CHAPTER 4:

CHARACTERIZATION OF RELATED IMPURITIES PRESENT IN DASATINIB

LIST OF FIGURES

LIST OF TABLES

4.6d	Wave numbers of corresponding groups from the recorded IR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate	145

4.1 Introduction:

Dasatinib as discussed in chapter 1 is one of the second-generation tyrosine kinase inhibitors used in imatinib resistance and/or intolerance, as well as in the frontline setting in patients with chronic myeloid leukemia-chronic phase, and also in patients with advanced disease. It is also utilized in Philadelphia chromosome-positive acute lymphocytic leukemia. Originally termed BMS-354825, Dasatinib (Sprycel®; Bristol-Myers Squibb, New York, NY, USA) is an orally potent, bioavailable inhibitor of BCR-ABL1 and was approved by the US Food and Drug Administration (FDA) in 2006 for the treatment of imatinib-resistant and -intolerant adults with CML-CP as well as Ph-positive acute lymphoblastic leukemia and advanced stages of disease (1,2).

Preliminary data, after the introduction of Dasatinib in clinical trials, in patients with CML, suggest that this drug is safe and well tolerated; Furthermore, majority of patients with imatinib-resistant disease achieved objective responses, although the durability of responses remains to be defined (3). It is largely metabolized in the liver, mainly by the cytochrome P450 isoenzyme CYP3A4. Moreover, the solubility of Dasatinib is pH-dependent, and long-term inhibition of gastric acid secretion reduces Dasatinib exposure (4). Dasatinib binds both the active and inactive forms of BCR-ABL1 and has in vitro activity against all currently described imatinib-resistant mutations except T315I. It may also overcome different resistance mechanisms to imatinib, including alternate signaling pathways involving the SFKs and MDR-1 gene overexpression. The FDA-approved dosages are 100 mg per day once daily orally for patients with CML-CP and 140 mg once daily for patients with advanced disease (5). Additionally, dosing modifications can be made based on toxicities. Dasatinib amorphous is a white to off-white powder and the drug substance is insoluble in water and slightly soluble in ethanol and methanol (6).

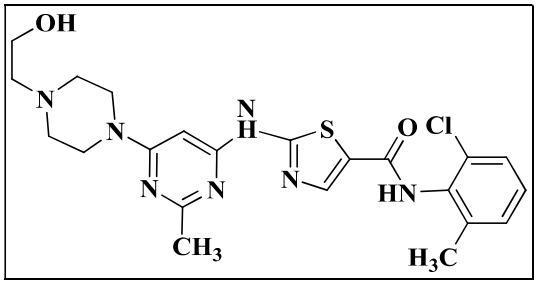


Fig 4.1 Structure of Dasatinib

Chemical name: N, N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl] amino]-5-thiazole carboxamide

Molecular formula: $C_{22}H_{26}ClN_7O_2S$

Molecular weight: 488.01

CAS Reg No: [302962-49-8]

4.2 Route of synthesis for Dasatinib amorphous

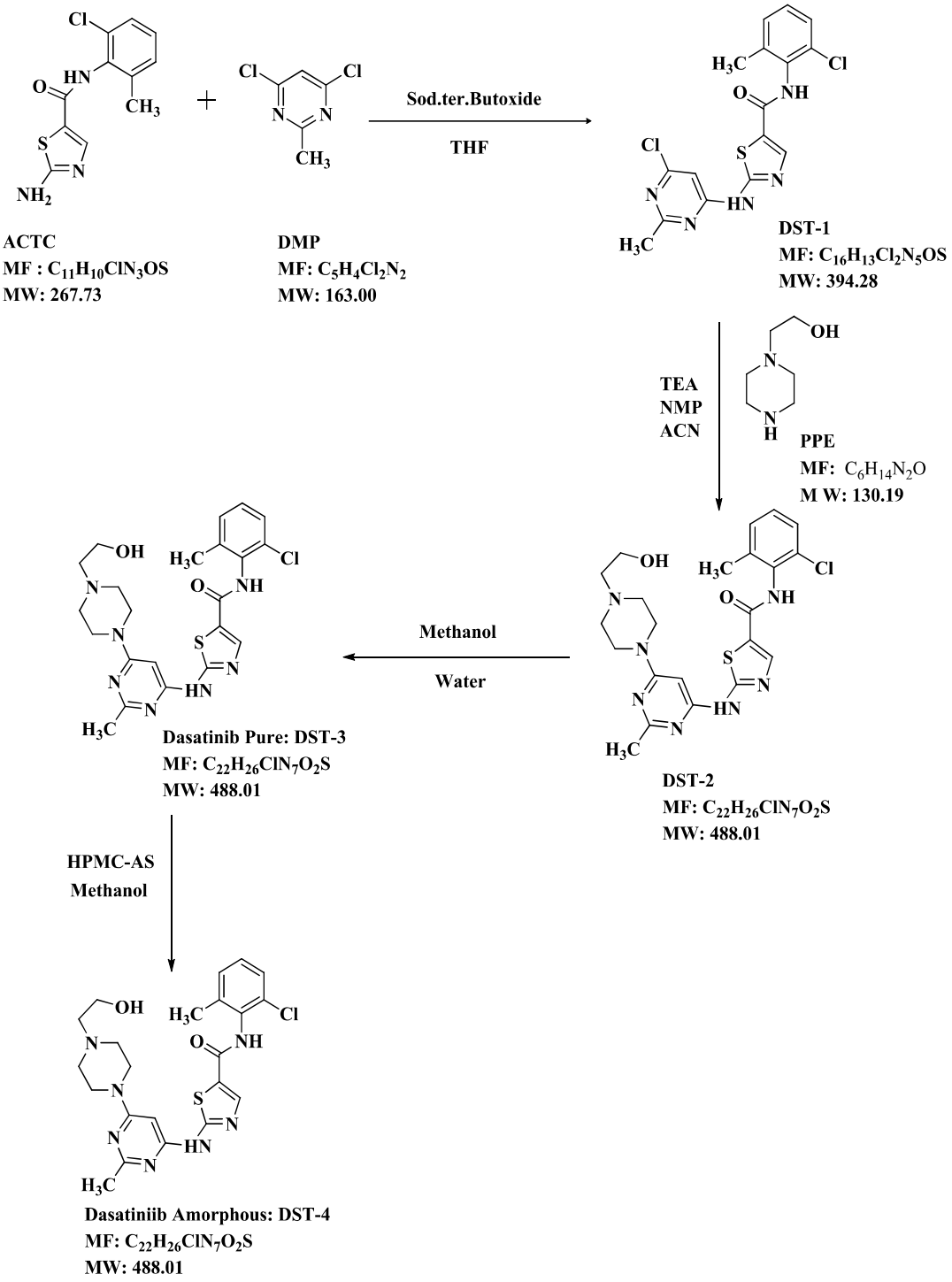


Fig 4.2 Route of synthesis for Dasatinib (26)

Impurities present in Dasatinib

There are six impurities present in dasatinib, amongst which two impurities are process related impurities and four impurities are degradation related impurities. All the known impurities present in Dasatinib were synthesized at Cadila healthcare Ltd., Ahmedabad, Gujarat, India and were characterized by Mass Spectroscopy, ^{1}H-NMR Spectroscopy, ^{13}C-NMR Spectroscopy and IR Spectroscopy.

4.3 Structure Elucidation and Characterization of Dasatinib Impurity-1

1. Mass Spectroscopy

Instrument used: Quattro Micro Mass

Make: Waters

Test (Sample) preparation: Test solution of concentration 1mg/mL of Dasatinib Imp-1 in methanol was prepared and used.

Procedure: Mass Spectra has been acquired of the above test solution under the specified conditions of the instrument.

Conditions:

Polarity: Positive & Negative (Separately)
Source Temperature: 150°C
Desolvation temperature: 350°C
Cone Gas Flow: 50L/h
Desolvation Gas Flow: 800 L/h

Same conditions have been applied for the characterization of other impurities. The Mass spectrum of Dasatinib Imp-1is represented in Fig 4.3a.

Interpretation:

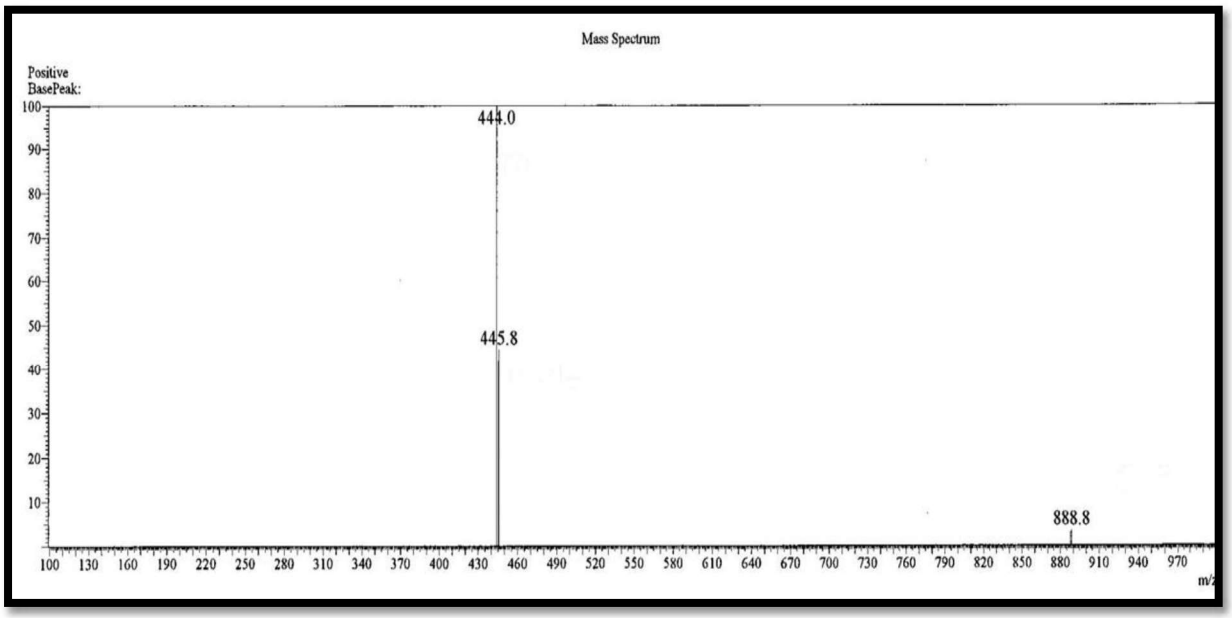


Fig 4.3a Mass spectrum of Dasatinib Imp-1

From the recorded Mass spectrum, the obtained spectral values are shown in Table 4.1a

