CHAPTER 2:

DEVELOPMENT AND VALIDATION OF DERIVATIZED CHROMATOGRAPHY METHOD TO ANALYSE GENOTOXIC IMPURITY HYDRAZINE HYDRATE IN IMATINIB MESYLATE API

LIST OF FIGURES

LIST OF TABLES

2.1 Introduction

2.1.1 Imatinib Mesylate

Imatinib mesylate is approved drug for treatment of gastrointestinal stroma tumor since 2002 and for chronic myelogenous leukemia since 2011 from United States food and drug administration (1,2). Reduction of nitro group of N-(2-Methyl-5-nitrophenyl)-4-(21yridine-3-yl)pyrimidin-2-amine(NMPPP) during the synthesis of Imatinib stage-1 can be achieved by several reducing reagents such as Fe/HCl, $SnCl_2$/HCl, hydrazine hydrate/Raney Ni, hydrazine hydrate/$FeCl_3$/C (3). The use of Fe/HCl and $SnCl_2$/HCl as reducing agents were not preferred due to formation of metallic hydroxide and emulsion formation during isolation process of Imatinib. Further $SnCl_2$ is an expensive reagent and also toxic (4). In comparison to other reduction processes, the reduction with hydrazine hydrate produces harmless byproducts such as nitrogen gas and water. Route of synthesis for Imatinib is depicted in Fig. 2.1a.

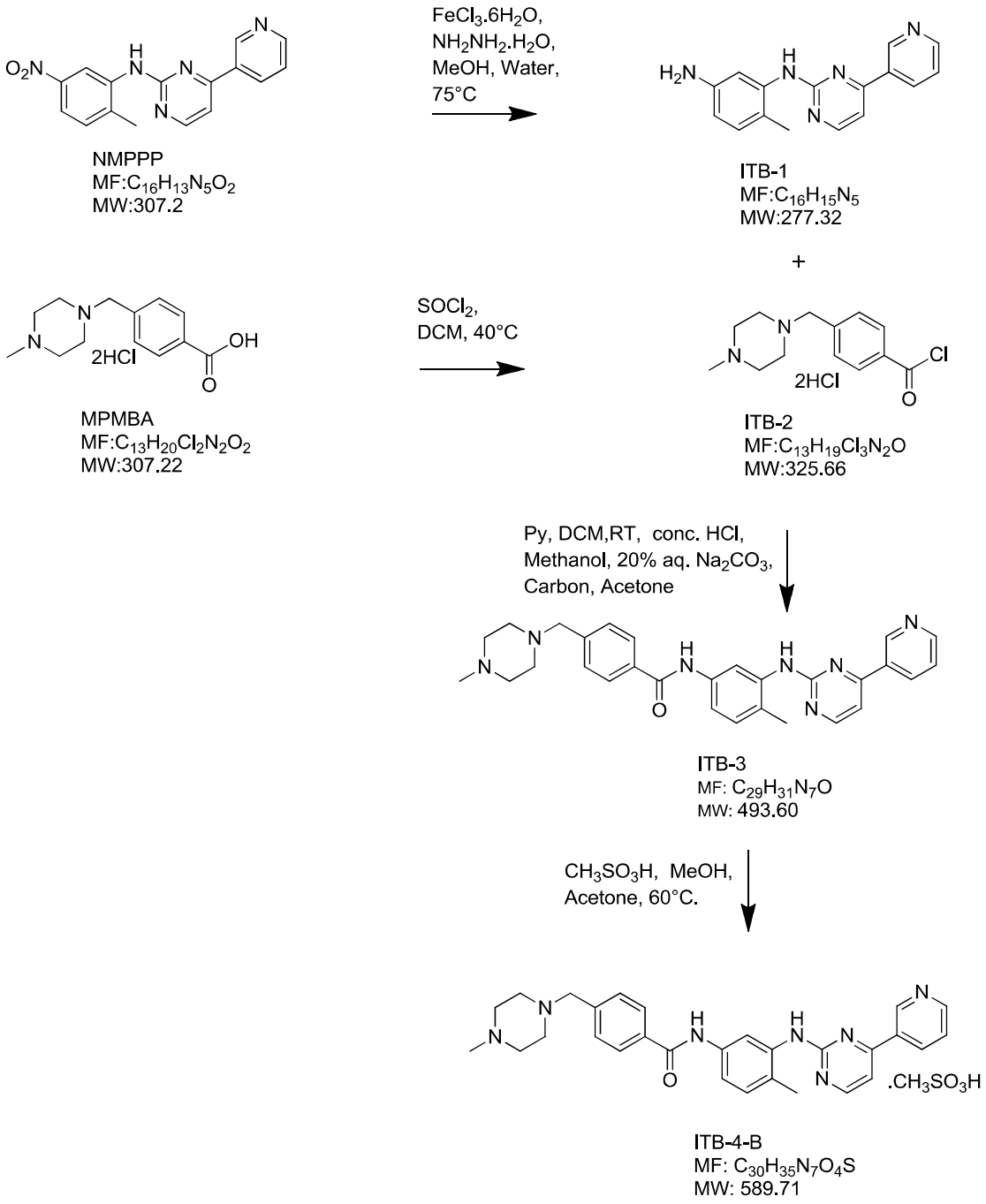

Fig 2.1a Route of synthesis of Imatinib

In the synthesis of Imatinib at stage 1; hydrazine hydrate played an important role as reducing agent and genotoxicity of hydrazine hydrate is proved (5).

2.1.2 Hydrazine hydrate

Hydrazine hydrate is highly reactive in nature and shows various carcinogenic effects to human and other creatures (6). Though it has hazardous property and is very dangerous to handle in solution form, it is used in manufacturing of variety of intermediates and pharmaceutical active ingredients (7). Physicochemical properties of hydrazine hydrate suggest that it is a colorless liquid which is flammable in nature with ammoniacal odor (8). Due to its structural specification, hydrazine hydrate accounts for hazardous genotoxicity and further its metabolites also potentiate its genotoxic properties (9).Intercalations of hydrazine hydratewith DNA produce highly reactive methyl diazonium ions and free methyl radicals (10). More dangerously, hydrazine hydrate also reacts with endogenous formaldehyde and produces formaldehyde hydrazone which also increase its genotoxic effect (11). Diazomethane, a metabolite of Hydrazine hydrate acts as alkylating agent; which can produce mutation in genes and worsen the condition (12). Quinoline derivatives are mostly synthesized by Knorr synthesis (13), Gabriel synthesis (14) and the wolff-kishner reaction (15). Carcinogen present in this reaction need to be controlled at Therapeutic Threshold Concentration (TTC) limit(16).

From an analytical point of view, hydrazine detection is very critical because it does not have chromophores for UV detection (HPLC), ionizable group for mass detection (LC-MS), carbon atoms for flame ionization detection (GC). Therefore derivatization becomes a mandatory approach to develop highly selective and sensitive method for determination of hydrazine (17). Number of methods such as GC-MS (18), LC-MS/MS (19-21), ion chromatography (22) and High performance liquid chromatography (23-25) have been used for the quantification and determination of hydrazine hydrate. Almost all the methods developed for estimation of hydrazine hydrate used derivatization approach. Zhang and associates developed a method having 0.25 ppm detection limit using 2-Hydroxy-1-Naphthalaldehyde as a derivatizing agent (26). At present there is not a single method available for hydrazine to quantify at a TTC (threshold of toxicological concern) level by HPLC (27). So, after intense research we can say that there is no method available for estimation of hydrazine hydrate at genotoxic level by high performance liquid chromatography.

2.1.3 Hypothesis

- Being polar molecule hydrazine hydrate ($N_2H_4.H_2O$) has no chromophores present in structure which can follow Lambert beer law, thus it is difficult to analyze.
- The method of quantification was developed by attaching chromophores to hydrazine with derivatization, which helped to increase sensitivity.

2.1.4 Objectives

To develop an accurate and highly sensitive reversed-phase liquid chromatography-UV derivatization method for determination of hydrazine in Imatinib Mesylate drug substance.

