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QUANTIFICATION OF POTENTIAL GENOTOXIC IMPURITY IN IMATINIB MESYLATE BY LC-MS/MS

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ABSTRACT

A sensitive and selective liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method was developed and validated for the trace analysis (> 1 ng/mL level) of 2-methyl-5-aminophenyl)-4-(3-pyridyl)-2-pyrimidine (Imp-A) a genotoxic impurity in imatinib mesylate drug substances. LC-MS/MS analysis of Imp-A was done on Inertsil C18 (150 mm × 4.6 mm) 5 µm column and 0.1% formic acid in 1000 mL of water was used as buffer in mobile phase A and acetonitrile in mobile phase B. Gradient program was developed for rapid analysis. 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. LC-MS/MS is able to quantitate up to 1 ng/mL of Imp-A.

Key words: Imatinib mesylate, LC-MS/MS, Genotoxic impurity, Gradient program.

INTRODUCTION

Pharmaceutical genotoxic impurities (PGIs) may induce genetic mutations, chromosomal breaks (rearrangements) and they have potential to cause cancer in human was observed by Bolt et al.[1] Muller et al.[2] Jacobson and McGovern[3] investigated that exposure to even low levels of such impurities present in final active pharmaceutical ingredient (API) may be of significant toxicological importance. Hence it is important for process chemists to avoid such genotoxic impurities in the manufacturing process[4]. However, it would be difficult or impossible to eliminate PGIs completely from the synthetic scheme. Therefore, it is a great challenge to analytical chemists to develop an appropriate analytical method to quantify the impurity accurately and control their levels in APIs. According to the European Medicines Agency (EMEA) and feedback from US Food and Drug Administration (USFDA) the proposed use of a threshold of toxicological concern (TTC), it is accepted that genotoxic impurities will be limited to a daily dose of 1.0–1.5 µg/day[5,6].

Imp-A is often used during the manufacture of imatinib mesylate (APIs), either as intermediate or as raw material[7]. In fact, aromatic amine is a known genotoxic functional group and it is genotoxic impurity

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and carcinogen in rats and mice reported by Fu et al.[8] The potential presence of these genotoxic impurities has attracted the attention of regulatory authorities; draft guidelines from the EMEA and feedback from the USFDA to the pharmaceuticals industry via responses to drug applications have influenced the industry to establish interim strategies. It is a great challenge to both analytical and synthetic chemists to prepare a drug dose of 1.5 g/day which does not cross 0.3 ppm of this genotoxic impurity and overview given by Snodin and McCrossen[9].

Though Imp-A is a well known carcinogen, this data would ascertain that the regulatory authorities may be expected to control the levels of Imp-A to be 0.3 ppm in the drug substance (assuming a 1.5 g/day daily dose). A method capable of such a lower level of detection is great challenge for analytical method development for controlling these genotoxic impurities. Ideally conventional analytical instruments in pharmaceutical industries such as HPLC with UV detection and GC with FID detection should be employed as the standards in first attempt for PGIs analysis and these methods were discussed by Klick[10] and Valvo et al.[11], but there are some drawbacks with above mentioned techniques because probability of co-elution at very trace level can change analytical result. When impurity standards are not available some method is needed to characterize the impurities on-line. Therefore, for accurate determination at ppm levels the above mentioned techniques are inadequate; consequently there is a great need to develop better analytical method for the analysis of such genotoxic impurities in pharmaceutical industries. As a result various kinds of chromatographic techniques and methodologies have been explored as useful approaches out of Hsieh and Korfmacher[12] and Lee and Kems[13] had discussed LC-MS/MS technique and application. It was felt necessary to develop simple, sensitive and validated method for estimation of Imp-A. The literature survey revealed that Raja et al.[14] developed and reported spectrophotometric methods for the determination of imatinib mesylate API. A method has been reported for the determination of Imp-A in imatinib mesylate API by RP-HPLC and reported by Kakde et al.[15] and Madireddy et al.[16]

The present study was undertaken to develop a sensitive and rapid LC-MS/MS method for the determination of Imp-A in imatinib mesylate API. Due to its higher selectivity and sensitivity LC-MS/MS has been adopted for quantification of Imp-A in imatinib mesylate, which is used to treat certain types of cancer. It is used in treating chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) and some other diseases. It blocks different abnormal enzyme found on tumor cells, there by curing the disease given by Zimmermann et al.[17]