Dasatinib Imp-1	**Impurity-1**
Molecular Formula	$C_{20}H_{22}ClN_7OS$
Theoretical Molecular Weight	444
Mass Fragmentation Peaks	444 (M), 445.8 (M^{+2})

Table 4.1a Obtained spectral values from the recorded Mass spectrum of Dasatinib Imp-1

Discussion: The most abundant positive ion peak is observed at m/e 444 indicating molecular ion peak (M^+) of Dasatinib Imp-1. Peaks observed at m/e 445.8 (M^{2+}) indicated presence of Cl atom. Dasatinib Imp-1confirmed the presence of one chlorine atom in the structure.

Conclusion: The positive ion Mass spectral analysis of Dasatinib Imp-1observed at m/e 444 (M^+) suggesting the possibility of Molecular formula $C_{20}H_{22}ClN_7OS$ which confirms the theoretical molecular weight of Dasatinib Imp-1.

2. ^{1}H-NMR spectroscopy

Instrument used: AVANCE II 400

Make: BRUKER

Test preparation: About 10 mg of Dasatinib Imp-1 was weighed, dissolved in 0.5 mL of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into a clean and dry NMR Tube.

Procedure: ^{1}H-NMR Spectra has been recorded of the above test solution under the specified operational conditions of the instrument. NMR Spectra has been recorded 400 (^{1}H), 100 (^{13}C), (100) with Bruker AVANCE-II 400MHz spectrometer with 5 mm BBO probe, TMS as internal reference. The instrumentation conditions are maintained same for the characterization of other impurities. The ^{1}H-NMR spectrum of Dasatinib Imp-1is represented in Fig 4.3 b.

Interpretation:

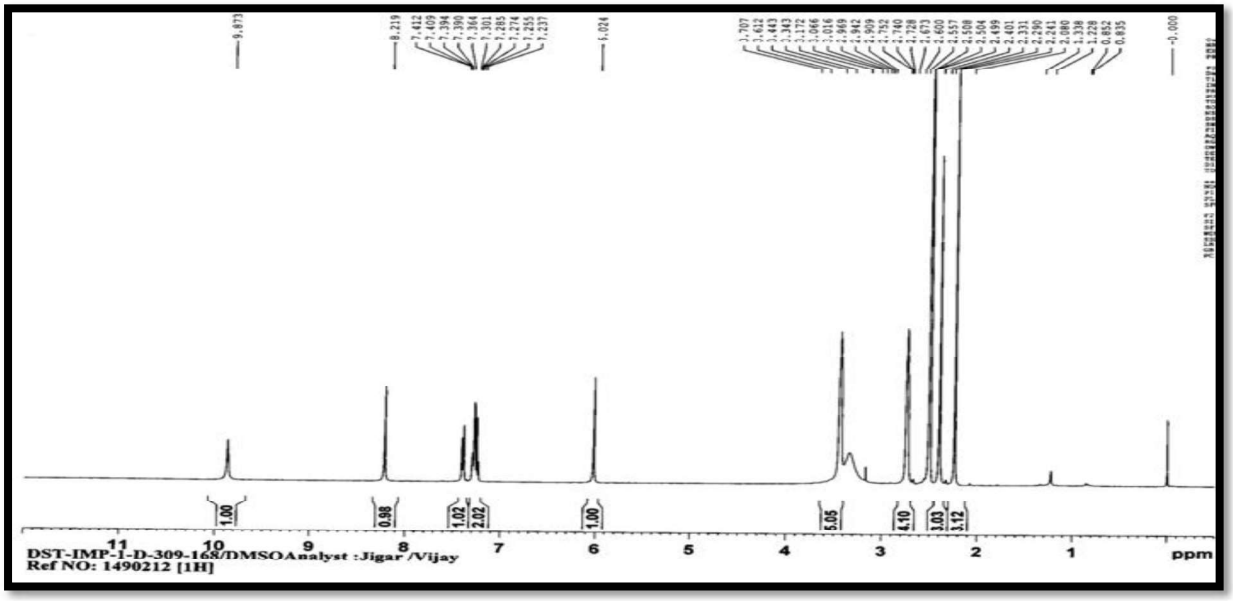


Fig 4.3b ^{1}H-NMR spectrum of Dasatinib Imp-1

From the recorded ^{1}H-NMR spectrum, chemical shifts and the multiplicity of the corresponding protons are shown in Table 4.1b.

Proton No. *	Chemical Shift (value in ppm)	Multiplicity	Assigned Proton
A	2.24	Singlet	3H of Methyl group (-CH_3) Aromatic ring
B	2.40	Singlet	3H of Methyl group (-CH_3) Pyrimidine ring
C	2.72-2.75	Triplet	4H of piperazine ring
d, e	3.44-3.70	Multiplet	4H of CH_2 and 1H of NH group of piperazine ring
F	6.02	Singlet	1H pyrimidine ring
g, h	7.23-7.30	Multiplet	2H of Aromatic ring

I	7.39-7.41	Quartet	1H of Aromatic ring
J	8.21	Singlet	1H of Thiazole ring
K	9.87	Singlet	1H of amine group

* Refer structure for Proton identification.

Table 4.1b Chemical shifts and the multiplicity of the corresponding protons from the recorded ^{1}H-NMR spectrum of Dasatinib Imp-1

Discussion: The ^{1}H-NMR result obtained for Dasatinib Imp-1is as below:

The protons of methyl group attached to aromatic ring appear at δ 2.24 ppm.

The protons of aromatic ring appear at δ 7.23-7.41 ppm.

The protons of pyrimidine ring appear at δ 6.02 ppm.

The protons of thiazole ring appear at δ 8.21ppm.

The protons of amine group of piperazine ring appear at δ 9.87ppm.

Conclusion: The data generated from the study of ^{1}H-NMR spectrum of Dasatinib Imp-1is in accordance with the following structure.

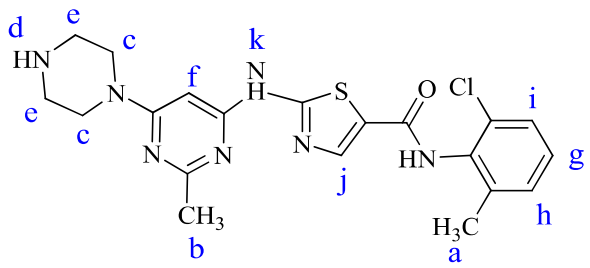


Fig 4.3c Structure elucidation from ^{1}H-NMR spectrum of Dasatinib Imp-1

3. ^{13}C-NMR Spectroscopy

Instrument used: AVANCE II 400

Make: BRUKER

Test preparation: About 40 mg of Dasatinib Imp-1 weighed, dissolved in 0.5 ml of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into clean and dry NMR Tube.

Procedure: ^{13}C-NMR Spectra has been recorded of the test solution under the specified operational conditions of the instrument. Other parameters for instrumentation are same as H^1 NMR technique.

The ^{13}C-NMR spectrum of Dasatinib Imp-1 is represented in Fig 4.3d.

Interpretation:

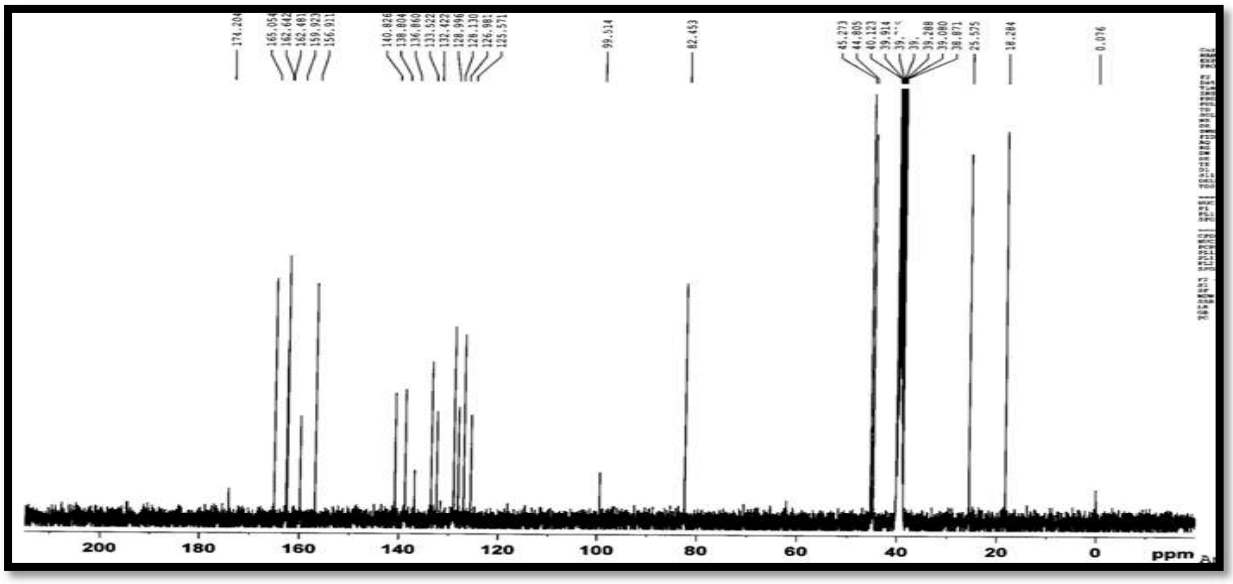


Fig 4.3d ^{13}C-NMR spectrum of Dasatinib Imp-1

From the recorded ^{13}C-NMR spectrum, chemical shifts of corresponding carbons are shown in Table 4.1c

