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Systematic Review

Quantitative Methods for the Trace level Determination of Six Potential Genotoxic impurities in anti-cancer Drug, Imatinib Mesylate Using Gas Chromatography With Mass Spectrometry

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ABSTRACT:
Two novel gas chromatography coupled with mass spectrometry (GC-MS-SIM) methods were developed for quantitation of trace levels of six potential genotoxic impurities (PGI's) namely 2-(Bromomethyl) benzonitrile (2-BBN), 3-(Bromomethyl) benzonitrile (3-BBN), 4-(Bromomethyl) benzonitrile (4-BBN). 2-(Dibromomethyl) benzonitrile (2-DBBN). 3-(Dibromomethyl) benzonitrile (3-DBBN) and 4-(Dibromomethyl) benzonitrile (4-DBBN) in Imatinib Mesylate (IMM) drug substance in selective ion monitoring mode. In these two methods, Chromatographic separation of potential genotoxic impurities (PGI's) were achieved on capillary GC column (Rtx-35, Fused silica capillary column; 30 m length; 0.32mm internal diameter, coated with 35% diphenyl and 65% dimethyl polysiloxane stationary phase of 1.0 µm film thickness) and passing helium as carrier gas with Electron Impact ionization (EI) in Selective Ion Monitoring (SIM) mode by using liquid-liquid extraction sample preparation technique for all six impurities. The mass fragments (m/z) were selected for the quantification of 2-BBN (m/z-116), 3-BBN (m/z-116), 4-BBN (m/z-119), 2-DBBN (m/z-194), 3-DBBN (m/z-194) and 4-DBBN (m/z-194). The performance of the methods validation was assessed by evaluating the specificity, linearity, sensitivity, precision and accuracy experiments. For 2-BBN, 3-BBN and 4-BBN, the limit of detection (LOD) and the limit of quantitation (LOQ) were 0.12 ppm and 0.38 ppm, respectively. For 2-DBBN, 3-DBBN and 4-DBBN impurities, the limit of detection (LOD) and the limit of quantitation (LOQ) were 0.12 ppm and 0.38 ppm, respectively. The correlation coefficient value of the linearity experiment were in the range of 0.9991–0.9999 for all six impurities. The average recoveries for the accuracy were in the range of 90.6–108.3% for all impurities. The validation results demonstrated the good linearity, precision and accuracy of the method which can be further adopted as an adequate quality control tool for quantitation of six potential genotoxic impurities (PGI's) at trace levels in Imatinib mesyalte drug substance.

Keywords: Trace level, Imatinib mesylate (IMM), GC-MS, Potential Genotoxic impurities (PGI's), Liquid-Liquid extraction and Method validation.

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INTRODUCTION

Imatinib mesylate is a tyrosine-kinase inhibitor used in the treatment of most particularly Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia‖ (CML) and multiple cancers. Imatinib acts by inhibiting BCR-Abl, a type of tyrosine-kinase, from phosphorylating subsequent proteins and starting the signalling cascade necessary for preventing the growth of cancer cells and leading to their death by apoptosis. The BCR-Abl tyrosine kinase enzyme exists only in cancer cells and not in healthy cells. Imatinib is a chemotherapy drug. It is

mainly used to treat certain leukemias, myelodysplastic syndromes and other cancers. It is also used in the treatment of specific digestive tract tumors called gastrointestinal stromal tumors (GISTs). Imatinib is one of the first anticancer drug to show the potential for such a targeted action and is often cited as a paradigm for research in cancer therapeutics. [1-3]

Imatinib mesylate is chemically known as 4-[(4-methyl-1-piperazinyl) methyl]-N- [4- methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]phenyl]benzamid methanesulfonate and has a chemical formula $C_{29}H_{31}N_7O.CH_3SO_3H$. Its molecular weight is 589.7 g/mol. The chemical structure of Imatinib mesylate is shown in **Figure 1**.

Control of impurities in active pharmaceutical ingredients (API) is an important aspect of drug development, ensuring product quality and minimizing safety risks. For impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantization/detection limit of the analytical procedures should be commensurate with the level at which the impurities should be controlled. Different impurities may be observed throughout the development lifecycle of an API.

According to Less-Than-Lifetime (LTL) exposures phenomena of ICH M7 [4] for mutagenic impurities in the pharmaceutical industry, the risk assessment for carryover into the drug substance from the raw materials and intermediates to be appropriately controlled to fulfil the regulatory requirements. To achieve this, genotoxic assessment (identification of genotoxic impurity, control of impurity and generated data presentation) is must w.r.t. analytical approach and control of identified impurities based on TTC concept.

According to TTC concept, we have developed a new GC-MS methods for quantification of six identified potential genotoxic impurities in Imatinib mesylate drug substance, as these impurities having alkyl halide structural alerts. Alkyl halides show conventional structural alerts for genotoxic potentiality [5]. Alerting structural elements are mainly based on the nature of the electrophilic character (as such or based on its metabolic activation) and/or genotoxicity data from representative compounds. Evaluation of drug substances for potential genotoxicity encompasses effects on both genes and chromosomes. Whereas, for impurities, testing is focused on the relation of the potential interaction with DNA. The chemical reactivity that makes them useful in chemical synthesis brings about the risk that, a number of such molecules may react with DNA, leading to carcinogenesis. These are called as potential genotoxic impurities (PGI's). As a result, PGT's can lead to mutations or cause cancer [6-8]. The issue of potential genotoxic impurities (PGI's) in pharmaceutical products has attracted increasing attention from the industry [9–10] as well as regulatory agencies [11–16]. To ensure these undesired PGI's are reduced to an acceptable level (often at low ppm) in the final product, it is critical to monitor them closely throughout the process. However, rapid development of analytical methods at such low levels remains a challenge for analytical chemists.

A molecule bearing an alkyl halide moiety is normally flagged by the most commonly used silico systems like Quantitative Structure-Activity Relationships (QSARs), Structure Activity Relationships (SARs) and consequently, an Ames assay test is carried out. The selected six impurities shows mutagenic activity based on these two alerts. Hence genotoxicity is assumed, staged and further TTC concept is applied [4] as per EMEA [14] and FDA [16] guidelines. According to the maximum daily dose (400 mg twice/day), the allowed limit is not more than 1.875 ppm for each impurity. Based on these two GC-MS methods developed and validated for the same limit.

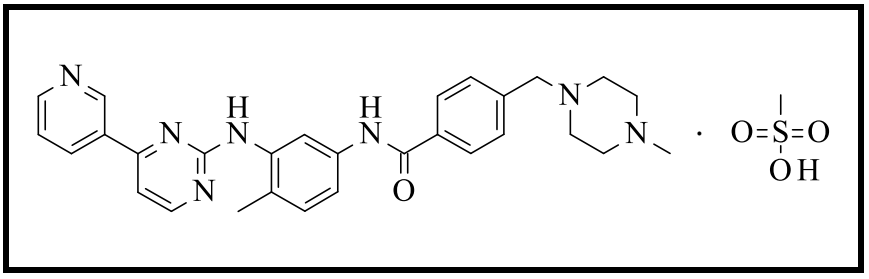


Figure 1. Chemical structure of Imatinib mesylate

Determination of trace levels of PGI's in API is often a great analytical challenge as an extremely sensitive, selective and robust analytical method is needed. Many traditional approaches such as HPLC-UV for non-volatile analytes and GC-FID for volatile analytes are usually not effective enough for impurity analysis at sub-ppm or trace levels [17]. Hyphenated techniques like GC-MS and LC-MS combining physical separation capabilities of chromatography (GC or HPLC) with mass spectrometry have higher sensitivity and specificity than conventional HPLC and GC methods. Their applications are oriented towards the potential identification and quantitation of trace levels of impurities in API. Several recent publications have reported systematic PGI method development and control strategies [13,18,19].

