CHAPTER 4

QUANTIFICATION OF GENOTOXIC IMPURITIES IN ANTI-CANCER DRUGS: ERLOTINIB HYDROCHLORIDE & IMATININB MESYLATE

4.1. ERLOTINIB HYDROCHLORIDE

4.1.1 INTRODUCTION

Erlotinib Hydrochloride (figure 4.1.1) [163] is targeted therapy and classified as epidermal growth factor receptor inhibitor-protein-tyrosine kinase inhibitor and used for pancreatic cancer and other type of cancer.

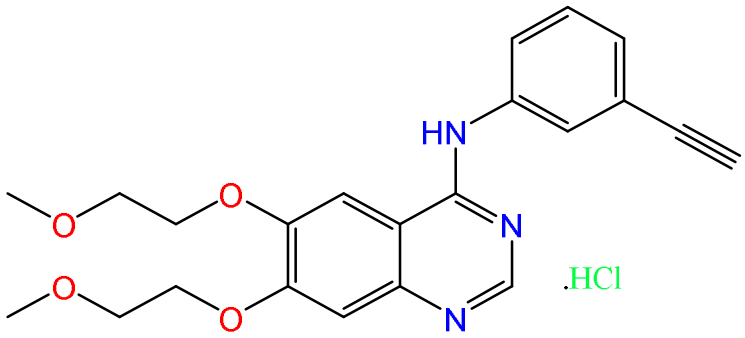
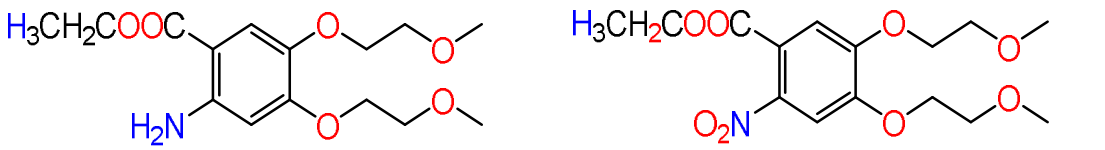


Figure 4.1.1: The structure of Erlotinib Hydrochloride

Chemical Name	**:**	N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy) quinazolin-4-amine
Molecular formula	**:**	$C_{22}H_{23}N_3O_4$.HCl
Molecular weight	**:**	429.9
CAS No.	**:**	183319-69-9
Melting point	**:**	223 to 225 ºC
Phase	**:**	Tablets
Appearance	**:**	white to pale yellow powder
Bioavailability	**:**	59%
Solubility	**:**	Soluble in dimethylsulfoxide and slightly soluble in methanol
Brand name	**:**	Tarceva

Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate and Ethyl 4,5-bis(2-methoxyethoxy) benzoate (figure 4.1.2) were identified that potential genotoxic impurities using DEREK nexus software. Through regulatory authorities proposed limit to be 10 ppm in the drug substance based on the daily dosage of drug substance.There was no literature available for the quantification of Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate (ERL ethyl ester) and Ethyl 4,5-bis(2-methoxyethoxy) benzoate (ERL nitro compound) at ppm level in Erlotinib Hydrochloride.



ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate (ERL ethyl ester)

ethyl 4,5-bis(2-methoxyethoxy)-2-nitrobenzoate (ERL nitro compound)

Figure 4.1.2: The structure of two genotoxic impurities

4.1.2 EXPERIMENTAL

4.1.2.1 Standards and chemicals

The following standards and chemicals were used for the evaluation, which is mentioned in the table 4.1.1.

Table 4.1.1: List of standards and chemicals

S.No.	Chemical/standard	Grade	Make
1.	Formic acid	LCMS	Merck, Mumbai, India.
2.	Methanol	LCMS	Merck, Mumbai, India.
3.	Acetonitrile	LCMS	Merck, Mumbai, India.
4.	Water	Mill Q water	Millipore, USA
5.	Erlotinib hydrochloride	---	Cipla, Research and development, India.
6.	ERL ethyl ester	---	Cipla, Research and development, India.
7.	ERL nitro compound	---	Cipla, Research and development, India.

4.1.2.2 Preparation of solutions

4.1.2.2.1 Sample preparation

10 mg of erlotinib hydrochloride sample transferred into 10 mL volumetric flask and dissolved and diluted upto the mark with diluent.

4.1.2.2.2 Preparation of standard stock solution

10 mg of ERL Ethyl Ester and ERL Nitro compound individually transferred into 100 mL volumetric flask, dissolved and diluted upto the mark with diluent. Transferred each 1 mL of the above solution to 100 mL volumetric flask and diluted upto the mark with diluent. Again, diluted 10 mL of above solution to 100 mL with diluent and mixed.

4.1.2.2.3 Preparation of standard Solution

1.0 mL standard stock solution transferred into 10 mL volumetric flask and diluted upto the mark with diluent, which is equivalent to 10 ppm with respect to test concentration of 1 mg/mL.

Calculations for impurity concentrations in ppm

$$\text{Concentration}\ (\text{ppm}) = \frac{\text{Std. weight}}{100} \times \frac{1}{100} \times \frac{10}{100} \times \frac{1}{10} \times \frac{1}{\text{Spl. conc.}\ (\text{mg/mL})} \times \frac{\text{Purity}}{100} \times 10^6$$

$$\text{ERL ethyl ester concentration}\ (\text{ppm}) = \frac{10}{100} \times \frac{1}{100} \times \frac{10}{100} \times \frac{1}{10} \times \frac{1}{1} \times \frac{98.9}{100} \times 10^6$$

$$\text{ERL nitro concentration}\ (\text{ppm}) = \frac{10}{100} \times \frac{1}{100} \times \frac{10}{100} \times \frac{1}{10} \times \frac{1}{1} \times \frac{99.1}{100} \times 10^6$$

4.1.2.2.4 Preparation of LOD and LOQ solution

Detection limit solution was prepared by diluted 0.3 mL of **s**tandard stock solution into 100 mL with diluent (equivalent 0.3 ppm). Quantification limit solution was prepared by diluted 0.1 mL of **s**tandard stock solution into 10 mL with diluent (equivalent to 1.0 ppm).

4.1.2.2.5 Solution preparation for accuracy

Accuracy at LOQ (1.0 ppm)

Triplicate samples were prepared by 10 mg of erlotinib hydrochloride transferred into 10 mL volumetric flask and added 0.10 mL of standard stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 50% (5 ppm)

Triplicate samples were prepared by 10 mg of erlotinib hydrochloride transferred into 10 mL volumetric flask and added 0.50 mL of standard stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 100% (10.0 ppm)

Six replicate samples were prepared by 10 mg of erlotinib hydrochloride sample transferred into 10 mL volumetric flask and added 1.0 mL of standard stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 150% (15.0 ppm)

Triplicate samples were prepared by 10 mg of erlotinib hydrochloride transferred into 10 mL volumetric flask and added 1.5 mL of standard stock solution, dissolved and diluted upto the mark with diluent.