Imatinib, a selective tyrosine kinase inhibitor that inhibits c-Abl, c-Kit, and PDGF receptors (PDGFR), has therapeutic efficacy in patients with chronic myeloid leukemia and gastrointestinal stromal tumors. The therapeutic benefit of imatinib in animal models of kidney diseases is reported and has largely been attributed to its effect on PDGFR[18], brief stability data was generated for the Imp-A in the water–acetonitrile (50 : 50, v/v) diluents.

EXPERIMENTAL

Reagents and standards

HPLC grade methanol and formic acid were purchased from Merck (Mumbai, India). Formic acid, trifluoroacetic acid and methanol were obtained in their analytical grade from S. d. fine Chemicals Limited (Mumbai, India). Purified water collected through Milli-Q Plus water purification system (Millipore, Milfordford, MA, USA). Reference substance of Imp-A and Pure Imatinib mesylate were obtained as gratis sample from Cipla Ltd. Research Laboratories (Mumbai, India).

Sample and standard preparation

0.1 mg/mL reference stock solution was prepared by dissolving Imp-A in water–methanol (50 : 50, v/v). Preparation of sub stock standard solution of 0.001 mg/mL was achieved on further dilution with water–acetonitrile (50 : 50, v/v). Finally desired concentration (20 ng/mL) of standard solution was prepared by diluting standard stock solution to 100 mL with water–acetonitrile (50 : 50, v/v). Linearity solution prepared from further dilution of standard solution of 1, 10, 15, 20, 25 and 30 ng/mL.

Instrumentation

The MS of LC-MS/MS system used was an Applied Biosystems Sciex QTRAP-5500 Model (Switzerland). LC was carried out on Agilent HPLC (1200 series, Germany) with photodiode array detector. The output signal was monitored and processed using Chromeleon software (ver no. 6.80SR10). In all the studies, separations were achieved on a Inertsil ODS 3V column (150 mm x 4.6 mm i.d., particle size 5 µm) procured from LCGC (Bangalore, INDIA). A pH/Ion analyzer (Labindia) was used to check and adjust the pH of buffer solutions. Other small equipment were PCI sonicator (22L500/CC/DTC), precision analytical balance (MX5, Mettler Toledo, Schwerzenbach, Switzerland).

Operating conditions of LC-MS/MS

The analytical column used in LC-MS/MS was Inertsil ODS 3V (150 mm × 4.6 mm) 5 µm column (LCGC Co., Bangalore-India) in gradient mode using 0.1 % formic acid as mobile phase-A and methanol as mobile phase-B. The flow rate was 1.0 mL/min, with the flow rate split down to 0.3 mL/min in to the MS source. The column oven temperature was maintained at 40ºC, sample cooler temperature was 10ºC and the wavelength was set at 230 nm. The injection volume was 20 µL. Positive ion electrospray ionization probe was operated with selective ion monitoring (SIM) or selective ion-recording (SIR) mode was used as MS method for quantification of Imp-A in imatinib mesylate drug substance. In this method Imp-A was monitored with its molecular ion $[M^+H]^+$ m/z 278.2 (protonated) and Imatinib mesylate was monitored with its molecular ion $[M^+H]^+$ m/z 495.1 (protonated). The ion spray voltage (V), declustering potential and entrance potential were kept as 5500 V, 60 V and 10 V, respectively. The curtain gas flow (psi), ion source gas1 and ion source gas2 nebulization pressure (psi) were maintained as 35, 50 and 45, respectively. This methodology has one of the advantages for the estimation of Imp-A in aqueous solutions, this approach is suitable for both API and intermediates. For gradient program (Table 1).