4-(Bromomethyl)benzonitrile or 4-Cyanobenzyl bromide is the starting material used in the manufacturing process of 4-(4-methylpiperazinomethyl) benzoic acid dihydrochloride which is the one of the intermediate in the synthesis of in Imatinib Mesylate. 4-(Bromomethyl)benzonitrile and its positional isomers i.e. (2-(Bromomethyl)benzonitrile) and (3-(Bromomethyl)benzonitrile) and dibromo impurities are having structural alerts. 2-(Dibromomethyl)benzonitrile, 3-(Dibromomethyl) benzonitrile and 4-(Dibromomethyl) benzonitrile are also possible impurities in 4-(Bromomethyl)benzonitrile and these impurities also having structural alerts.

Based on literature and evaluation by Derek software, these six compounds are found to be potential genotoxic impurities. The European Agency for the Evaluation of Medicinal products (EMEA) [20], United States Food and Drug Administration (USFDA), ICH Q3A/B and ICH M7 issued the guidelines and draft guidance have established a threshold of toxicological concern (TTC) of 1.5 µg/day (1.5 ppm, assuming a daily dose of 1 g/day) for each GTI as an acceptable threshold for any marketing authorization application [21, 22]. As per the toxicological threshold concern (TTC) approach and based on the maximum daily dosage of Imatinib Mesyalte (400mg twice a day), these potential genotoxic impurities (2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN) should be <1.875 ppm (as per TTC concern)[17, 23-24]. To attain the best quality of IMM drug, these potential genotoxic impurity levels should be monitored and controlled with appropriate analytical methods in IMM drug substance. Hence, in order to meet the regulatory agencies requirements and best quality of IMM, it is essential to develop a sensitive analytical method.

There are few references for Imatinib Mesylate (IMM), literature survey revealed that currently there is no method for the low level quantification of this six PGI's (2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN) in IMM drug substance till to date. Hence, it is aimed to develop and validate a sensitive and specific method for the trace level determination of the six PGI's in IMM drug substance by GC-EI-MS with selective ion monitoring (SIM) mode. The chemical structures of this six PGI's are shown in **Figure 2 (a)** to **2 (f)** and its mass spectrums are shown in **Figure 2 (g)** to **2 (l)**.

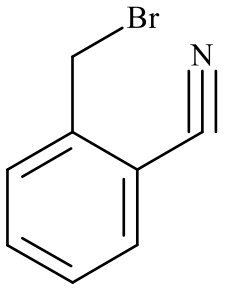


Fig. 2(a) 2-Bromomethyl benzonitrile (2-BBN)

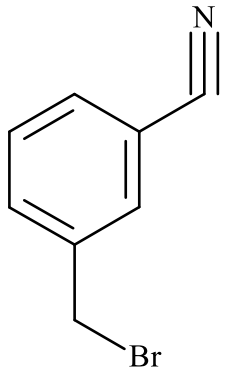


Fig. 2(b) 3-Bromomethyl benzonitrile (3-BBN)

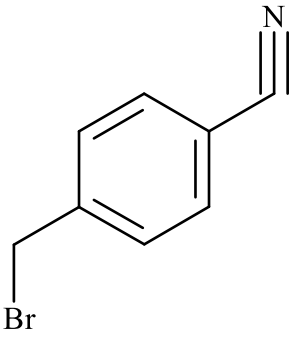


Fig. 2(c) 4-Bromomethyl Benzonitrile (4-BBN)

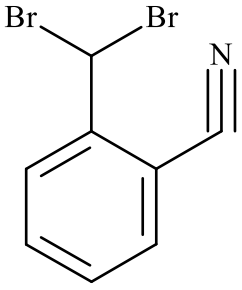


Fig. 2 (d) 2-(Dibromomethyl) benzonitrile (2-DBBN)

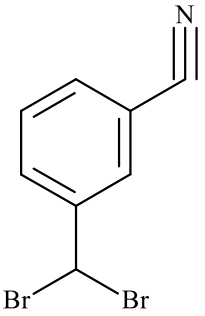


Fig. 2(e) 3-(Dibromomethyl) benzonitrile (3-DBBN)

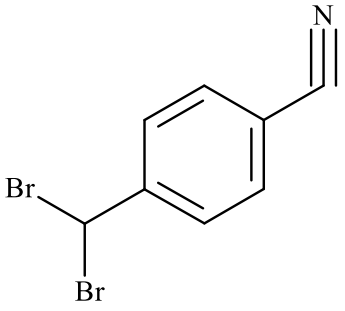


Fig. 2(f) 4-(Dibromomethyl) benzonitrile (4-DBBN)

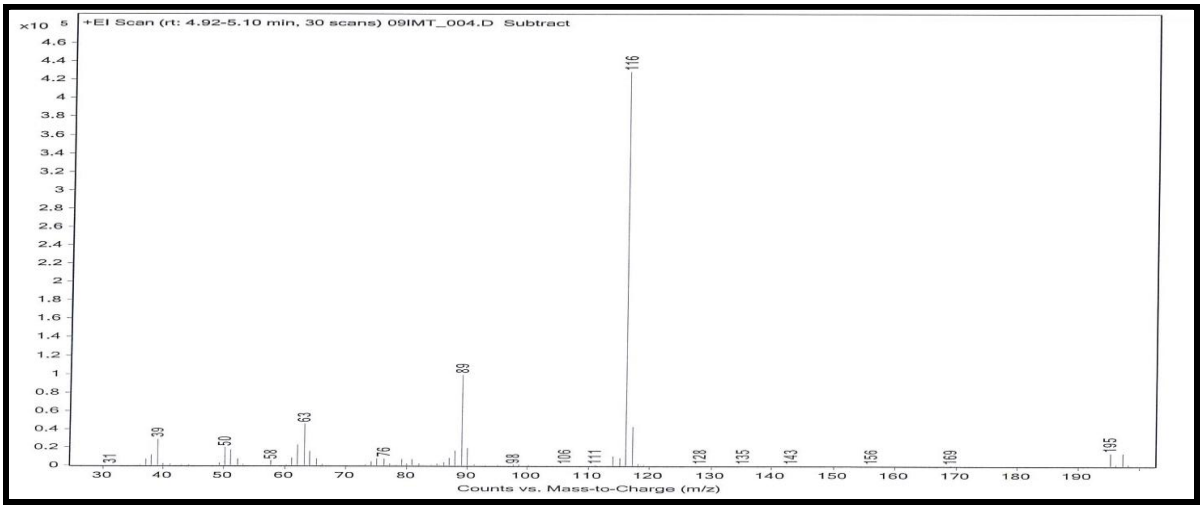


Fig. 2(g): Mass Spectrum of 2-Bromomethyl benzonitrile (2-BBN)

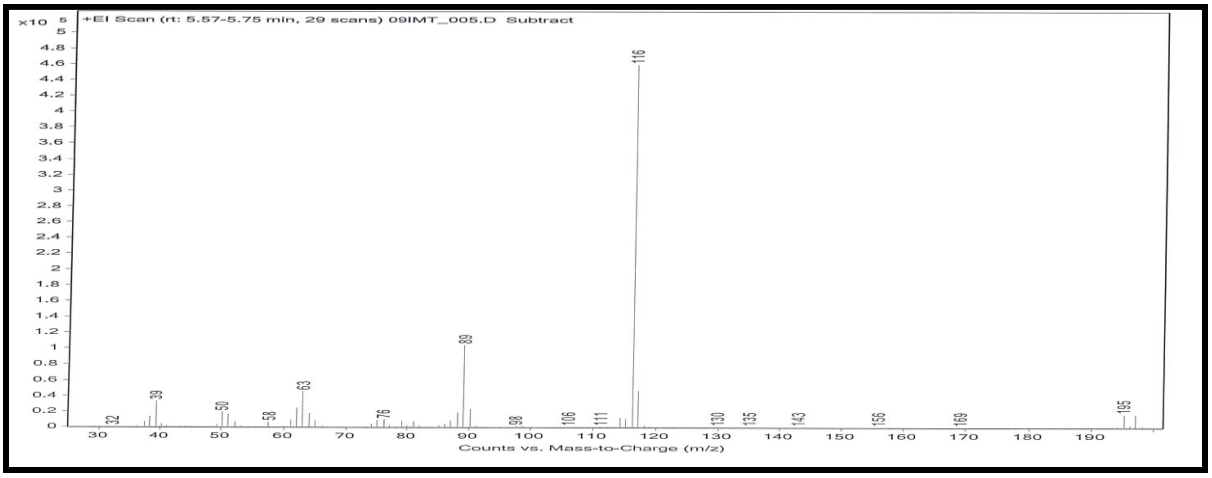


Fig. 2(h): Mass Spectrum of 3-Bromomethyl benzonitrile (3-BBN)