4.1.2.2.6 Solution preparation for linearity

LOQ solution (1.0 ppm)

Transferred 0.1 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent (equivalent to 1.0ppm with respect to test concentration 1mg/mL).

50% Linearity solution (5.0 ppm)

Transferred 0.5 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent.

75% Linearity solution (7.5 ppm)

Transferred 0.75 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent.

100% Linearity solution (10.0 ppm)

Transferred 1.0 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent.

125% Linearity solution (12.5 ppm)

Transferred 1.25 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent.

150% Linearity solution (15.0 ppm)

Transferred 1.5 mL of standard stock solution into 10 mL volumetric flask and diluted upto the mark with diluent.

4.1.2.2.7 Solution preparation for method precision, intermediate precision and robustness study

100% spiked sample solution used for the method precision, intermediate precision and robustness study. Intermediate precision was performed with different instrument, different lots of solvents and different column in different day.

4.1.2.2.8 Preparation for stability of analytical solution

100% spiked sample solution and standard solution were prepared by using the above methods and both kept in cooler temperature at 15°C to check the solution stability.

4.1.2.3 Instrumentation

The list of instrument/equipment was used for the present investigation discussed inchapter 3, table 3.2.

4.1.3 DISCUSSION ON RESULTS

4.1.3.1 Method development

The main objective of LC-MS/MS method was to quantify and separation between GTI's and Erlotinib Hydrochloride active pharmaceutical ingredient. The impurities were soluble in acetonitrile, but the peak shape of ERL ethyl ester was not good in pure acetonitrile. ERL ethyl ester and ERL nitro compound both good peak shapes were achieved by water adding to the methanol. So, methanol and water in the ratio of 50:50 (v/v) was used as diluent throughout the study. Initial trails were performed in HPLC with different buffers (TFA, Phosphate, volatile buffers etc.). But required sensitivity was not obtained. LC-MS technique used for better sensitivity and the finally chromatographic separation was achieved and final chromatographic parameters and mass parameters mentioned in the table 4.1.2.

4.1.3.2 LC-MS/MS operating condition

The final operating condition of the current method LC and mass parameters mentioned in table 4.1.2.

Table 4.1.2:LC and mass parameters for genotoxic impurities in erlotinib

LC parameters	
Mode of flow	Isocratic
Column	Purosphere star RP 18 e (100 mm X 4.6 mm, 3.0 µm)
Buffer	0.1% formic acid in water
Mobile phase	Buffer: acetonitrile 42:58 (*v/v*)
Flow	1.0 mL/min
Inj.volume	10 µL
Column oven temperature	25°C
Sampler cooler temperature	15°C
Run time	4 min.
Sample concentration	1.0 mg/mL
Diluent	Methanol:Water (50:50, *v/v*)

Mass parameters		
Parameter	**ERL ethyl ester**	**ERL nitro compound**
Probe	ESI	ESI
Polarity	+Ve (Positive)	+Ve (Positive)
Declustering potential	50 (volts)	55 (volts)
Collision energy	17 (volts)	18 (volts)
Collagen exit potential	13 psi	23 psi
Ion spray voltage	5500 (volts)	5500 (volts)
Source temperature	450ºC	450 ºC
Entrance potential	10 (volts)	15 (volts)
Curtain gas	40 psi	40 psi
GS1	50 psi	50 psi
GS2	50 psi	50 psi
Scan Type	MRM	MRM
MRM Transition	361.1 >298.1	314.2 > 268.2

4.1.3.3 Validation study

The developed method was validated in terms of specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, robustness and solution stability.

4.1.3.3.1 System suitability

The standard solution was prepared limit level (10 ppm) with respect to test concentration and injected six times for system precision, before starting the sample analysis. The %RSD value was calculated for areas and observed less than 2.0% and corresponding results were presented in table 4.1.3.

Table 4.1.3: System suitability results

Injection no	ERL ethyl ester peak area	ERL nitro compound peak area
1	645458	567427
2	641007	573310
3	632115	566754
4	649054	563322
5	636088	568904
6	657014	576555
Avg. area	643456	569379
Std. dev.	9034.0	4786.7
% RSD	1.4	0.8

4.1.3.3.2. Specificity

The specificity of the method was checked by injecting 1.0 ppm of erlotinib hydrochloride, Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate and Ethyl 4,5-bis(2-methoxyethoxy) benzoate with respect to the test concentration. The retention time of Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate, Ethyl 4,5-bis(2-methoxyethoxy) benzoate and erlotinib were eluted at retention time of 2.12, 2.88 and 1.02 minutes respectively. There was no interference at the retention time of Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate and Ethyl 4,5-bis(2-methoxyethoxy) benzoate, which is indicating that the method was specific.

4.1.3.3.3 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate and Ethyl 4,5-bis(2-methoxyethoxy) benzoate were obtained with 0.3 ppm and 1.0 ppm respectively, which was shown in table 4.1.4.

Table 4.1.4: LOD and LOQ data for both GTI's

Impurity name	LOD Concentration (ppm)	S/N Ratio	LOQ Concentration (ppm)	S/N Ratio
ERL ethyl ester	0.3	3.2	1.0	9.9
ERL nitro compound	0.3	3.9	1.0	10.6

4.1.3.3.4 Linearity

The optimised method was checked for linearity over a concentration of 1.0 – 15.0 ppm (1.0, 5, 7.5, 10, 12.5 and 15 ppm). The concentration of ppm in X-axis and peak areas in Y-axis checked for calibration curve.The correlation coefficient, intercept and slope values were derived from linear least-square regression analysis and linearity graph was shown in figure 4.1.3 and 4.1.4 and data were presented in table 4.1.5.

