) owing to their ability to alkylate DNA bases (on N-7 of guanine and N-3 of adenine) [1–5].

Production safety is a top priority for scientists, chemists, engineers, and formulators when manufacturing pharmaceutical products for use in industry or clinical trials. APIs [6–8] perform a prominent role in the safety, purity, and quality of raw materials. Various low-level impurities are frequently present in pharmacological substances and must be investigated and controlled at permissible levels (parts per million (ppm)). When compared with the expected health benefits, it is possible to accept a certain amount of patient risk, even if it is doubtful that the pharmaceutical material itself is completely safe.

Pharmaceutical companies and regulatory agencies must carefully assess this risk-to-reward trade-off. However, impurities are considered to only be harmful and to have no beneficial effects. To eliminate genotoxic impurities, manufacturers must create and implement their own analytical strategies and limits [9–13]. Human cancer due to genetic mutations, chromosomal breakages, or chromosomal rearrangements [14–16] was observed as a result of pharmaceutical PGIs. Serious toxicological consequences occur owing to exposure to trace amounts of PGIs present in the final drug products. Therefore, chemical scientists should consider methods to reduce the production and use of genotoxic compounds [11,17–19]. It may not always be possible to stop using these drugs entirely or to stop producing pollutants with DNA reactivity. Although present in small concentrations, PGIs are essential for drug evolution [20] and, if appropriately addressed, could lead to a delay in clearance by regulatory authorities [21].

Analytical scientists must develop the required techniques to precisely analyze and regulate the amounts of PGIs in drugs [22–26]. Appropriate analytical methods are necessary to develop reliable manufacturing processes and ensure patient safety. In addition to the contamination of drugs during processing, PGIs can be produced by them during formulation or storage. Genotoxic substances, including hydrolytic substances, pose numerous obstacles to the development of new drugs [27–31]. Genotoxicity is defined as an adverse destructive effect on the DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) of a cell, jeopardizing the health of the cell. Genotoxic chemicals are also known as genotoxins. Teratogens, mutagens, and carcinogens are substances that can cause birth deformities, create mutations, and cause cancer, respectively [32–35].

The EMEA, ICH Q3A/B, and USFDA guidelines are followed to restrict the genotoxic impurities in pharmaceutical substances. Four types of impurities are listed by the FDA, ICH, and USP [36–38], respectively. Owing to certain reactions, including the removal of carbon dioxide, dehydration, and oxidation, the first category of impurities associated with APIs is further divided into two groups. Due to the relationship between their structure and activity, impurities associated with APIs may be carcinogenic, mutagenic, or genotoxic [39,40].

Genotoxic impurities may originate from different sources; however, they are often introduced by the starting materials used to synthesize pharmaceuticals and genotoxic impurities, by-products, or intermediates. Additionally, because solvents, catalysts, and reagents are used in pharmaceutical synthesis, genotoxic impurities are present in pharmacological compounds. Drug impurities accumulate owing to the degradation of drugs during storage, air oxidation, hydrolysis, and exposure to light. During the manufacturing of stereoselective pharmaceuticals, chiral impurities are produced in pharmacological compounds [41–43].

Genotoxicity statistics are helpful for evaluating the risks associated with pharmaceutical substances, food, consumer goods, and industrial products. Information on genotoxicity is essential for determining the risks posed by naturally occurring environmental toxins. Genetic alterations have severe adverse consequences for health, even at modest levels of vulnerability. Roto-oncogenes, tumor suppressor genes, and DNA damage response genes can be mutated by a variety of carriers, including both chemical and physical agents. Somatic cells with damaged DNA also contribute to degenerative conditions, such as accelerated aging, lowered immunity, and cardiovascular and neurological problems. The assessment of mutagenic potential is an essential component of chemical risk evaluation to prevent the negative effects of genetic alteration on human health [44–46].

Regulatory bodies worldwide require managerial data on the genotoxic potential of pharmaceutical products to evaluate the products and procedures for safety. Therefore,

pre-symptomatic investigations are often conducted to assess basic toxicological data of new chemical entities (NCE). Additionally, such data helps identify genotoxicity risks that can cause DNA damage and fixation [47–49].

The posaconazole intermediate, (5R-cis)-toluene-4-sulfonic acid 5-(2,4 -difluorophenyl)-5-(1H-1,2,4-triazol-1-yl) methyl tetra hydrofuran-3-ylmethyl ester, is a key compound in its synthesis (Figure 1). Posaconazole is sold under the trade name, Noxafil® and is an azole antifungal agent (Figure 1). It has been approved for the treatment and prophylaxis of invasive Aspergillus and Candida infections in patients in the United States [50,51]. In high-risk immunity individuals, posaconazole is often used to prevent invasive yeast and mold infections, such as invasive aspergillosis.