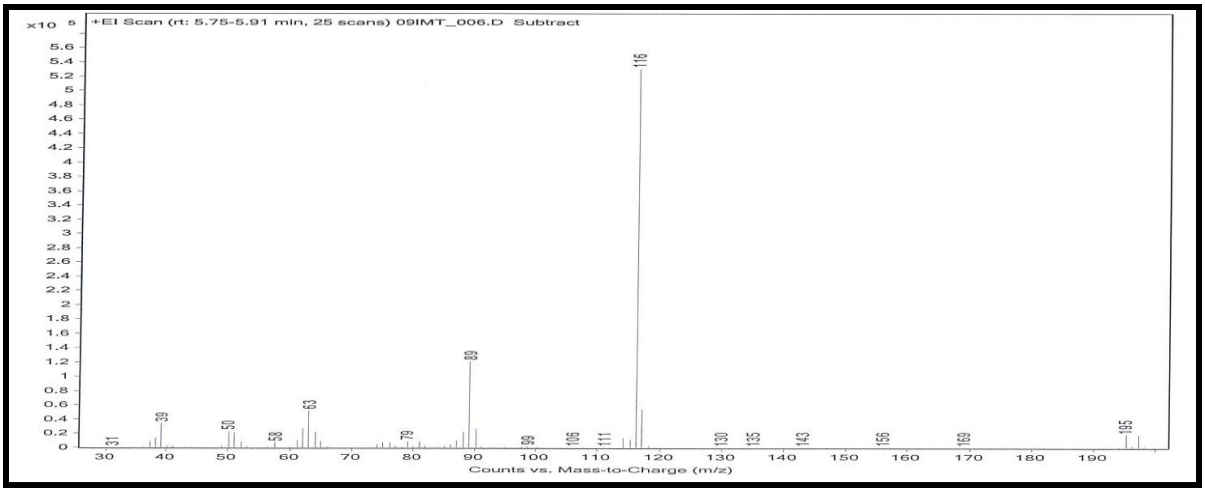


Fig. 2(i): Mass Spectrum of 4-Bromomethyl benzonitrile (4-BBN)

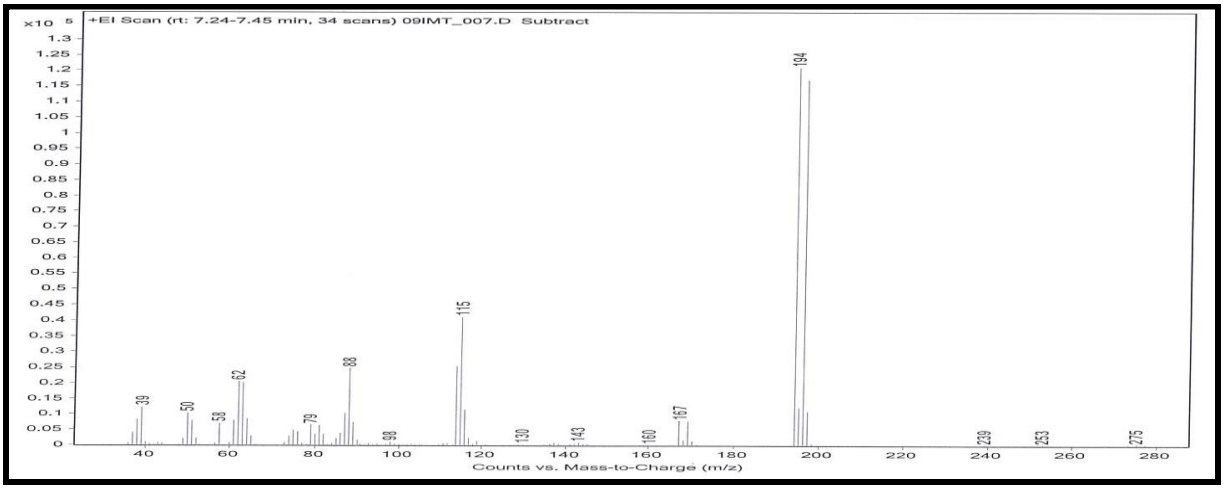


Fig. 2(j): Mass Spectrum of 2-(Dibromomethyl) benzonitrile (2-DBBN)

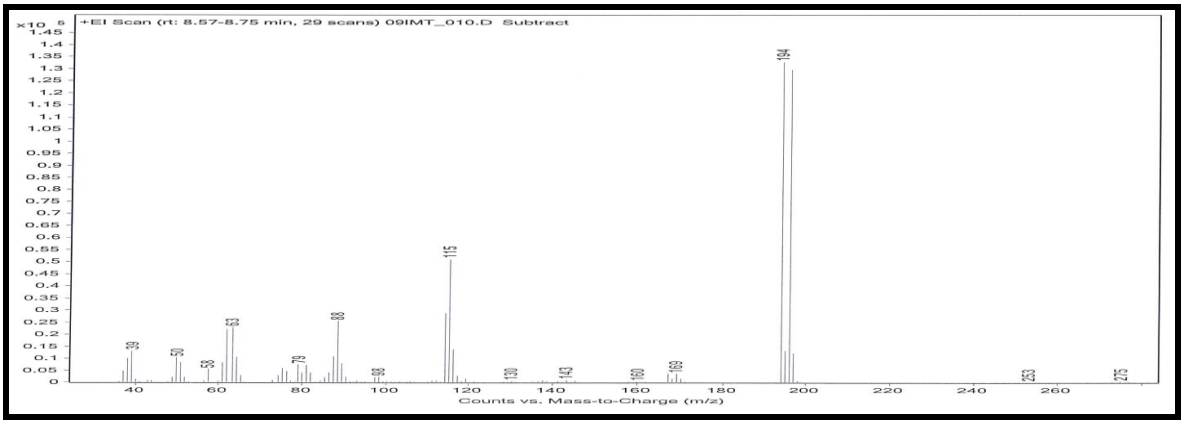


Fig. 2(k): Mass Spectrum of 3-(Dibromomethyl) benzonitrile (3-DBBN)

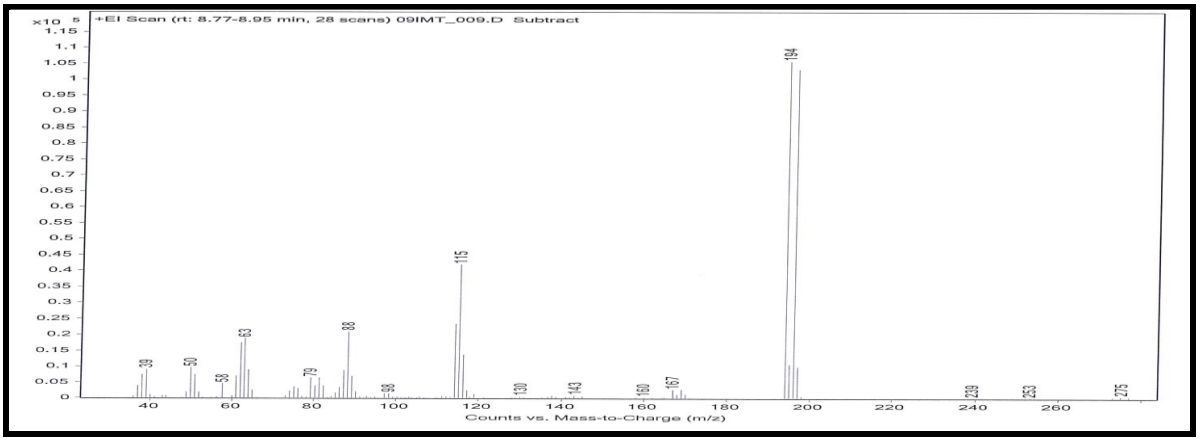


Fig. 2(l): Mass Spectrum of 4-(Dibromomethyl) benzonitrile (4-DBBN)

EXPERIMENTAL

Chemicals and reagents:

2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN and pure samples of IMM were obtained from Chemical Research Division of APL Research Centre laboratories (A division of Aurobindo Pharma Ltd., Hyderabad, India). Formic acid (Grade: EMPARTA ACS) was procured from Merck, India. Water (Grade: HPLC) and Dichloromethane (Grade: GC) were procured from Rankem, India.