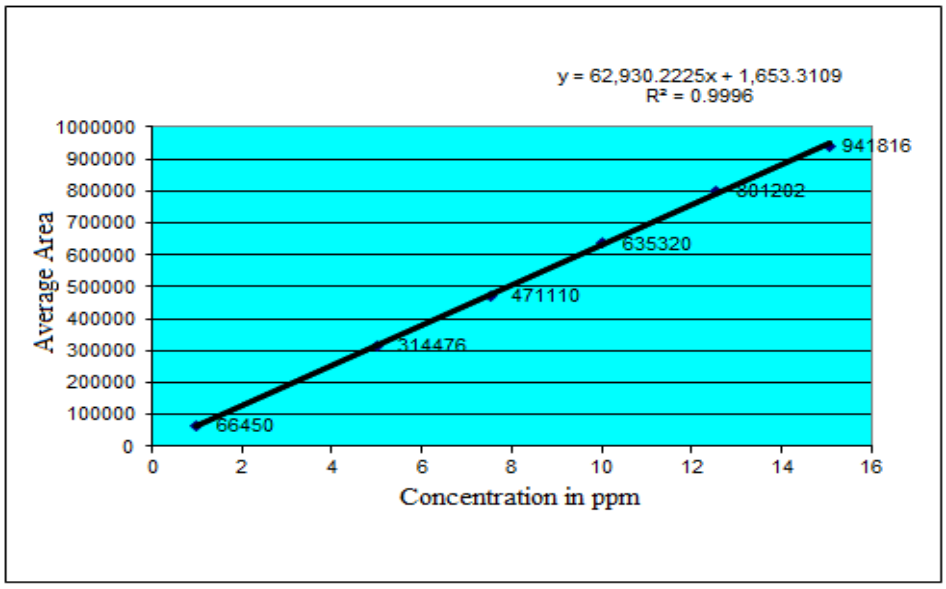


Figure 4.1.3: Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate linearity graph.

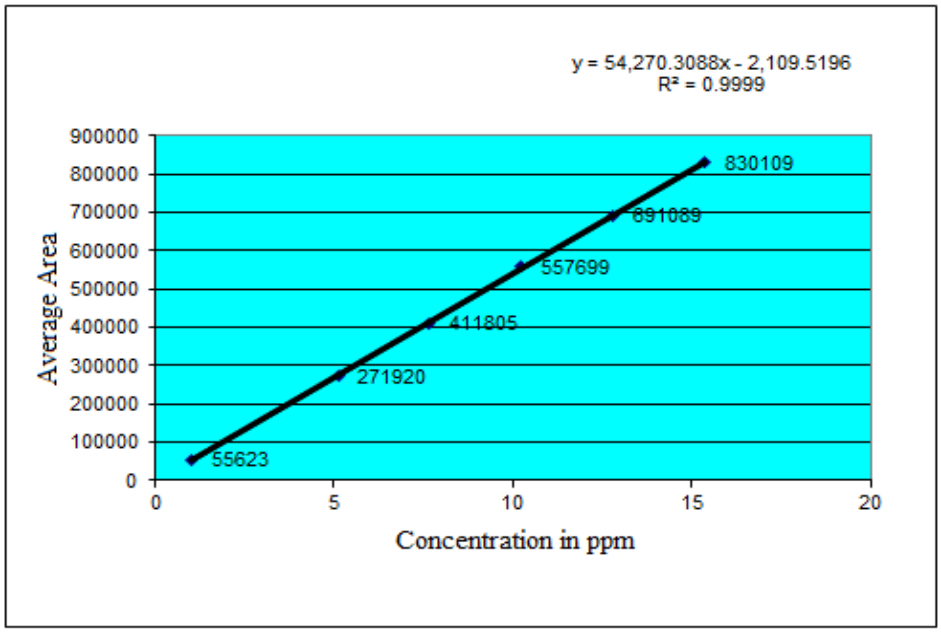


Figure 4.1.4: Ethyl 4,5-bis(2-methoxyethoxy) benzoate linearity graph.

Table 4.1.5: Linearity data for both GTI's

	ERL ethyl ester		ERL nitro compound	
Level	**Conc. (ppm)**	**Peak area**	**Conc. (ppm)**	**Peak area**
LOQ	1.003	66450	1.023	55623
50%	5.017	314476	5.114	271920
75%	7.526	471110	7.671	411805
100%	10.034	635320	10.228	557699
125%	12.543	801202	12.785	691089
150%	15.052	941816	15.342	830109
	Correlation	0.9996		0.9999
	Slope	62930		54270
	Intercept	1653		-2110

4.1.3.3.5 Accuracy

The accuracy of the method was determined six replicates at limit level and rest of the levels LOQ, 50% and 150% in triplicate. The recovery values was found well within the limit for both GTI's and corresponding recovery values were represented in table 4.1.6 and corresponding chromatograms were shown in figure. 4.1.5 and 4.1.6.

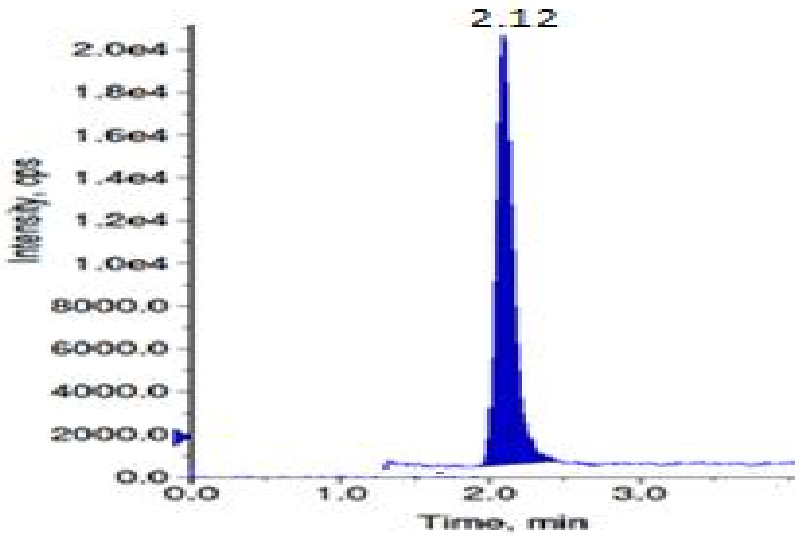


Figure 4.1.5:LOQ accuracy chromatogram for Ethyl 2-amino-4,5-bis(2-methoxyethoxy) benzoate.

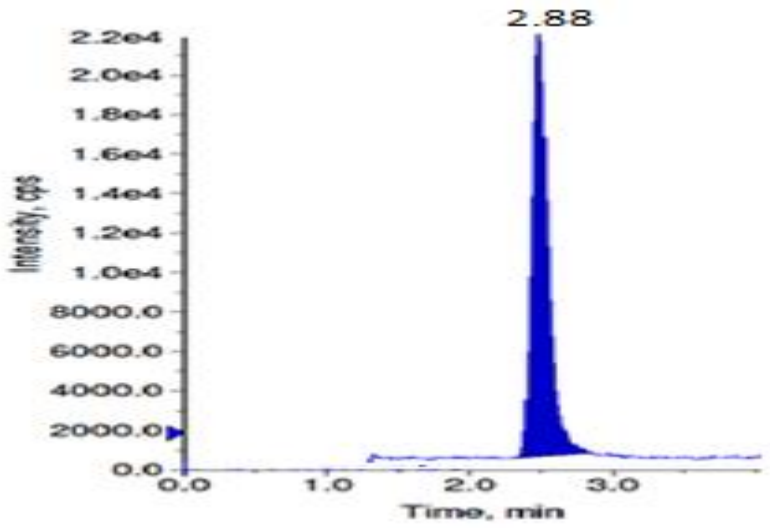


Figure 4.1.6:LOQ accuracy chromatogram for Ethyl 4,5-bis(2-methoxyethoxy) benzoate

