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A Selective and Sensitive Method Development and Validation by LC–MS/MS Approach for Trace Level Quantification of Potential Genotoxic Impurity IMT-01 in Imatinib Mesylate Drug Substance

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Abstract: ***A novel reversed-phase selective and sensitive liquid chromatography coupled with tandem mass spectrometric (LC-MS/MS) method was developed and validated for the trace analysis of (>0.5 ng/mL level) of N-(2-methtyl-5-nitrophenyl)-4-(pyridine-3-yl)-pyrimidine-2-amine (IMT-01) genotoxic impurity in imatinib mesylate drug substance. The method utilizes Inertsil C18 column (150 mm×4.6 mm, 5.0 µm) with electrospray ionization in multiple reaction monitoring (MRM) detection mode. Isocratic program was developed for rapid analysis and mobile phase was 0.1% formic acid in water and acetonitrile in the ratio of 30:70(v/v). The flow rate was 1.0 mL/min and elution was monitored by mass spectrophotometer. The method was validated as per International Conference on Harmonization (ICH) guidelines and was able to quantitate up to 0.5 ng/mL of IMT-01.***

Keywords: Imatinib mesylate, LC-MS/MS, Genotoxic impurity, Isocratic program, multiple reaction monitoring (MRM)

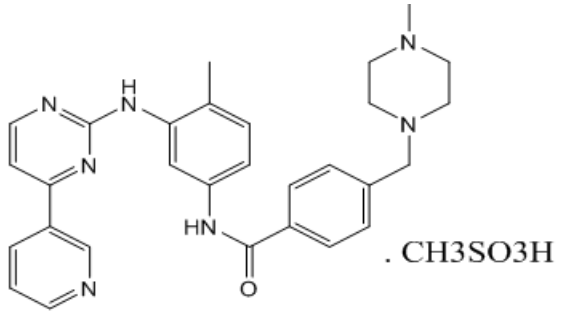
1. Introduction

Imatinib Mesylate chemically Known as 4-[(4-methylpiperazin-1-yl) methyl]-N-(4-methyl-3-{[4-(pyridin-3-yl) pyrimidin-2-yl] amino} phenyl) benzamide, has an empirical formula of C29H31N7O.CH3SO3H and a molecular weight of 589.72. Imatinib Mesylate is a tyrosine-kinase inhibitor used in the treatment of multiple cancers, most notably Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML) [1]. Like all tyrosinekinase inhibitors, Imatinib Mesylate works by preventing a tyrosine kinase enzyme, in this case BCR-Abl, from phosphorylating subsequent proteins and initiating the signaling cascade necessary for cancer development, thus preventing the growth of cancer cells and leading to their death by apoptosis [2]. The chemical structure of Imatinib mesylate and genotoxic impurity are shown in figure 1.

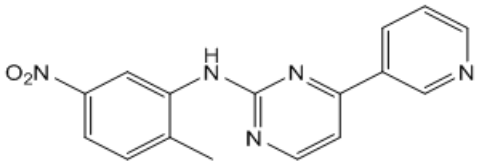
Due to the advanced technical capability in identifying the potential genotoxic impurities (PGIs) and their potential impact on human health, regulatory issues related to the presence of PGIs have been arisen [3]. Starting materials, intermediates, process impurities and by-products are often found as impurities in drug substances. Some of these known impurities are potential mutagens or carcinogensand they have potential to cause cancer in human was observed by Bolt et al[4]. Muller et al [5] Jacobson and McGovern [6]. But it is difficult/impossible to eliminate them completely from the synthetic scheme. As per the guidelines from the European Medicines Agency on the limits of genotoxic impurities, a threshold of toxicological concern (TTC) value of 1.5 µg/day intake of a genotoxic impurity is considered to be associated with an acceptable riskfor most of the pharmaceuticals [7,8]. Testing and control of genotoxic impurities at trace levels presents challenges to the pharmaceutical industry with regard to both analytical and process controls [9-10].

