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Simultaneous Quantitation of Two Potential Genotoxic Impurities In Imatinib by Liquid Chromatography Mass Spectrometry

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ABSTRACT

A new, simple, accurate and sensitive method was developed for the quantification of two potential genotoxic impurities 1-(2-methyl-5-nitro-phenyl)guanidine nitrate and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl)pyrimidin-2-amine at low level (2 ppm) in Imatinib using liquid chromatographic mass spectrometry. The chromatographic separation was achieved on Kromasil 100-3.5-C18 (150 x 2.1) mm column with gradient programme and elution was monitored by mass spectrometer in selective ion monitoring mode using electrospray ionization. The LOD and LOQ values found to be 0.2 ppm and 0.6 ppm for both the impurities with respect to the test concentration 5 mg/ml. The method was linear (r^2>0.99), precise (RSD<2%), accurate and well within acceptable ICH limits.

Keywords: Potential genotoxic impurities, Imatinib, LC-MS, Selective Ion Monitoring (SIM), Threshold of Toxicological Concern (TTC)

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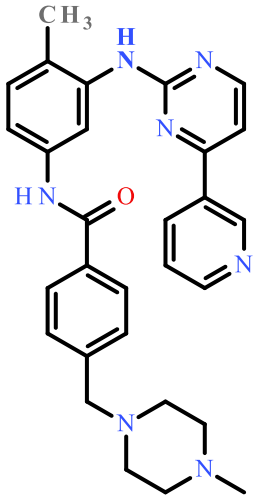
INTRODUCTION

Identification and control of genotoxic and Potential Genotoxic Impurities (PGI's) in active pharmaceutical ingredients (API's) is of utmost importance to ensure patient safety. Genotoxic impurities are residues of starting materials, reagents, intermediates, degradation products and bi products of synthesis that have a demonstrated ability to damage DNA, thus having the potential to cause a mutagenic and possibly a carcinogenic response [1]. Genotoxic impurities are defined as impurities demonstrated to be genotoxic in an appropriate test model like bacterial gene mutation test (Ames test). A potential genotoxic impurity, defined by EMA as "an impurity that shows a structural alert for genotoxicity but that has not been tested in an experimental test model" may or may not be present in the drug substance. The allowable levels of PGI's are determined by a staged toxicological threshold of concern (TTC) based on both the dose and duration of the intended clinical study. This allowable amount can be in low ppm range, which is much lower than the allowable levels of non-PGI impurities controlled under ICH Q3A guideline. This TTC value was estimated to be 1.5 μg/person/day. For example a drug dosed at 1g/day the genotoxic impurity level would be 1.5ppm [2-13].

The main focus of the genotoxic risk assessment is on impurities that may arise particularly in the penultimate and final synthetic stages. Imatinib 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 $C_{29}H_{31}N_7O$ and a molecular weight of 493.6. Imatinib Mesylate is used in chronic myelogenous leukemia, gastrointestinal stromal tumors (GISTs) and a number of other malignancies. The U.S. Food and Drug Administration (FDA) have approved Imatinib Mesylate as first line treatment for Philadelphia chromosome (Ph)-positive CML, both in adults and children. The synthetic intermediates 1-(2-methyl-5-nitro-phenyl) guanidine nitrate and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine are present in small amounts as process impurities in API. These intermediates are identified as potential genotoxic impurities because of the presence of nitro and aromatic amine groups and hence should be controlled to ppm level in the final product. The chemical structure of Imatinib and these impurities are shown in Figure 1, 2 and 3. Based on the maximum daily dose of 800 mg of Imatinib, its potential genotoxic impurities are required to be controlled at a limit of 2ug/g (2ppm).