Table 4.1.6: Accuracy data for both GTI's

	ERL ethyl ester				ERL nitro compound			
% Spiked	**Theoretical conc. (ppm)**	**Found conc. (ppm)**	**% Recovery**	**% RSD**	**Theoretical conc. (ppm)**	**Found conc. (ppm)**	**% Recovery**	**% RSD**
LOQ	0.9890	0.9208	93.1	**5.6**	0.9910	1.0643	107.4	**6.6**
	0.9890	1.0295	104.1		0.9910	0.9890	99.8	
	0.9890	0.9633	97.4		0.9910	0.9325	94.1	
50% Level	4.9450	4.8313	97.7	**3.3**	4.9550	5.1383	103.7	**3.2**
	4.9450	4.8560	98.2		4.9550	4.8163	97.2	
	4.9450	4.9203	99.5		4.9550	4.9847	100.6	
100% Level	9.8900	9.7318	98.4	**2.7**	9.9100	9.8605	99.5	**1.8**
	9.8900	9.6032	97.1		9.9100	9.8208	99.1	
	9.8900	10.3746	104.9		9.9100	9.5433	96.3	
	9.8900	9.8406	99.5		9.9100	9.8704	99.6	
	9.8900	9.8109	99.2		9.9100	9.8010	98.9	
	9.8900	9.9296	100.4		9.9100	10.0983	101.9	
150% Level	14.8350	14.9982	101.1	**2.4**	14.8650	15.0731	101.4	**2.8**
	14.8350	14.6125	98.5		14.8650	14.6272	98.4	
	14.8350	14.3009	96.4		14.8650	15.4745	104.1	

4.1.3.3.6 Precision

Six different sample solutions were prepared by spiking limit level GTI's with erlotinib hydrochloride with respect to test concentration 1 mg/mL for method precision and intermediate precision. Intermediate precision was performed with different instrument, different lots of solvents and different column in different day.The %RSD was observed to less than 2.0% for both the impurities in intermediate and method precision, which results confirmed the method is precise.

Table 4.1.7: The precision data for both GTI's

	Method precision		**Intermediate precision**	
Sample id	**ERL ethyl ester (ppm)**	**ERL nitro compound (ppm)**	**ERL ethyl ester (ppm)**	**ERL nitro compound (ppm)**
1	10.1324	10.1476	10.2416	10.0065
2	10.1016	10.3088	10.1019	10.2214
3	10.1146	10.1285	10.4466	10.0147
4	10.1088	10.1142	10.0122	10.1078
5	10.1641	10.1408	10.2275	10.1123
6	10.1968	10.2218	10.4388	10.1989
Avg.	10.1364	10.1770	10.2448	10.1103
Std. dev.	0.037	0.075	0.175	0.090
% RSD	**0.4**	**0.7**	**1.7**	**0.9**

4.1.3.3.7 Robustness

Robustness of the method was performed by making small and deliberate changes in operational parameters. The mobile phase flow rate waschanged by 1.1 and 0.9 mL/min(changed by ±0.1 mL/min) and column oven temperature 23°C and 27°C (changed by ±2°C) wereperformed. The %RSD values were calculated and found to be well within the limit, demonstrate that the method was robust. Results for robustness at various conditions (flow rate and temperature) including %RSD represented in table 4.1.8 and 4.1.9, which indicated that the chromatographic performance was not affected by these changes. The %RSD was found to be less than 3.0%.

Table 4.1.8: Robustness data for various flow rates

Injection no	Actual flow		Low flow		High flow	
	ERL ethyl ester peak area	ERL nitro compound peak area	ERL ethyl ester peak area	ERL nitro compound peak area	ERL ethyl ester peak area	ERL nitro compound peak area
1	645458	567427	692239	579880	611044	568008
2	641007	573310	690199	586026	610887	561083
3	632115	566754	678140	571238	606753	570112
4	649054	563322	685480	574453	612370	567895
5	636088	568904	691019	573010	601446	566710
6	657014	576555	653045	575401	618766	561004
Avg. area	643456	569379	681687	576668	610211	565802
Std. dev.	9034.0	4786.8	14954.7	5427.1	5792.8	3845.4
% RSD	**1.4**	**0.8**	**2.2**	**0.9**	**0.9**	**0.7**

Table 4.1.9: Robustness data for various column temperatures

Injection no	Actual temperature		Low temperature		High temperature	
	ERL ethyl ester peak area	ERL nitro compound peak area	ERL ethyl ester peak area	ERL nitro compound peak area	ERL ethyl ester peak area	ERL nitro compound peak area
1	645458	567427	633047	560156	688143	571410
2	641007	573310	641015	560342	681032	570987
3	632115	566754	641266	559197	684422	572444
4	649054	563322	633780	560224	678045	571004
5	636088	568904	638843	561675	689044	572231
6	657014	576555	631002	563011	687563	570847
Avg. area	643456	569379	636492	560768	684708	571487
Std. dev.	9034.0	4786.8	4430.3	1354.5	4398.7	688.2
% RSD	**1.4**	**0.8**	**0.7**	**0.2**	**0.6**	**0.1**

4.1.3.3.8 Solution Stability

The solution stability was performed for both GTI'sand quantitatively spiked at limit level concentration with sample and limit level GTI's and stored at 15ºC. The standard solution and spiked sample were injected initially and at different intervals. The % recoveries were calculated as per calculations given below and corresponding results represented in table 4.1.10. No significant change observed for sample and standard solutions, this indicates that the standard solution and sample solution were stable upto 48hours at 15ºC.

Calculations for recovery in solution stability studies

$$\text{Found conc. (ppm)} = \frac{\text{Area observed at various conditions}}{\text{Standard area}} \times \text{Theoretical conc. (ppm)}$$

$$\%\ \text{Recovery} = \frac{\text{Found conc. (ppm)}}{\text{Theoretical conc. (ppm)}} \times 100$$

Table 4.1.10: The solution stability data for both GTI's and spiked sample at different conditions

Conditions	**ERL ethyl ester Found. conc.(ppm)**	**ERL nitro compound (% Recovery)**	**ERL ethyl ester Found conc.(ppm)**	**ERL nitro compound (%Recovery)**
Standard initial	10.0881	99.8	10.1344	99.0
Spiked initial	10.1184	100.1	10.2086	99.7
Standard 24 hours	10.0429	99.4	10.0863	98.6
Spiked 24 hours	10.1014	99.9	10.1689	99.4
Standard 48 hours	10.0006	98.9	10.0012	97.7
Spiked 48 hours	10.0246	99.2	10.0144	97.9

4.1.3.3.9 Method Application

If the sample has any detectable ERL ethyl ester and ERL nitro compound, then we can calculate in terms of ppm by using the equation discussed in chapter 3, 3.3.3.9 and both impurities were not detected in sample solution.