Carbon No.*	Chemical Shift (δ value in ppm)	Nature of Carbon	Assigned Carbon
A	18.28	Primary	Methyl group (-CH_3) of Aromatic ring
B	25.57	Primary	Methyl group (-CH_3) of pyrimidine ring
C	44.80	Secondary	piperazine ring
D	45.27	Secondary	piperazine ring
E	82.45	Secondary	pyrimidine ring
F	125.57	Tertiary	Thiazole ring
G	126.98	Tertiary	Aromatic ring
H	128.13	Tertiary	Aromatic ring
I	128.99	Tertiary	Aromatic ring
J	132.42	Quaternary	Aromatic ring
K	133.52	Quaternary	Aromatic ring
L	136.86	Quaternary	Thiazole ring
M	138.80	Quaternary	Aromatic ring

N	140.82	Quaternary	Thiazole ring
O	156.91	Quaternary	Amide group
P	159.92	Quaternary	Pyrimidine ring
Q	162.48	Quaternary	Pyrimidine ring
R	165.05	Quaternary	Pyrimidine ring

* Refer structure for Carbon identification.

Table 4.1c Chemical shifts of corresponding carbons from the recorded ^{13}C-NMR spectrum of Dasatinib Imp-1

Conclusion: The data generated from the study of ^{13}C-NMR spectrum of Dasatinib Imp-1is in accordance with the following structure.

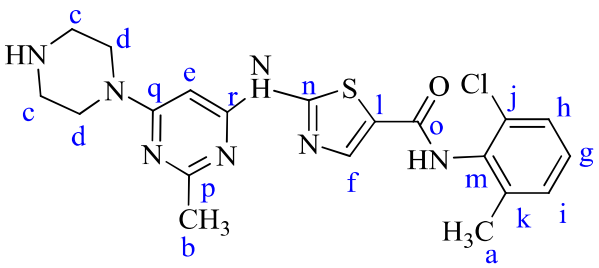


Figure 4.3e Structure elucidation from ^{13}C-NMR spectrum of Dasatinib Imp-1

4. IR Spectroscopy

Instrument used: FT-IR-8300

Make: Shimadzu

Test preparation: About 2 mg of Dasatinib Imp-1 triturated with 300 mg of finely powdered and dried potassium bromide. The mixture was carefully ground, spread uniformly in a suitable die and submitted in vacuum to a pressure of about 800 MPa (80 kg/cm^2).

Procedure: The infrared absorption spectrum of the above potassium bromide pellet was recorded between 4000 cm^{-1} and 400 cm^{-1} under the specified operational conditions of the instrument.

The IR spectrum of Dasatinib Imp-1 is represented in Fig 4.3f.

Interpretation:

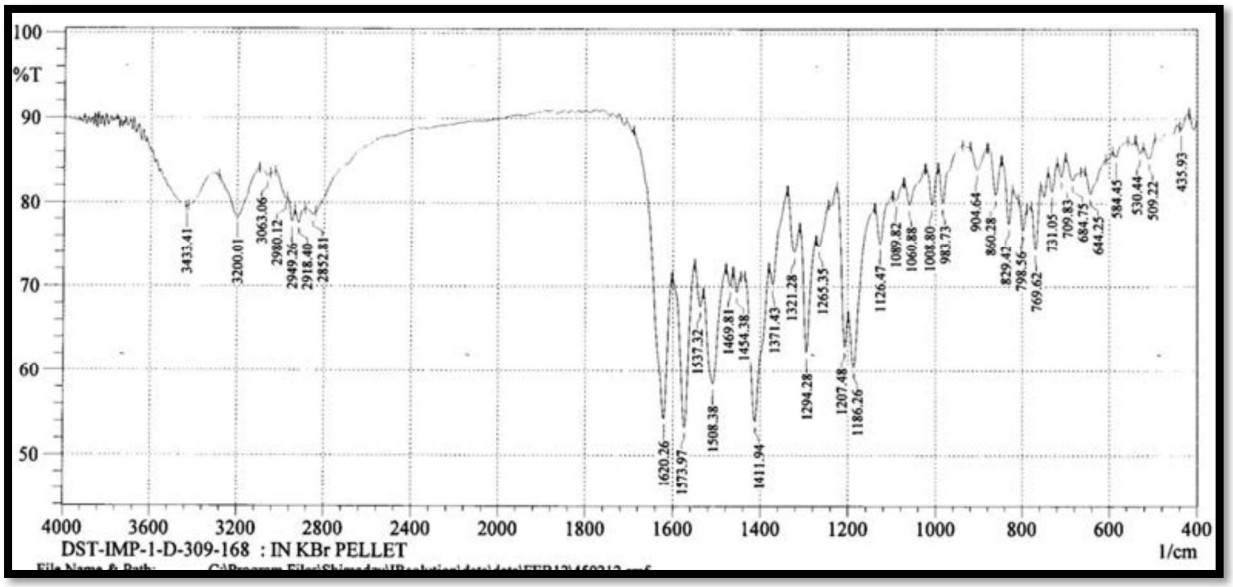


Fig 4.3f IR spectrum of Dasatinib Imp-1

From the recorded IR spectrum, the wave numbers of corresponding groups are shown in Table 4.1d.

Wave number (cm^{-1})	Assignment
3433	-NH Asymmetric stretching of amine
3200	-NH symmetric stretching of amine
3063	Ar-CH Stretching
2980	-CH stretching of CH_3
1620	-CO stretching of amide
1573	-C=C Stretching of Aromatic ring
1537	C=N stretching pyrimidine ring
1411	-C=C Stretching of Aromatic ring
769	N-H Wagging

Table 4.1d Wave numbers of corresponding groups from the recorded IR spectrum of Dasatinib Imp-1

Conclusion: The data generated from the study of IR spectrum of Dasatinib Imp-1 is in accordance with the following structure.

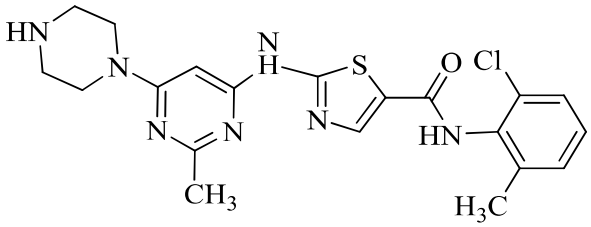


Fig 4.3g Structure elucidation from IR spectrum of Dasatinib Imp-1

5. Summary of study: The structural characterization study carried out for Dasatinib Imp-1 using Mass, ^{1}H-NMR, ^{13}C-NMR and IR spectroscopy reveals the following structure of Dasatinib Imp-1, which matches with the structure given in literature.

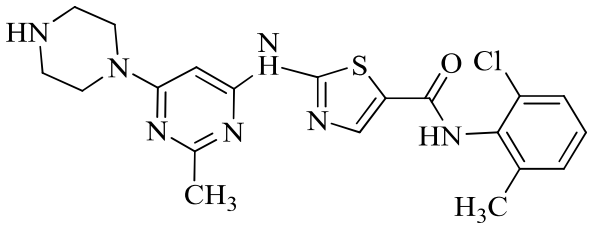


Fig 4.3h Structure of Dasatinib Imp-1

6. **Remarks:** Therefore, compound Dasatinib Imp-1 is qualified as in-house reference standard for intended use.

4.4 Structure Elucidation and Characterization of Dasatinib Impurity-2

The sample of DasatinibImp-2 (Synthesized at cadila healthcare ltd., Ahmedabad) as primary reference standard has been analyzed using different techniques.

1. Mass Spectroscopy

Test preparation: Test solution of concentration 1mg/ml of Dasatinib Imp-2 in methanol was prepared and used.

Procedure: Mass Spectra has been acquired of the above test solution under the specified conditions of the instrument.

The Mass spectrum of Dasatinib Imp-2 is represented in Fig 4.4a.

Interpretation:

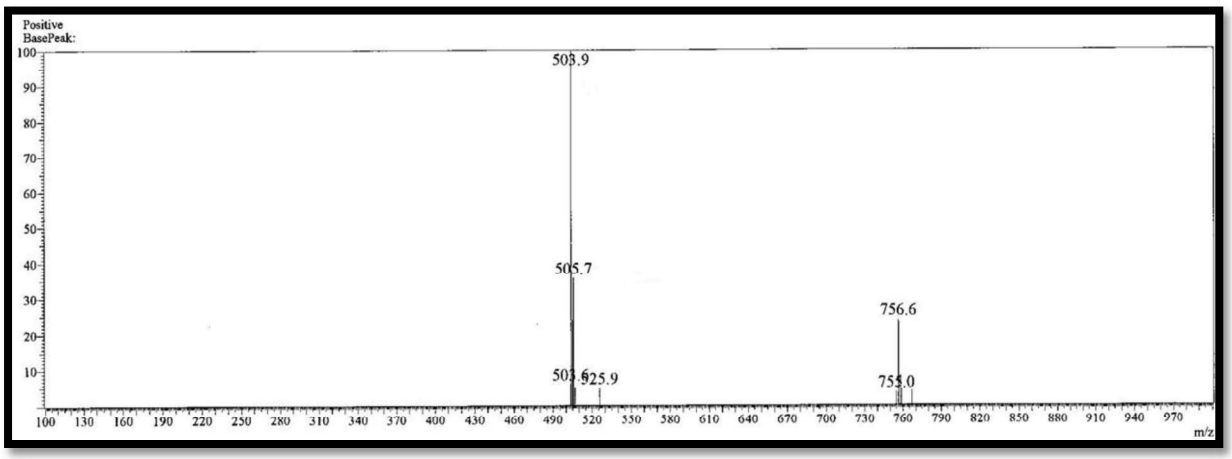


Fig 4.4a Mass spectrum of Dasatinib Imp-2

From the recorded Mass spectrum, the obtained spectral values are shown in Table 4.2a.