2.2 Materials and Equipments

2.2.1 Materials

Materials and Reagents	**Sources**
Imatinib Mesylate	Cadila Healthcare Ltd. Ahmedabad, India.
Hydrazine hydrate (>99%)	Sigma-Aldrich. Darmstadt, Germany.
Benzaldehyde solution (>99%)	Sigma-Aldrich. Darmstadt, Germany.
Glacial acetic acid	Merck. Darmstadt, Germany.
Methanol HPLC Grade	Merck. Darmstadt, Germany.
Acetonitrile HPLC Grade	Merck. Darmstadt, Germany.
High purity HPLC Grade water by Milli-Q water purification system	Millipore. Darmstadt, Germany.

Table 2.1 List of Materials

2.2.2 Equipments

Equipment/Instrument	**Manufacture**
Prominence HPLC-DAD system	Shimadzu, JAPAN.
Inertsil ODS-3V HPLC column (5.0 µm, 4.6 × 250 mm)	GL Sciences Inc, Japan.
Micro balance CP225D	Sartorious, Germany
Sonicator	PCI Analytics, India
Electrospray LC–MS system	Shimadzu, JAPAN.

Table 2.2 List of Equipments used

2.3 Preparation of solutions

2.3.1 Derivatization solution preparation

1% benzaldehyde solution in methanol was prepared by mixing 1 mL of benzaldehyde solution into methanol and making up to 100 mL in methanol.

2.3.2 Acetic acid solution preparation

0.06% acetic acid solution in methanol was prepared by gentle mixing of 0.06 mL of acetic acid solution which was diluted with methanol up to 100 mL.

2.3.3 Standard solution preparation

Standard stock solution of 1.5mg/mL hydrazine was prepared with methanol. Second stock solution was prepared by diluting standard stock solution to achieve concentration of 0.15 ppm (Parts per million) with methanol as diluting agent. From the second stock 5 mL solution was taken in a 50 mL volumetric flask to which, 1ml of benzaldehyde solution and 2 mL acetic acid solution was added followed by heating at 50°C-55°C for 1 h. After that samples were cooled to room temperature and dilutions were made up to 50 mL with methanol. Prepared solutions were directly injected to HPLC.

2.3.4 Test solution preparation

400 mg of Imatinib drug substance was taken in 50ml volumetric flask. 5ml methanol was added to dissolve Imatinib. 1ml of benzaldehyde solution and 2ml acetic acid solution was added to it. The solution was heated at 50°C-55°C for 60 min. Samples were removed, cooled to room temperature and made up with methanol and injected directly to HPLC.

2.4 Selection of derivatizing agent

For the selective, sensitive and efficient analysis of hydrazine hydrate; selection of a derivatization agent is a critical parameter. The derivatization agent should accelerate conversion of free impurity (hydrazine hydrate) to the derivatized product (1,2-dibenzylidenehydrazine) (28). The absorption of derivatized product should be far away from the absorption of reagents and solvents so the interference should be minimum(29). For derivatized product 1,2-dibenzylidenehydrazine a strong UV absorption at 300 nm is noted which is far away from any interference of solvents and reactive species. A derivatized product 1,2-dibenzylidenehydrazine thus seems to be highly suitable for HPLC due to its high lipophilicity which could also facilitate retention in C18, C8 and also in C4 columns.

For the conversion of hydrazine to 1,2-dibenzylidenehydrazine; benzaldehyde is needed which is easily available and commercially avialable. So, it can be used for analysis of multiple

batches at considerable economic rate (29). Also, the excess amount of benzaldehyde present in product mixture did not interfere with further analysis. The proposed scheme for the reaction is depicted in Fig.2.1b which shows together; one molecule of benzaldehyde and one molecule of hydrazine form one molecule of 1,2-dibenzylidenehydrazine. During the reaction, methanol was used as a solvent for 1,2-dibenzylidenehydrazine and even for reactant Imatinib Mesylate and benzaldehyde. Sensitivity, selectivity and reproducibility of method were performed for the optimization.

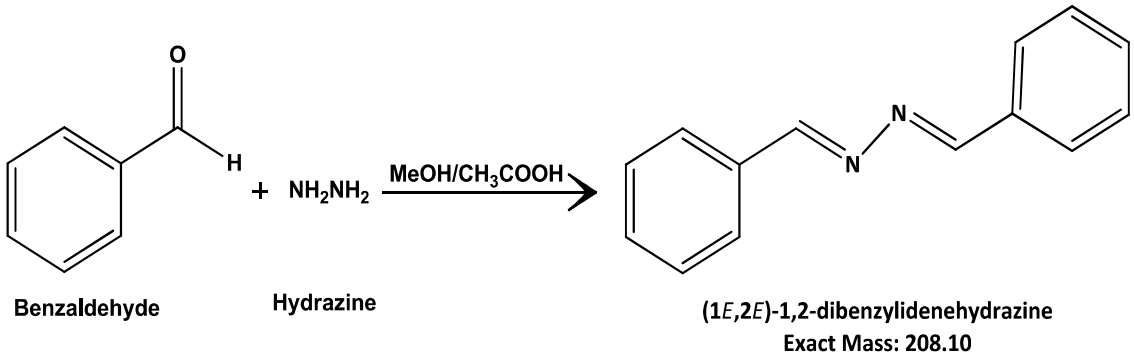

Fig 2.1b Reaction scheme of Benzaldehyde with Hydrazine in acidic condition for Derivatization

2.5 Detection of impurity by LC-MS

For identification of derivatized product 1,2-dibenzylidenehydrazine an electrospray LC–MS system (Shimadzu Prominence HPLC coupled with Triple Quadropole Mass Spectrometer LCMS-8040 with lab solution software, version 5.72, Japan) was used. An Inertsil ODS-3V HPLC column 250-mm, 4.6 mm and 5 µm particle size column from GL Sciences Inc. (Japan) was used for chromatography. A mixture of Acetonitrile: Water-9:1 %v/v was used as mobile phase for the separation of derivatized product and better resolution. Pump flow rate of HPLC system was set at 1.5 ml/min. The LC system was operated in isocratic mode consisting premix ratio of 30% Solvent A and 70% Solvent B. The column temperature was maintained at 40°C. Methanol was used as a diluent. Injection volume was fixed at 50 µL. The analysis was carried out by using electro spray ionization mode (positive). Capillary voltage at 3500 V and collision energy -35V. Desolvation temperature was 250°C with nebulizing gas flow rate of 180 L/h. The resultant LC-MS Chromatograms are given in Fig.2.3.

2.6 HPLC instrumentations and working conditions

The derivatized 1,2-dibenzylidenehydrazine was analyzed with a reversed-phase chromatographic technique. Numerous reverse phase columns were used during the process of method development with different stationary phase and different make such as Alltima C18, Waters Symmetry C18, X-bridge Shield C18, Eclipse XDB Phenyl, Hypersil BDS C18, Zorbax SB-Phenyl and YMC Triat C18. The stationary phase in which Imatinib API and their related substance are eluted near to dead time and maximum resolution achieved between the derivatized 1,2-dibenzylidenehydrazine product and Matrix of API was chosen as optimized chromatographic conditions.

2.7 Analytical procedure

A 20.0 µL of blank, six replicate injections of system suitability solution and test sample solution were separately chromatographed. The resolution of not less than 5.0 between impurity and Imatinib Mesylate was set as a system suitability requirement in system suitability solution. The relative standard deviation (RSD) of not more than 5.0 % for peak areas obtained from six replicate injections of system suitability solution was used to verify the system precision. All the known related substances of impurities were determined against mean area of respective impurities obtained from replicate injections of system suitability solution.

2.8 Method validation

The proposed method was validated for content of hydrazine hydrate in Imatinib Mesylate by HPLC as per ICH guideline. The method was validated for its specificity, linearity, range, accuracy and precision to demonstrate that the method is suitable for its intended use as per ICH Q2 (R1) guideline. Method validation data are summarized in Table-2.6.

2.9 Result and discussion

2.9.1 Optimization of derivatizing agent

Change in pH, temperature and time were optimized for the derivatization reaction and the results of all the experiments are shown in Figs.2.2a and 2.2b. Fig 2.2a shows the effect of time while Fig 2.2b shows the effect of temperature. Respectively, at basic pH, process of derivatization is found inconsistent (shown in Figure 2.2c) while at acidic pH nature of derivatization is found consistent, linear and reproducible which suggest accurate reaction efficiency (Shown in linearity Figure 2.7a and 2.7b). Acidic pH was achieved by adding 0.06% acetic acid in methanol during reaction and basic pH was achieved with addition of 1 % sodium hydroxide in methanol to reaction mixture. In addition, increasing temperature of reaction mixture up to 50°C improved the yield of 1,2-dibenzylidenehydrazine. Kinetics of reaction was monitored up to 4 h at 50°C. Results indicated reaction was completed in 1h.