4.2. IMATININB MESYLATE

4.2.1 INTRODUCTION

Imatinib is a small molecule kinase inhibitor used to treat different types of cancer.Imatinib mesylate (figure 4.2.1) [164] is used in treating gastrointestinal stromal tumors, chronic myelogenous leukemia and a number of other malignancies.

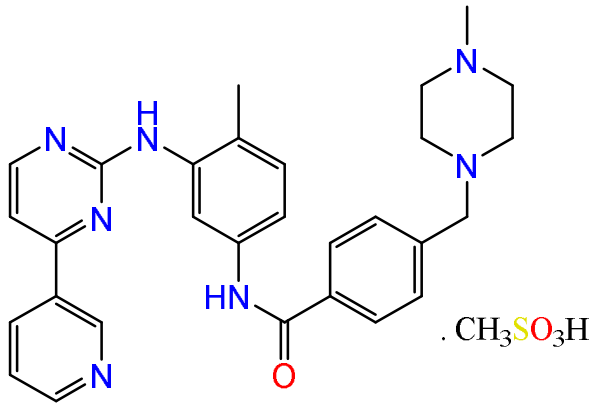


Figure 4.2.1: The structure of imatinib mesylate

Chemical Name	**:**	N-(4-methyl-3-((4-(pyridin-3-yl)pyrimidin-2-yl)amino)phenyl)-4-((4-methylpiperazin-1-yl)methyl)benzamide methanesulfonate.
Molecular formula	**:**	$C_{29}H_{31}N_7O.CH_3SO_3H$
Molecular weight	**:**	589.72
CAS No.	**:**	220127-57-1
Melting point	**:**	214 to 224 ºC
Phase	**:**	Tablets
Appearance	**:**	Off white solid
Bioavailability	**:**	98%
Solubility	**:**	Soluble in dimethylsulfoxide and slightly soluble in methanol
Brand name	**:**	Gleevec

N-(2-methtyl-5-nitrophenyl)-4-(pyridine-3-yl)-pyrimidine-2-amine (IMT-01) (figure 4.2.2) was potential genotoxic impurity identified from DEREK nexus software. Through regulatory authorities proposed limit to be 1.8 ppm in the drug substance based on daily dosage of drug substance. There was no literature available for the quantification of IMT-01 at ppm level in imatinib mesylate.

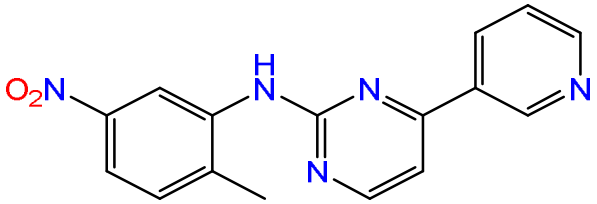


Figure 4.2.2: The structure of IMT-01

4.2.2 EXPERIMENTAL

4.2.2.1 Standards and chemicals

The following standards and chemicals were used for the evaluation, which is mentioned in the table 4.2.1.

Table 4.2.1: List of standards and chemicals

S.No.	Chemical/standard	Grade	Make
1.	Formic acid	LCMS	Merck, Mumbai, India.
2.	Acetonitrile	LCMS	Merck, Mumbai, India.
3.	Water	Mill Q water	Millipore, USA
4.	Imatinib mesylate	---	Cipla, Research and development, India.
5.	IMT-01	---	Cipla, Research and development, India.

4.2.2.2 Preparation of solutions

4.2.2.2.1 Sample preparation

Weighed and transferred 25 mg of imatinib mesylate sample into 25 mL volumetric flask, dissolved and diluted upto the mark with diluent.

4.2.2.2.2 Preparation of standard IMT-01 stock solution

10 mg of IMT-01 transferred into 100 mL volumetric flask, dissolved and diluted to upto the mark. Further diluted 1 mL of above solution to 100 mL with diluent and again diluted 1 mL of above solution to 20 mL with diluent and mixed.

4.2.2.2.3 Preparation of Standard Solution

Diluted 1 mL of IMT-01 stock solution to 25 mL with diluent, which is equivalent to 2.0 ppm with respect to test concentration of 1 mg/mL.

Calculations for impurity concentration in ppm

$$\text{Concentation}\ (\text{ppm}) = \frac{\text{Std. weight}}{100} \times \frac{1}{100} \times \frac{1}{20} \times \frac{1}{25} \times \frac{1}{\text{Spl. conc.}\ (\text{mg/mL})} \times \frac{\text{Purity}}{100} \times 10^6$$

$$\text{IMT - 01 content}\ (\text{ppm}) = \frac{\text{Std. weight}}{100} \times \frac{1}{100} \times \frac{1}{20} \times \frac{1}{25} \times \frac{1}{1.0\ (\text{mg/mL})} \times \frac{99.4}{100} \times 10^6$$

4.2.2.2.4 Preparation of LOD and LOQ solution

Detection limit solution was prepared by diluted 0.15 mL of IMT-01 stock solution into 50 mL with diluent (equivalent 0.15 ppm). Quantification limit solution was prepared by diluted 0.25 mL standard stock solution into 25 mL with diluent (equivalent to 0.5 ppm).

4.2.2.2.5 Preparation of accuracy solutions

Accuracy at LOQ (0.5 ppm)

Six replicate samples were prepared by 25 mg of imatinib mesylate transferred into 25 mL volumetric flask and added 0.25 mL of IMT-01 stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 50% (1.0 ppm)

Triplicate samples were prepared by 25 mg of imatinib mesylate transferred into 25 mL volumetric flask and added 0.50 mL of IMT-01 stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 75% (1.5 ppm)

Triplicate samples were prepared by 25 mg of imatinib mesylate transferred into 25 mL volumetric flask and added 0.75 mL of IMT-01 stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 100% (2.0 ppm)

Triplicate samples were prepared by 25 mg of imatinib mesylate transferred into 25 mL volumetric flask and added 1.0 mL of IMT-01 stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 200% (4.0 ppm)

Triplicate samples were prepared by 25 mg of imatinib mesylate transferred into 25 mL volumetric flask and added 2.0 mL of IMT-01 stock solution, dissolved and diluted upto the mark with diluent.

Accuracy at 300% (6.0 ppm)

Triplicate samples were prepared by 25 mg of imatinib mesylate transferred into 25 mL volumetric flask and added 3.0 mL of IMT-01 stock solution, dissolved and diluted upto the mark with diluent.