Standard solutions for Method-1:

Diluent: Dichloromethane

Standard stock solution:

Weigh accurately about each 23.4 mg of 2-(Bromomethyl) benzonitrile (2-BBN), 3-(Bromomethyl) benzonitrile (3-BBN) and 4-(Bromomethyl) benzonitrile (4-BBN) standards into a 25 mL of volumetric flask half-filled with diluent and make up to volume with diluent and mix well. Transfer 0.5 mL of this solution into a 50 mL volumetric flask and make up to the volume with diluent.

Standard solution:

Transfer 0.5 mL of the above standard stock solution into a 50 mL volumetric flask and dilute to volume with diluent and mix well.

Standard solution vial:

Transfer 1.0 mL of Formic acid into a clean glass centrifuge tube followed by add 1.0 mL of water and shake the solution. To this, add 2.0 mL of the above standard solution and shake the solution about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

Blank solution vial:

Transfer 1.0 mL of Formic acid into a clean glass centrifuge tube followed by add 1.0 mL of water and shake the solution. To this, add 2.0 mL of Dichloromethane and shake the solution about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

Test solution vial:

Accurately weigh and transfer about 100 mg of test sample into a clean glass centrifuge tube, add 1.0 mL of Formic acid and dissolve. To this, add 1.0 ml of water and shake the solution about 1 min and add 2.0 mL of Dichloromethane and shake the solution for about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

Standard solutions for Method-2:

Diluent: Dichloromethane

Standard stock solution:

Weigh accurately about each 23.4 mg of 2-(Dibromomethyl)benzonitrile (2-DBBN), 3-(Dibromomethyl)benzonitrile (3-DBBN) and 4-(Dibromomethyl)benzonitrile (4-DBBN) standard into a 25 mL of volumetric flask half-filled with diluent and make up to volume with diluent and mix well. Transfer 0.5 mL of this solution into a 50 mL volumetric flask and make up to the volume with diluent.

Standard solution:

Transfer 1.0 mL of the above standard stock solution into a 50 mL volumetric flask and dilute to volume with diluent and mix well.

Standard solution vial:

Transfer 1.0 mL of Formic acid into a clean glass centrifuge tube followed by add 1.0 mL of water and shake the solution. To this, add 2.0 mL of the above standard solution and shake the solution about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

Blank solution vial:

Transfer 1.0 mL of Formic acid into a clean glass centrifuge tube followed by add 1.0 mL of water and shake the solution. To this, add 2.0 mL of Dichloromethane and shake the solution about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

Test solution vial:

Accurately weigh and transfer about 200 mg of test sample into a clean glass centrifuge tube, add 1.0 mL of Formic acid and dissolve. To this, add 1.0ml of water and shake the solution about 1 min and add 2.0 mL of Dichloromethane and shake the solution for about 1 min. Allow the two phases to separate. Collect the lower layer (Dichloromethane layer) and use for analysis.

GC-MS Conditions:

The analysis was carried out on the Agilent GCMS-5977A and GCMS-5977B gas chromatograph equipped with 7890B GC System auto sampler and data handling system having Mass Hunter solution software. The instrument was run in EI mode. Rtx-35, (30m × 0.32 mm I.D, 1.0 µm film thickness, Agilent Technologies, USA) column consists of 35% diphenyl and 65% dimethyl polysiloxane as a stationary phase. Chromatographic method conditions used were as follows **(Tables 1-3).**

RESULTS AND DISCUSSION

Optimization of chromatographic parameters:

The objective of the present work is, to establish a simple GC-MS-SIM methods for the determination of Mono bromo impuritites i.e.2-BBN, 3-BBN, 4-BBN and Dibromo impurities i.e. 2-DBBN, 3-DBBN and 4-DBBN contents in Imatinib mesyalte drug substance. In the synthesis process of IMM drug substance, 4-BBN was used as a key raw material. The positional isomers, i.e. 2-BBN, 3-BBN and dibromo impurities i.e. 2-DBBN, 3-DBBN and 4-DBBN may give corresponding potential impurities in IMM drug substance. Method development activity was initiated based on the solubility studies of IMM drug substance and six PGI's. IMM drug substance and 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN analytes having chromophore for UV or Fluorescence detection. Initially these analytes tried in HPLC, UPLC, LC-MS and LC-MS/MS techniques. But in these techniques, the required levels are not able to achieved. Moreover, based on the tendency of volatility and polarity of the analytes, there is a possibility to develop a chromatography method by GC equipped with flame ionization detector (FID). We made few trials by changing different diluents and chromatographic conditions in GC with FID. Due to the lower response of these impurities by GC-FID technique, we have chosen a gas chromatography electron ionization mass spectrometry (GC-MS-EI) technique in SIM mode for good

separation and desired sensitivity. No analytical methods available in literature to quantifying this PGI's in IMM drug substance by GC-MS till date.

Due to high boiling points of 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN, the peaks were not eluted in head-space technique. Further, development trials were initiated in direct-liquid injection technique using the stationary phase, 6% cyanopropyl and 94% dimethyl polysiloxane (DB-624; Make: Agilent). The sample solution was prepared by dissolving the sample in diluent (i.e. Methanol used as diluent) and injecting into the GC-MS. Background interference was encountered in this trial and peak shapes were also not good. After cleaning the inlet port (to avoid ghost peaks), a broad peak shape of analytes was observed, which suggests another type of sample preparation required to reduce the interference from the sample matrix and proper peak shapes. During optimization procedure we have tried with few of diluents i.e. chloroform, diethyl ether and ethyl acetate and different columns. Finally, Methylene chloride extraction is used for sample preparation and using the stationary phase, 35% diphenyl and 65% dimethyl polysiloxane (Rtx-35; Make: Restek). Sample dissolved in Formic acid and extracted with Methylene chloride has given satisfactory results. But in this extraction procedure, Dibromo impurities response was low when compare to Monobromo impurities. Due to this reason for Dibromo impurities quantifications method was slightly modified. In final methods, m/z-116 ion selected for quantification of 2-BBN, 3-BBN and 4-BBN and m/z-194 ion selected for quantification of 2-DBBN, 3-DBBN and 4-DBBN.

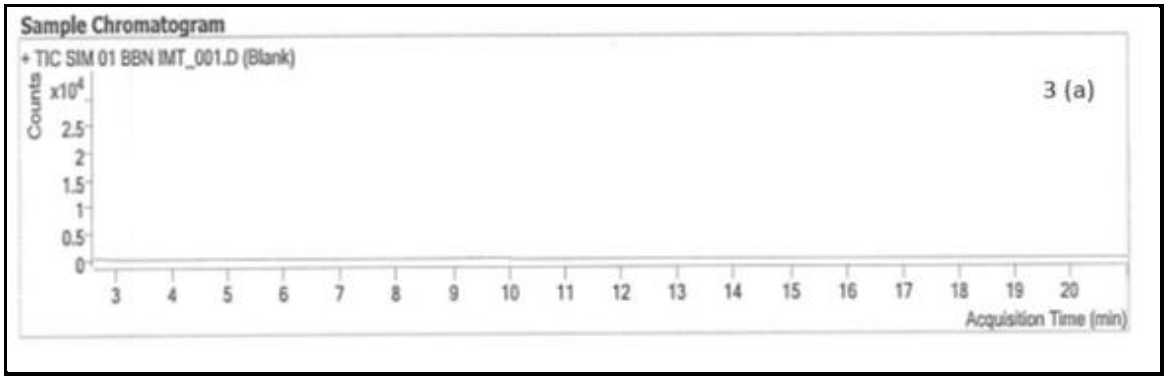
A well resolved, satisfactory chromatographic GC-MS-EI methods were developed by using Rtx-35, 30m long with 0.32mm i.d.,1.0µm particle diameter column consists of 35% diphenyl and 65%-dimethylpolysiloxane as stationary phase and passing helium as carrier gas. Dichloromethane used as diluent for two methods. In the Quantification of six impurities in two method used as same temperature programme. The temperature of column oven is used initially 180°C is maintained for 4 min and then increased to 240°C at a rate of 10°C/min followed by holding at 240°C for 11 min. The developed methods were used for validation study to evaluate its performance characteristics. The present investigation was initiated for the quantification of 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN, by GC-MS-EI technique in IMM drug substance.