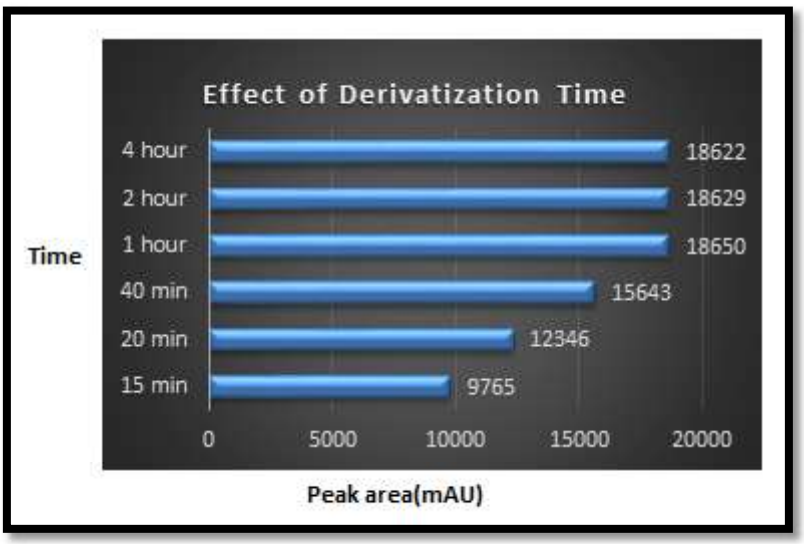

Fig 2.2a Effect of derivatization time in acidic condition at 50°C

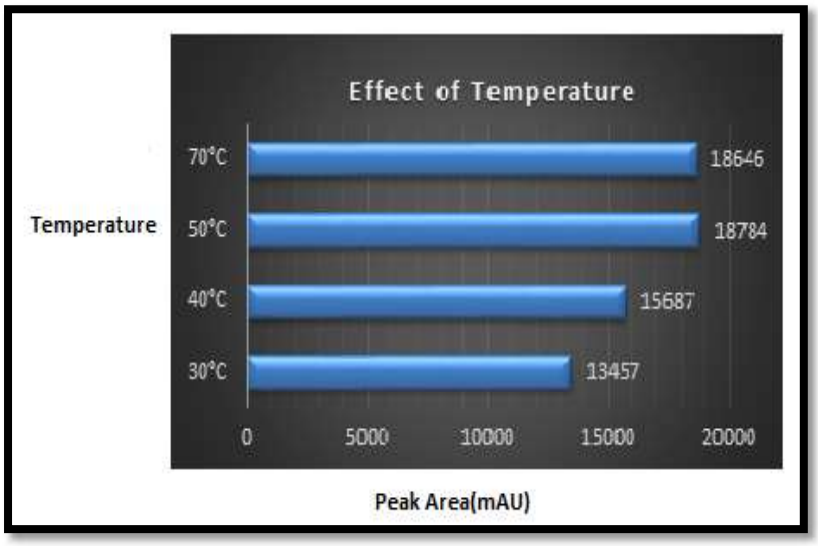

Fig 2.2b Effect of derivatization with increasing temperature in acidic condition for derivatization

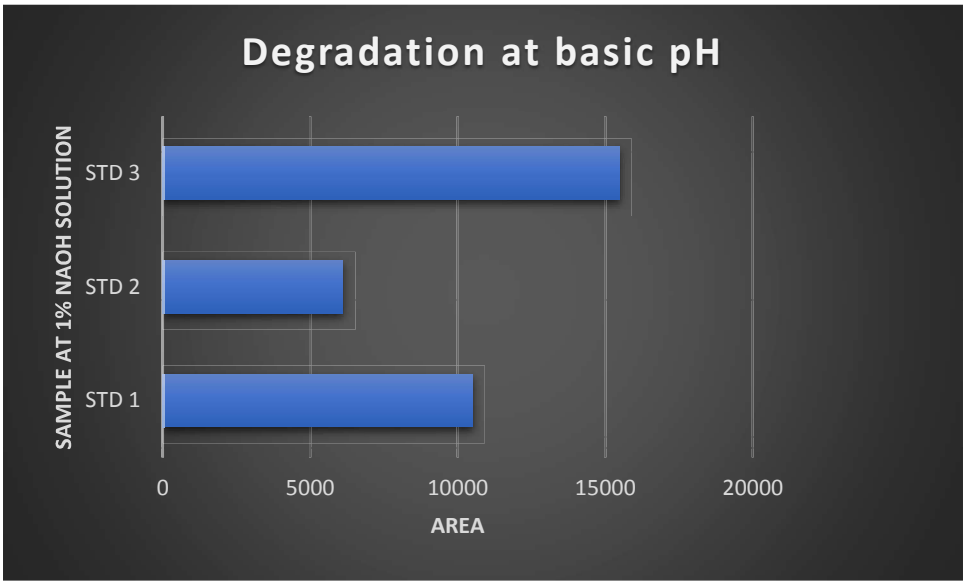

Figure 2.2c Effect of derivatization in basic pH for derivatization

Based on above results, the optimal reaction conditions were chosen to be 50°C in a water bath for 60 min using 1% methanolic benzaldehyde solution as the derivatizing solution with 0.06% acetic acid in solution. Derivatized 1,2-dibenzylidenehydrazine product was confirmed by LC-MS.

2.9.2 Identification of impurity by LC-MS:

LC-MS chromatogram exhibited molecular ion peak at m/z 209.05 (M^++H) which confirmed that the resultant product was 1,2-dibenzylidenehydrazine.