. CH_3SO_3H

Figure 1: Imatinib Mesylate

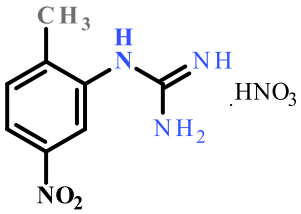


Figure 2: 1-(2-methyl-5-nitro-phenyl) guanidine nitrate

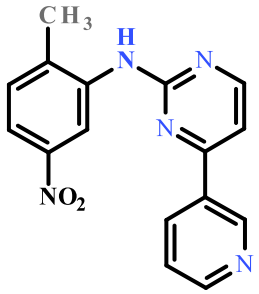


Figure 3: N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine

From the literature survey of this product Yadav et al., reported a RPHPLC method with PDA detector for the evaluation of the genotoxic impurities N-(2-methyl-5-aminophenyl)-4-(pyridyl)-2-pyrimidineamine (impurity A) and N-[4-methyl-3-(4-methyl-3-ylpyrimidin-2-ylamino)-phenyl]-4-chloromethyl benzamide (impurity B) (14). Veera Reddy et.al has reported a LC-MS/MS method for the determination of Methyl-3-N[4-(3-Pyridinyl)-2-pyrimidinyl]-1,3-benzenediamine(PNMP) and 4-(4-Methyl piperazinomethyl) benzoic acid dihydrochloride(MPBA) [15]. A Stability-indicative, validated, fast HPLC method for quantification of two genotoxic impurities N-(2-methyl-5-aminophenyl)-4-(3-pyridyl)-2-pyrimidine amine and 4-(4-methyl piperazinyl methyl) benzoic acid dihydrochloride in imatinib mesylate was reported by Venkatramanna et.al [16]. Vaibhav Bhatt et.al have reported a LCMSMS method for quantification of 2-methyl-5-aminophenyl)-4-(3-pyridyl)-2-pyrimidine [17]. Ramakrishna et.al have reported GC–MS method for the determination of methyl methanesulfonate and ethyl methanesulfonate in Imatinib Mesylate [18].

There are currently no methods reported in literature for the analysis of the two potential genotoxic impurities 1-(2-methyl-5-nitro-phenyl) guanidine nitrate and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine in Imatinib Mesylate.

Therefore a novel LCMS method was developed and validated which could adequately resolve these two PGIs from the matrix interferences of the API and simultaneously quantify them in ppm level.

MATERIALS AND METHOD

Samples of Imatinib and its potential genotoxic impurities 1-(2-methyl-5-nitro-phenyl) guanidine nitrate and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine were obtained from Natco Pharma Ltd, Natco Research Centre, and Hyderabad, India. LC-MS grade ammonium bicarbonate was purchased from Sigma-Aldrich. HPLC grade acetonitrile was purchased from JT Baker (Mumbai, India). Purified water collected through Milli-Q Plus water purification system (Millipore, USA)

Instrumentation:

The LC-MS method development and validation was done using Agilent 1200 series HPLC system connected with Agilent mass spectrometer G6120B Single Quad, equipped with electrospray ionization in positive mode.

LC-MS chromatographic conditions:

The analysis was carried out using Kromasil 100-3.5-C18 (150 x 2.1) mm with a flow rate of 0.3 ml/min. The mobile phase used was a mixture of 10mM Ammonium bicarbonate and acetonitrile using a gradient programme of Time (mins) / %A: 0/60, 5/60, 10/20, 20/20, 25/60, 30/60. The column temperature was maintained at 40°C and the injection volume was 10µl. Mass spectrometer was operated in electrospray ionization with positive ion mode with a capillary voltage of 3800V. The Fragmentor was set at 90, gain was 1, the drying gas flow was 12 L/min with a temperature of 330° C and nebulizer pressure was 55 psig. Under these conditions impurities 1-(2-methyl-5-nitro-phenyl) guanidine nitrate and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl)pyrimidin-2-amine in Imatinib were quantified by SIM mode.1-(2-methyl-5-nitro-phenyl) guanidine nitrate was monitored at m/z 195.2(protonated) and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl)pyrimidin-2-amine was monitored at m/z 308.2(protonated) .

Preparation of standards and test sample solutions:

The standard stock solutions of 1-(2-methyl-5-nitro-phenyl) guanidine nitrate and N-(2-methyl-5-

nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine were prepared approximately at 100 ng/ml in diluent. The Imatinib test samples were typically prepared at 5mg/ml in diluent.

RESULTS AND DISCUSSION

Method development:

As the actual goal of the present work was to develop a method that could successfully resolve and estimate the two potential genotoxic impurities in Imatinib in a single run, different stationary phases and mobile phases were used.

An isocratic elution using 0.1% Formic acid and Acetonitrile was tried where the peak shape for both the impurities was broad and the sensitivity was very poor. By adding 0.1% formic acid in the acetonitrile and in gradient elution the impurities could not be separated.

Then the buffer was changed to Ammonium bicarbonate and isocratic elution was tried but there was no separation for the impurities from Imatinib. In the same buffer trials with gradient elution was carried out and finally the desired chromatographic separation was achieved on a Kromasil 100-3.5-C18 (150 x 2.1) mm with a flow rate of 0.3 ml/min. The mobile phase used was a mixture of 10mM Ammonium bicarbonate and acetonitrile using a gradient programme of Time(mins)/%A: 0/60, 5/60, 10/20, 20/20, 25/60, 30/60.