Method validation study parameters:

To order to determine the contents of 2-BNN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN in IMM drug substance, the developed methods were validated as per the ICH guidelines [25] individually in terms of specificity, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy and precision (system precision, method precision and intermediate precision) and robustness and system suitability.

Specificity:

The specificity of the developed GC-MS-EI methods was indicated by showing the m/z peaks in the method as 116 for all 2-BBN, 3-BBN and 4-BBN, 194 for all 2-DBBN, 3-DBBN and 4-DBBN. Specificity is the ability of the method to measure the analyte response in presence of all impurities (2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN) in IMM drug substance. To evaluate the specificity experiment, all impurity solutions (2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN) were prepared individually and injected into GC-MS to confirm the retention times. Further, blank, control sample (IMM sample) and spiked sample solutions (IMM sample spiked with 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN) were prepared as per methodology and injected into GC-MS. From the chromatograms of all individual injection solutions (2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN), blank solution, control sample solution and spiked sample solutions, it was observed that 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN peaks were well resolved from each other and there was no other interference (co-elution) from the sample matrix indicated that the method is selective and specific for the determination of 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN contents in IMM drug substance. A typical representative overlaid GC-MS chromatograms of Method-(1) and Method-(2) are shown in **Figure 3 (a)** to **3 (e)** and **Figure 4 (a)** to **4 (e)**.



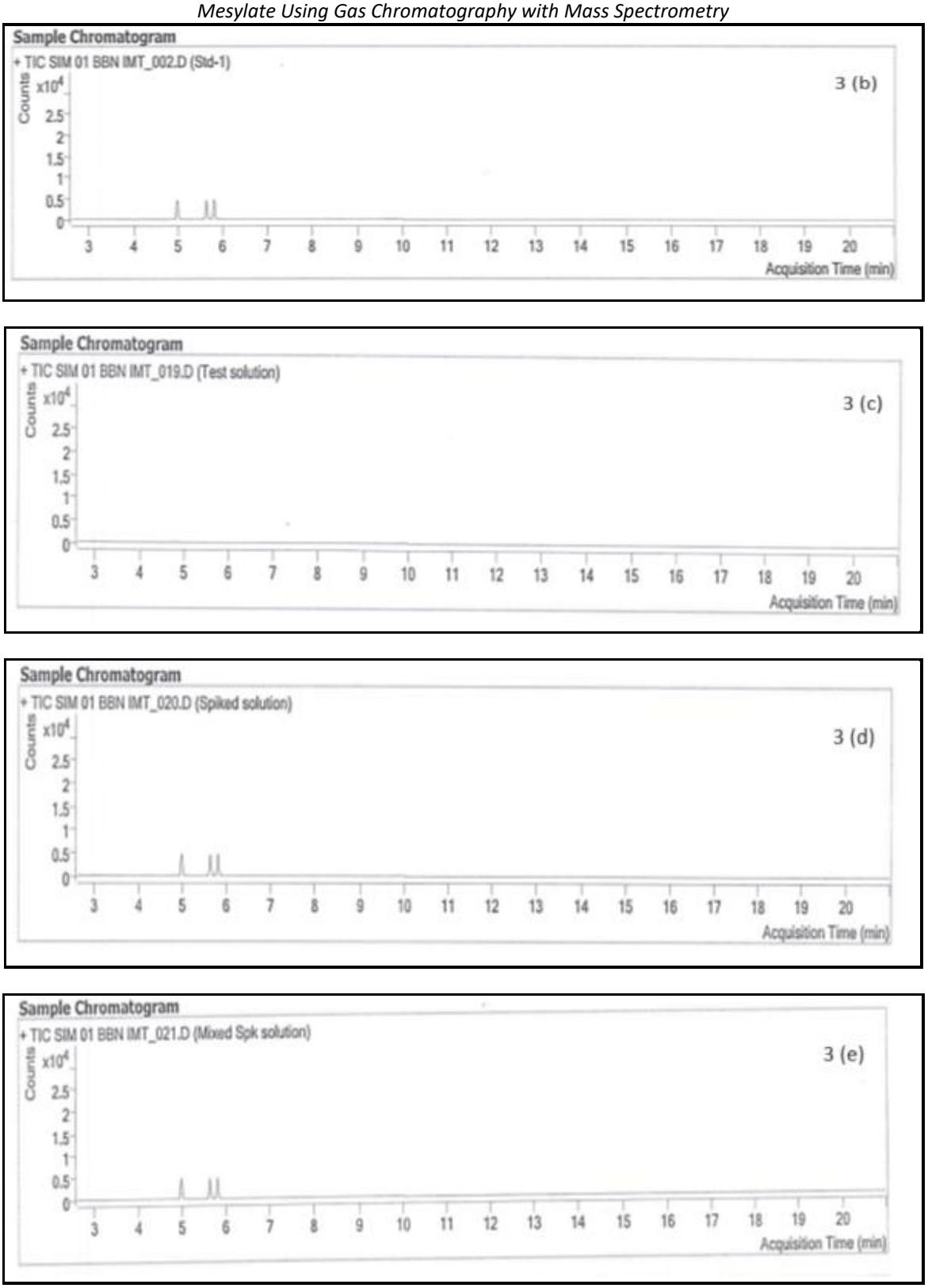


Figure 3: Typical GC-MS chromatograms of Method-(1) a) Blank solution, (b) Standard solution, (c) Imatinib mesylate drug substance (as such sample), (d) Imatinib mesylate drug substance spiked with 2-BBN, 3-BBN and 4-BBN (spiked sample) and (e) Imatinib mesylate drug substance spiked with 2-BBN, 3-BBN and 4-BBN including all residual solvents (all spiked sample).