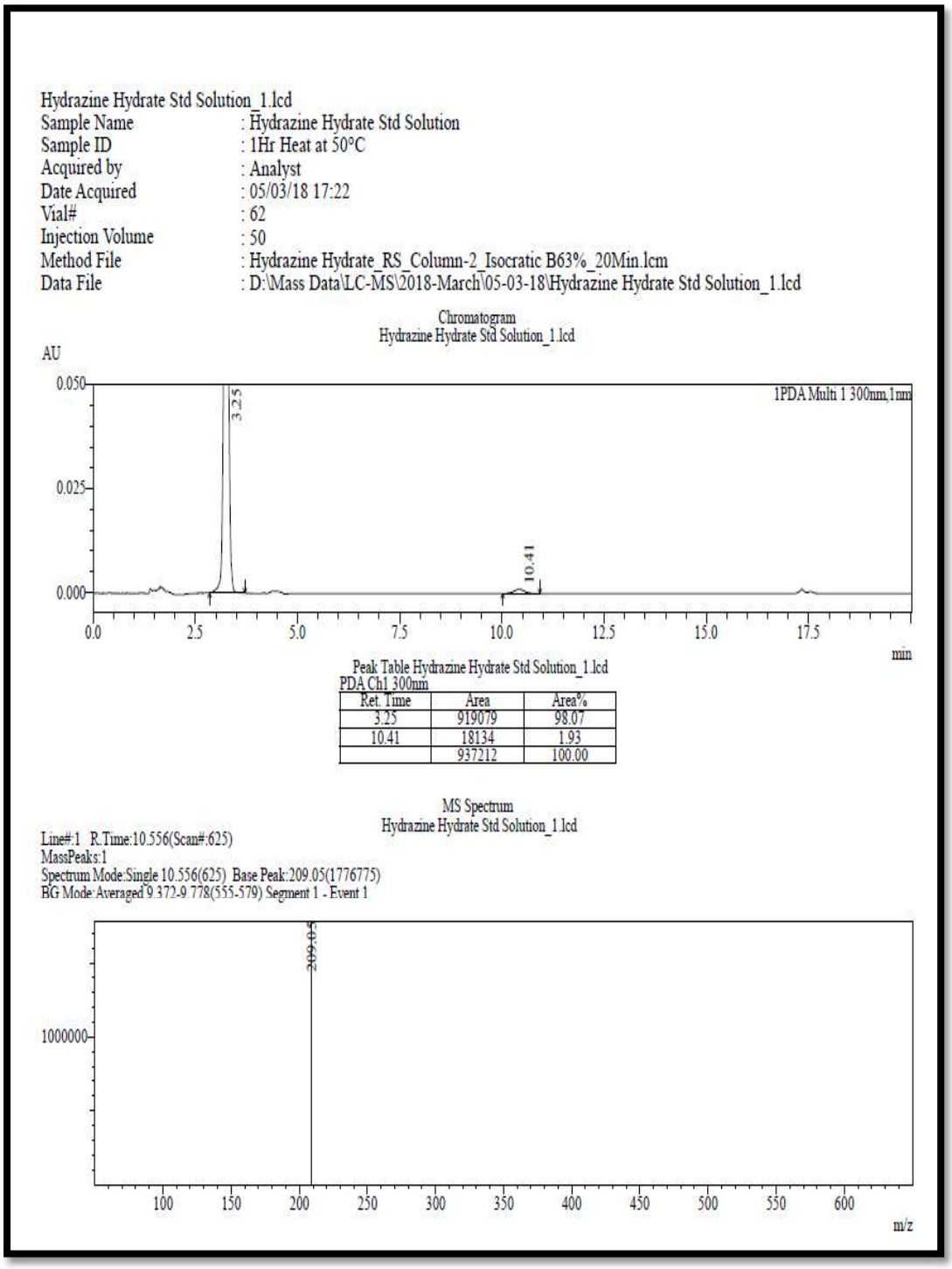

Fig 2.3 Identification of impurity by LC-MS.

2.9.3 Optimized HPLC parameters and Peak purity

Due to high retention time of the 1,2-dibenzylidenehydrazine product and optimum resolution from the API peak and derivatizing agent (benzaldehyde) peak as seen in Fig. 2.4 & Fig.2.5 respectively, Inertsil ODS-3V column was selected. A chromatogram with perfectly resolved peaks between Imatinib Mesylate and 1,2-dibenzylidenehydrazine (resolution was 23.6) is shown in Fig 2.4. Similarly, Fig.2.5. shows a chromatogram of standard solution with separation between benzaldehyde and 1,2-dibenzylidenehydrazine peak. The shape of peak and resolution of the 1,2-dibenzylidenehydrazine product was excellent with this column. The optimized parameters, for reversed-phase LC method water: acetonitrile was the use of mobile phase ratio of 90:10% v/v wherein separation was done at system flow rate of 1.5 ml/min. Volume of injection was fixed to 50 µL while the column temperature was set at 40°C. Detection wavelength of 300 nm UV was selected for detection of derivatized 1,2-dibenzylidenehydrazine. Total run time was set as 20 min. The isocratic flow was selected in the ratio of MP-A:MP-B-30:70%v/v. The HPLC method parameters are summarized in Table 2.3 and peak purity results are summarized in Table 2.4.

Peak purity plot and peak 3D Plot is provided in Fig2.6a and 2.6b.

Parameter	**Conditions**
HPLC Column	Inertsil ODS-3V, 5µm, 4.6 × 250 mm
Mobile Phase	A: water
	B: Acetonitrile:Water-90:10 %v/v
Injection Volume	50µL
Isocratic	Solvent A:Solvent B-30:70
Flow Rate	1.5ml/min
Column Oven Temp.	40°C
UV Wavelength	300nm
Run Time (min)	20 min

Table 2.3 Summary of HPLC method parameter condition

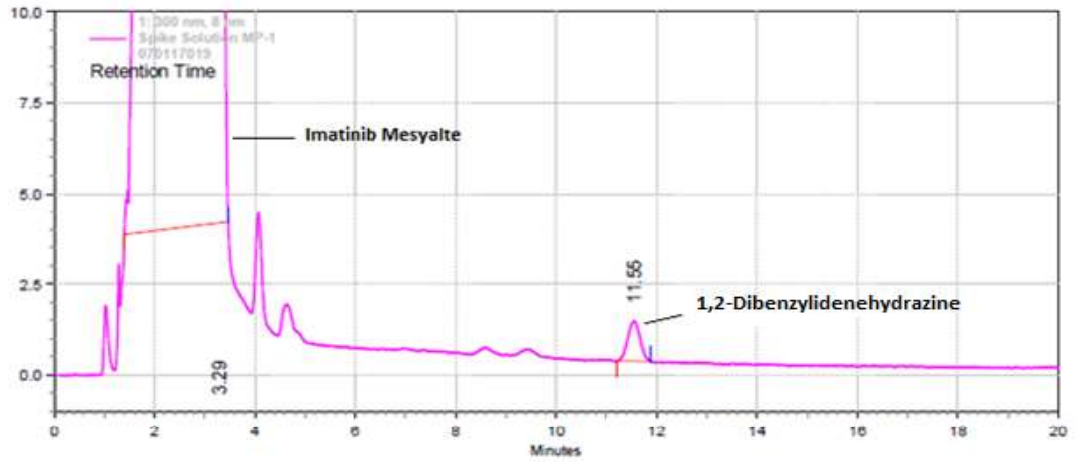

Fig 2.4 Chromatogram of spike solution having 0.015 µg/g hydrazine spiked in 8000µg/g Imatinib Mesylate

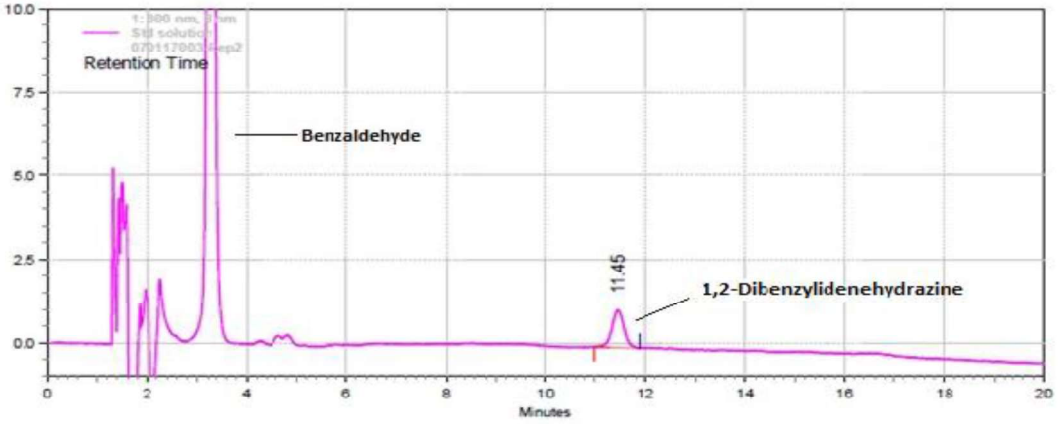

Fig 2.5 Chromatogram of standard solution having 0.015 µg/g derivatized 1,2-dibenzylidenehydrazine