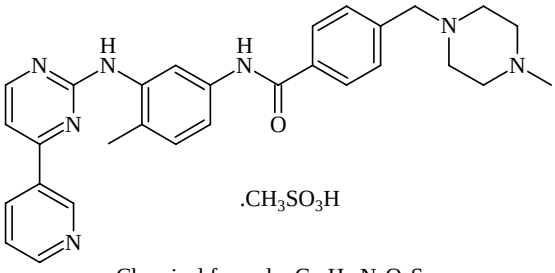
Table 1: Gradient program

Time (mins.)	% A (Mobile phase-A)	% B (Mobile Phase-B)
0.01	60	40
5	60	40
7	20	80
10	20	80
11	60	40
14	60	40

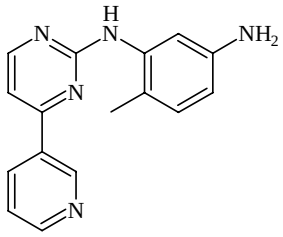
RESULTS AND DISCUSSION

Method development

The main aim of the LC-MS/MS method in this study was to separate and quantify Imp-A in the imatinib mesylate active pharmaceutical ingredient structure are shown in Fig. 1. Different reversed phase stationary phases have been assessed which included C18, C8 and cyano phases. In addition different mobile phase additives such as formic acid, trifluoroacetic acid, acetonitrile and methanol have been tested. Chromatographic separation was finally achieved on a Inertsil ODS 3V (150 mm × 4.6 mm) 5 µm column (LCGC Co, India) in gradient mode using 0.1% formic acid as mobile phase-A and acetonitrile as mobile phase-B. The flow rate was 1.0 mL/min, with the flow rate split down to 0.3 mL/min in to the MS source MS parameter set to get maximum sensitivity for Imp-A.



Chemical formula: $C_{30}H_{35}N_7O_4S$
Exact mass: 589.25



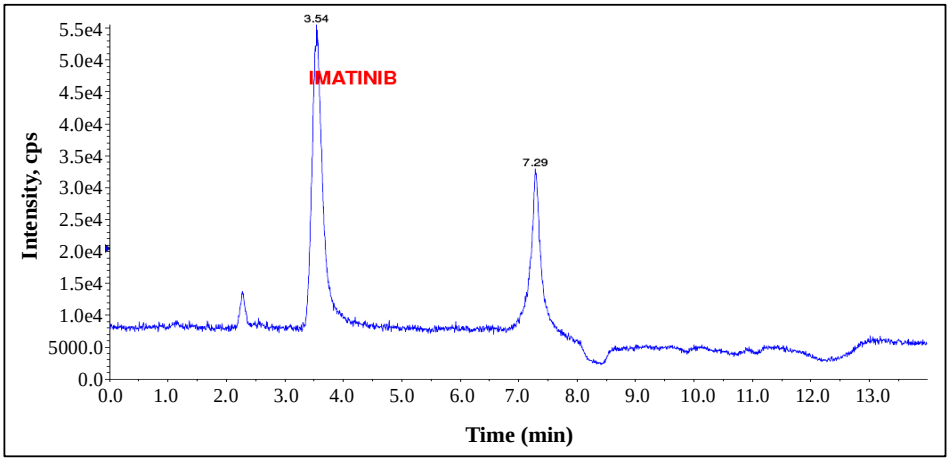
Chemical formula: $C_{16}H_{15}N_5$
Exact mass: 277.13

Fig. 1: Structure of imatinib mesylate and (2-methyl-5-aminophenyl)-4-(3-pyridyl)-2-pyrimidine (Imp-A)

Method validation

Specificity

Imatinib mesylate and Imp-A solutions were prepared individually at a concentration of about 0.001 mg/mL in the diluent and a solution of imatinib mesylate spiked with Imp-A was also prepared. Blank and specificity chromatograms are shown in Fig. 2. The robustness of the method was studied with deliberate modifications in flow rate of mobile phase and column temperature. The flow rate of mobile phase was altered by 0.2 units, i.e. 0.8–1.2 mL/min. The effect of column temperature on resolution was studied at 37ºC and 43ºC (temperature altered by 3 units), the results revealed that these changes do not impact on chromatographic performance.