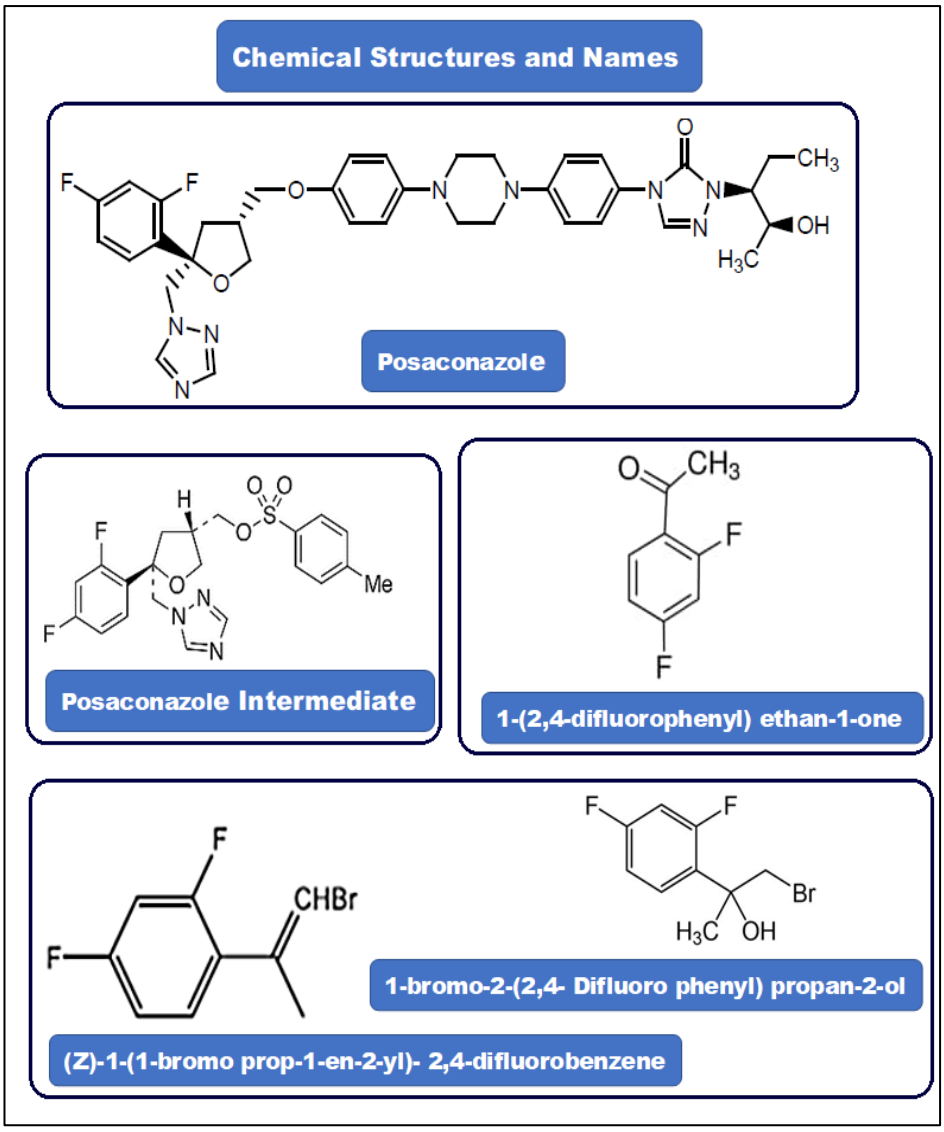


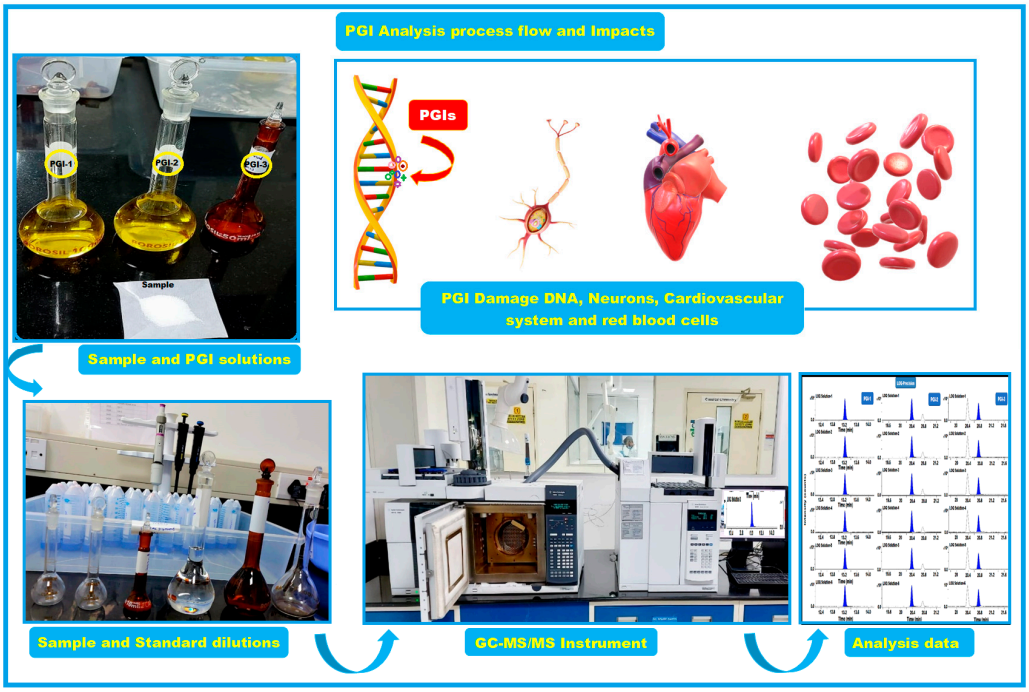
Figure 1. Structures of posaconazole, posaconazole intermediate, PGI-1, PGI-2, and PGI-3.

Impurities must be assessed to establish upper limits for those that are considered PGIs. According to the ICH Q3A guidelines, the ideal limits for PGIs must be well below those for common impurities, and it is necessary to create improved analytical methods that can detect and assess PGIs at the ppm level. Class-1–5 impurities are distinguished based on their ability to cause cancer and mutations. According to the toxicological concern-based (TTC-based) threshold, the preferred daily intake of PGIs is 1.5 µg per person per day; this value can be used, along with the length of pharmacological treatment, to establish an acceptable limit for impurities in pharmaceutical products according to ICH M7.

The impurities PGI-1 ($C_8H_6F_2O$), PGI-2 ($C_9H_7BrF_2$), and PGI-3 ($C_9H_9BrF_2O$) (Figure 1) in antifungal agents are toxic. These PGIs are Class-3 (ICH M7) impurities and are alkyl halides [2]. Any PGIs should be measured in accordance with the recommendations of regulatory bodies; otherwise, they would become hazardous over time. To detect and measure these contaminants, a sensitive and reliable analytical technique is needed. Although there are numerous methods for posaconazole analysis in the prior literature, including HPLC [52], HPLC/UV and bioassay [53], HPLC-DAD [54], and LC-MS/MS [55,56], these methods described the content of posaconazole in other substances and have not described for trace-level analysis and cannot be used to analyze the low content of PGIs in posaconazole [57]. The established LOD and LOQ are higher. Additionally, in a previously published study, Chen et al., have applied HR/MS/MS and online H/D exchange LC/MS methods to study the degradation product of posaconazole. In this study, the accurate mass value has significantly improved the possibility of the identification of unknown structures formed due to the degradation of posaconazole, whereas the online H/D exchange LC-HR/MS experiments have facilitated the structural identifications of four degradants during the degradation process [58]. Similarly, in a recently published study, Li et al. have successfully identified the degradation products of lurasidone using LC-PDA/UV-MS technique, and they have suggested that this technique can also be readily applied to rationalize the formation of posaconazole degradant [59].

Neither the detection of PGIs nor their quantification in posaconazole and its intermediate has been disclosed by prior methods. In contrast, a more precise method is more suitable for the detection of PGIs in trace levels. Additionally, the proposed method uses less solvent and has a shorter overall quantification time. This approach was evaluated in accordance with the ICH guidelines, and the analysis method was straightforward, sensitive, and repeatable.

No new approach was disclosed for the quantification of the three PGIs in posaconazole after reviewing the reported methodologies. To determine these three PGIs, a specific and sensitive approach using GC-MS/MS was evaluated and validated according to the ICH Q2 (R1) guidelines [60]. The current GC-MS/MS method for the identification and quantification of the three PGIs is novel, advanced, and industrially feasible (Scheme 1). This method is highly sensitive with the lowest LOD and LOQ detection.



Scheme 1. Graphical representation of the GC-MS/MS method for the identification and quantification of PGIs.

2. Results

2.1. Optimization of Mass Spectrometric Parameters

The Q1 and Q3 values were determined for PGI-1, PGI-2, and PGI-3 through mass tuning. The solubility of each analyte was evaluated to identify the impurities present in the posaconazole and its intermediate. Posaconazole and its impurities and intermediates are soluble in alcohol.

The mass parameters were obtained by tuning the mass spectrometry with diluted solutions of each PGI. The EI acts as an ion source to establish mass detection and ascertain both the Q1 and Q3 values. MRM-1 (m/z) and MRM-2 (m/z) were established for each impurity.