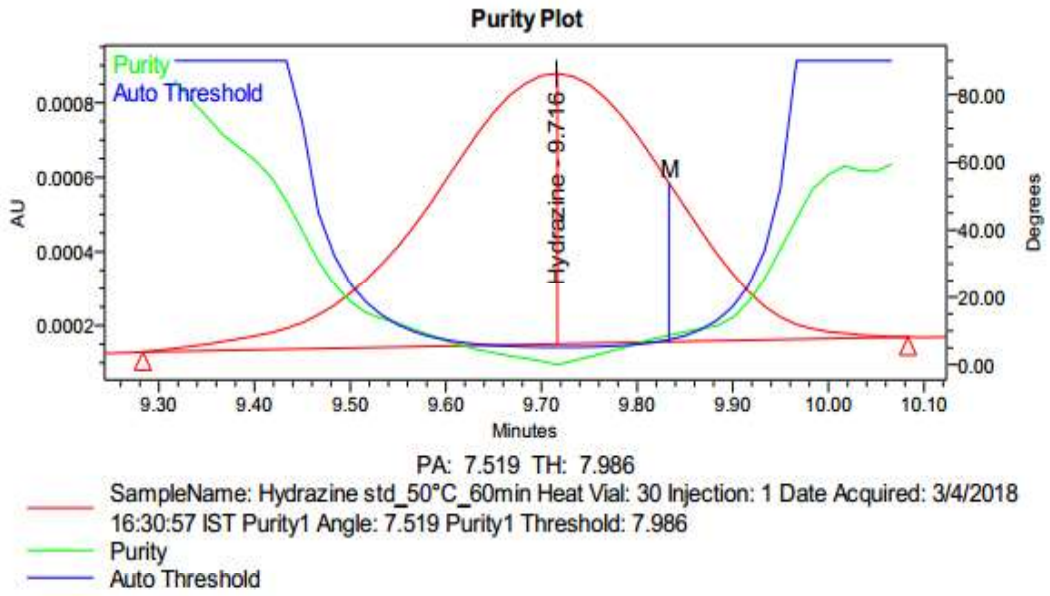

Fig 2.6a Peak purity plot

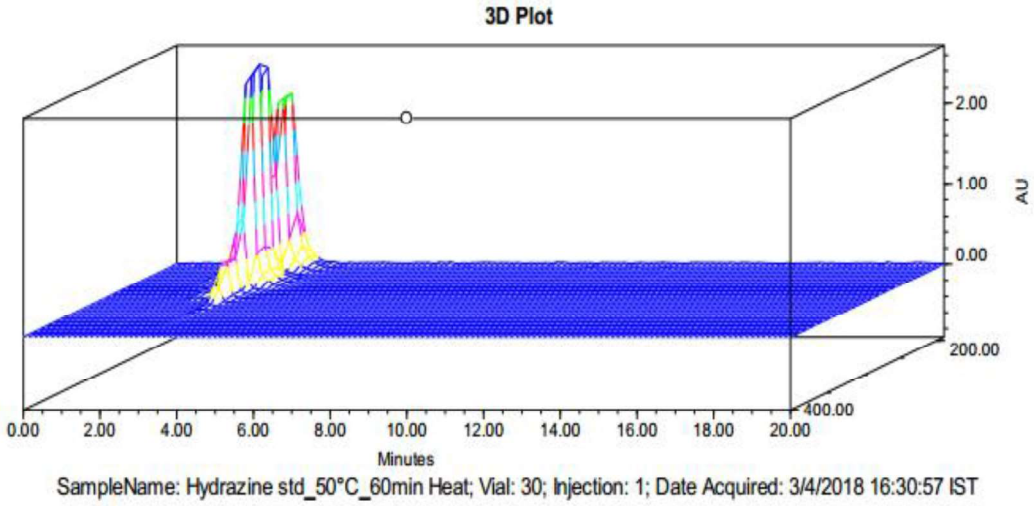

Fig 2.6b 3D plot of Peak purity

Parameters	Observed results	Acceptance criteria	Remarks
Purity angle	7.519	Purity angle should be less than purity threshold	Peak is pure
Purity threshold	7.986		

Table 2.4. Results of peak purity

2.9.4 System Suitability Criteria

A suitability of systems can be defined on the basis of results obtained from number of repetitive chromatograms(30). For the acceptance of system column efficiency determined from the analyte peak >10000, the tailing factor should be more than 2.0, and RSD for impurity areas in six replicate injection of system suitability solution was <5.0%(31). A resolution between any two compounds was >5 in SST (system suitability test) solution(32). All the system suitability criteria during validation study and batch analysis study were noticed within the acceptable limit. The results of system suitability are depicted in Table 2.6.

Area of standard solution	
Injection	Area
1	18944
2	18679
3	18758
4	18374
5	18483
6	18046
Avg.	18547
SD	237
%RSD	**1.30**

Table 2.5 Area of Standard solution

Parameters	Observed Results (n=6)	Acceptance Criteria	Remarks
Theoretical plates	11223	> 2000	Method passes the system suitability test
Tailing factor	1.03	T ≤ 1.5	
Repeatability (% RSD)	1.70	%RSD <5	
Resolution	23.6	Rs <2	

Table 2.6 Results for system suitability parameters by RP-HPLC

2.9.5 Validation of the method

2.9.5.1 Selectivity(Specificity)

The specificity of this method was demonstrated by separation of Imatinib Mesylate API and derivatized 1,2-dibenzylidenehydrazine. Chromatogram of solution containing 8000 µg/g Imatinib Mesylate spiked with 0.015 µg/g hydrazine and a standard solution of 0.015 µg/g derivatized 1,2-dibenzylidenehydrazine were recorded with help of UV detector. From the chromatogram, we can clearly conclude that there is no interference of other substance in chromatogram of spiked 1,2-dibenzylidenehydrazine. The resolution of the 1,2-dibenzylidenehydrazine derivative from Imatinib and benzaldehyde is greater than 2.5. The representative chromatograms are already showed in Fig. 2.4 and Fig. 2.5.

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

Sensitivity of the method was proved by establishing the LOD and LOQ for hydrazine hydrate with a signal-to-noise ratio of 3:1 and 10:1, respectively. Accuracy at LOQ level was verified by injecting three individual preparations of Imatinib Mesylate spiked with hydrazine hydrate at LOQ level and by calculating % recoveries of hydrazine hydrate content. AS/N ratio range from4-6 was obtained at the limit of detection of 0.0020 µg/g, while a range of 15-20 of S/N ratio was observed for the quantitation limit of 0.0040 µg/g. The quantitation limit was also validated with sample matrix where 0.0040 µg/g of hydrazine was spiked in Imatinib Mesylate. The average percent of recovery of three replicate injections at LOQ level was 101.3% with a %RSD of 0.87%.

2.9.5.3 Linearity and range

Linearity

Linearity is an ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range (30).

Range

The interval between the upper and lower levels of analyte(inclusive) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the method as written (31).

Methodology

- **Linearity**
  - ✓ Demonstrate across the entire range of the analytical procedure.
  - ✓ A minimum of five concentrations is recommended.
- **Range**
  - ✓ Verify that the method provides acceptable precision, accuracy, and linearity when applied to samples at the extreme as well as within the range.
  - ✓ Recommended minimum ranges:
    - Assay of Drug Substance or Finished Product
    - From 80–120% of the test concentration.
  - ✓ Determination of an Impurity
    - From 50–120% of the specification.
  - ✓ Content Uniformity
- A minimum of 70–130% of the test concentration unless a wider or more appropriate range is justified based upon the dosage form.
  - ✓ Dissolution Testing
    - +/- 20% over the specified range of the dissolution test.

Preparation of Linearity solutions					
Sample ID	**Level**	**Volume taken from Stock solution**	**Dilution (mL)**	**Volume taken (mL)**	**Dilution (mL)**
Linearity solution-1	25%	1.25	50	5	50
Linearity solution-2	50%	2.5	50	5	50
Linearity solution-3	80%	4	50	5	50
Linearity solution-4	100%	5	50	5	50
Linearity solution-5	120%	6	50	5	50
Linearity solution-6	150%	7.5	50	5	50