4.2.2.2.6 Preparation of linearity solutions

LOQ solution (0.5 ppm)

Transferred 0.25 mL of IMT-01 stock solution into 25 mL volumetric flask, and diluted upto the mark with diluent (equivalent to 0.5 ppm with respect to test concentration 1 mg/mL).

50% Linearity solution (1.0 ppm)

Transferred 0.5 mL of IMT-01 stock solution into 25 mL volumetric flask, and diluted upto the mark with diluent.

75% Linearity solution (1.5ppm)

Transferred 0.75 mL of IMT-01 stock solution into 25 mL volumetric flask, and diluted upto the mark with diluent.

100% Linearity solution (2.0ppm)

Transferred 1.0 mL of IMT-01 stock solution into 25 mL volumetric flask, and diluted upto the mark with diluent.

200% Linearity solution (4.0ppm)

Transferred 2.0 mL of IMT-01 stock solution into 25 mL volumetric flask, and diluted upto the mark with diluent.

300% Linearity solution (6.0 ppm)

Transferred 3.0 mL of IMT-01 stock solution into 25 mL volumetric flask, and diluted upto the mark with diluent.

4.2.2.2.7 Solution preparation for method precision, intermediate precision and robustness

100% spiked sample solution used for the method precision, intermediate precision and robustness study.Intermediate precision was performed with different instrument, different lots of solvents and different column in different day.

4.2.2.2.8 Preparation for stability of analytical solution

100% spiked sample solution and standard solution were prepared by using the above methods and both kept in cooler temperature 10°C to check the solution stability.

4.2.2.3 Instrumentation

The list of instrument/equipment was used for the present investigation discussed in chapter 3, table 3.2.

4.2.3 RESULTS AND DISSCUSSION

4.2.3.1 Method Development

The main aim of method development was to attain efficient separation between imatinib mesylate and IMT-01. We have developed the method by the following objects.

- Genotoxic impurity separation from the main peak
- Impurity detection at lower concentrations (ppm)
- Impurity quantification in presence of higher imatinib mesylate sample Concentration (i.e. mg/mL)
- Control of drug substance in mass source and detectors
- Optimization of the tuning parameters for better sensitivity

Different stationary phases have been evaluated which included C4, C8, C18, phenyl and Cyano phases. In addition, different mobile phase additives such as ammonium acetate, formic acid, methanol and acetonitrile have been verified. Finally, chromatographic separation was attained on following LC/MS conditions.

4.2.3.2 Operating conditions of LC/MS/MS

Final optimization conditions of LC and MS are presented in table 4.2.2.

Table 4.2.2: LC and mass parameters for IMT-01 content in Imatinib mesylate.

LC parameters	
Mode of flow	Isocratic
Column	Inertsil ODS 3V,150 mm x 4.6 mm, 5 µm
Buffer	0.1% formic acid in water
Mobile phase	Buffer: Acetonitrile 30:70 (v/v)
Flow	1.0 mL/min
Injection volume	10 µL
Column chamber temperature	25°C
sample cooler temperature	10°C
Run time	5 min.
Sample concentration	1.0 mg/mL
Diluent	Water and acetonitrile, 50:50 v/v.
Mass parameters	
Probe	ESI (Electro spray ionization)
Polarity	+Ve (Positive)
Declustering potential	50 (volts)
Entrance potential	8 (volts)
Collision exit potential	10 (volts)
Ion spray voltage	5500 (volts)
Collision energy	45 (volts)
Source temperature	450°C
Curtain gas	40 psi
GS1	50 psi
GS2	50 psi
Scan Type	MRM (Multiple reaction monitoring)
Transition (m/z)	308.1 > 261.1

4.2.3.3 Validation Study

The developed method was validated as per ICH guidelines and checked in terms of specificity, precision, limit of detection (LOD), limit of quantification (LOQ), Accuracy, robustness and solution stability.

4.2.3.3.1 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 and imatinib and IMT-01 were eluted at retention time of 1.28 and 3.28 minutes respectively and specificity chromatograms shown in the figure 4.2.3 to 4.2.5.