MRM-1 used 156–141 amu, 233–153 amu, and 152–127 amu for the quantification of PGI-1, PGI-2, and PGI-3, respectively. MRM-2 used 156–113 amu, 233–150 amu, and 152–132 amu for the qualification of PGI-1, PGI-2, and PGI-3, respectively (Figures 2–4).

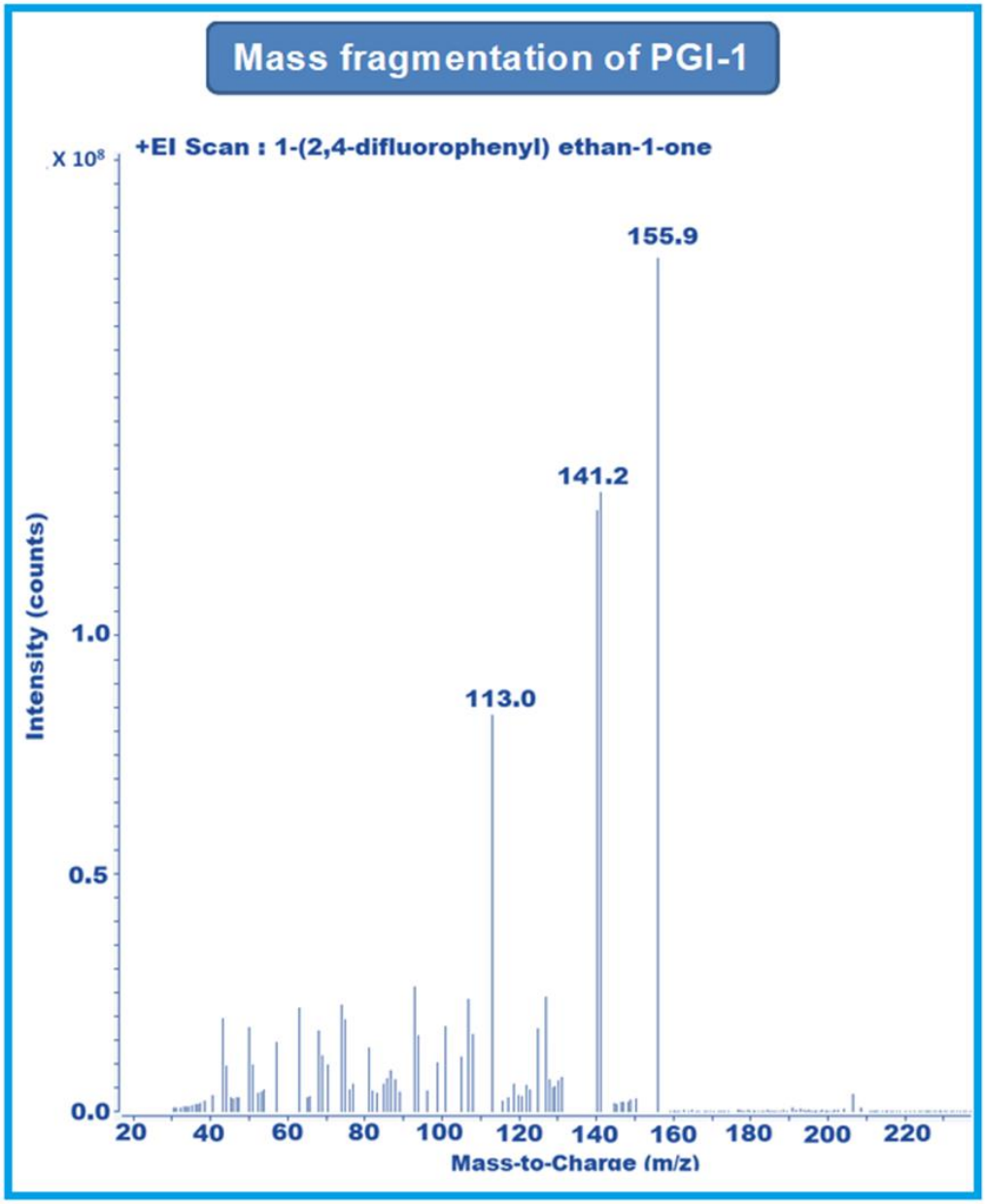


Figure 2. Mass fragmentation of PGI-1.

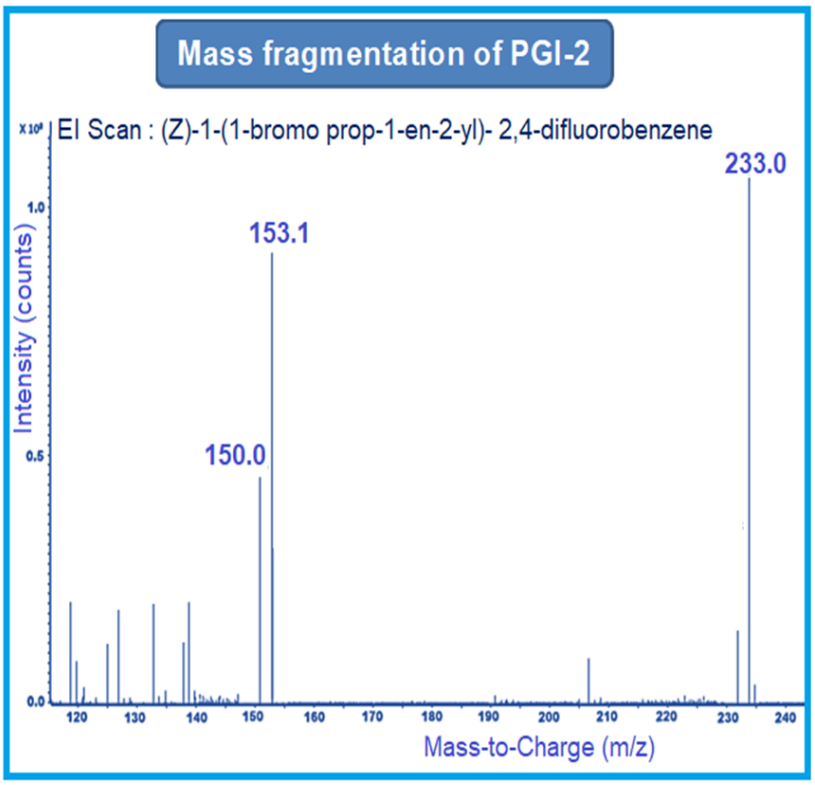


Figure 3. Mass fragmentation of PGI-2.