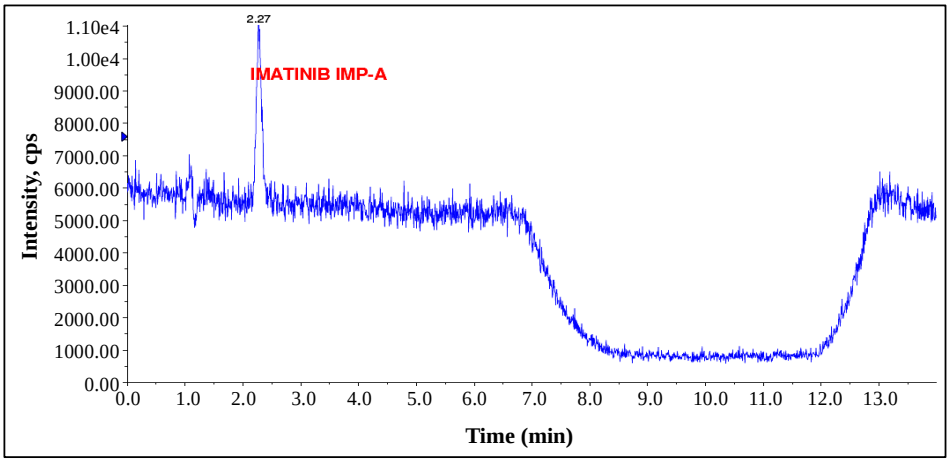


Fig. 2: Specificity of imatinib, (2-methyl-5-aminophenyl)-4-(3-pyridyl)-2-pyrimidine (i.e-Imp-A)

Determination of LOD and LOQ

The LOD and LOQ were calculated from S/N (signal to noise) ratios. Now to determine LOD and LOQ values Imp-A concentrations were reduced sequentially such that they yield S/N ratio as 13 and 12, respectively. The determined LOD and LOQ chromatograms were shown in Fig. 3. Data generated from six injections of Imp-A (with out API) containing 0.3 ppm of each Imp-A with respect to an API sample concentration ng/mL. The LOQ of 0.3 ppm is typical for the Imp-A, with a LOD approximately three times less than LOQ. In addition, the relative efficiency of SIM versus MRM (SRM) modes in sensitivity improvement was also evaluated. We found that, in SIM mode the LOD was 0.3 ng/mL, whereas with SRM/MRM it was 1.0 ng/mL, the corresponding chromatogram was shown in Fig. 4.

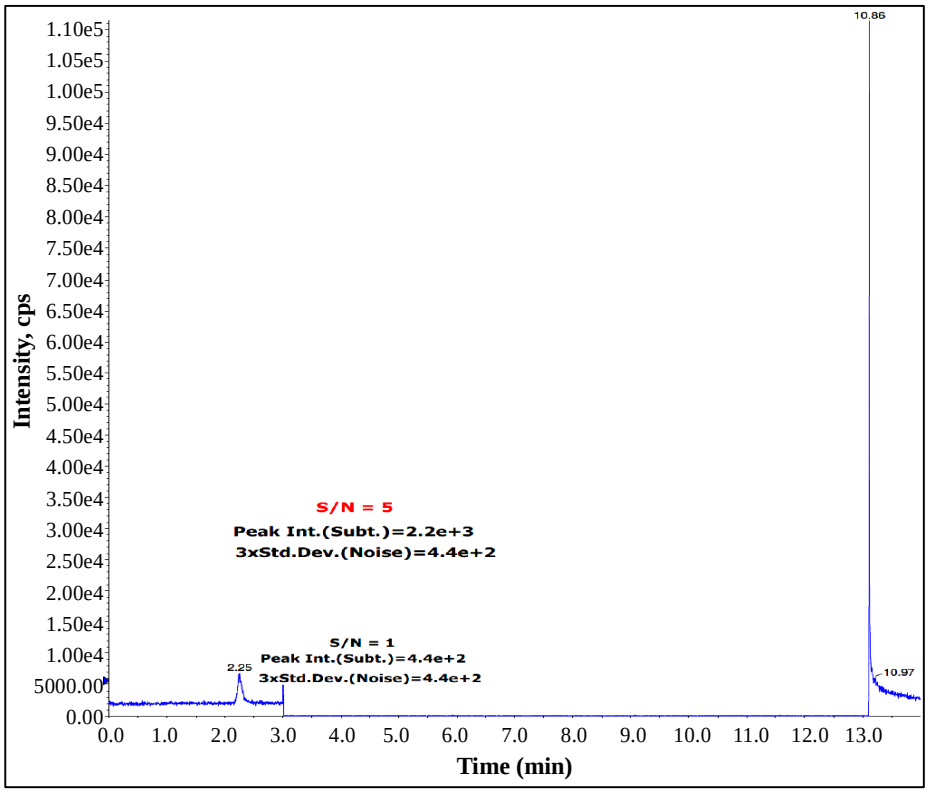


Fig. 3: LOD (Limit of detection)