Though IMT-01is a well known carcinogen, this data would ascertain that the regulatory authoritiesmay be expected to control the level of IMT-01to be 2 ppm in the drug substance (assuming a 1.5 µg/daydaily dose). A method capable of such a lower level of detection is great challenge for analytical methoddevelopment for control the genotoxic impurity. Ideally conventional analytical instruments inpharmaceutical industries such as HPLC with UV detection and GC with FID detection should be employedas the standards in first attempt for PGIs analysis and these methods were discussed by Klick [11] and Valvoet al.[12], but there are some drawbacks with above mentioned techniques because probability of co-elution atvery trace level can change analytical result. When impurity standards are not available some method isneeded to characterize the impurities on-line. Therefore, for accurate determination at ppm levels the abovementioned techniques are inadequate; consequently there is a great need to develop better analytical methodfor the analysis of such genotoxic impurities in pharmaceutical industries. As a result various kinds ofchromatographic techniques and methodologies have been explored as useful approaches out of Hsieh andKorfmacher [13] and Lee and Kems [14] had discussed LC-MS/MS technique and application. It was feltnecessary to develop simple, sensitive and validated method for estimation of IMT-01. The literature surveyrevealed that Raja et al. [15] developed and reported spectrophotometric methods for the determination ofImatinib mesylate API.

We have developed a simple LC-MS/MS method that can quantitate at permitted limit level of impurity in Imatinib mesylate. This method to be validated as per ICH guidelines [16] in terms of limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, specificity and robustness.



Chemical Formula: $C_{29}H_{31}N_7O$
Exact Mass: 589.25
IMATINIB MESILATE



Chemical Formula: $C_{16}H_{13}N_5O_2$
Exact Mass: 307.11
N-(2-methtyl-5-nitrophenyl)-4-(pyridine-3-yl)-pyrimidine-2-amine (IMT-01)

Figure 1: The chemical structure of Imatinib mesylate and genotoxic impurity

2. Experimental

2.1 Reagents and standards

Formic acid and acetonitrile were obtained in their LCMS grade from Merck (Mumbai, India). Purified water collected through Milli-Q Plus water purification system (Millipore, Milford ford, MA, USA). Imatinib mesylate and IMT-01were obtained from Cipla Ltd (R&D, Bangalore, India).

2.2 Instrumentation

The mass spectrometer LC-MS/MS system used was an Applied Bio system Sciex QTRAP-5500 Model (Switzerland). LC was carried out on Agilent HPLC (1200 series, Germany) with photodiode array detector. Other small equipment were PCI sonicator (22L500/CC/DTC), precision analytical balance (MX5, Mettler Toledo, Schwerzenbach, Switzerland). Data acquisition and processing were conducted using the Analyst 1.5.1 software on a dell computer (Digital equipment Co).

2.3 Chromatographic conditions

All chromatographic experiments were carried out on Agilent HPLC (1200 series, Germany) with photodiode array detector system coupled with MS/MS (Applied Bio system Sciex QTRAP-5500 model, Rotkreuz, Switzerland). The analytical column used was Inertsil ODS 3V column (150 mm x 4.6 mm i.d., particle size 5 µm) procured from LCGC (Bangalore, INDIA). The mobile phase flow operated in isocratic mode using 0.1 % formic acid in water and acetonitrile in the ratio of 30:70 (v/v). The flow rate was 1.0 mL/min, with the flow rate split down to 0.4 mL/min in to the MS source. The column oven temperature was maintained at 25ºC, sample cooler temperature was 10ºC. The injection volume was 10 µL. Positive ion electrospray ionization probe was operated with multiple reaction monitoring (MRM) detection mode. Electrospray ionisation in positive mode was used with MRM monitoring for m/z 308.1>261.1 transition for IMT-01 and 494.2 > 394.3 transition for Imatinib. The Declustering potential (V), entrance potential (V), Collision energy (V), Collision exit potential (V) and ion spray voltage (V) were kept as 50, 8, 45, 10 and 5500, respectively. The source temperature (°c), curtain gas flow (psi), ion source gas1 and ion source gas2 were maintained as 450, 40, 50 and 50, respectively.