Table 2.7 Preparation of Linearity solutions

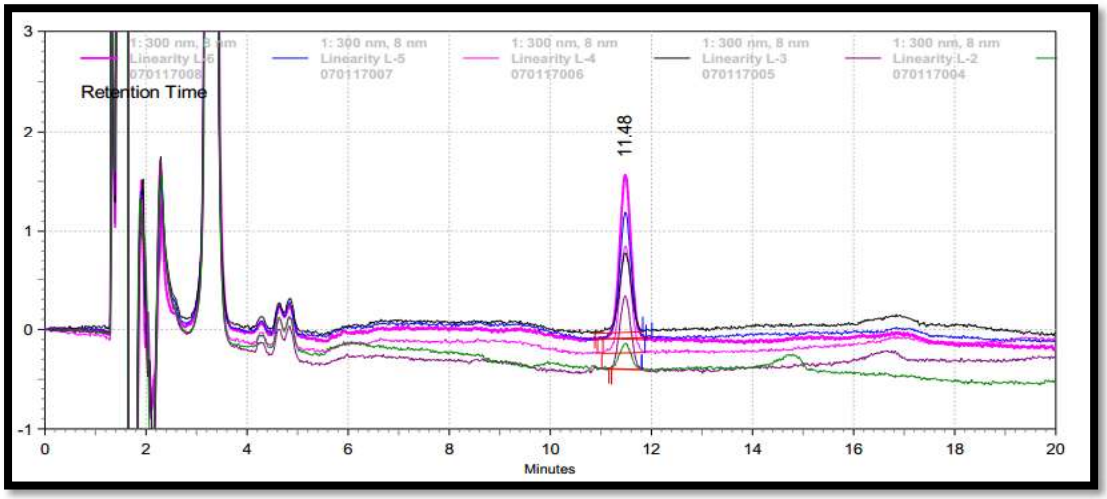

Fig2.7a. Linearity overlay chromatogram-1

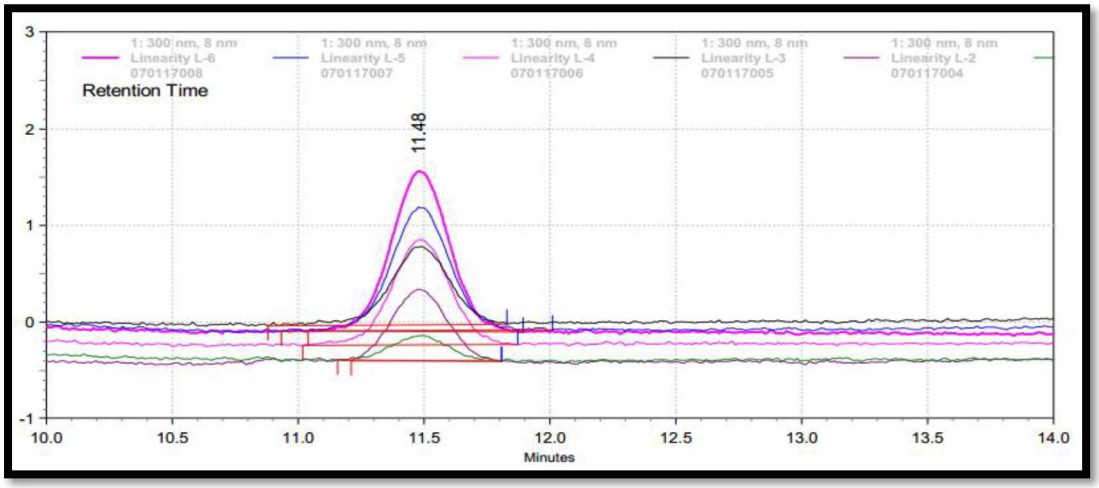

Fig 2.7b. Linearity overlay chromtogram-2(close-up)

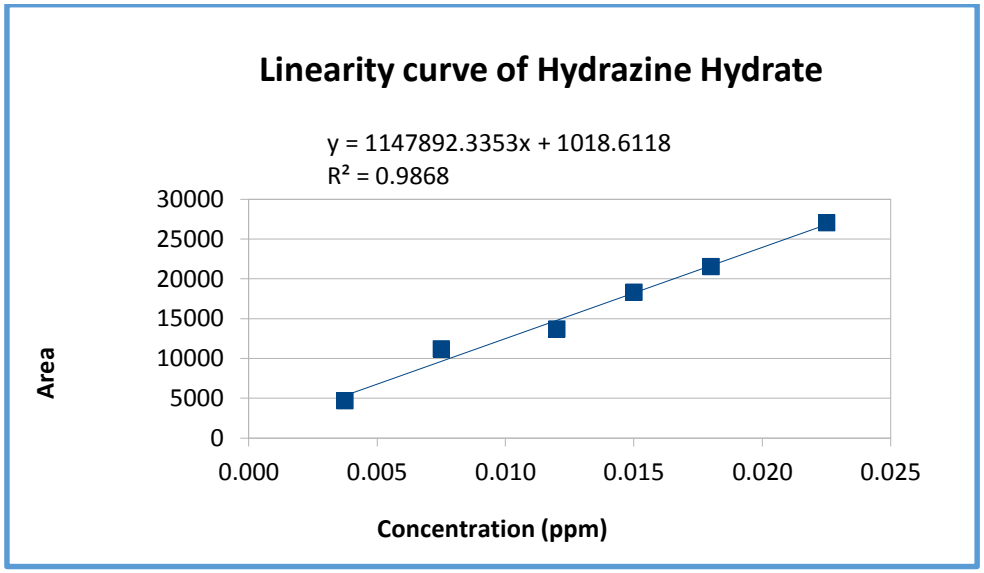

Fig2.8 Linearity curve of Hydrazine Hydrate

To establish linearity of the method, solutions have been prepared by diluting the hydrazine hydrate impurity second stock solution of 0.15ppm to obtain the required concentrations at six different levels ranging from LOQ to 150% (i.e. LOQ (0.0040), 0.0075, 0.0120, 0.0150, 0.0180 and 0.0225µg/g). The hydrazine linearity curves are shown in Fig2.8, the correlation coefficient, slope and y-intercept of the calibration curve were calculated which is shown in table 2.7. The method exhibits good linearity and range with a linear regression fit of R2 = 0.9930 with a best fit equation of y=1147892.335x+1018.6, linearity overlay chromatograms are shown in Fig.2.7a and 2.7b. The method was demonstrated to be linear in a range of 25% to 150% level from 0.0040µg/g to 0.0225µg/g.

2.9.5.4 Precision:

Precision is the degree of agreement among individual test results when an analytical method is used repeatedly to multiple samplings of a homogeneous sample (32).

❖ **Intermediate precision**

Results from within-laboratory variations due to random events such as different days, analysts, equipment, etc. Experimental design should be employed so that the effects (if any) of the individual variables can be monitored.

❖ **Reproducibility**

Results of collaborative studies between laboratories.

❖ Methodology

Weigh accurately seven replicate, 400 mg of Imatinib samples in 50 mL volumetric flasks. Amongst the 7 replicate samples one is control sample and six is for recovery preparation. The control sample has been diluted up to mark with diluent while 5ml of standard solution stock-2 was added to the rest of the six samples, before making up with diluent. .

Sr. No	Level	Wt. of sample (mg)	Sample Dilution	Vol. Of stock-2 (mL)	Dilution (mL)- Recovery
1	Control sample	400.50	50.00	0.00	50.00
2	Precision Set-1	400.60	50.00	5.00	50.00
3	Precision Set-2	401.30	50.00	5.00	50.00
4	Precision Set-3	402.10	50.00	5.00	50.00
5	Precision Set-4	401.20	50.00	5.00	50.00
6	Precision Set-5	400.60	50.00	5.00	50.00
7	Precision Set-6	400.50	50.00	5.00	50.00