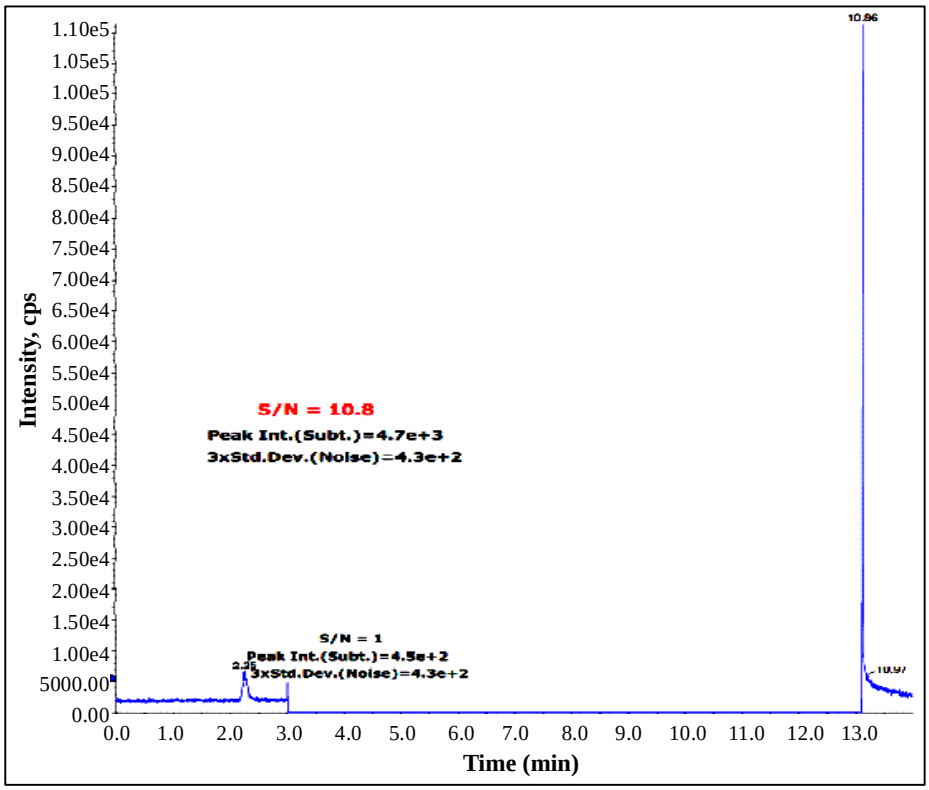


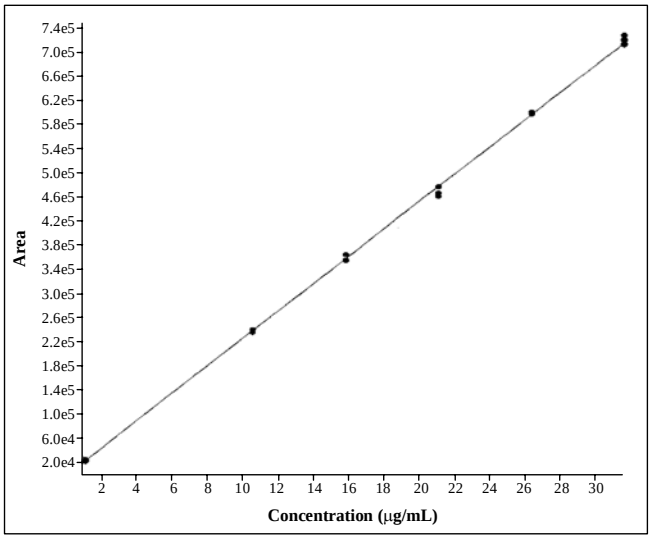
Fig. 4: Sensitivity L-LOQ (Lower-Limit of quantification) in SRM mode