2.4 Standard and sample preparation

The diluent used as water and acetonitrile in the ratio of 50:50 (v/v). 0.1 mg/mL reference stock solution was prepared by dissolving IMT-01in diluent, Preparation of sub stock standard solution of 0.001 mg/mL was achieved on further dilution with diluent. Finally desired concentration (2 ng/mL) of standard solution was prepared by diluting standard sub stock solution to 100 mL with diluent. Linearity solution prepared from further dilution of standard solution of 0.5, 1, 1.5, 2, 4 and 6 ng/mL.The testing API sample was typically prepared at approximately 1 mg/mL with diluent.

3. Results and Discussion

3.1 Method development

The main objective of method development was to achieve efficient separation between Imatinib mesylate and IMT-01. Different stationary phases have been assessed which included C18, C8, phenyl and Cyano phases. In addition different mobile phase additives such as formic acid, ammonium acetate, acetonitrile and methanol have been tested. Chromatographic separation was finally achieved on Inertsil ODS 3V (150 mm × 4.6 mm) 5 µm column (LCGC Co, India) in isocratic mode using 0.1 % formic acid in water and acetonitrile in the ratio 30:70 (v/v). The flow rate was 1.0 mL/min, with the flow rate split down to 0.4 mL/min in to the MS source and mass spectrometer parameter set to get maximum sensitivity for IMT-01.

3.2 Method validation

3.2.1 Specificity

The specificity of the method was checked by injecting 0.5 ppm of Imatinib mesylate and IMT-01 with respect to the test concentration, the retention times shown in table 1.

Blank and Specificity chromatograms are shown in the figure 2 to 4.