Table 2.8 Sample Preparation for method precision

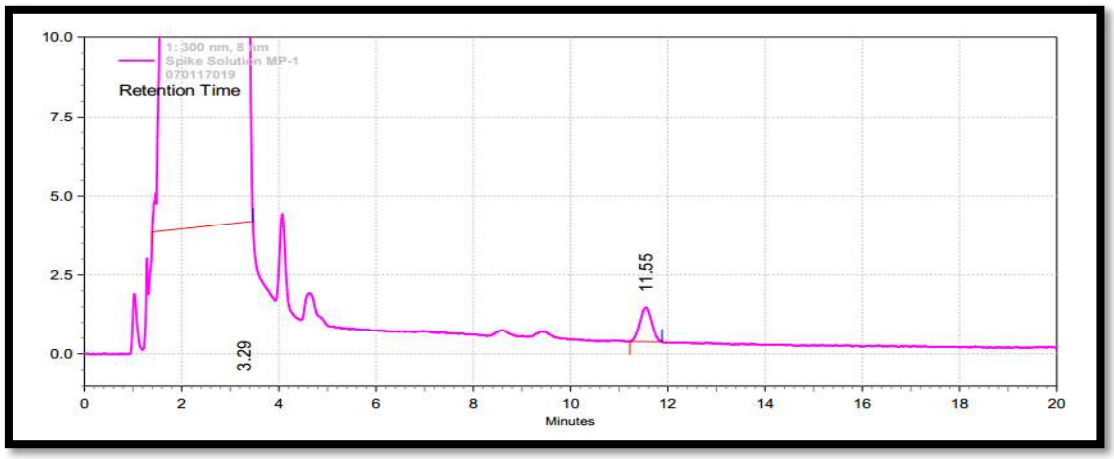

Fig 2.9 Method Precision chromatogram -1

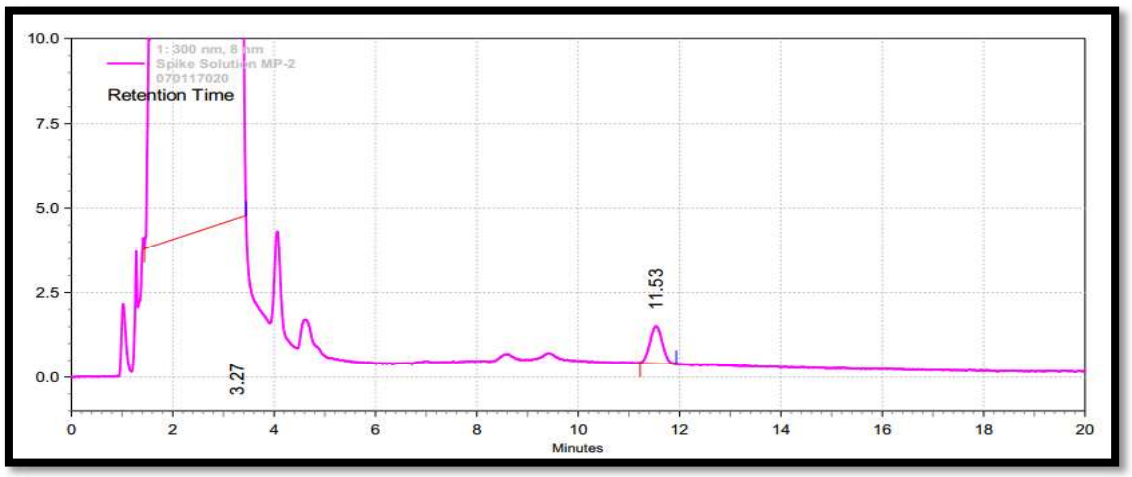

Fig 2.10 Method Precision chromatogram -2

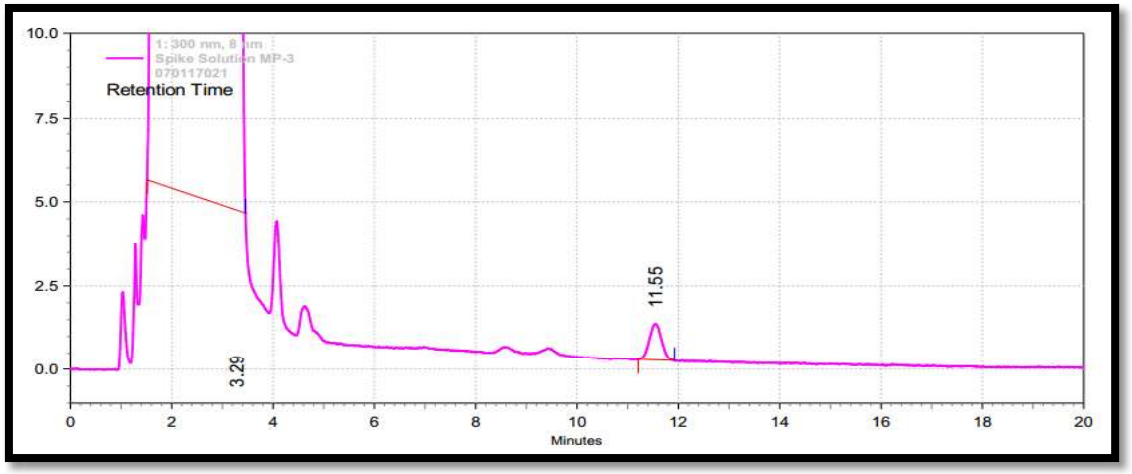

Fig 2.11 Method Precision chromatogram -3

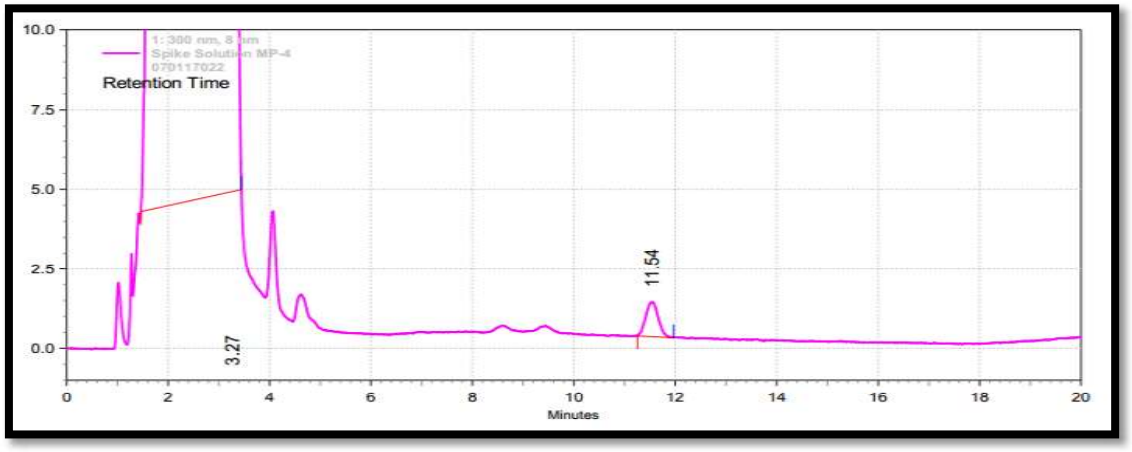

Fig 2.12 Method Precision chromatogram -4

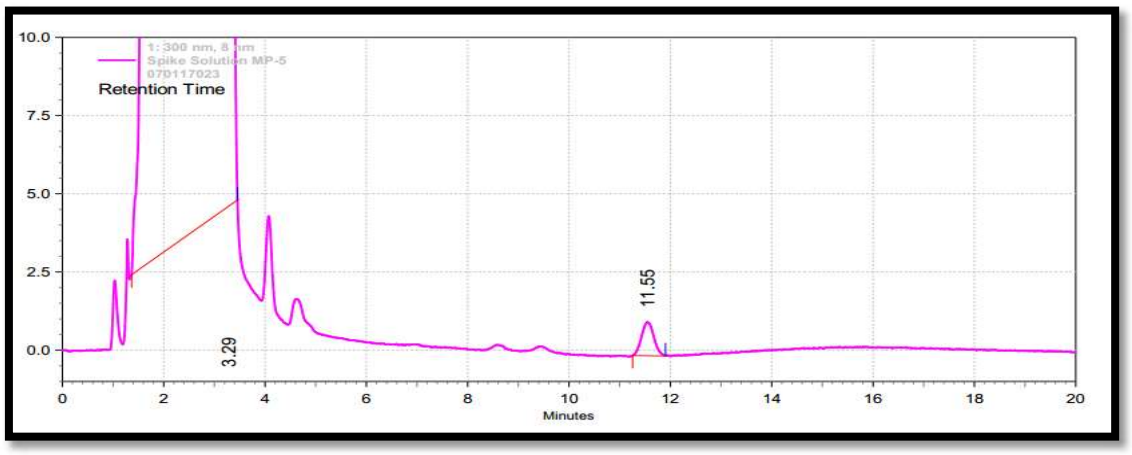

Fig 2.13 Method Precision chromatogram -5

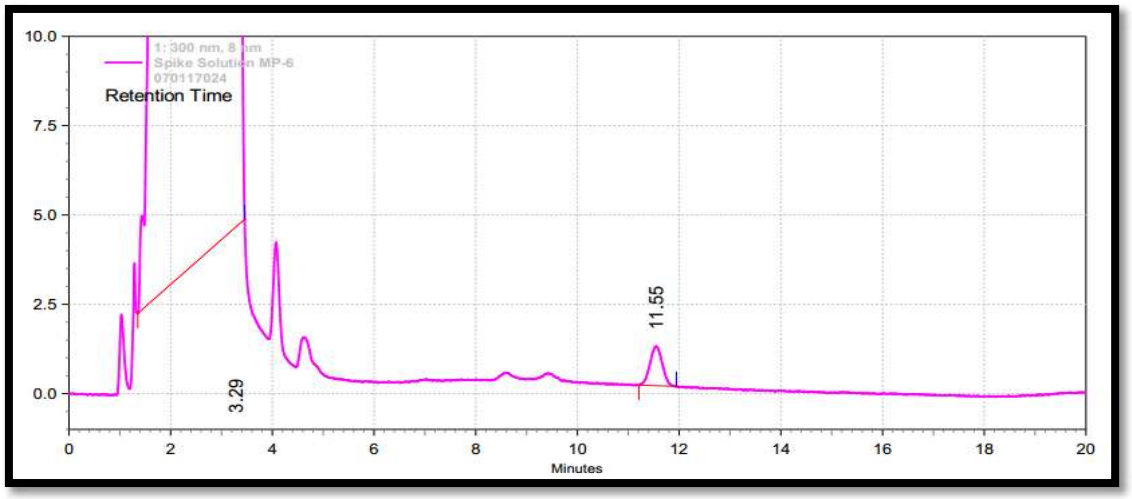

Fig 2.14 Method Precision chromatogram -6

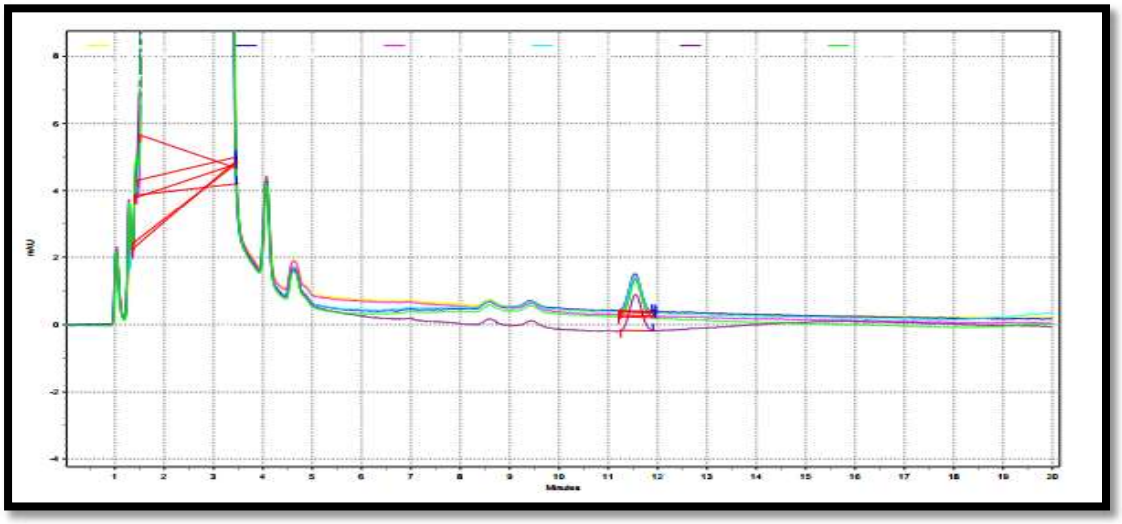

Fig 2.15 Method precision overlay chromatogram

Accuracy Calculation for Hydrazine Hydrate						
Sr.No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**
			ppm	**ppm**	**Ppm**	**(%)**
1	Control sample	0	0.000	0.0	0.000	0
2	Method Precision Set-1	18620	1.9	1.9	1.88	100.2
3	Method Precision Set-2	18150	1.9	1.8	1.83	97.5
4	Method Precision Set-3	18774	1.9	1.9	1.89	100.7
5	Method Precision Set-4	18892	1.9	1.9	1.91	101.6
6	Method Precision Set-5	18334	1.9	1.9	1.85	98.7
7	Method Precision Set-6	18642	1.9	1.9	1.88	100.4
					Overall Mean	99.85
					Overall SD	1.463
					Overall RSD (%)	1.47

Table 2.9 Calculation for method precision.

Repeatability of the method was checked by analyzing six replicate samples of 8000 µg/g Imatinib mesylate spiked with 0.0150 µg/g of hydrazine hydrate (at 100% level). The %RSD was calculated for hydrazine hydrate for its content. The percent relative standard deviation for recovery of six replicate injections at spike level was 1.47%. All the graphs for the method precision are shown in figures 2.9 to2.14. Overlay for the method precision are shown in Fig.2.15. and tabulated in table 2.9.

2.9.5.5 Accuracy:

Accuracy is the closeness of test results to the true value.

Methodology

- **Drug substance**
  - Comparison of the results with the analysis of a standard reference material.
  - Comparison to a second, well-characterized method.
- **Drug product**
  - Evaluate by analyzing synthetic mixtures of known amounts or samples spiked with known quantities of components.
  - Comparison to a second, well-characterized method.
- **Quantization of impurities**
  - Analyze samples (drug substance or drug product) spiked with known amounts of impurities. (If impurities are not available, see specificity.)

Sr. No	Level	Wt of sample (mg)	Sample dilution	Vol. Of stock solution (ml)	Dilution (ml)
1	Control sample	400.50	50	0	50
2	Level-(150%) Sample Prep.-1	400.10	50	7.5	50
3	Level-(150%) Sample Prep.-1	400.20	50	7.5	50
4	Level-(150%) Sample Prep.-1	400.30	50	7.5	50
5	Level-(100%) Sample Prep.-1	400.60	50	5.0	50
6	Level-(100%) Sample Prep.-2	401.30	50	5.0	50
7	Level-(100%) Sample Prep.-3	402.10	50	5.0	50
8	Level-(50%) Sample Prep.-1	403.20	50	2.5	50
9	Level-(50%) Sample Prep.-2	400.60	50	2.5	50
10	Level-(50%) Sample Prep.-3	402.90	50	2.5	50
11	Level-(25%) Sample Prep.-1	400.90	50	1.25	50
12	Level-(25%) Sample Prep.-2	401.20	50	1.25	50
13	Level-(25%) Sample Prep.-3	401.30	50	1.25	50

Table 2.10 Sample Preparation for accuracy