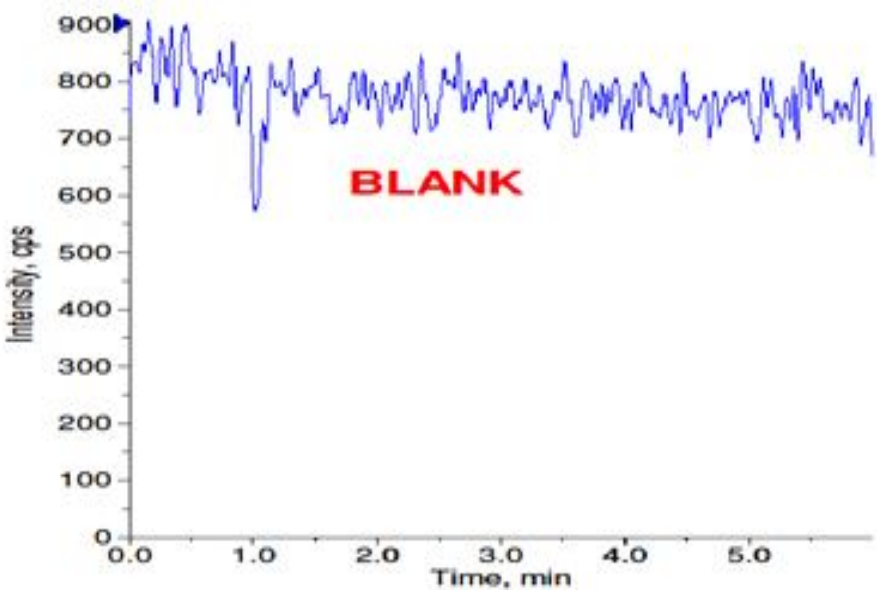


Figure 4.2.3: Blank chromatogram for specificity

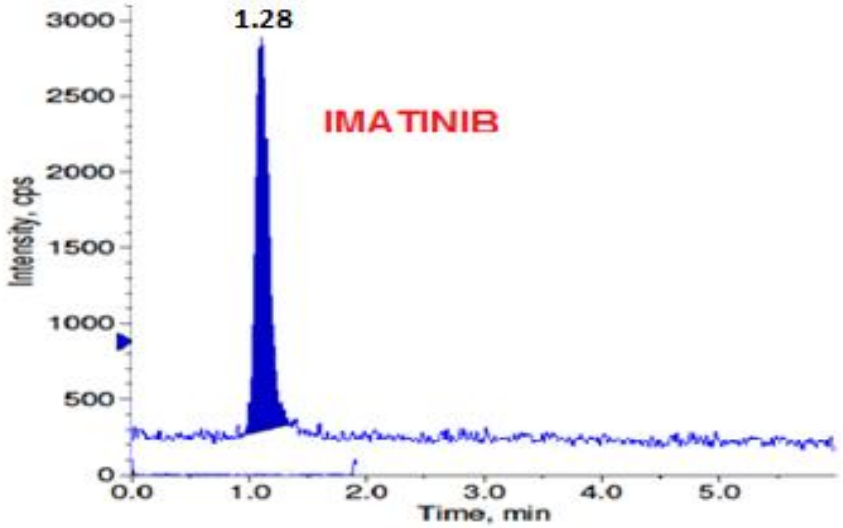


Figure 4.2.4: Imatinib chromatogram for specificity

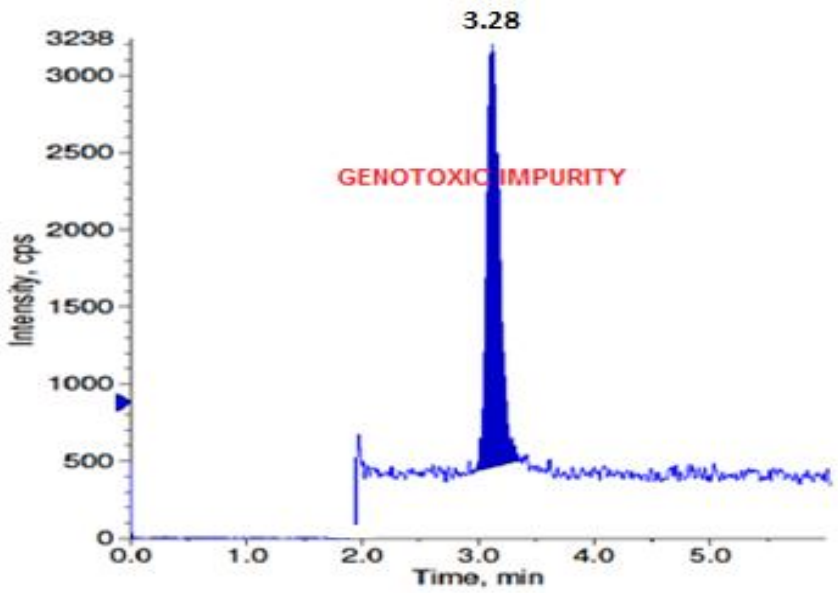


Figure 4.2.5: IMT-01 chromatogram for specificity

4.2.3.3.2 Determination of LOD and LOQ

The LOD and LOQ were calculated from S/N (signal to noise) ratio. To determine LOD and LOQ values of IMT-01 concentration were reduced sequentially and found LOD and LOQ were 0.15 ppm and 0.5 ppm with signal to noise ratio 4.1 and 10.9 respectively.

4.2.3.3.3 Recovery Studies

The accuracy of the method was analysed in six replicates at LOQ level (0.5 ppm) and rest of the levels (1.0, 1.5, 2.0, 4.0 and 6.0 ppm) triplicate in bulk drug sample. The percentage recoveries were calculated andexcellent recovery values were found for all five levels (90-109%). % RSD was calculated for LOQ level of six preparations and which was shown in table 4.2.3 and spiked LOQ chromatograms was shown in figure 4.2.6.

Table 4.2.3: Accuracy data for IMT-01

Injection No.	0.5 ppm	1.0 ppm	1.5 ppm	2.0 ppm	4.0 Ppm	6.0 ppm
1	104.2	90.4	98.8	100.6	108.5	99.5
2	103.8	92.7	99.6	101.8	106.4	99.2
3	104.9	91.8	98.3	102.9	106.8	98.9
4	103.4	---	---	---	---	---
5	103.9	---	---	---	---	---
6	103.1	---	---	---	---	---
Average	103.9	91.6	98.9	101.8	107.2	99.2
Std. dev.	0.6	1.2	0.7	1.2	1.1	0.3
%RSD	**0.6**	**1.3**	**0.7**	**1.1**	**1.0**	**0.3**

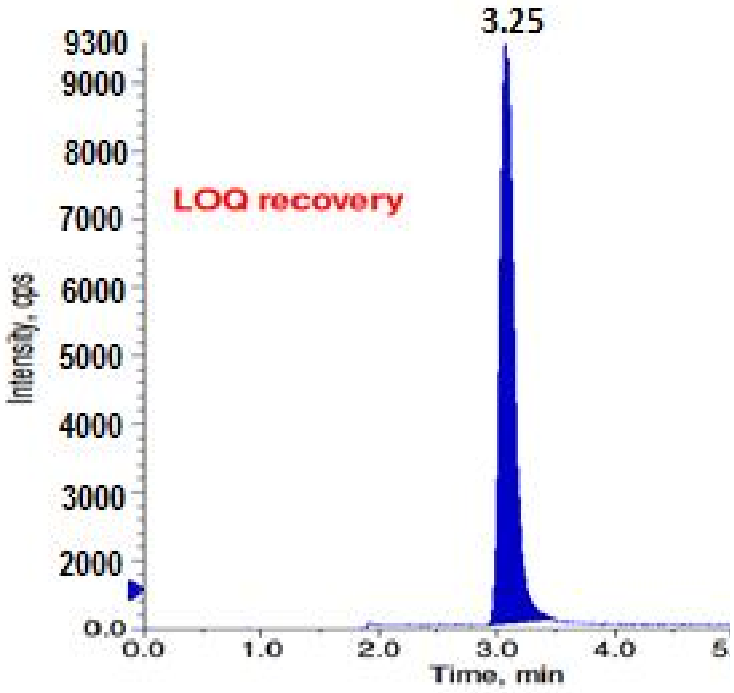


Figure 4.2.6: LOQ level accuracy chromatogram

4.2.3.3.4 Linearity

The optimised method was checked linearity over a six concentration levels LOQ (0.5ppm), L1 solution (1 ppm), L2 Solution (1.5ppm), L3 solution (2ppm), L4 solution (4ppm) and L5 solution (6ppm) for IMT-01. Six replicates were injected for LOQ and L5 solution and L1, L2, L3 and L4 solutions were injected three times. The concentration of ppm in X-axis and peak areas in Y-axis checked for calibration curve.The co-relation coefficient was found to be 0.9999 and shown in table 4.2.4 and shown in the figure 4.2.7

Table 4.2.4: Linearity data for IMT-01

Expected concentration	Sample Name	Number of values used	Mean (peak area)	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