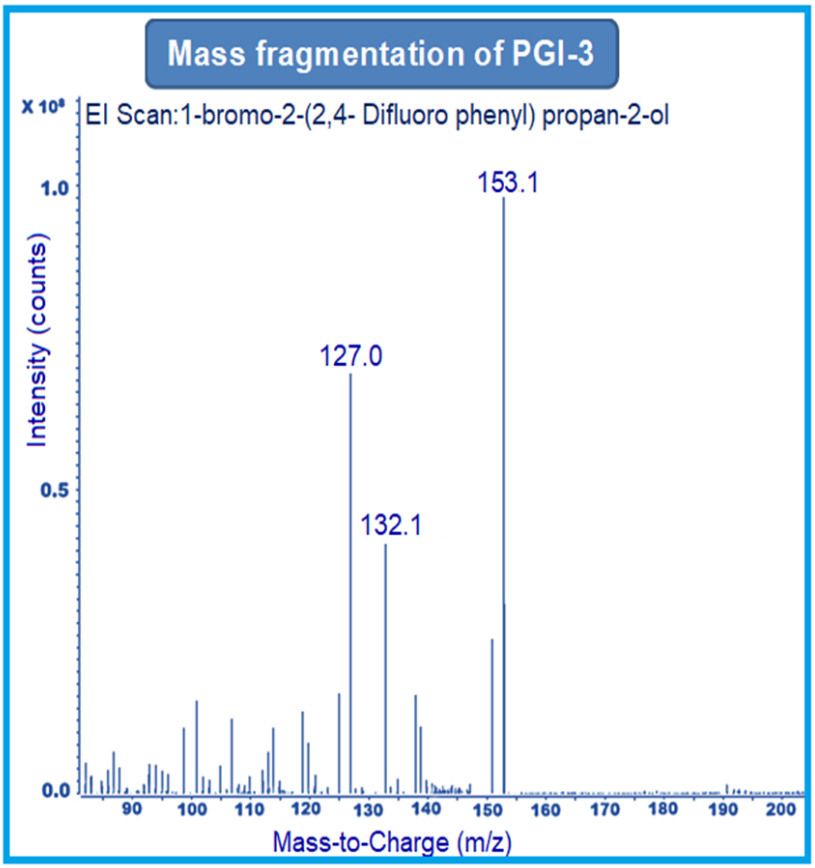


Figure 4. Mass fragmentation of PGI-3.

2.2. Optimization of Chromatographic Conditions

Different solvents were used as diluents for the preparation of the standards and samples. To conduct the diluent compatibility study, polar and nonpolar solvents and low and high boiler solvents were considered, including dimethyl sulfoxide, dimethylformamide, N-methyl-2-pyrrolidone, ethanol, dichloromethane, acetonitrile, methanol, and hexane.

We observed solvent interference, split peaks, broad peak shapes, and poor responsiveness of PGI impurities to various diluents during development. Methanol was the most suitable diluent. In methanol, no interference was observed, and each PGI responded very well at ppm concentrations.

Choosing the right column was crucial for developing this method. Different column chemistries, such as DB-wax, DB-5, DB-624, and DB-1, were employed for optimization during development. We observed that DB-624 was most suitable because each PGI peak was very sharp and well ionized, with good resolution.

The final method was improved by using helium as the carrier gas. The Detector off (MS-off) program was used before and after PGI peak elution. The retention times for PGI-1, PGI-2, and PGI-3 were approximately 13, 20, and 21 min, respectively.

2.3. Method Validation Study

To demonstrate that the established analytical method was suitable for its intended purpose, validation was conducted in compliance with the ICH Q2 (R1) requirements. The method was validated in terms of system suitability, specificity, the limit of detection (LOD), limit of quantification (LOQ), LOQ precision, linearity/range, method precision, intermediate precision, accuracy/recovery, robustness, and solution stability to ascertain the presence of PGIs in posaconazole and its intermediate.

2.4. System Specificity and Suitability

The capacity of an analytical method to evaluate a target constituent precisely and selectively within a mixture of contaminants is known as specificity. The resolution between neighboring peaks in the reference solution must be measured. Specificity is a key component of this strategy because multiple PGIs must be studied concurrently. In this method, specificity refers to the ability to quantify the analyte response in the presence of impurities (PGI-1, PGI-2, and PGI-3) in the posaconazole and its intermediate. To assess the specificity, all impurity solutions (PGI-1, PGI-2, and PGI-3) were independently produced and injected into the GC-MS instrument to determine the retention time. Additionally, according to the methodology, blank, sample, and spiked sample solutions were created, then injected into the GC-MS/MS (Table 1) (Figure 5).

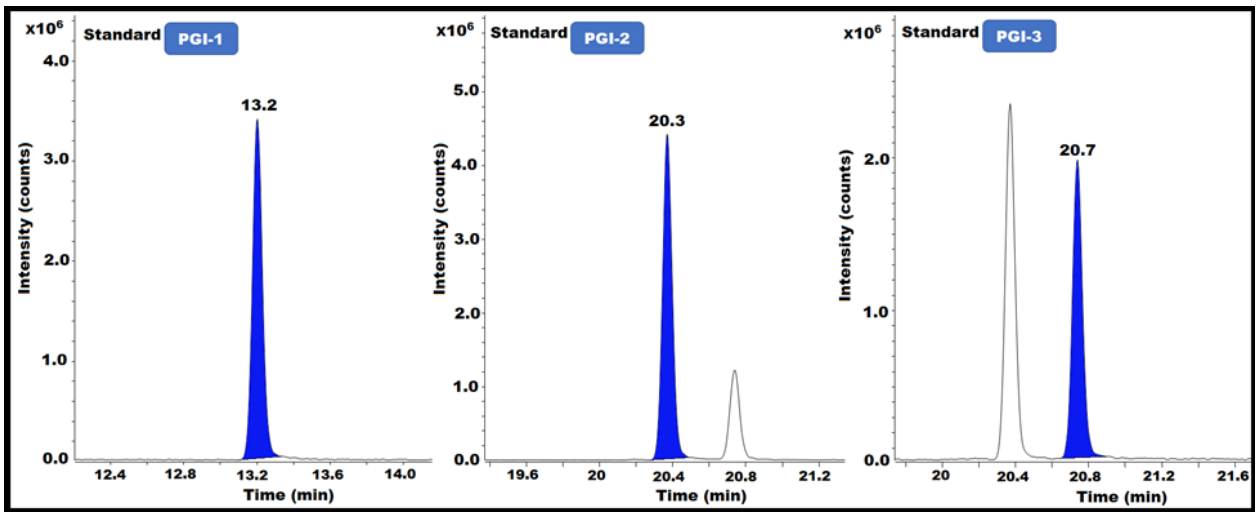


Figure 5. PGI-1, PGI-2, and PGI-3 standard GC-MS/MS chromatograms.

Table 1. Detailed results and their validation.