Method validation:

The method has been validated for the quantification of potential genotoxic impurities 1-(2-methyl-5-nitro-phenyl) guanidine nitrate and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine in Imatinib to ensure that the performance characteristics of the method meet the requirements for its intended analytical applications. During the method validation the assessed parameters were specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, precision and accuracy.

Limit Of Detection (LOD) and Limit Of Quantification (LOQ):

The LOD and LOQ were calculated with signal to noise ratios of 3:1 & 10:1 respectively and by injecting a dilute solution having known concentrations of impurities and established the minimum level at which the impurities can be reliably detected. The LOD & LOQ results obtained for impurities are listed in Table 1.

Table 1: LOD and LOQ values

Impurity	***LOD***		***LOQ***	
	Conc.(ppm)	**S/N ratio**	**Conc.(ppm)**	**S/N ratio**
1-(2-methyl-5-nitro-phenyl) guanidine nitrate N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl)pyrimidin-2-amine	0.2	3.1 : 1	0.6	10.1 : 1

Specificity:

The specificity of the optimized method was performed by injecting stock solutions of individual impurities to check resolution among the impurities and Imatinib under the same conditions mentioned in LC-MS chromatographic conditions. The specificity is represented in the Figure 4. Summary of retention time and m/z value for Imatinib and its impurities are mentioned in the given Table 2.

Table 2: Summary of retention time and m/z values

Compound	Retention Time(Min)	Mass(m/z) value
1-(2-methyl-5-nitro-phenyl) guanidine nitrate	1.6	195.2 (+ve)
N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl)pyrimidin-2-amine	8.9	308.2 (+ve)
Imatinib	3.4	494.1(+ve)

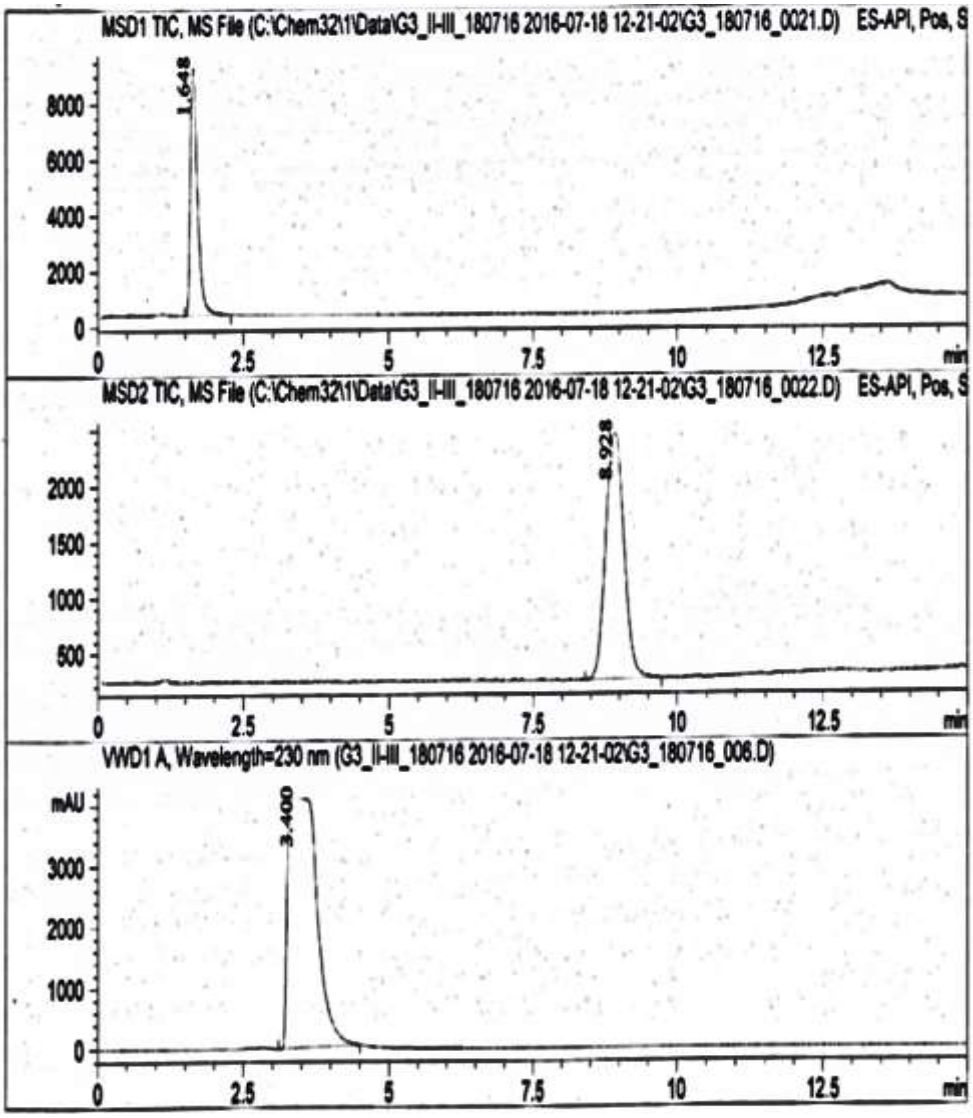


Figure 4: **Specificity chromatogram**

Linearity:

Linearity of the method was checked by preparing the solutions at 6 concentration levels from LOQ to 150% of specification limit (4.5, 7.5, 12.25, 15.0, 18.0, 22.5 ng/ml). The responses recorded for each impurity were plotted against concentration. The correlation coefficient of linear regression was found to be greater than 0.99 for each impurity, indicating good linearity. Corresponding linearity graphs are shown in Figure 5 and 6 and data is represented in the Table 3.

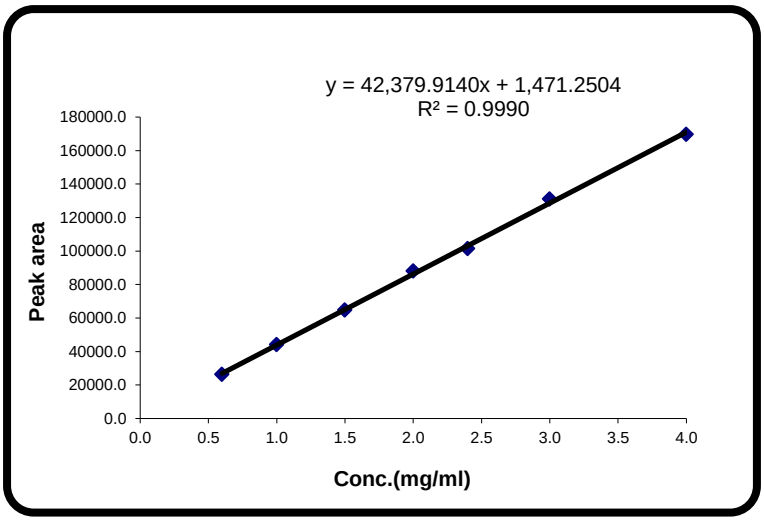


Figure 5: Linearity graph for 1-(2-methyl-5-nitro-phenyl) guanidine nitrate

Table 3: Linearity data

Level	1-(2-methyl-5-nitro-phenyl) guanidine nitrate hydrochloride **peak area**	N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl)pyrimidin-2-amine **peak area**
LOQ	26227.6	21990.4
50%	43984.1	36669.5
75%	64652.7	54541.6
100%	87916.9	73529.1
120%	101366.8	87359.8
150%	131001.0	110175.6
200%	169658.4	145749.2
Slope	42379.9	36479.8
R^2	**0.9990**	**0.9999**

Accuracy:

Accuracy of the method was evaluated by using four solutions containing Imatinib base spiked with the impurities at LOQ, 50%, 100% and 150% of the specification limit (2 ppm). Each concentration level was prepared in triplicate. The percentage recovery results obtained for the two impurities are listed in Table 4. A representative spiked chromatogram is shown in Figure 7.

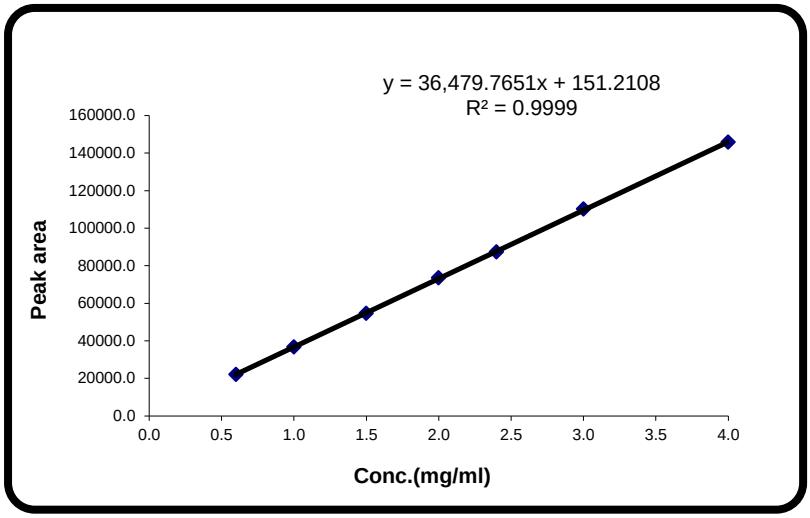


Figure 6: Linearity graph for N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine