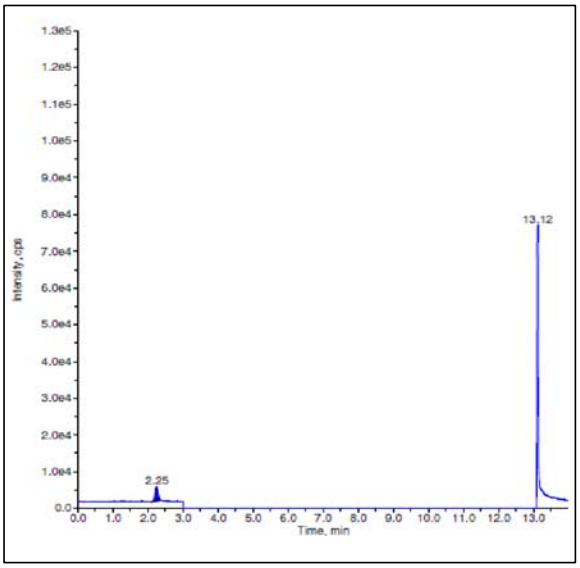
Linearity

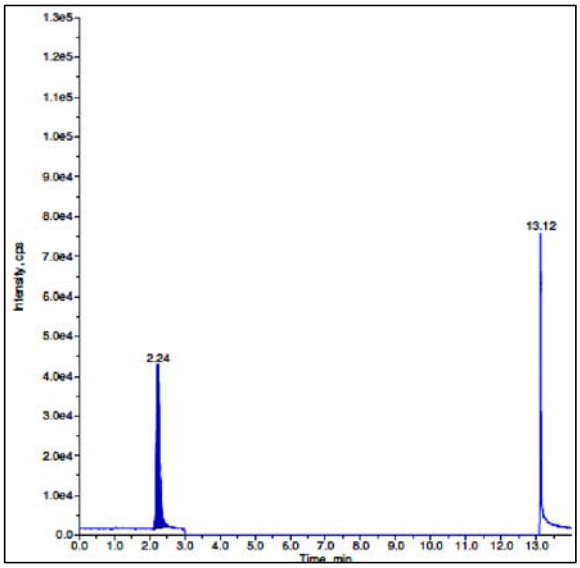
Linearity of the method was checked by preparing solutions at six concentration levels of 1 ng/mL (L-LOQ), 10 ng/mL (L1 solution), 15 ng/mL (L2 solution), 20 ng/mL (L3 solution), 25 ng/ml (L4 solution) and 30 ng/mL (L5 solution) for Imp-A. L1 solution and L5 solution were injected six times were as L2, L3, L4 and L5 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, which indicates good linearity. Corresponding linearity chromatogram shown in Fig. 5 and also shown in Table 2.

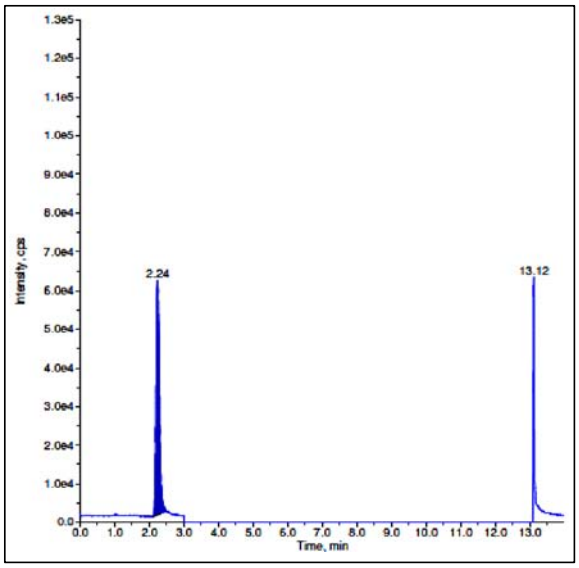
Table 2: Linearity 1 ng/mL to 30 ng/mL. Each level % CV for six and three replicate and correlation coefficient