Accuracy Calculation for Hydrazine Hydrate							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			ppm	**ppm**	**ppm**	**(%)**	**(%)**
1	Control sample	0	0.000	0.00	0.000	NA	NA
2	Level-(150%) Sample Prep.-1	26207	2.81	2.65	2.65	94.2	**95.7**
3	Level-(150%) Sample Prep.-1	26761	2.81	2.71	2.71	96.1	
4	Level-(150%) Sample Prep.-1	26933	2.81	2.72	2.72	96.7	
5	Level-(100%) Sample Prep.-1	18620	1.88	1.88	1.88	100.2	**99.5**
6	Level-(100%) Sample Prep.-2	18150	1.88	1.83	1.83	97.5	
7	Level-(100%) Sample Prep.-3	18774	1.88	1.89	1.89	100.7	
11	Level-(50%) Sample Prep.-1	10111	0.94	1.01	1.01	108.2	**107.5**
12	Level-(50%) Sample Prep.-2	9791	0.94	0.99	0.99	105.4	
13	Level-(50%) Sample Prep.-3	10175	0.94	1.02	1.02	108.9	
14	Level-(25%) Sample Prep.-1	4732	0.47	0.48	0.48	101.8	**101.3**
15	Level-(25%) Sample Prep.-2	4689	0.47	0.47	0.47	100.8	
16	Level-(25%) Sample Prep.-3	4712	0.47	0.48	0.48	101.3	
						Overall Mean	101.00
						Overall SD	4.627
						Overall RSD (%)	4.58

Table 2.11 Calculation for accuracy determination.

Recovery studies has been carried out for the determination of the accuracy by analyzing the spiked samples. Known amounts of hydrazine hydrate was spiked in triplicate at four different

concentration levels of 0.0040, 0.0075, 0.0150 and 0.0225 µg/g to a previously analyzed Imatinib mesylate drug substance sample. The percentage of recoveries for hydrazine hydrate was calculated. The accuracy and precision was validated on a Imatinib Mesylate spiked with hydrazine hydrate at four concentration levels covering the specified range with 6 replicates for 0.0150 µg/g and 3 replicates for 0.0040, 0.0075 and 0.0225 µg/g. Imatinib mesylate was prepared at a concentration of 8000 µg/g. The percent recovery was calculated by spiking hydrazine in Imatinib Mesylate API. Calculation for accuracy determination is shown in table 2.11. The individual percent recoveries for all preparations were from 97.3-105.2% and the %RSD for all injections was 1.2%.

2.10 Conclusion:

The presented method provides specific and meticulous quantization of hydrazine hydrate in a variety of pharmaceutical product and active ingredients using a derivatization technique which is very simple in nature and with help of HPLC. Derivatizing a hydrazine hydrate proved a key approach for the detection of impurity. A methanolic solution of benzaldehyde performed role of derivatizing agent and was able to meet the requirements of all analytical tools. As hydrazine hydrate doesn't have chromophores in its structure, derivatization by benzaldehyde helped to shift its wavelength to the detectable UV range. In addition, derivatized product can be easily resolved by HPLC from Active pharmaceutical ingredients' peak. Moreover, the suitability of the current method was proved on the basis of linearity, range, accuracy, specificity and precision. All the statistical results present i.e. R.S.D., % recovery and mean seem to be in acceptable criteria. In addition the developed method can be used for final formulation of active pharmaceutical ingredients.

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