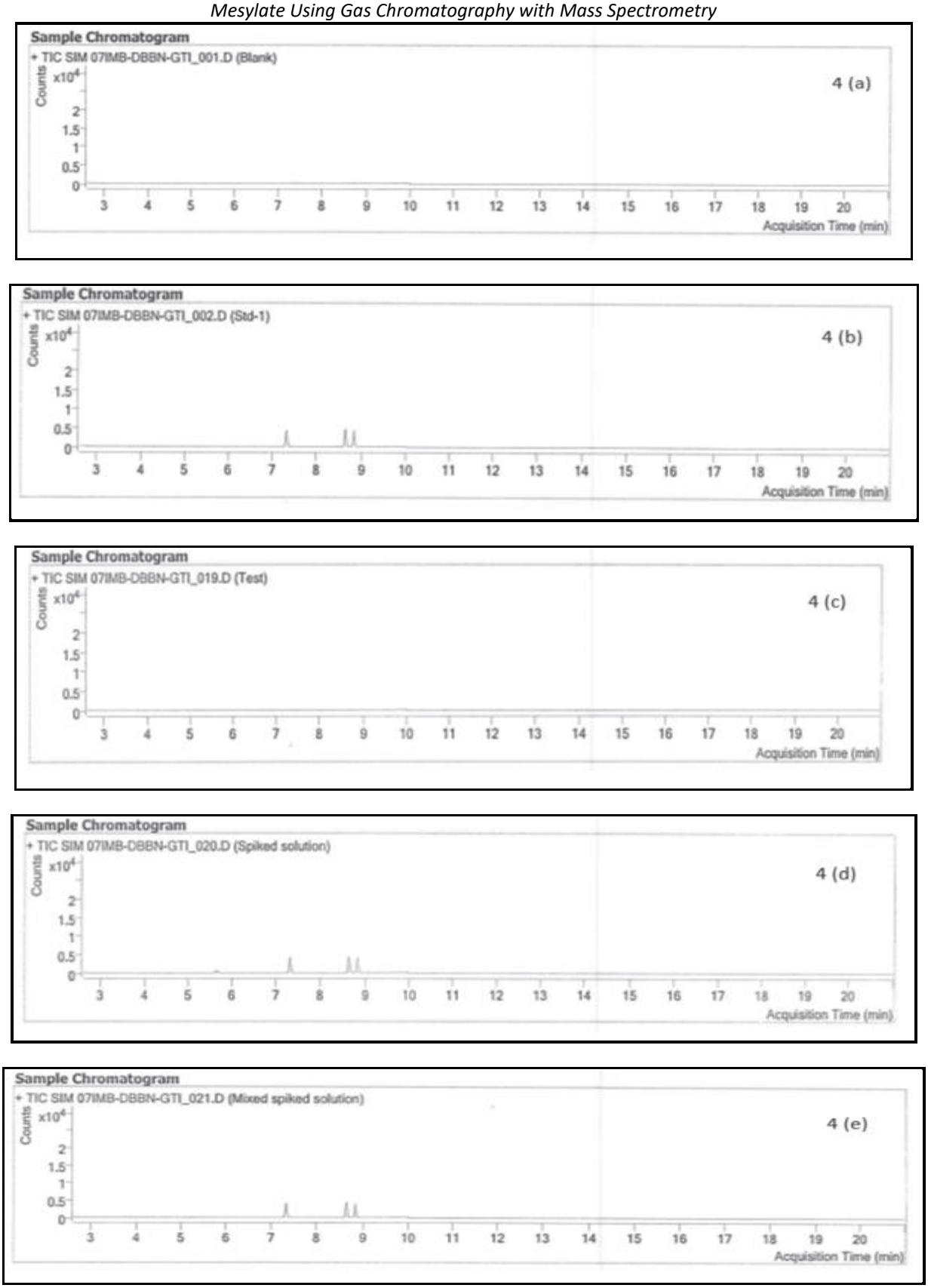


Figure 4: Typical GC-MS chromatograms of Method-(1) a) Blank solution, (b) Standard solution, (c) Imatinib mesylate drug substance (as such sample), (d) Imatinib mesylate drug substance spiked with 2-DBBN, 3-DBBN and 4-DBBN (spiked sample) and (e) Imatinib mesylate drug substance spiked with 2-DBBN, 3-DBBN and 4-DBBN including all residual solvents (all spiked sample).

Limit of detection and Limit of quantification

In these both methods, Specification level standard solution was injected in to GC-MS and S/N ratios for all analytes were recorded. Based on these values, the LOD and LOQ values of 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN were predicted. At LOQ level S/N ratio was $\geq$ 10 and LOD level S/N ratio was $\geq$ 3 for all analytes. Each predicted concentration was verified for precision by preparing the solutions containing 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN about its detection limit and quantification limit concentrations. The LOD and LOQ solutions were injected six replicates into GC-MS. The relative standard deviation [% RSD (n = 6)] for LOD precision of 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN were 3.2, 1.1, 2.6, 2.6, 2.4 and 2.5; for LOQ precision 0.5, 0.8, 1.0, 1.2, 0.6 and 2.3 respectively. The details of the précised LOD and LOQ values are shown in Table 4. The overlaid GC-MS chromatograms of Method-(1) LOD solution and LOQ solution are shown in **Figure 5 (a)** and 5 **(b)**. The overlaid GC-MS chromatograms of Method-(2) LOD solution and LOQ solution are shown in **Figure 6 (a)** and **6 (b).**

Linearity and Range

The linearity was evaluated by measuring the response of 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN seven different concentrations were prepared across the range concentrations were studied in the range of LOQ to 150% of the specification level (~0.37–2.87 ppm). The linearity level solutions of 2-BBN (~0.37-2.77 ppm), 3-BBN (~0.37–2.81 ppm), 4-BBN (~0.38-2.87 ppm), 2-DBBN (~0.36-2.68 ppm), 3-DBBN (~0.37-2.75 ppm) and 4-DBBN (~0.38-2.84 ppm) were prepared and injected each in duplicate injections into GC-MS. The data was subjected to statistical analysis using a linear-regression model. The statistical parameters slope, intercept, residual standard on deviation and correlation coefficient values were calculated. The derived correlation coefficients were in the range of 0.9991–0.9999 indicating the best fitness of the linearity curves of the developed methods. The calculated statistical results are shown in **Table 4.**