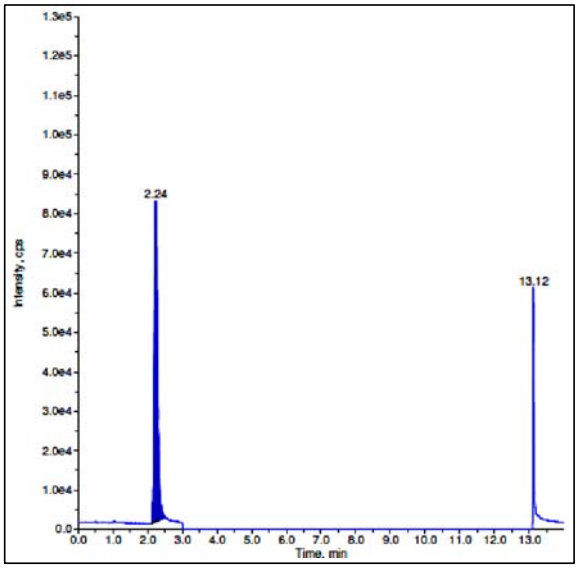
Expected Concentration	Sample Name	Number of values used	Mean	Standard Deviation	% CV
1.04	L-LOQ	6 of 6	22866.67	886.146239	3.93
10.44	L1 Solution	3 of 3	239333.33	2886.75	1.21
15.66	L2 Solution	3 of 3	380000.00	5291.50	1.47
20.89	L3 Solution	3 of 3	470333.33	8082.90	1.72
26.11	L4 Solution	3 of 3	603000.00	1732.05	0.29
31.34	L5 Solution	6 of 6	723833.33	6112.83	0.84


Area
Concentration (µg/mL)


Intensity, cps
2.25
13.12
Time, min


Intensity, cps
2.24
13.12
Time, min


Intensity, cps
2.24
13.12
Time, min


Intensity, cps
2.24
13.12
Time, min

Cont…

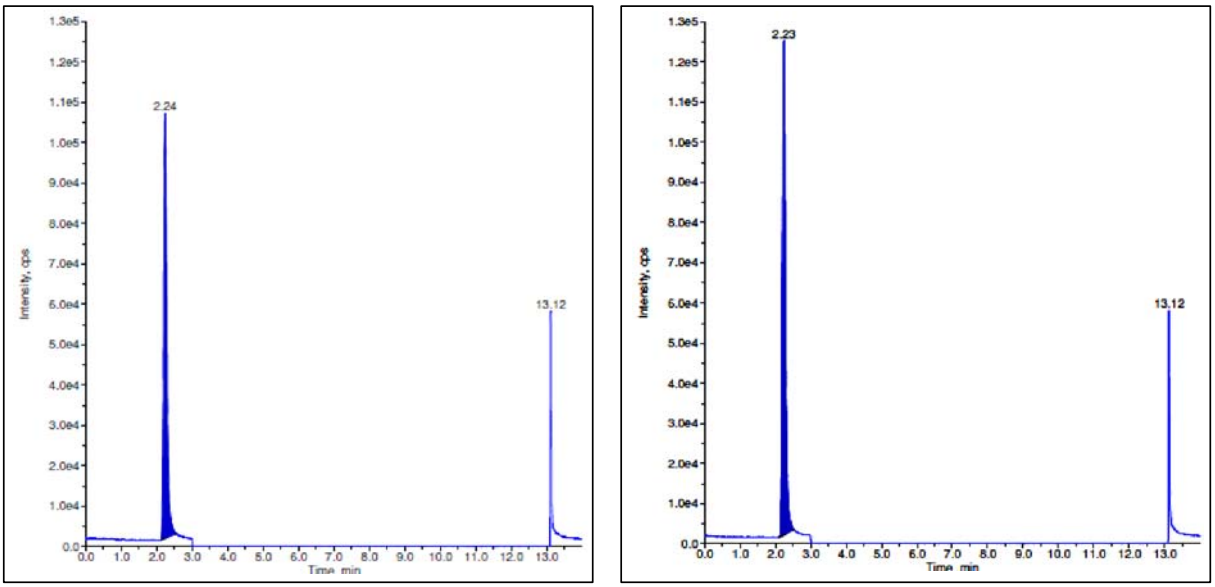


Fig. 5: Linearity 1 ng/mL to 30 ng/mL (L-LOQ, L1 solution, L2 solution, L3 solution, L4 solution & L5 solution)

Accuracy

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 L-LOQ level & L5 level, and remaining level (level 1, 2, 3 & 4) triplicate in bulk drug sample. Then the percentage recoveries were calculated. Excellent recovery values of Imp-A (109–110%) was obtained at L-LOQ level. At such a low levels these recoveries and % CV is < 1.0 was satisfactory. Sample and accuracy at L-LOQ chromatograms are shown in Fig. 6, and the coefficient variant, % CV were calculated from the average of six and three plicate analysis, which were shown in Table 3.