Parameters	Typical Acceptance Limits	Results		
		PGI-1	**PGI-2**	**PGI-3**
System suitability	%RSD ≤ 20.0 for each PGI	4.0	3.7	4.4
Specificity	RT of PGIs in all the solutions	13.2	20.3	20.7
	blank Interference at PGIs RT	No interference		
LOD	Concentration in ppm	0.010	0.011	0.010
	s/n ≥ 3	7.6	9.4	7.8
LOQ	Concentration in ppm	0.0251	0.0250	0.0251
	s/n ≥ 10	18.2	28.4	19.4
LOQ precision	%RSD ≤ 20.0 for each PGI	5.5	6.1	4.8
Linearity	Range (ppm)	0.0251 to 0.151	0.0250 to 0.150	0.0251 to 0.151
	(R) ≥ 0.99	0.998	0.997	0.996
MP	% RSD ≤ 20.0	4.2	5.5	5.9
IP	% RSD ≤ 20.0	5.0	4.2	6.1
MP and IP (n = 12) spike	% RSD ≤ 20.0	≤20.0	≤20.0	≤20.0
Accuracy	Spiked samples average recovery between 80 to 120%.			
	LOQ spiked solutions	95.4	96.7	95.9
	50% spiked solutions	96.7	95.8	96.4
	100% spiked solutions	95.6	96.3	95.8
	150% spiked solutions	96.2	95.9	95.2
Robustness	(+) Flow: concentration and RT	3.9% 12.10 min	2.7% 19.23 min	2.3% 19.68 min
	(−) Flow: concentration and RT	3.0% 13.98 min	2.8% 21.43 min	1.5% 21.86 min
	(+) Oven 122 °C: concentration and RT	3.2% 13.01 min	2.4% 20.02 min	2.2% 20.43 min
	(−) Oven 118 °C: concentration and RT	3.7% 13.32 min	2.2% 20.61 min	3.2% 21.00 min
Solution Stability	Report the results	Solutions are Stable up to 24 h		

2.5. LOQ, LOD, and Precision at LOQ

The minimum quantity of analyte required for validation was used to calculate the LOQ for each sample. The LOD is the lowest measurable concentration of any analyte in the samples. The signal-to-noise (s/n) ratios of the test were approximately 3 and 10, which allowed the separation of the LOD and LOQ. The LOD and LOQ were determined by injecting diluted PGI solutions in triplicates and measuring the known levels of each impurity. To determine LOQ precision, replicate injections of the LOQ solution (n = 6) were employed. The results showed that for the LOD and LOQ solutions, the s/n ratios were greater than 3 and 10, respectively. For each PGI, the % RSD of duplicate injections with LOQ precision was <15.0%. The technique accurately determined the amounts of PGI-1, PGI-2, and PGI-3 in posaconazole and its intermediate at the LOQ level (Table 2) (Figure 6).

Table 2. Final instrument conditions for the GC-MS/MS technique.

Instrument Parameters	**Final Condition Details**		
Gas chromatography conditions			
system of chromatography	Agilent Technologies 7890B GC system		
GC Column	USP phase G43, 60 m in length, 0.32 mm inner diameter, and 1.8 µm film thickness		
Carrier Gas	Helium		
Column Mode	Standard Flow		
Flow rate	1.5 mL/min		
Injector (Heater) temperature	200 °C		
Injection volume	2 µL		
Oven Programming	Ramping (°C/min)	Temperature (°C)	Hold time (min)
	-	120	5
	5	250	6
Run time	38 min		
Mass spectrometry conditions			
MS system	Agilent Technologies 7010B GC/TQ		
Ion source and Detection mode	EI and MRM		
Impurity	PGI-1	PGI-2	PGI-3
For qualification (m/z)	156 amu 113 amu	233 amu 150 amu	153 amu 132 amu
For quantification (m/z)	156 amu 141 amu	233 amu 153 amu	153 amu 127 amu
Collision energy (CE)	0	15	25
Gain Factor	20		
Dwell time (in milliseconds)	100		
Detector off (MS -Off)	As required		
MS Source temperature	240 °C		
MS Transfer Line temperature	270 °C		
MS Quad temperature	150 °C		

2.6. Linearity

The linearity of a test method is defined as its ability to obtain a linear measurement value within a specified range in relation to the amount (or concentration) of each analyte. Linearity was performed from the LOQ level to 0.15 ppm. With respect to the sample concentrations, the linearity solution concentrations were LOQ (0.025 ppm), 50% (0.05 ppm), 75% (0.075 ppm), 100% (0.1 ppm), 120% (0.12 ppm), and 150% (0.15 ppm). Each solution was injected in duplicate into the GC-MS/MS system. We also established the range and correlation coefficient by plotting the peak area responses versus the concentration (R). The analytical method was demonstrated to be linear for each PGI, with all findings being no less than 0.99 for the correlation coefficient (R) for each PGI, indicating that the method is linear for the determination of PGI-1, PGI-2, and PGI-3 content in posaconazole and its intermediate (Table 1) (Figure 7).

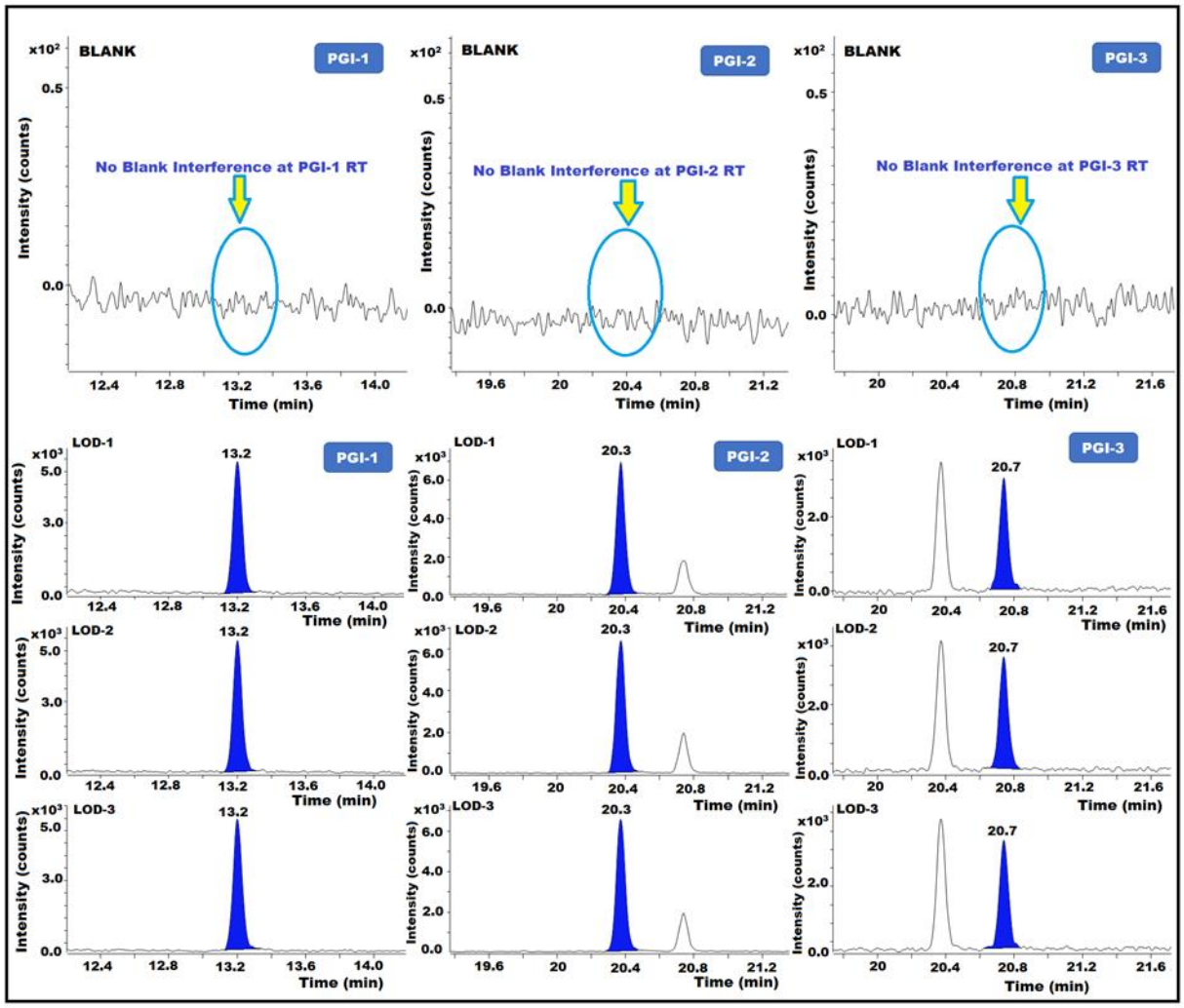


Figure 6. GC-MS/MS chromatograms of PGI-1, PGI-2, and PGI-3 show the limit of detection (LOD).