Dasatinib Imp-2	**Impurity 2**
Molecular Formula	$C_{22}H_{26}ClN_7O_3S$
Theoretical Molecular Weight	504
Mass Fragmentation Peaks	503.9 (M^+), 505.7 (M^{+2})

Table 4.2a Obtained spectral values from the recorded Mass spectrum of Dasatinib Imp-2

Discussion: The most abundant positive ion peak is observed at m/e 503.9 indicating molecular ion peak (M) of Dasatinib Imp-2. Peaks observed at m/e 505.7 indicating (M^{2+}), these two fragment i.e.M & M^{2+}indicate the presence of Cl atom.

Conclusion: The positive ion Mass spectral analysis of Dasatinib Imp-2 observed at m/e **503.9** (M) suggesting the possibility of Molecular formula $C_{22}H_{26}ClN_7O_3S$ which confirms the theoretical molecular weight of Dasatinib Imp-2.

2. ^{1}H-NMR spectroscopy

Instrument used: AVANCE II 400

Make: BRUKER

Test preparation: About 10 mg of DasatinibImp-2 was weighed, dissolved in 0.5 mL of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into a clean and dry NMR Tube.

Procedure: ^{1}H-NMR spectra has been recorded of the above test solution under the specified operational conditions of the instrument.

The ^{1}H-NMR spectrum of DasatinibImp-2 is represented in Fig 4.4b.

Interpretation:

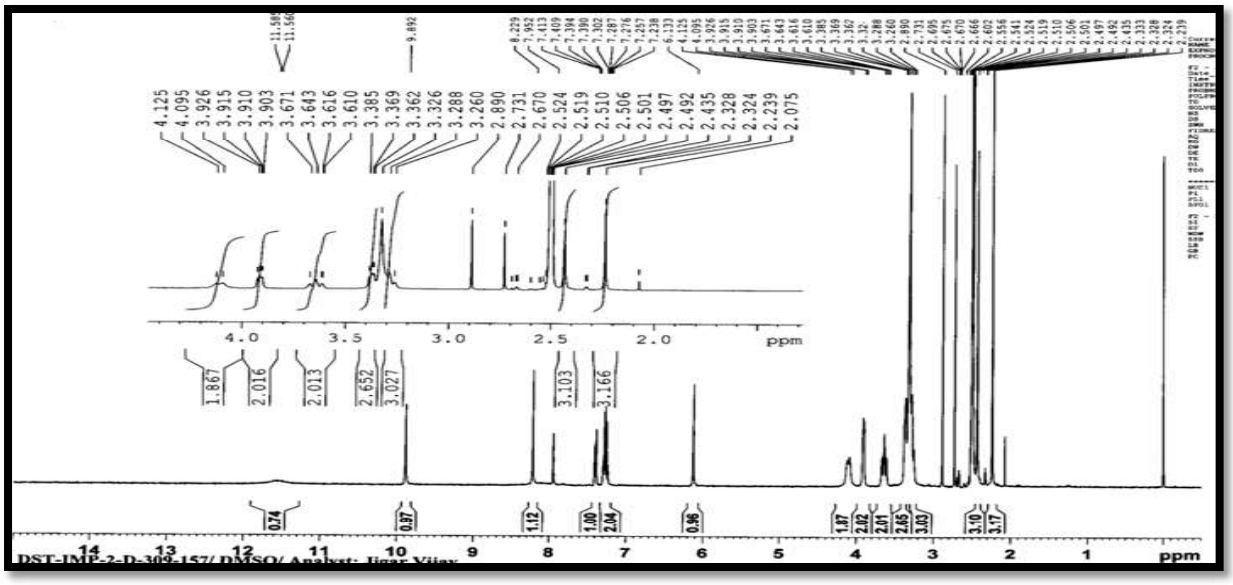


Fig 4.4b 1H-NMR spectrum of Dasatinib Imp-2

From the recorded ^{1}H-NMR spectrum, chemical shifts and the multiplicity of the corresponding protons are shown in Table 4.2b.

Proton No. *	Chemical Shift (δ value in ppm)	Multiplicity	Assigned Proton
A	2.23	Singlet	3H of Methyl group (Ar-CH_3)
B	2.43	Singlet	3Hof Methyl group (-CH_3) of pyrimidine
c, d	3.26-3.38	Multiplet	2H of Ethanol & 4H of piperazine ring
E	3.61-3.92	Multiplet	4H of piperazine ring (-CH_2)
F	4.09-4.12	Triplet	2H of Ethanol
G	6.13	Triplet	1H of pyrimidine ring
h, i	7.23-7.30	Multiplet	2H of aromatic ring
J	7.39-7.41	Quartet	1H of aromatic ring
K	8.22	Singlet	1H of thiazole ring
L	9.89	Singlet	1H of amine group
M	11.56-11.58	Broad	1H of amide group

Table 4.2b Chemical shifts and the multiplicity of the corresponding protons from the recorded ^{1}H-NMR spectrum of Dasatinib Imp-2 (* Refer structure for Proton identification)

Discussion: The ^{1}H-NMR result obtained for DasatinibImp-2is as below:

The protons of methyl group attached to aromatic ring appear at δ 2.23ppm.

The protons of methyl group attached to pyrimidine ring appear at δ 2.43ppm.

The protons of aromatic ring appear at δ 7.23-7.41ppm.

The protons of thiazole ring appear at δ 8.22 ppm.

Conclusion: The data generated from the study of ^{1}H-NMR spectrum of DasatinibImp-2 is in accordance with the following structure.

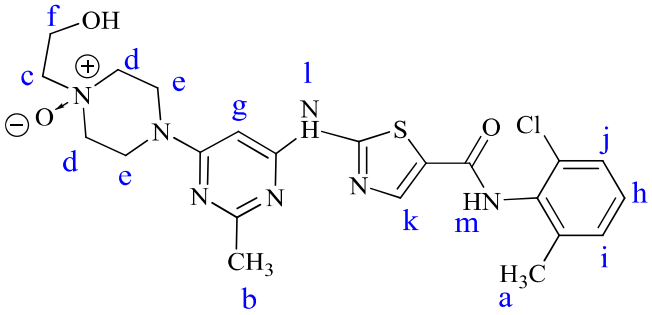


Fig 4.4c Structure elucidation from ^{1}H-NMR spectrum of Dasatinib Imp-2

3. ^{13}C-NMR Spectroscopy

Instrument used: AVANCE II 400

Make: BRUKER

Test preparation: About 40 mg of Dasatinib Imp-2 weighed, dissolved in 0.5 mL of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into clean and dry NMR Tube.

Procedure: ^{13}C-NMR spectra has been recorded of the test solution under the specified operational conditions of the instrument.

The ^{13}C-NMR spectrum of Dasatinib Imp-2 is represented in Fig 4.4d.

Interpretation:

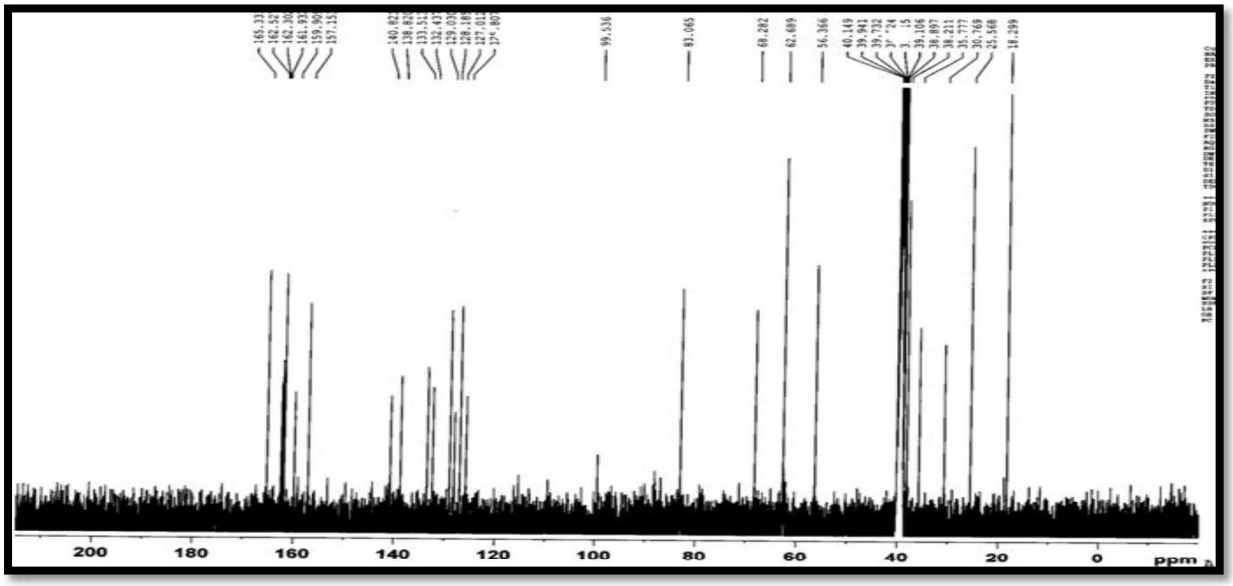


Fig 4.4d ^{13}C-NMR spectrum of Dasatinib Imp-2

From the recorded ^{13}C-NMR spectrum, chemical shifts of corresponding carbons are shown in Table 4.2c.