Table 1: Determination of specificity

S. No.	Name	Retention time (min)
1	Imatinib	1.28
2	IMT-01	3.28

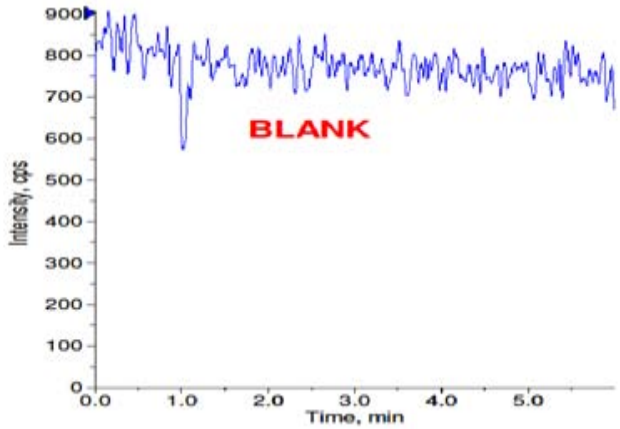


Figure 2: Blank chromatogram for specificity

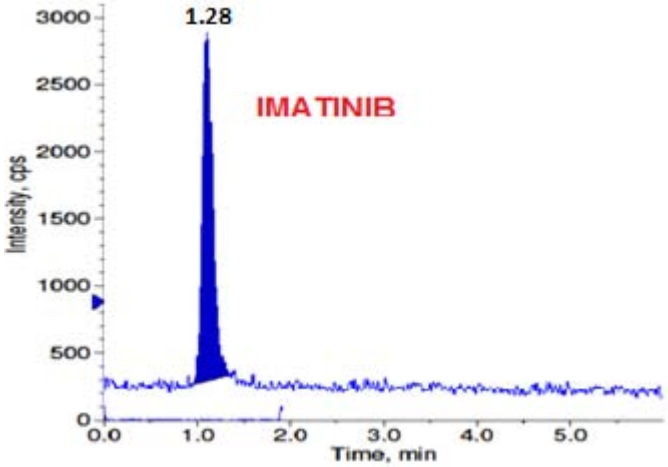


Figure 3: Imatinib chromatogram for specificity

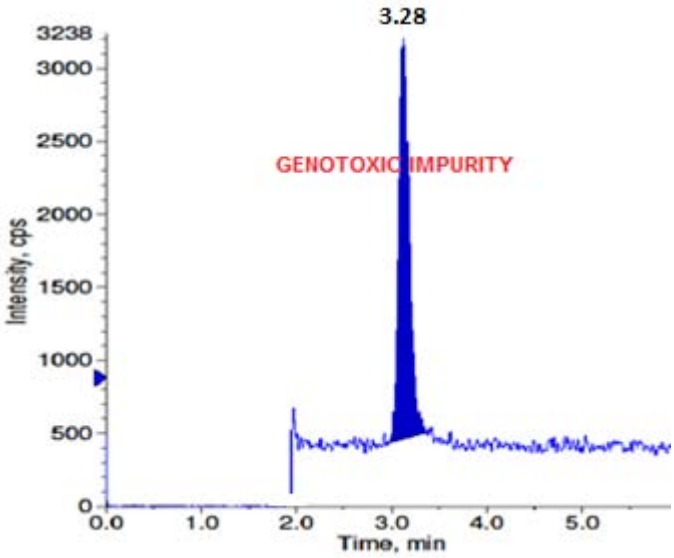


Figure 4: IMT-01 chromatogram for specificity

3.2.2 Determination of LOD and LOQ

The LOD and LOQ were calculated from S/N (signal to noise) ratio. Now to determine LOD and LOQ values of IMT-01concentrationwere reduced sequentially such that they yield S/N ratio as 4.1 and 10.9 respectively. The LOQ of 0.5 ppm is typical for the IMT-01, with LOD approximately three times less than LOQ.

3.2.3 Linearity

Linearity of the method was checked by preparing solutions at six concentration levels of 0.5 ng/mL (LOQ), 1 ng/mL (L1 solution), 1.5 ng/mL (L2 solution), 2 ng/mL (L3 solution), 4 ng/ml (L4 solution) and 6 ng/mL (L5 solution) for IMT-01. LOQ solution and L5 solution were injected six times and L1, L2,L3 and L4 solution were injected three times. The mean responses recorded for each impurity were plotted against concentration. The correlation coefficient was found to be 0.9999 and shown in figure5 and also shown in table 2.

Table 2: Linearity 0.5ppm to 6ppm and each level % RSD

Expected concentration	*Sample Name*	*Number of values used*	*Mean*	*Standard deviation*	*%RSD*
0.509	LOQ	1 of 6	34247.0	644.1	2.79
1.018	L1 Solution	1 of 3	67041.2	1893.4	1.14
1.528	L2 Solution	1 of 3	98078.3	1646.9	1.58
2.037	L3 Solution	1 of 3	128988.1	1380.8	1.15
4.074	L4 solution	1 of 3	255876.2	3183.1	1.28
6.110	L5 Solution	1 of 6	386435.6	9568.9	0.84

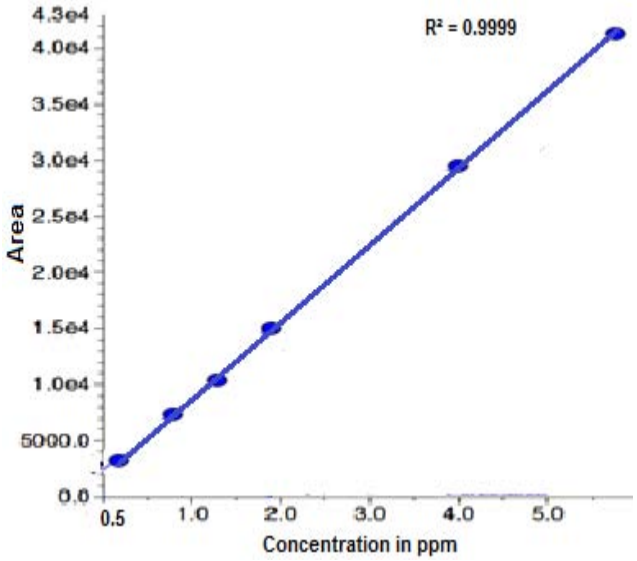


Figure 5: Linearity graph of IMT-01

3.2.4 Recovery

Hence the recovery studies by the standard addition method were performed to evaluate accuracy and specificity, accordingly the accuracy of the method was determined in six replicate at LOQ level and remaining level (level 1, 2, 3, 4 and level 5) triplicate in bulk drug sample. Then the percentage recoveries were calculated. Excellent recovery values of IMT-01 (90–109%) was obtained. Spiked LOQ chromatograms of accuracy was shown in figure 6 and %RSD was calculated from the average of six replicate analysis, which was shown in table 3.