Table 3: Accuracy/Recovery for 1 ng/mL

Sample area	Standard area	Spiked area	Theoretical concentration (mg/mL)	Measured concentration	% Recovery
51333	25467	76800	0.00010	0.00011	109.6
47533	25467	73000	0.00010	0.00011	109.3
47333	25467	72800	0.00010	0.00011	109.9
46633	25467	72100	0.00010	0.00011	109.9
44933	25467	70400	0.00010	0.00011	110.0
50333	25467	75800	0.00010	0.00011	110.8
			Average		109.91
			Standard deviation		0.51
			%CV		0.46

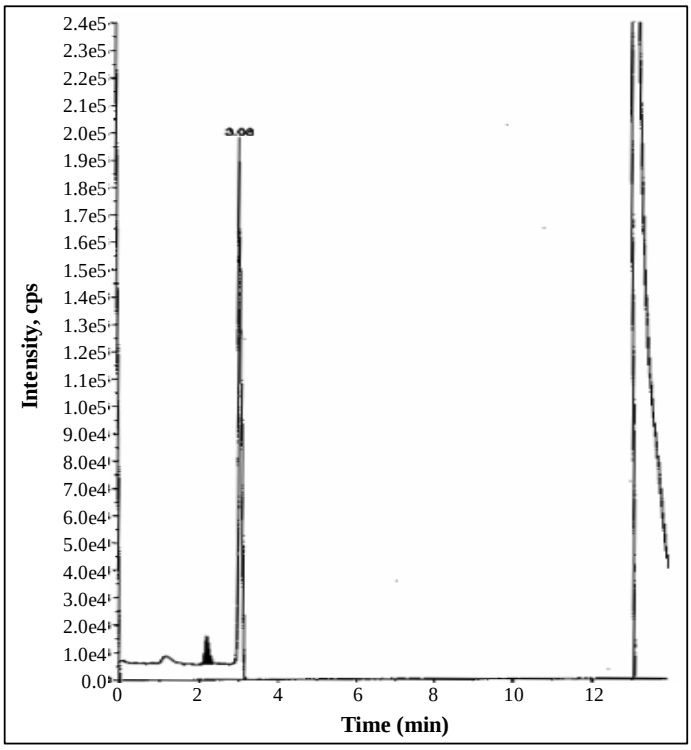


Fig. 6: Accuracy/Recovery at L-LOQ

System and method precision

Infact Imp-A in imatinib was checked for repeatability. The sample was prepared by spiking imatinib with the impurity at a concentration of 20 ng/mL with target analyte concentration and injected six times. The % CV was found to be less than 5.0% for system precision.

To determine the method precision six independent solutions were prepared by spiking imatinib with the impurity at a concentration of 20 ng/mL with respect to target analyte concentration. Each solution was injected once. The variation in the results for the two impurities was expressed in terms of % RSD. The values calculated were found to be below 15.0% RSD for impurities, indicating satisfactory method precision.

CONCLUSION

The desired goal of this study was to develop highly sensitive and more accurate analytical method using LC-MS/MS for the quantification of Imp-A in APIs. It has been demonstrated that it is highly sensitive with a limit of detection (L-LOQ) of 1 ng/mL. Trace level of formic acid is added to the mobile phase to enhance ionization and detection. Dramatic differences in stability were noted, therefore it is recommended that recovery/standard addition studies may be carried to rule out stability or ion suppression issues so this impurity is process as well as degrading impurity. The method, which is able to quantify them at ppm level is developed and validated. The information presented here could be very useful for monitoring of Imp-A in imatinib mesylate in its pure and tablet form.

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