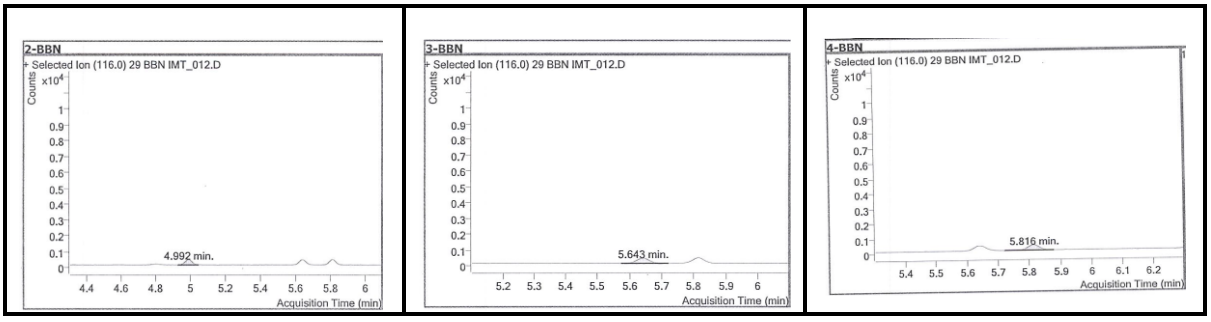


Figure. 5 (a): Typical GC-MS chromatograms of Method-(1) LOD solution

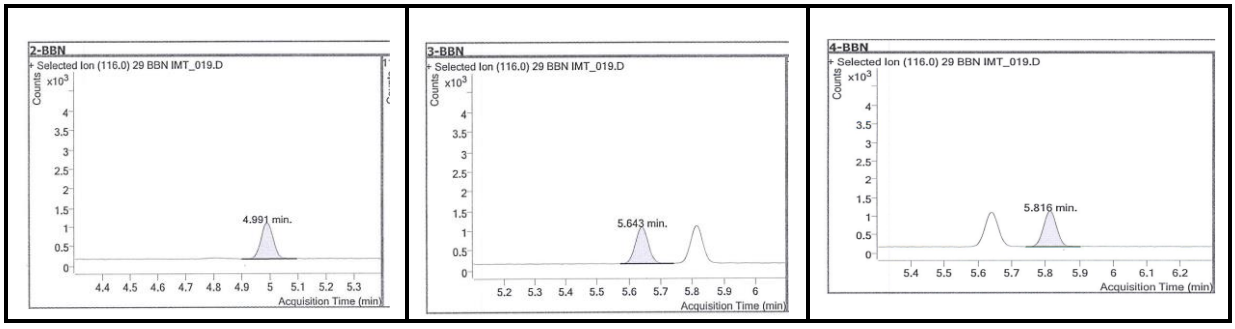


Figure. 5 (b): Typical GC-MS chromatograms of Method-(1) LOQ solution

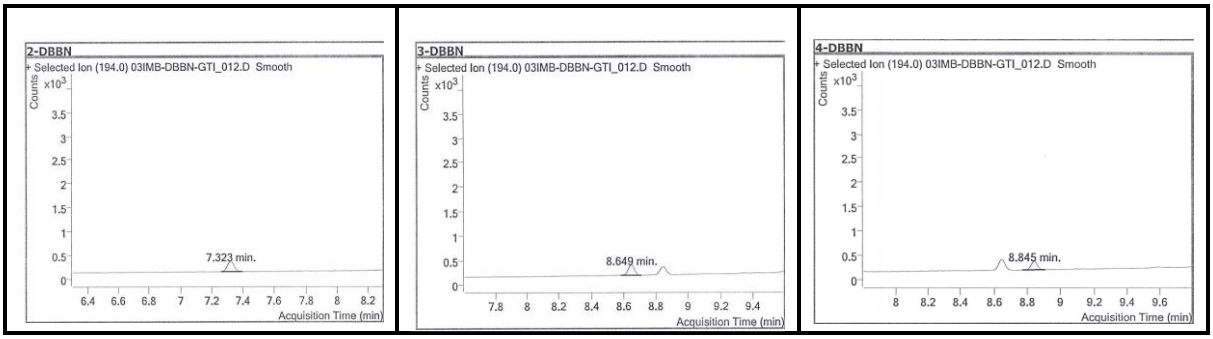


Figure. 6 (a): Typical GC-MS chromatograms of Method-(2) LOD solution

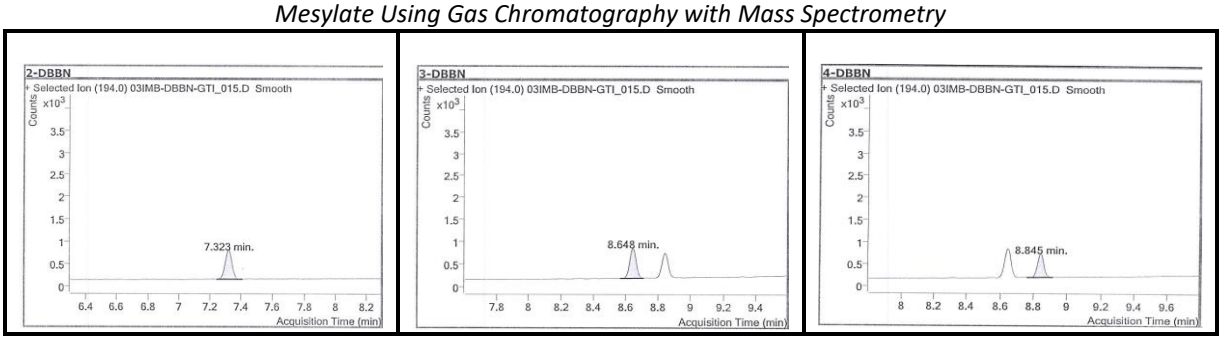


Figure. 6 (b): Typical GC-MS chromatograms of Method-(2) LOQ solution

Accuracy

Accuracy experiment was performed by spiking the known amounts of 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN at LOQ level, 50%, 100% and 150% levels (with respect to 1.88 ppm limit) into IMM drug substance. In the accuracy experiment, IMM sample solutions (control sample) were prepared without spiking any impurity in triplicate and injected into GC-MS. Further, IMM sample solutions (spiked sample) were prepared in triplicate by spiking with the all the impurities (2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN) at LOQ level, 50% level (0.94 ppm), 100% level (1.88 ppm) and 150% level (2.82 ppm) and injected into GC-MS. Control samples, Spiked samples were analysed and the percentage recoveries were calculated. The average % recovery values of four levels (LOQ, 50%, 100% and 150% levels) for twelve determinations for 100.9 (2-BBN), 100.3 (3-BBN), 100.6 (4-BBN), 99.8 (2-DBBN), 104.4 (3-DBBN) and 99.9 (4-DBBN). The complete validated accuracy results are shown in **Table 5.**

Precision

The precision was the study of the method using repeatability (Method precision). The performance of the method was evaluated with replicate injections of standard and sample solutions. Standard solution was analysed six times for checking the performance of the GC-MS system under test method conditions on the day tested (System Precision). The relative standard deviation results obtained for the system precision experiment were 2.5 (2-BBN), 2.5 (3-BBN), 2.6 (4-BBN), 1.8 (2-DBBN), 1.7 (3-DBBN) and 1.9 (4-DBBN) respectively. Repeatability (Method Precision) experiment was performed by prepared six sample solutions were using single batch of IMM drug substance spiked with 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN about known concentration (1.88 ppm) level and injected into GC-MS. The relative standard deviation for the content results of the Method precision experiment 2.7 (2-BBN), 2.7 (3-BBN), 2.7 (4-BBN), 2.3 (2-DBBN), 2.8 (3-DBBN) and 3.2 (4-DBBN). The intermediate precision was the inter-day variation (ruggedness) defined as the degree of reproducibility obtained by following the same procedure as mentioned for Method precision experiment. Ruggedness of the method was evaluated by preparing six individual sample preparations (same sample which was used in Method precision experiment) by spiking 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN to IMM drug substance and injected into different column, different instrument and different analyst on different days. The achieved precision (System precision, Method precision and Intermediate precision) experiment results are shown in **Table 6.**

Robustness

Robustness of the method was evaluated by deliberately altering the method conditions from original method parameters and verifying compliance to the system suitability parameters. The impact of variation of column oven temperature and flow rate of carrier gas on system suitability was conducted. In robustness verification of test method, one parameter changed while keeping the other unchanged from actual parameter. The study was carried out with respect to column flow variation of carrier gas initial flow rate ±10% and column oven initial temperature ± 2°C as follow mentioned in **Table 7 (a)** and **Table 7 (b)**. Results of peak areas for with 2-BBN, 3-BBN, 4-BBN, 2-DBBN, 3-DBBN and 4-DBBN are summarized in **Table 8**.

Solution stability:

Standard solutions, controlled sample and sample spiked with PGI's at 1.875 ppm was prepared and kept at room temperature as well as in the refrigerator at 2-8 ºC. The results indicates that Mono bromo impuritites i.e.2-BBN, 3-BBN, 4-BBN and Dibromo impurities i.e. 2-DBBN, 3-DBBN and 4-DBBN in standard solution, the sample solution and spiked solution were stable upto 24 h at 2-8 ºC as well as at room temperature. The results were statistically identical with the initial value without measurable loss and moreover % difference between the standard and spiked sample areas of each impurity was below the 10.

Conclusion

The main goal of this study is to find a way to measure the amount of six potential genotoxic impurities (PGI's) in Imatinib mesylate drug substance using GC-MS and a mass analyzer which is extremely new, selective, fast, sensitive, accurate, linear, rugged and reliable. The ICH guidelines were followed to validate the developed and optimized GC-MS methods. The %RSD of precision indicates the methods are highly precise in reproducibility. The retention time of the peaks indicates that there is no interference and the PGI's are well separated from each other indicating specificity. The values derived from linear least square regression reveal a good correlation between concentrations and areas. The % recovery and %RSD from triplicate samples indicate the diluent selected is more appropriate for extracting the PGI's from the drug substance. The results obtained in this study demonstrated that the developed method is precise, rugged, specific, linear, accurate

and sensitive for the determination of six PGI's in Imatinib mesylate drug substance. The stability data of the methods indicates the present method can be effectively used for routine development analysis, quality control testing, thereby the method is suitable for the intended use.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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Table 1: Gas chromatograph conditions for PGI's analysis:

	Method-1			**Method-2**		
Instrument	Agilent 7890B			Agilent 7890B		
Column	Rtx-35, 30 m × 0.32 mm I.D. × 1.0 µm Film thickness			Rtx-35, 30 m × 0.32 mm I.D. × 1.0 µm Film thickness		
Carrier gas	Helium			Helium		
Injector temperature (°C)	240°C			240°C		
Injection type	ALS mode (Auto liquid sampler)			ALS mode (Auto liquid sampler)		
Column oven program	Heating rate (°C/min)	Initial temperature (°C)	Hold time (min)	Heating rate (°C/min)	Initial temperature (°C)	Hold time (min)
		180	4		180	4
	10	240	11	10	240	11
Flow rate (mL/min)	3.0			3.0		
Injection volume (µL)	2.0			2.0		
Split ratio	10			5		
Run time (min)	21			21		

Table 2: Gas chromatography mass spectrometer conditions for PGI's analysis:

	Method-1	**Method-2**
Instrument	Agilent GCMS-5977A and GCMS-5977B Single Quad MS	Agilent GCMS-5977A and GCMS-5977B Single Quad MS
MS Transfer line temperature (°C)	250	250
MS Source temperature (°C)	230	230
MS Quad temperature (°C)	150	150
Function type	SIM (selective ion monitoring)	SIM (selective ion monitoring)
Gain factor	5	5

Table 3: SIM Time segments:

Method-1					**Method-2**				
Solvent delay time	Group Name	Resolution	Mass (m/z)	Dwell time (ms)	Solvent delay time	Group Name	Resolution	Mass (m/z)	Dwell time (ms)
2.5 min	2-BBN	Low	116*	100	2.5	2-DBBN	Low	194*	100
	3-BBN		116*	100		3-DBBN		194*	100
	4-BBN		116*	100		4-DBBN		194*	100
***Quantifier ion**					***Quantifier ion**				
Timed MS Detector**:** The MS must be ''Detector Off'' after 10 min.					Timed MS Detector**:** The MS must be ''Detector Off'' after 10 min.				