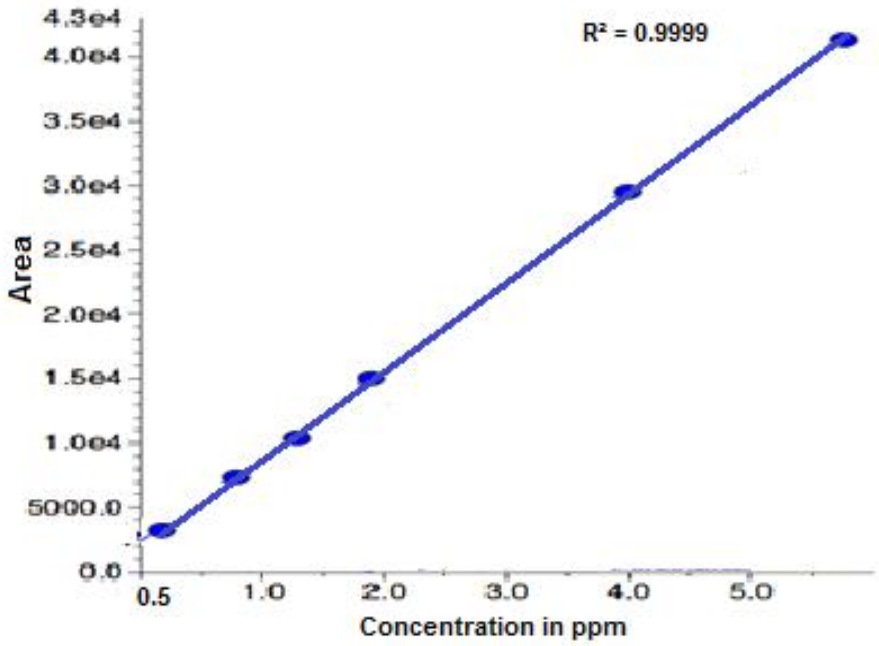


Figure 4.2.7: Linearity graph for IMT-01

4.2.3.3.5 System Suitability

The IMT-01 standard solution was prepared limit level (2ppm) with respect to test concentration and injected six times for system precision, before starting the sample analysis. The % RSD value was calculated for area and observed less than 2.0% and corresponding results were presented in table 4.2.5.

Table 4.2.5: System suitability results

Injection no	**IMT-01 Peak Area**
1	128988
2	126991
3	124984
4	130101
5	128453
6	126589
Avg. area	127684
Std. dev.	1849.5
%RSD	**1.4**

4.2.3.3.6 Precision

The precision was verified through method precision and intermediate precision six independent solutions were prepared by spiking IMT-01 with imatinib mesylate at a concentration of 2 ppm with respect to test concentration. Intermediate precision was performed with different instrument, different lots of solvents and different column in different day. %RSD of both above determinations were calculated and found below 2% and values were represented in table 4.2.6.

Table 4.2.6: Precision data for IMT-01

Sample Id	Method precision		Intermediate precision	
	Recovery (%)	**Recovery (ppm)**	**Recovery (%)**	**Recovery (ppm)**
1	103.2	2.074	96.3	1.978
2	102.8	2.051	94.6	1.955
3	103.1	2.071	96.9	1.968
4	99.8	1.998	95.3	1.920
5	101.2	2.039	95.9	1.926
6	101.7	2.041	98.9	1.999
Average	102.0	2.046	96.3	1.958
Std. dev.	1.3	0.0	1.5	0.0
% RSD	**1.3**	**1.3**	**1.6**	**1.6**

4.2.3.3.7 Robustness

Robustness of the method was performed by making small and deliberate changes in operational parameters. The mobile phase flow rate waschanged by 1.1 and 0.9 mL/min(changed by ±0.1 mL/min) and column oven temperature 23℃ and 27℃ (changed by ±2℃) wereperformed and the results presented in table 4.2.7 and 4.2.8. The %RSD values were calculated and found to be below 2% for IMT-01 and do not impact on chromatographic changes, demonstrate that the method was robust. The summary of all robustness parameters represented in table 4.2.9.

Table 4.2.7: Robustness data for flow rate changes

Injection no	IMT-01 peak area		
	Actual flow (1.00 mL/min)	**Low flow (0.90 mL/min)**	**High flow (1.10 mL/min)**
1	128988	120146	118009
2	126991	121234	121032
3	124984	120989	120989
4	130101	123111	118098
5	128453	120098	119675
6	126589	121934	121939
Avg. area	127684	121252.0	119957.0
Std. dev.	1849.5	1144.2	1641.9
% RSD	**1.4**	**0.9**	**1.4**

Table 4.2.8: Robustness data for column temperature changes

Injection no	IMT-01 peak area		
	Actual column temp(25°C)	**Low column temp (23°C)**	**High column temp (27°C)**
1	128988	125412	123146
2	126991	123489	121032
3	124984	125965	124091
4	130101	126167	122099
5	128453	124183	122980
6	126589	123045	123546
Avg.area	127684	124710	122815.7
Std. dev.	1849.5	1321.5	1094.6
% RSD	**1.4**	**1.1**	**0.9**

Table 4.2.9: Robustness data at various conditions

Conditions	Retention time (min)	IMT-01 (ppm)		Recovery of IMT-01 (%)
		Added	Found	
Actual condition	3.25	1.997	1.989	99.5
Low flow (0.90 mL/min)	3.47	1.998	2.013	100.7
High flow (1.10 mL/min)	3.06	2.016	2.021	101.1
Low temperature (23°C)	3.29	2.003	2.019	101.0
High temperature (27°C)	3.27	1.997	2.010	100.5

4.2.3.3.8 Solution Stability

The IMT-01 was 2.0 ppm quantitively spiked at limit level concentration and stored at 10°C. The standard solution limit level and spiked sample were injected into system initially and at various time intervals. The %recovery of IMT-01 in the initial and each interval were calculated by using the following equation and respective results were presented in table 4.2.10. Good recovery values observed in the range of 95.2-100.7. This indicates that the standard solution and spiked sample solution were stable upto 48 hours at 10°C.

Calculations for recovery in solution stability studies

$$\text{Found conc. (ppm)} = \frac{\text{Area observed at various conditions}}{\text{Standard area}} \times \text{Theoretical conc. (ppm)}$$

$$\%\,\text{Recovery} = \frac{\text{Found conc.(ppm)}}{\text{Theoretical conc.(ppm)}} \times 100$$

Table 4.2.10: Solution stability of both IMT-01and spiked sample

Conditions	Found (ppm)	Recovery (%)
Standard – Initial	1.9833	99.3
Spiked – Initial	2.0121	100.7
Standard – 24 hrs	1.9588	98.0
Spiked – 24 hrs	1.9532	97.8
Standard–48 hrs	1.9101	95.6
Spiked – 48 hrs	1.9018	95.2

4.2.3.3.9 Application of the method

If the sample has any detectable IMT-01, then we can calculate in terms of the ppm by using the equation discussed in chapter 3, 3.3.3.9 and IMT-01 was 1.0 ppm detectedin sample solution.