Carbon No.*	Chemical Shift (value in ppm)	Nature of Carbon	Assigned Carbon
A	18.29	Primary	Methyl group of Aromatic
B	25.56	Primary	Methyl group of pyrimidine
C	30.76	Secondary	-CH_2 of piperazine ring
D	35.77	Secondary	-CH_2 of piperazine ring
E	56.36	Secondary	-CH_2 of Ethanol

F	62.68	Secondary	-CH_2 of Ethanol
G	68.28	Secondary	-CH of Pyrimidine ring
H	83.06	Secondary	-CH of Thiazole ring
I	125.80	Tertiary	Aromatic ring
J	127.01	Tertiary	Aromatic ring
K	128.18	Tertiary	Aromatic ring
L	129.03	Tertiary	Aromatic ring
M	132.43	Tertiary	Aromatic ring
N	133.51	Quaternary	Thiazole ring
O	138.82	Quaternary	Aromatic ring
P	140.82	Quaternary	Thiazole ring
Q	157.15	Quaternary	Amide Group
R	159.90	Quaternary	Pyrimidine ring
S	161.93	Quaternary	Pyrimidine ring
T	165.33	Quaternary	Pyrimidine ring

* Refer structure for Carbon identification.

Table 4.2c Chemical shifts of corresponding carbons from the recorded ^{13}C-NMR spectrum of Dasatinib Imp-2

Conclusion: The data generated from the study of ^{13}C-NMR spectrum of Dasatinib Imp-2 is in accordance with the following structure.

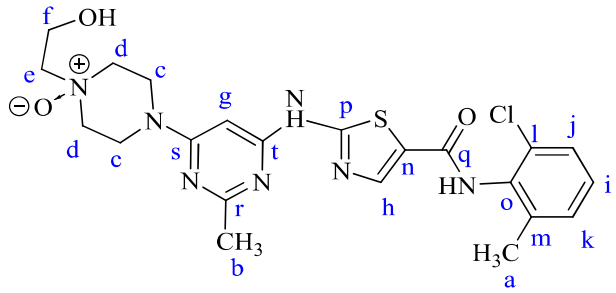


Fig 4.4e Structure elucidation from ^{13}C-NMR spectrum of Dasatinib Imp-2

4. IR Spectroscopy

Instrument used : FT-IR 8300

Make : Shimadzu

Test preparation: About 2 mg of Dasatinib Imp-2 triturated with 300 mg of finely powdered and dried potassium bromide. The mixture was carefully grinded, spread uniformly in a suitable die and submitted in vacuum to a pressure of about 800 MPa (80 kg/cm^2).

Procedure: The infrared absorption spectrum of the above potassium bromide pellet was recorded between 4000 cm^{-1} and 400 cm^{-1} under the specified operational conditions of the instrument.

The IR spectrum of DasatinibImp-2 is represented in Fig 4.4f.

Interpretation:

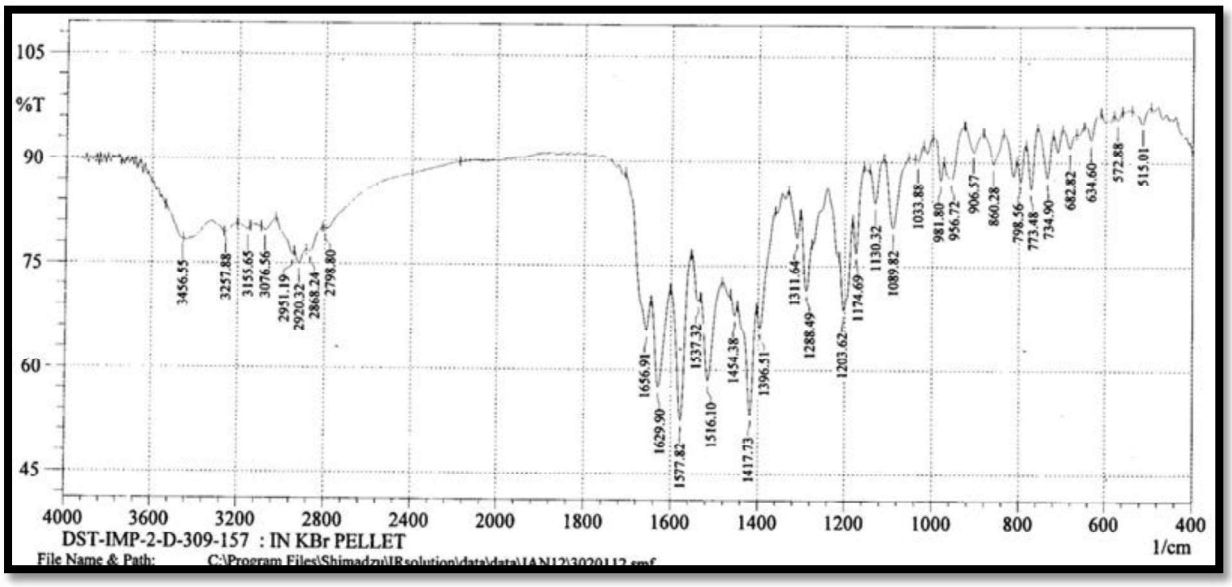


Fig 4.4f IR spectrum of Dasatinib Imp-2

From the recorded spectrum, the wave numbers of corresponding groups are shown in Table 4.2d.

Wave number (cm^{-1})	Assignment
3456	-OH Stretching of Hydroxy group
3257	-NH stretching of Amine
3076	-CH Stretching of Aromatic proton
2951	-CH Stretching of $–CH_3$
1629	-C=O stretching of amide
1577	-C=C stretching of aromatic ring
1516	-C=N stretching of aromatic ring
773	-N-H Wagging

Table 4.2d Wave numbers of corresponding groups from the recorded IR spectrum of Dasatinib Imp-2

Conclusion: The data generated from the study of IR spectrum of DasatinibImp-2 is in accordance with the following structure.

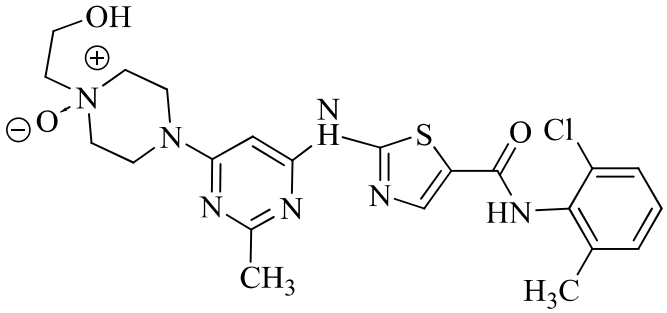


Fig 4.4g Structure elucidation from IR spectrum of Dasatinib Imp-2

5. Summary of study: The structural characterization study carried out using Mass, ^{1}H-NMR, ^{13}C-NMR and IR spectroscopy reveals the following structure of Dasatinib Imp-2, which matches with the structure given in literature.

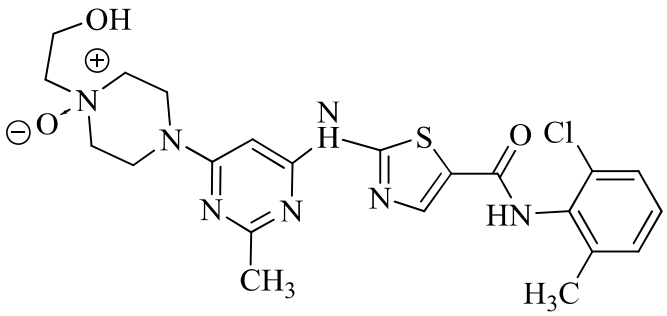


Fig 4.4h Structure of Dasatinib Imp-2

6. Remarks: Therefore compound Dasatinib Imp-2is qualified as in-house reference standard for intended use.

4.5 Structure Elucidation and Characterization of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (Impurity 3)

The sample of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (Synthesized at cadila healthcare ltd., Ahmedabad) as primary reference standard has been analyzed using following techniques.

1. Mass Spectroscopy

Instrument used : Quattro Micro Mass

Make : Waters

Test preparation: Test solution of concentration 1mg/ml of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamidein methanol was prepared and used.

Procedure: Mass Spectra has been acquired of the above test solution under the specified conditions of the instrument.

The Mass spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide) is represented in Fig 4.5a.