Table 4: LOD, LOQ and Linearity experiments results:

Statistical parameters	**Results**					
	Method-1			**Method-2**		
	2-BBN	**3-BBN**	**4-BBN**	**2-DBBN**	**3-DBBN**	**4-DBBN**
Correlation coefficient	0.9991	0.9993	0.9992	0.9999	0.9998	0.9995
Concentration range (ppm)	0.37 – 2.77	0.37 – 2.81	0.38 – 2.87	0.36 – 2.68	0.37 – 2.75	0.38 – 2.84
Calibration points	7	7	7	7	7	7
% Y-Intercept	0.05	0.21	-0.43	-3.03	-4.30	-6.89
Slope(S)	7019.4525	6441.6581	6405.6760	6297.4556	5846.9589	5157.8778
STEYX	289.10	247.97	255.76	97.80	113.71	172.59

LOD (ppm)	0.12	0.12	0.13	0.12	0.12	0.13
LOQ (ppm)	0.37	0.37	0.38	0.36	0.37	0.38
Precision at LOD level (%R.S.D)	3.2	1.1	2.6	2.6	2.4	2.5
Precision at LOQ level (%R.S.D)	0.5	0.8	1.0	1.2	0.6	2.3

Table 5: Accuracy experiment results

Identification	**2-BBN**				**3-BBN**			
Control sample	ND				ND			
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)
*Added (µg/g)	0.368	0.92	1.84	2.77	0.372	0.93	1.87	2.82
*Found (µg/g)	0.366	0.93	1.86	2.82	0.366	0.94	1.88	2.85
Recovery (%)	99.5	101.1	101.1	101.8	98.4	101.1	100.5	101.1
% RSD	2.4	1.7	2.9	0.9	2.2	1.0	2.6	0.9
Identification	**4-BBN**				**2-DBBN**			
Control sample	ND				ND			
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)
*Added (µg/g)	0.379	0.93	1.86	2.80	0.357	0.90	1.80	2.70
*Found (µg/g)	0.377	0.94	1.87	2.84	0.353	0.85	1.83	2.81
Recovery (%)	99.5	101.1	100.5	101.4	98.9	94.4	101.7	104.1
% RSD	2.8	2.1	2.9	0.9	1.2	1.8	2.4	1.6

Identification	**3-DBBN**				**4-DBBN**			
Control sample	ND				ND			
	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)	LOQ Level	Level-I (50%)	Level-II (100%)	Level-III (150%)
*Added (µg/g)	0.367	0.93	1.87	2.81	0.379	0.96	1.92	2.88
*Found (µg/g)	0.362	0.88	1.92	2.97	0.371	0.87	1.97	3.12
Recovery (%)	98.6	94.6	102.7	105.7	97.9	90.6	102.6	108.3
% RSD	0.9	0.7	1.8	1.5	1.4	1.2	2.0	1.6

*Average of three replicates. ND: Not Detected.

Table 6: Statistical Data of Precision experiment

Repeatability (System precision) Area	**2-BBN**	**3-BBN**	**4-BBN**	**2-DBBN**	**3-DBBN**	**4-DBBN**
1	14660	13837	13791	10725	9940	8773
2	14585	13714	13830	10659	9939	8839
3	14204	13392	13426	10507	9725	8698
4	14257	13386	13430	10253	9600	8476
5	14013	13160	13223	10306	9560	8466
6	13727	12941	12899	10622	9864	8772
Average	**14241**	**13405**	**13433**	**10512**	**9771**	**8671**
STDEV	**349.8**	**334.1**	**350.7**	**194.2**	**168.1**	**161.0**
%RSD	**2.5**	**2.5**	**2.6**	**1.8**	**1.7**	**1.9**
	Reproducibility (Method precision) (µg/g)					
1	1.92	1.94	1.94	1.79	1.90	1.96
2	1.83	1.85	1.85	1.73	1.80	1.85

3	1.82	1.85	1.83	1.74	1.82	1.88
4	1.80	1.82	1.81	1.70	1.76	1.81
5	1.80	1.82	1.81	1.69	1.77	1.81
6	1.79	1.82	1.81	1.71	1.80	1.86
Average	**1.83**	**1.85**	**1.84**	**1.73**	**1.81**	**1.86**
STDEV	**0.05**	**0.05**	**0.05**	**0.04**	**0.05**	**0.06**
%RSD	**2.7**	**2.7**	**2.7**	**2.3**	**2.8**	**3.2**
	Reproducibility (Intermediate Precision) (µg/g)					
1	1.95	1.91	1.91	1.81	1.90	1.95
2	1.87	1.83	1.82	1.80	1.90	1.95
3	1.80	1.77	1.76	1.88	1.96	2.02
4	1.81	1.77	1.76	1.81	1.90	1.94
5	1.81	1.78	1.77	1.84	1.93	1.98
6	1.90	1.85	1.85	1.82	1.91	1.95
Average	**1.86**	**1.82**	**1.81**	**1.83**	**1.92**	**1.97**
STDEV	**0.06**	**0.06**	**0.06**	**0.03**	**0.02**	**0.03**
%RSD	**3.2**	**3.3**	**3.3**	**1.6**	**1.0**	**1.5**
	Overall statistical data (n=12)					
Average	**1.84**	**1.83**	**1.83**	**1.78**	**1.86**	**1.91**
STDEV	**0.05**	**0.05**	**0.06**	**0.06**	**0.07**	**0.07**
%RSD	**2.7**	**2.7**	**3.3**	**3.4**	**3.8**	**3.7**

Table 7 (a): Flow variations: -

Column Flow (ml/min)		
	Method-1	**Method-2**
As per Methodology	3.0	3.0
-10% Flow variation	2.7	2.7
10% Flow variation	3.3	3.3

Table 7 (b): Column Oven Temperature variations: -

Column Oven Temperature for Method-1	
As per Methodology	10°C/min 180°C (4min) ⟶ 240°C (11min)
-2°C Column Oven Temperature variation	8°C/min 178°C (4min) ⟶ 240°C (11min)
+2°C Column Oven Temperature variation	12°C/min 182°C (4min) ⟶ 240°C (11min)

Column Oven Temperature for Method-2	
As per Methodology	10°C/min 180°C (4min) ⟶ 240°C (11min)
-2°C Column Oven Temperature variation	8°C/min 178°C (4min) ⟶ 240°C (11min)
+2°C Column Oven Temperature variation	12°C/min 182°C (4min) ⟶ 240°C (11min)

Table 8: Robustness experiment results

Robustness condition	**System suitability criteria (% RSD)**					
	2-BBN	**3-BBN**	**4-BBN**	**2-DBBN**	**3-DBBN**	**4-DBBN**
As per methodology	2.5	2.5	2.6	1.8	1.7	1.9
Flow variation						
-10%	1.2	2.1	2.4	3.1	2.3	3.8
+10%	0.4	2.8	2.6	2.2	1.8	3.1
Temperature variation - Initial Oven						
-2°C	0.6	4.2	4.1	5.2	2.2	5.5
+2°C	0.8	3.6	3.5	3.1	1.6	2.7