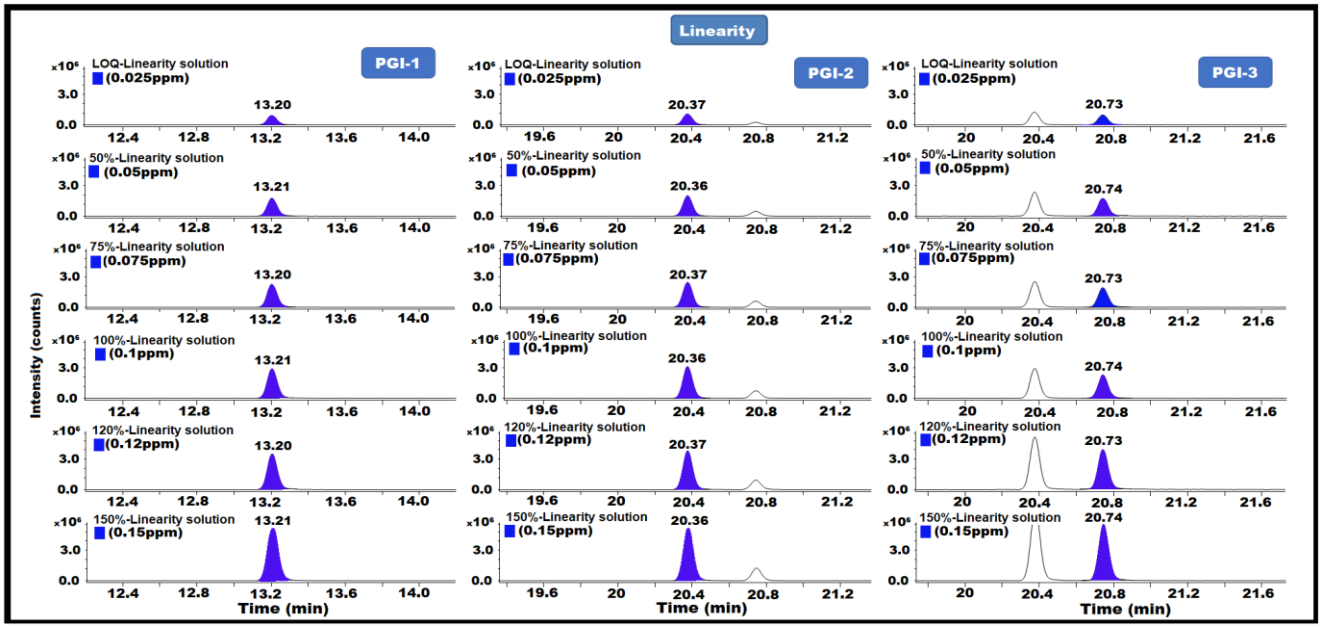


Figure 7. GC-MS/MS chromatograms of PGI-1, PGI-2, and PGI-3 show linearity.

2.7. Repeatability (Method Precision) (MP)

The repeatability of the approach was investigated in terms of method precision. The effectiveness of the procedure was assessed through repeated injections of the standard, sample, and spiked sample solutions. Six analyses of the standard solution were performed to evaluate the performance of the GC-MS/MS system on the day of the test, using the parameters of the test method (system precision). For each PGI, the system precision experimental results for the relative standard deviation were provided in the system suitability parameters. Six sample solutions were prepared using a single batch of Posaconazole, and each PGI was added at a specified level for the MP experiment. These solutions were injected into the GC-MS/MS apparatus. Each prepared solution was administered only once. In the sample solution, the impurity content and % RSD were calculated; the findings showed that % RSD $\leq$ 20.0% (Table 1).

2.8. Intermediate Precision

The term "intermediate precision" (IP) refers to the consistency of results from variances within the laboratory caused by unpredictable occurrences, such as different days, analysts, or equipment, that may occur during the procedure. Different analysts, days, and columns were used to establish an IP in line with the MP. The PGI content and % RSD of the sample and spiked solutions were calculated. The % RSD for the spiked sample solution was <15.0% (n = 6). The % RSD for the MP- and IP-spiked sample solutions at the prescribed level was <20.0% (n = 12). The findings show that this technique is reliable for determining the amounts of PGI-1, PGI-2, and PGI-3 in posaconazole and its intermediate (Table 1).

2.9. Accuracy

Accuracy is the degree to which a measured value is within a certain range of true or standard values. Recovery analysis of a matrix API that had been spiked with a PGI standard was used to assess the accuracy. Therefore, PGI-1, PGI-2, and PGI-3 were added to the posaconazole at the following concentrations: LOQ (0.025 ppm), 50% (0.05 ppm), 100% (0.1 ppm), and 150% (0.15 ppm). Posaconazole samples without impurities were prepared in triplicate for the accuracy experiment, then injected into the GC-MS/MS.

The % recoveries were calculated after analyzing the control and spiked samples. The proposed analytical method was used to determine the analyte content in the spiked sample solutions, and the recovery was estimated for each solution. All obtained results passed the acceptance requirements and were within 80–120% recovery for all PGIs. The results indicate that the method is accurate for the determination of PGI-1, PGI-2, and PGI-3 content in the posaconazole and in its intermediate (Table 1) (Figure 8).

2.10. Robustness

The robustness of a method is its capacity to survive small, deliberate modifications to its input parameters. Together with the original column oven temperatures of +122 and −118 °C, the actual column flow rate was modified to plus (+) (1.65 mL/min) and minus (−) (1.35 mL/min) flows. The results for the spike, standard, and MP data were compared with the solutions for concentration and retention time (RT). The RT of each impurity showed no irregularities, and there was a discrepancy of <10% in the impurity content between the MP and the robustness study results. The findings show that this technique is reliable for determining the amounts of PGI-1, PGI-2, and PGI-3 in Posaconazole and in its intermediate (Table 1).

2.11. Solution Stability

Solution stability studies were performed for up to 24 h at ambient laboratory temperature (25 ± 5 °C) and under refrigeration (8 ± 2 °C) using secondary intermediate stock solutions of PGI-1, PGI-2, and PGI-3 impurities and spiked samples with impurities at 100% concentration levels (0.10 ppm). Stability studies were performed using the percent recovery of freshly prepared primary standard solutions of the impurities and spiked

samples. The findings demonstrate that these solutions are reliable for determining the amounts of PGI-1, PGI-2, and PGI-3 in the posaconazole intermediate and posaconazole (Table 1). Additional method validation chromatograms and FT-IR and NMR data are provided in the Supplementary Materials (Figures S1–S25).