Interpretation:

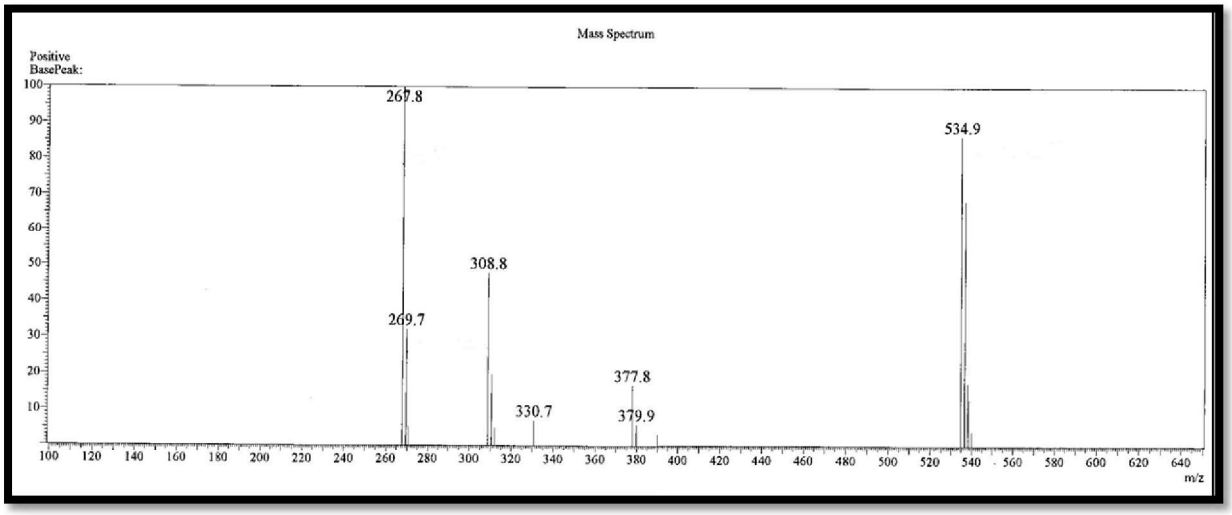


Fig 4.5a Mass spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

From the recorded Mass spectrum, the obtained spectral values are shown in Table 4.3a.

2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide	**Impurity -3**
Molecular Formula	$C_{11}H_{10}ClN_3OS$
Theoretical Molecular Weight	267.73
Mass Fragmentation Peaks	267.8 (M), 269.7 (M^{+2})

Table 4.4a Obtained spectral values from the recorded Mass spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

Discussion: The most abundant positive ion peak is observed at m/e 267.8 indicating molecular ion peak (M^+) of 2-a mino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide. Peaks observed at m/e 269.7 (M^{2+}) of 2-Amino-N-(2-chloro-6-methylphenyl) thiazole-5-carboxamide confirmed the presence of one chlorine atoms in the structure.

Conclusion: The positive ion mass spectral analysis of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide observed at m/e 267.8 (M^+) suggesting the possibility of molecular formula $C_{11}H_{10}ClN_3OS$ which confirms the theoretical molecular weight of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide.

2. ^{1}H-NMR spectroscopy

Instrument used : AVANCE II 400

Make : BRUKER

Test preparation: About 10 mg of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide was weighed, dissolved in 0.5 ml of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into a clean and dry NMR Tube.

Procedure: ^{1}H-NMR spectra has been recorded of the above test solution under the specified operational conditions of the instrument.

The ^{1}H-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide is represented in Fig 4.5b.

Interpretation:

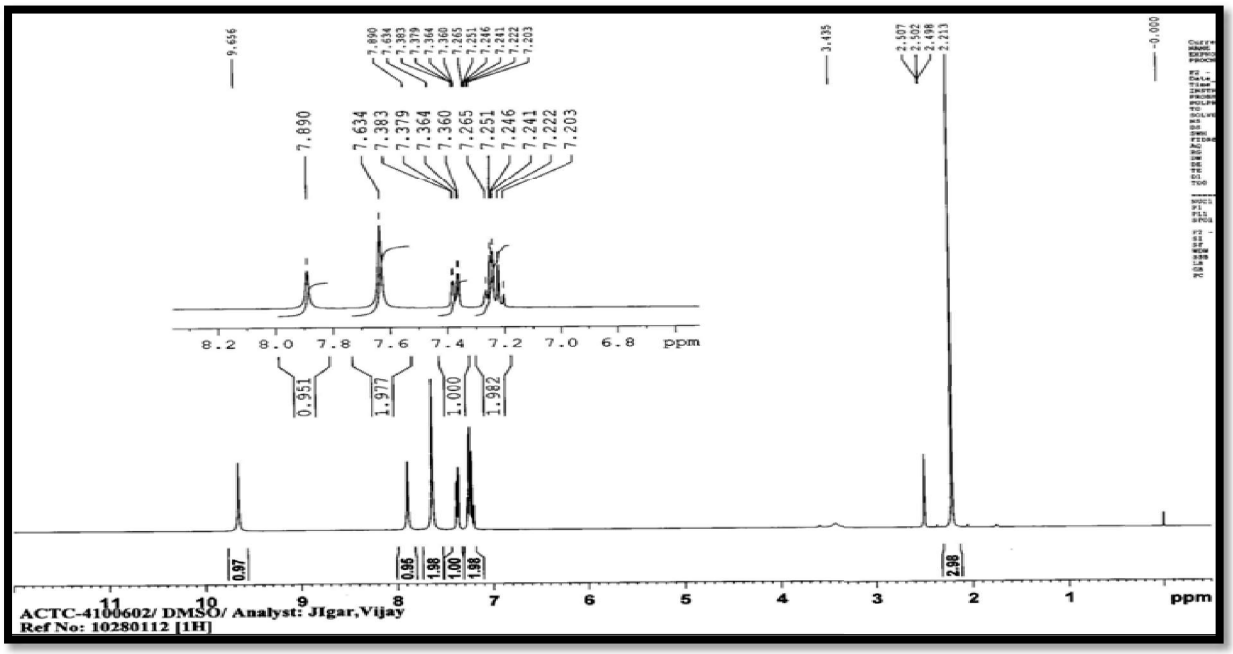


Fig 4.5b ^{1}H-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

From the recorded ^{1}H-NMR spectrum, chemical shifts and the multiplicity of the corresponding protons are shown in Table 4.3b

Proton No. *	Chemical Shift (δ value in ppm)	Multiplicity	Assigned Proton
A	2.21	Singlet	3H of Methyl group (-CH_3)
b, c	7.20-7.26	Multiplet	1H of Aromatic group
D	7.36-7.38	Doublet of doublet	1H Aromatic ring
E	7.63	Singlet	2H of Amine group
F	7.89	Singlet	1H of Thiazole ring
G	9.65	Singlet	1H of amide proton

* Refer structure for Proton identification.

Table 4.3b Chemical shifts and the multiplicity of the corresponding protons from the recorded ^{1}H-NMR spectrum 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

Discussion: The ^{1}H-NMR result obtained for 2-Amino-N-(2-chloro-6-methylphenyl) thiazole-5-carboxamide is as below:

The protons of methyl group appear at δ 2.21 ppm.

The protons of aromatic ring appear at δ 7.20-7.38 ppm.

The protons of thiazole ring appear at δ 7.89 ppm

The protons of amide group appear at δ 9.65 ppm

Conclusion: The data generated from the study of ^{1}H-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide is in accordance with the following structure.

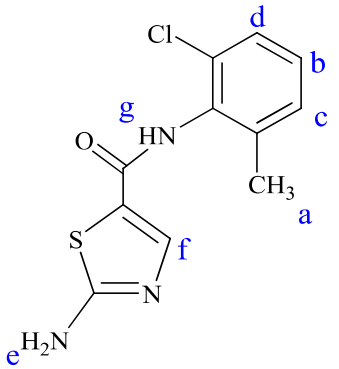


Fig 4.5c Structure generated from ^{1}H-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

3. ^{13}C-NMR Spectroscopy

Instrument used : AVANCE II 400

Make : BRUKER

Test preparation: About 40 mg of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide weighed, dissolved in 0.5 mL of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into clean and dry NMR Tube.

Procedure: ^{13}C-NMRSpectra has been recorded of the test solution under the specified operational conditions of the instrument.

The ^{13}C-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide is represented in Fig 4.5d.

Interpretation:

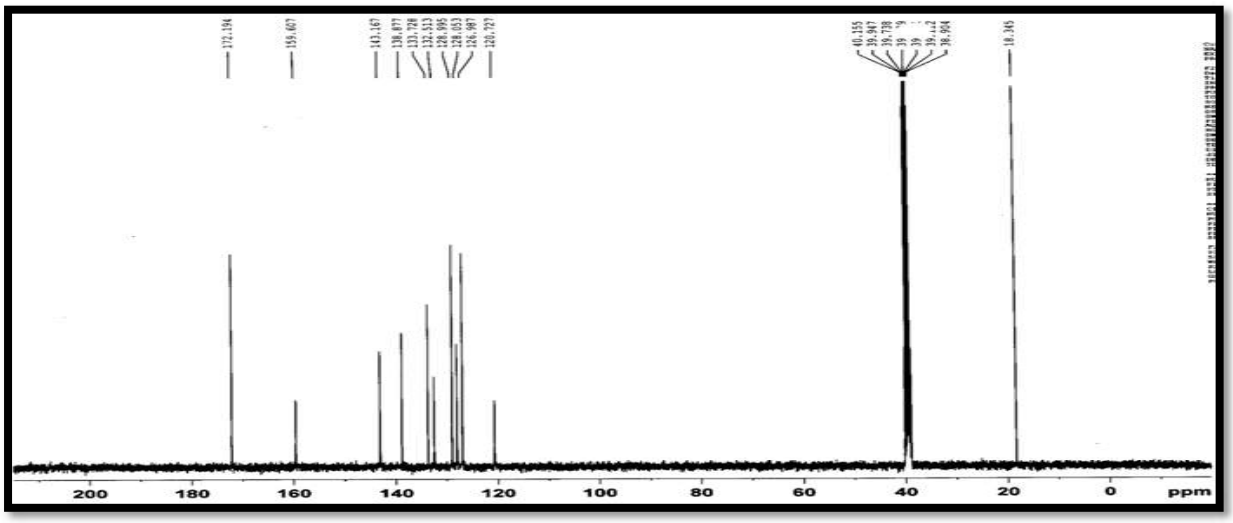


Fig 4.5d ^{13}C-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

From the recorded ^{13}C-NMR spectrum, chemical shifts of corresponding carbons are shown in Table 4.3c.