Table 3: Accuracy/Recovery for 0.5 ppm

Sample Area	*Standard Area*	*Spiked Area*	*Theoretical Concentration (mg/ml)*	*Measured Concentration*	*% Recovery*
66009	34247	102098	0.000050	0.000051	104.2
66009	34247	101171	0.000050	0.000051	103.8
66009	34247	102711	0.000050	0.000051	104.9
66009	34247	101019	0.000050	0.000051	103.4
66009	34247	101199	0.000050	0.000051	103.9
66009	34247	101159	0.000050	0.000051	103.1
Average			103.9		
Standard deviation			0.63		
% RSD			0.61		

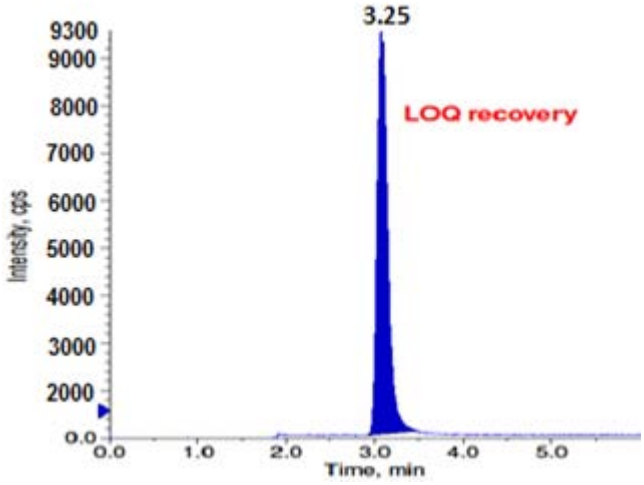


Figure 6: Accuracy/Recovery at LOQ

3.2.5 System, method and intermediate precision

Infact IMT-01 in imatinib mesylate was checked for repeatability. The standard solution was prepared 2 ng/mL (limit level) with respect to test concentration and injected six times for system precision. The % RSD was found to be less than 5.0%. To determine the method precision six independent solutions were prepared by spiking Imatinib mesylate with the impurity at a concentration of 2 ppm with respect to target analyte concentration. Intermediate precision was studied with different column, different instrument and different day. % RSD of all the above determinations were calculated and found below 10.

3.2.6 Robustness

Robustness of the method was determined by making slight and deliberate changes in experimental conditions. The flow rate of mobile phase was altered by 0.1 units i.e. 1.0 to 1.1 mL/min, 1.0 to 0.9 and effect of temperature on resolution was also studied at 23°C and 27°C (altered by 2 units).The cumulative % RSD values from method precision and robustness study (altered conditions) were calculated. The % RSD values calculated were found to be below 10 for IMT-01 demonstrates that the method was robust.

3.2.7 Stability of analytical solution

Sample solution was prepared as per the proposed method. The IMT-01 was quantitatively spiked at limit level concentration and stored at 10°C. The spiked sample was injected into the system initially and at various time intervals. The % variation of IMT-01 in the initial and each interval was calculated and found to be below 10. This indicates that the sample solution was found to be stable up to 48 hours at 10 ºC.

4. Conclusion

The results of the present study indicated that the newly developed LC-MS/MS method is simple, rapid, cost-effective, linear, accurate, precise and robust over the specified range and selective for the quantification of IMT-01 in Imatinib mesylate drug substance at low concentration levels. The LOD and LOQ of the developed method were quite satisfactory. In addition to this method can be employed conveniently, reliably and successfully for the estimation of IMT-01 for routine quality control and stability studies.

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