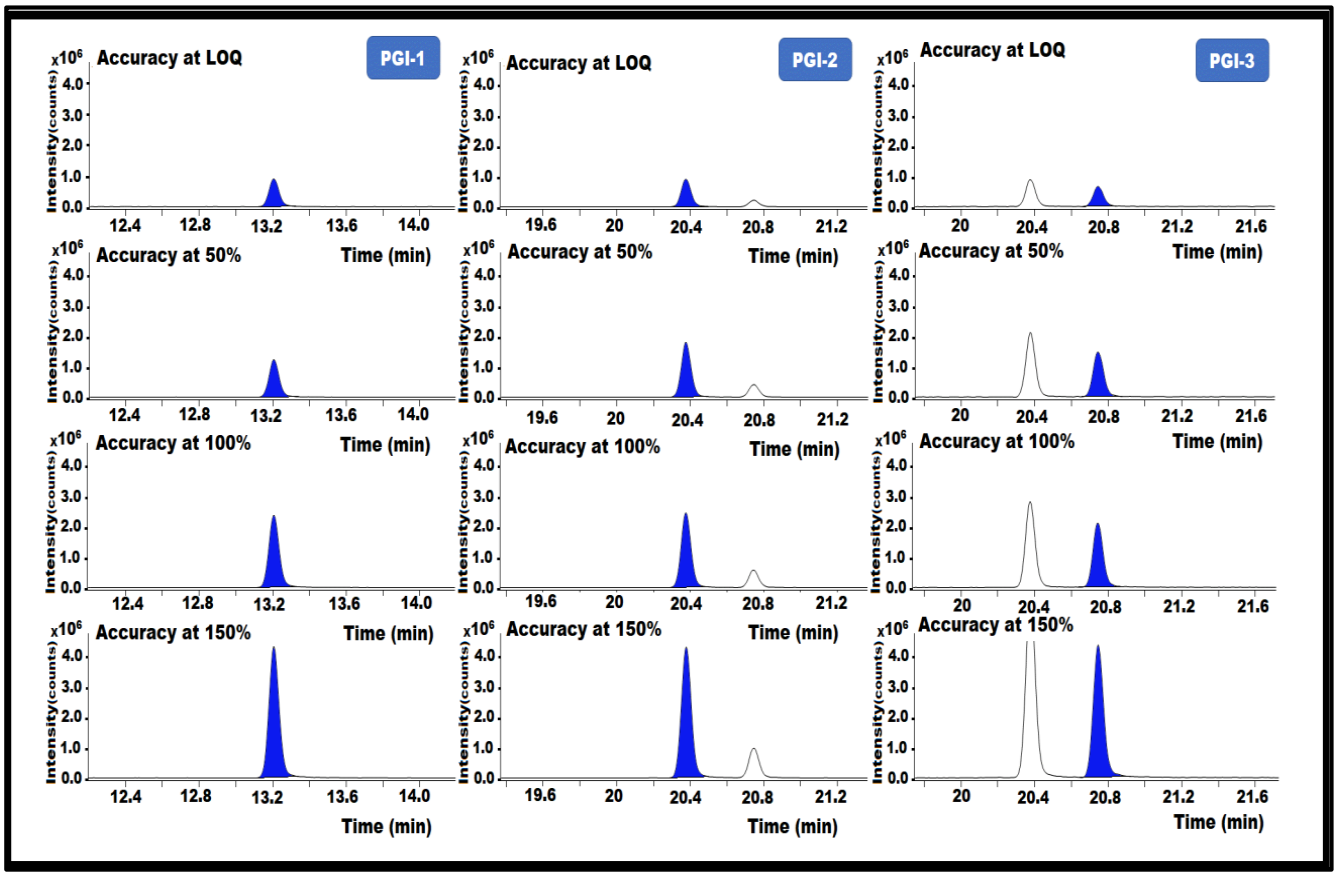


Figure 8. GC-MS/MS chromatograms of PGI-1, PGI-2, and PGI-3 show the accuracy of the method used in this study.

3. Discussion

Gas chromatography with electron ionization mass spectrometry is a powerful analytical technique in the pharmaceutical industry for the highly specific and quantitative measurement of trace levels of analytes and impurities. Among various commonly encountered genotoxic impurities, alkylating agents, such as alkyl halides, alkyl sulfonates, and other related structures, poses significant challenge to analytical scientists for the development of suitable analytical methodologies for their accurate measurement at trace level [61]. These impurities, particularly alkyl halides, are typically generated during the chemical synthesis and processing of APIs, including Posaconazole, which often result in cytotoxicity. For instance, Posaconazole is a member of triazole derivatives, which potentially involve the utilization of alkyl halide during their synthesis process [62]. An optimized GC-MS/MS method was developed to determine the PGI-1, PGI-2, and PGI-3 content in posaconazole and in its intermediate. Due to the molecular mass and fragmentation are specific to each compound and impurity, there was no interference with the impurity retention time because of the sample and blank solutions. This method has the advantage of detecting impurities at the trace and ppm levels, whereas previous methods [54–58], such as HPLC, HPLC/UV and bioassay, HPLC-DAD, and LC-MS/MS, are not focused on the content and determination of impurities. These methods are complex and describe the content of posaconazole in other substances.

In view of the prior arts and analytes polarity, there was a probability of developing an analytical method by using different analytical techniques, including HPLC-PDA and LC-MS/MS. We conducted a few experiments by changing different diluents and chromatographic conditions in LC-MS/MS. Different ion sources, including ESI positive and negative and APCI positive and negative, were used for mass tuning. All ionization conditions were found to produce extremely poor fragmentation and response. The development trials were conducted by using C8 and C18 columns with various lengths, diameters, and particle sizes and different pH buffers (acidic, basic, and neutral) to measure these PGIs; however, poor peak response was noticed. We concluded from the findings that the LC-MS/MS approach would not be suitable for quantifying these three PGIs. In HPLC experiments, the PGI-1, PGI-2, and PGI-2 peaks detected more than 100 ppm concentration standard solution; hence, we concluded from the findings that the HPLC approach would not be suitable for quantifying these three PGIs in trace levels.

To measure these three PGIs, a specific and sensitive approach using GC-MS/MS with a triple quadrupole mass spectrometry detector was evaluated. Different column chemistries, such as DB-wax, DB-5, DB-624, and DB-1, were employed for optimization during development. We observed that DB-624 (USP phase G43) was suited to one since each PGI peak was very sharp and well ionized with good resolution. Furthermore, the use of helium as the carrier gas improved the method. To enhance the response rate of these PGIs, we conducted the diluent study with different solvents, such as dimethyl sulfoxide, dimethylformamide, N-methyl-2-pyrrolidone, ethanol, dichloromethane, acetonitrile, methanol, and hexane. However, we observed that other than methanol, the response rate of the PGI impurities was poor and found split peaks and broad peak shapes.