Carbon No.*	Chemical Shift (δ value in ppm)	Nature of Carbon	Assigned Carbon
A	18.34	Primary	Methyl group (-CH_3)
B	120.72	Tertiary	Thiazole ring

C	126.98	Tertiary	Aromatic ring
D	128.05	Tertiary	Aromatic ring
E	128.99	Tertiary	Aromatic ring
F	132.51	Quaternary	Aromatic ring
G	133.72	Quaternary	Aromatic ring
H	138.87	Quaternary	Thiazole ring
I	143.16	Quaternary	Aromatic ring
J	159.60	Quaternary	Thiazole ring
K	172.19	Quaternary	Amide group

* Refer structure for Carbon identification.

Table 4.3c Chemical shifts of corresponding carbons from the recorded ^{13}C-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

Conclusion: The data generated from the study of ^{13}C-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide is in accordance with the following structure.

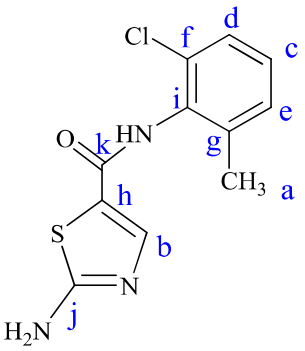


Fig 4.5e Structure generated from ^{13}C-NMR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

4. IR Spectroscopy

Instrument used : FT-IR-8300

Make : Shimadzu

Test preparation: About 2 mg of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamidetriturated with 300 mg of finely powdered and dried potassium bromide. The

mixture was carefully grinded, spread uniformly in a suitable die and submitted in vacuum to a pressure of about 800 MPa (80 kg/cm^2).

Procedure: The infrared absorption spectrum of the above potassium bromide pellet was recorded between 4000 cm^{-1} and 400 cm^{-1} under the specified operational conditions of the instrument.

The IR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide is represented in Fig 4.5f.

Interpretation:

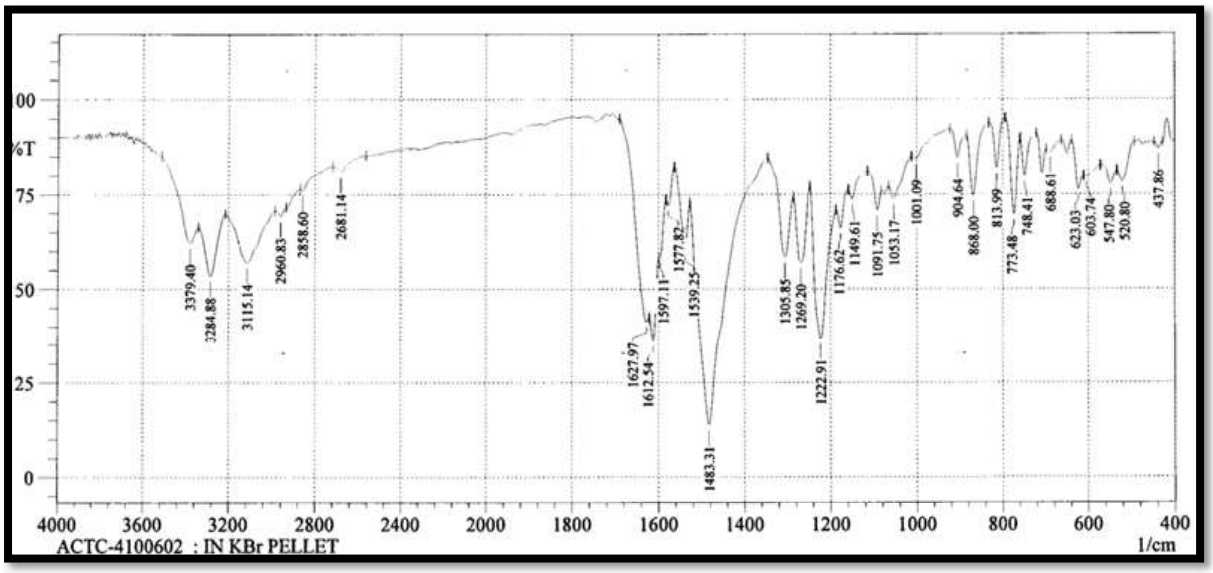


Fig 4.5f IR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

From the recorded IR spectrum, the wave numbers of corresponding groups are shown in Table 4.3d.

Wave number (cm^{-1})	Assignment
3379	-NH Asymmetric stretching of amine
3284	-NH Symmetric stretching of amine
1627	-CO stretching of amide
1597	-C=C Stretching of Aromatic ring
1483	-C=C Stretching of Aromatic ring
1091	-C-Cl Stretching

Table 4.3d Wave numbers of corresponding groups from the recorded IR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

Conclusion: The data generated from the study of IR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide is in accordance with the following structure.

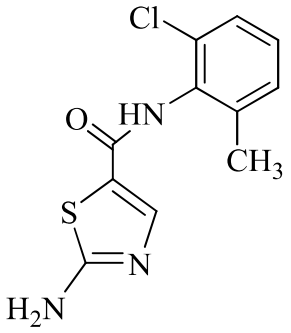


Fig 4.5g Structure generated from IR spectrum of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

5. Summary of study: The structural characterization study carried out for 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide using Mass, ^{1}H-NMR, ^{13}C-NMR and IR spectroscopy reveals the following structure of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide, which matches with the structure given in literature.

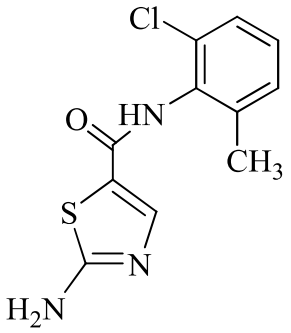


Fig 4.5h structure of 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide

6. **Remarks:** Based on these studies and data compound 2-Amino-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide is qualified as in-house reference standard for intended use.

4.6 Structure Elucidation and Characterization of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid (Impurity-4)

The sample of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid (Synthesized at cadila

healthcare ltd., Ahmedabad) as primary reference standard has been analyzed by using following analytical techniques to characterize and conclude the structure.

1. Mass Spectroscopy
2. ^{1}H-NMR Spectroscopy
3. ^{13}C-NMR Spectroscopy
4. IR Spectroscopy

1. Mass Spectroscopy

Instrument used : Quattro Micro Mass

Make : Waters

Test preparation: Test solution of concentration 1mg/ml of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acidin methanol was prepared and used.

Procedure: Mass Spectra has been acquired of the above test solution under the specified conditions of the instrument.

The Mass spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid is represented in Fig 4.6a.

Interpretation:

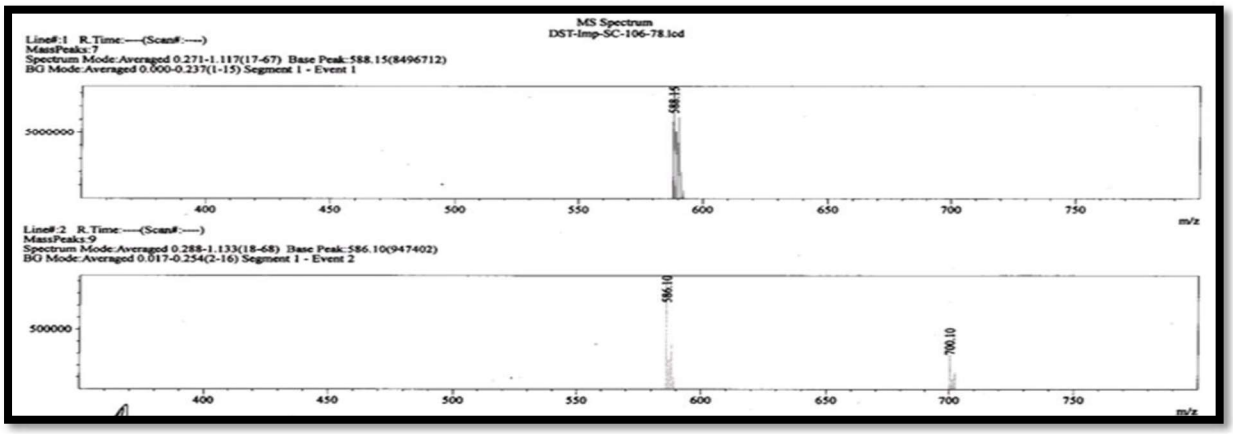


Fig 4.6a Mass spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

From the recorded Mass spectrum, the obtained spectral values are shown in Table 4.4a.

Dasatinib Imp-4	**Impurity-4**
Molecular Formula	$C_{26}H_{30}ClN_7O_5S$
Theoretical Molecular Weight	588.08
Mass Fragmentation Peaks	588.15 (M^+)

Table 4.4aObtained spectral values from the recorded Mass spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

Discussion: The most abundant positive ion peak is observed at m/e 588.15 indicating molecular ion peak (M^+) of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid.

Conclusion: The positive ion Mass spectral analysis of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid observed at m/e 588.15 (M^+) suggesting the possibility of Molecular formula $C_{22}H_{26}ClN_7O_2S$ which confirms the theoretical molecular weight of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid.

2. ^{1}H-NMR spectroscopy

Instrument used : AVANCE II 400

Make : BRUKER

Test preparation: About 10 mg of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid) was weighed, dissolved in 0.5 ml of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into a clean and dry NMR Tube.

Procedure: ^{1}H-NMRSpectra has been recorded of the above test solution under the specified operational conditions of the instrument.

The ^{1}H-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid is represented in Fig 4.7b.