Table 4: Recovery

Test + Spiked (n=3)	**% Recoveries for 1-(2-methyl-5-nitro-phenyl) guanidine nitrate**	**% Recoveries for N-(2-methyl-5-nitro-phenyl)-4-(pyridyl)pyrimidin-2-amine**
Test+LOQ spiked (0.6 ppm)	94.3	94.1
Test+50% spiked (1 ppm)	94.6	97.7
Test+100% spiked (2 ppm)	93.2	96.6
Test+150% spiked (3 ppm)	93.0	98.0

System precision and system suitability:

The precision and system suitability was performed by injecting six replicates of the working standard solution (2ppm of the two impurities). The %RSD for the peak areas obtained was calculated. The data presented in the Table 5 establishes system precision.

Table 5: **System suitability and system precision**

S.No	**Peak area**	
	1-(2-methyl-5-nitro-phenyl) guanidine nitrate	N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl)pyrimidin-2-amine
1	82939.602	72653.242
2	83291.844	72670.750
3	84764.719	73049.172
4	84006.688	73596.398
5	84552.563	73900.703
6	83975.578	73822.328
Mean	83921.83	73282.10
% RSD	**0.84**	**0.77**

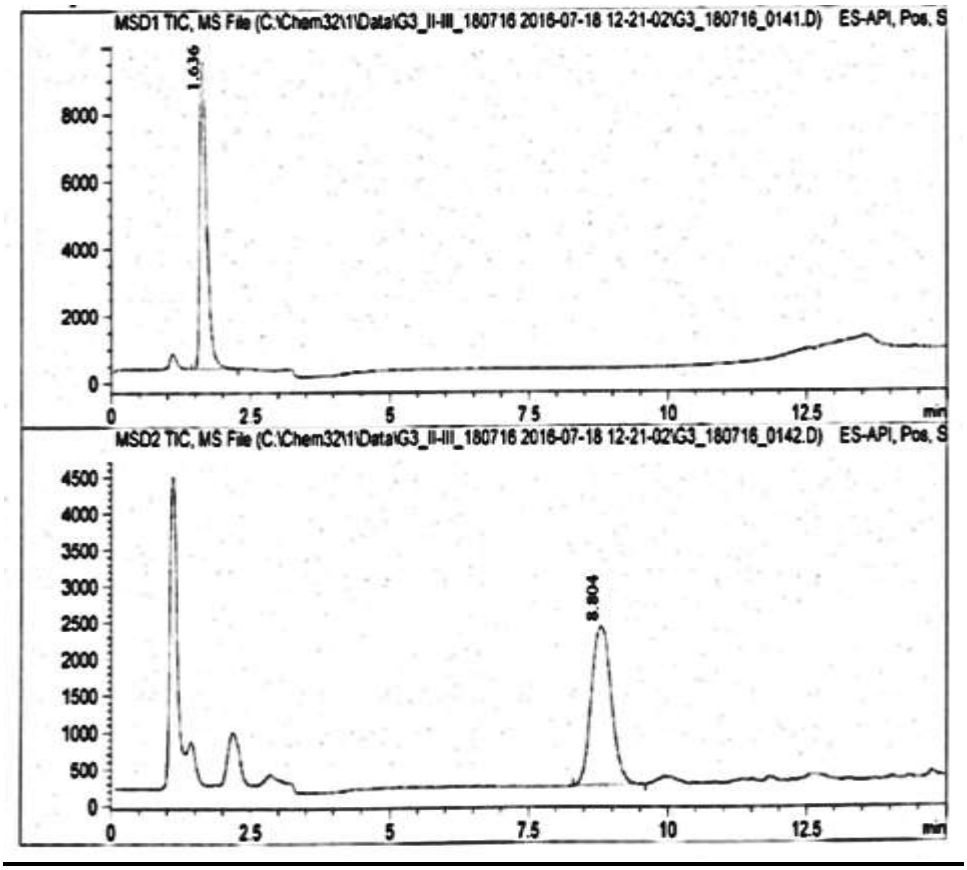


Figure 7: Representative spiked chromatogram

CONCLUSION

The proposed LCMS method is simple, sensitive and accurate to quantify simultaneously the two impurities 1-(2-methyl-5-nitro-phenyl) guanidine nitrate and N-(2-methyl-5-nitro-phenyl)-4-(3-pyridyl) pyrimidin-2-amine at ppm level present in Imatinib base. The validated parameters are well within the limits and this method is found suitable for routine quality control test of Imatinib base.

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