The developed method has the following advantages over the other methods mentioned. Detection via GC-MS-MS would be more accurate and reliable. The sensitivity was assessed using the LOQ. For each PGI, the LOQ was determined to be 0.025 ppm. This method is as good as or better than the methods described in the other published articles.

The developed method was used for the study of validation to sleuth its performance characteristics.

4. Experimental

4.1. Materials and Reagents

PGI-1, PGI-2, and PGI-3 were purchased from HTS Biopharma Pvt. Ltd. (ALEAPIndustrial Estate, near Pragathi Nagar, Hyderabad, India). Methanol of GC grade was obtained from Merck (India). Posaconazole and its intermediate were received as gifts from Jisai Pharma Pvt. Ltd. (Phase-4, Plot No. 12, IDA, Cherlapally, Hyderabad, India).

4.2. Equipment

Using an Agilent 7890 B GC system (Agilent, Santa Clara, CA, USA) coupled to an Agilent 7010 B GC/TQ triple quadrupole outfitted with electron impact ionization (EI) as the MSD ion source and multiple reaction monitoring (MRM) modes, data were gathered using Mass Hunter software. Method development and validation were performed using this GC-MS/MS device. The standards and samples were weighed using an analytical balance (Mettler Toledo ME204E, Zürich, Switzerland). Samples and standards were mixed using a vortex mixer (Remi, India). A Thermo Scientific variable micropipette, Finn pipette F2, was used for dilution (Thermo Scientific, Vantaa, Finland).

4.3. Chromatographic Conditions

The GC-MS/MS system was optimized using USP phase G43, a mid-polar 6% cyanopropyl, 94% polydimethylsiloxane with a length of 60 m, inner diameter of 0.32 mm, and film thickness of 1.8 µm. The temperature in the column oven was initially set to 120 °C and held for 5 min. The temperature was gradually increased to 250 °C at a rate of 5 °C/min and was maintained for 6 min. As the carrier gas, 1.5 mL/min of helium was selected. The

injector heater was kept at a temperature of 200 °C, and a split ratio of 1:1 was used for the injection volume of 2 µL.

4.4. Mass Spectrometer Conditions

The multiple reaction monitoring (MRM) mode of the GC-MS/MS system was used while considering each PGI precursor ion (Q1) and production (Q3). MRM-1 (m/z) and MRM-2 (m/z) were established for each PGI based on its fragmentation pattern and response, and MRM-1 was used to quantify each PGI. MRM-1 (m/z) values were 156–141 amu for PGI-1, 233–153 amu for PGI-2, and 152–127 amu for PGI-3. The mass source and quad temperatures were 240 and 150 °C, respectively (Table 2).

4.5. Impurity Standard and Test Sample Solution Preparation

Each PGI standard (0.1 ppm with respect to sample concentration) was prepared in methanol (diluent). A posaconazole or posaconazole intermediate test sample (200 mg/mL) was prepared and diluted. The solutions were thoroughly incorporated after 5 min in a vortex. The specified concentration for each PGI was 0.1 ppm/(µg/mL) with respect to sample concentration is 100% of the specification limit. The sample concentration was optimized based on the accuracy findings obtained during technique development. Recovery was attained when the impurity was spiked at different sample concentrations, using a sample concentration of 200 mg/mL.

5. Conclusions

A sensitive and simultaneous GC-MS analytical method for three alkyl halides as PGIs in posaconazole and posaconazole intermediate was successfully developed. This study emphasizes the utility and efficiency of implementing an advanced analytical approach for the development of an analytical method using GC-MS/MS to quantify PGIs with low detection limits. This work presents a sensitive, effective, and reproducible GC-MS/MS method that is useful for determining and quantifying traces of PGI-1, PGI-2, and PGI-3 in posaconazole intermediate and posaconazole. The proposed method was validated according to the ICH guidelines; it met the criteria of acceptance for analytical parameters, such as specificity and system suitability, LOD, LOQ, LOQ precision, linearity and range, method precision, accuracy, ruggedness, robustness, and solution stability. This method can detect 0.01 ppm and quantify each PGI at 0.025 ppm and, thus, is useful for determining these PGIs in the routine analysis of posaconazole and its intermediate.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10050295/s1, Figure S1: 1-(2,4-difluorophenyl) ethan-1-one (PGI-1) standard certificate of analysis; Figure S2: PGI-1 Purity by HPLC Chromatogram; Figure S3: PGI-1 standard ^{1}H-NMR spectrum data; Figure S4: PGI-1 standard ^{13}C-NMR spectrum data; Figure S5: PGI-1 standard FT-IR spectrum; Figure S6: PGI-1 standard TGA graph; Figure S7: (Z)-1-(1-bromo prop-1-en-2-yl)-2,4-difluorobenzene (PGI-2) standard certificate of analysis; Figure S8: PGI-2 standard purity by HPLC chromatogram; Figure S9: PGI-2 standard ^{1}H-NMR spectrum graph; Figure S10: PGI-2 standard ^{13}C-NMR spectrum graph; Figure S11: PGI-2 standard FT-IR spectrum; Figure S12: PGI-2 standard TGA graph; Figure S13: 1-bromo-2-(2,4-Difluoro phenyl) propan-2-ol (PGI-3) standard certificate of analysis; Figure S14: PGI-3 standard purity by HPLC chromatogram; Figure S15: PGI-3 standard ^{1}H-NMR spectrum; Figure S16: PGI-3 standard ^{13}C-NMR spectrum; Figure S17: PGI-3 standard FT-IR spectrum; Figure S18: PGI-3 standard TGA graph; Figure S19: Blank solution chromatogram; Figure S20: Standard solution chromatogram; Figure S21: LOQ precision chromatogram; Figure S22: Method precision chromatogram; Figure S23: Sample solution chromatogram; Figure S24: Intermediate precision chromatogram; Figure S25: Linearity chromatogram; Figure S26: Method development experiments result; Figure S27: Plausible Fragmentation pattern of PGI-1, PGI-2, and PGI-3 impurities.

Author Contributions: Conceptualization, H.N.P.R.C. and J.V.S.K.; methodology, H.N.P.R.C. and J.V.S.K.; formal analysis, M.R.S., T.H.O., M.K. (Mujeeb Khan), M.K. (Merajuddin Khan) and B.S.; investigation, H.N.P.R.C., J.V.S.K. and M.R.S.; resources, H.N.P.R.C. and J.V.S.K.; data curation, H.N.P.R.C., J.V.S.K. and M.R.S.; validation, A.B. and M.K. (Mujeeb Khan); writing—original draft preparation, H.N.P.R.C., J.V.S.K. and A.B.; writing—review and editing, H.N.P.R.C. and M.R.S.; supervision, J.V.S.K.; project administration, J.V.S.K.; funding acquisition, M.R.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Researchers Supporting Project number (RSPD2023R665), King Saud University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data contained within the article and supplementary file.

Acknowledgments: The authors acknowledge funding from the Researchers Supporting Project number (RSPD2023R665), King Saud University, Riyadh, Saudi Arabia.

Conflicts of Interest: The authors declare no conflict of interest.

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