Interpretation:

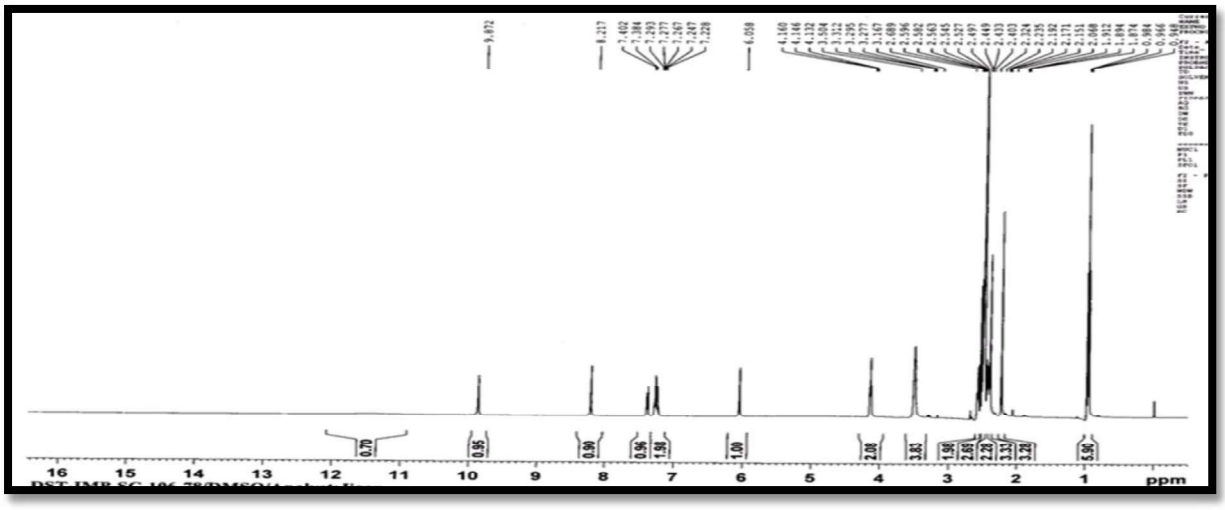


Fig 4.6b ^{1}H-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

From the recorded ^{1}H-NMR spectrum, chemical shifts and the multiplicity of the corresponding protons are shown in Table 4.4b.

Proton No. *	Chemical Shift (δ value in ppm)	Multiplicity	Assigned Proton
a,b	0.96	Singlet	3Hx2 of Methyl group (Ar-CH_3)
C	2.06-2.19	Multiplet	2Hof -CH2-
D	2.32	Singlet	2Hof -CH2-
E	2.49	Multiplet	2Hof -CH2-N
F	2.52-2.59	Multiplet	2H of -CH2-O
G	3.50	Singlet	2H of Piperazine ring
H	4.13-4.16	Triplet	2H of Piperazine ring
I	6.05	Singlet	1H of pyrimidine ring
J	7.22-7.29	Multiplet	1H of aromatic ring
K	7.38-7.40	Multiplet	1H of aromatic ring
L	8.21	Singlet	1H of thiazole group
M	9.87	Singlet	1H of amide group
N	11.5	Singlet	1H of acid group

* Refer structure for Proton identification.

Table 4.4b Chemical shifts and the multiplicity of the corresponding protons from the recorded ^{1}H-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

Discussion: The ^{1}H-NMR result obtained for Impurity-4 is as below:

The protons of methyl group appear at δ 0.96 ppm.

The methylene protons of piperazine appear at δ 3.5 and δ 4.13-4.16 ppm.

The protons of aromatic ring appear at δ 7.22-7.40 ppm.

The protons of thiazole ring appear at δ 8.21 ppm.

Conclusion: The data generated from the study of ^{1}H-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid is in accordance with the following structure.

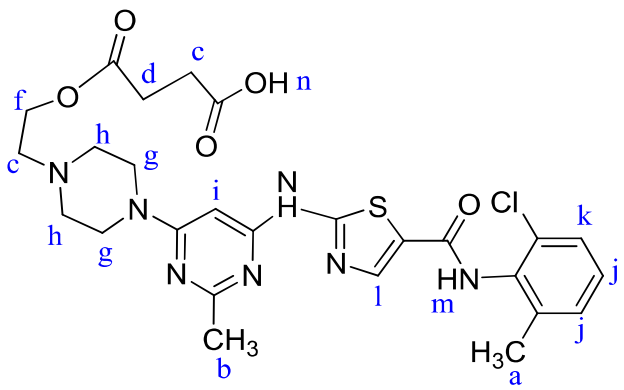


Fig 4.6c Structure elucidation from ^{1}H-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

3. ^{13}C-NMR Spectroscopy

Instrument used : AVANCE II 400

Make : BRUKER

Test preparation: About 40 mg of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid weighed, dissolved in 0.5 mL of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into clean and dry NMR Tube.

Procedure: ^{13}C-NMRSpectra has been recorded of the test solution under the specified operational conditions of the instrument.

The ^{13}C-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid is represented in Fig 4.6d.

Interpretation:

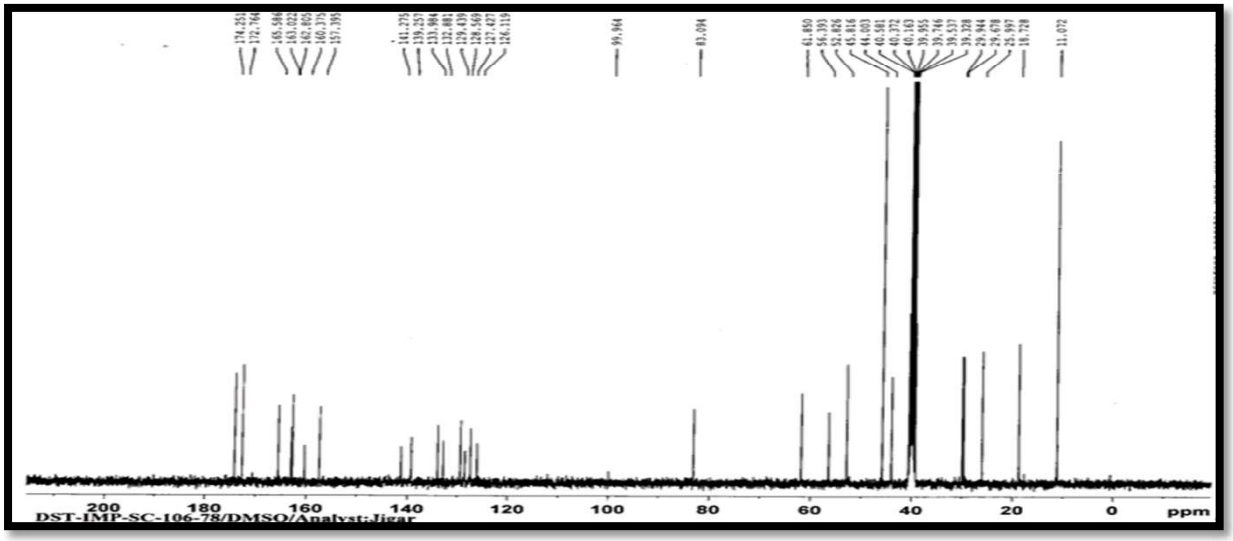


Fig 4.6d ^{13}C-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

From the recorded ^{13}C-NMR spectrum, chemical shifts of corresponding carbons are shown in Table 4.4c.

Carbon No.*	Chemical Shift (δ value in ppm)	Nature of Carbon	Assigned Carbon
A	11.07	Primary	Methyl group
B	18.72	Primary	Methyl group
C	29.67	Secondary	-CH_2- group
D	29.94	Secondary	-CH_2- group
E	44.0	Secondary	-CH_2- group of piperazine ring
F	45.81	Secondary	-CH_2- group of piperazine ring
G	56.39	Secondary	-CH_2- group
H	61.85	Secondary	-CH_2- group
I	83.09	Tertiary	Pyrimidine ring
J	126.11	Tertiary	Thiazole ring

K	127.42	Tertiary	Aromatic ring
L	128.56	Tertiary	Aromatic ring
M	129.43	Tertiary	Aromatic ring
N	132.88	Quaternary	Aromatic ring
O	133.98	Quaternary	Aromatic ring
P	139.25	Quaternary	Thiazole ring
Q	141.27	Quaternary	Aromatic ring
R	157.39	Quaternary	Thiazole ring
S	160.37	Quaternary	Amide Group
T	162.80	Quaternary	Pyrimidine ring
U	163.02	Quaternary	Pyrimidine ring
V	165.58	Quaternary	Pyrimidine ring
W	172.76	Quaternary	Carbonyl group
X	174.25	Quaternary	Carbonyl group

* Refer structure for Carbon identification.

Table 4.4c Chemical shifts of corresponding carbons from the recorded ^{13}C-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

Conclusion: The data generated from the study of ^{13}C-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid) is in accordance with the following structure.

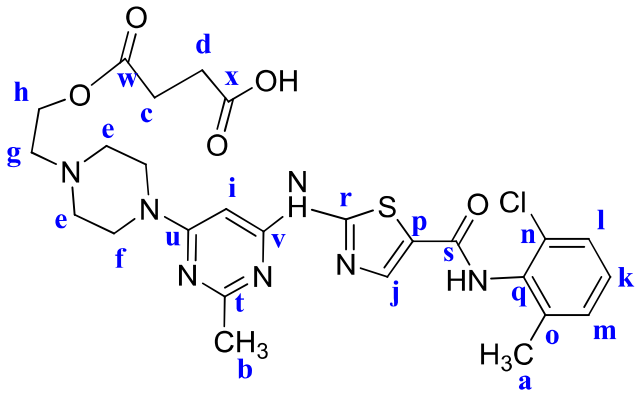


Fig 4.6e Structure elucidation from ^{13}C-NMR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

5. IR Spectroscopy

Instrument used : FT-IR-8300

Make : Shimadzu

Test preparation: About 2 mg of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid triturated with 300 mg of finely powdered and dried potassium bromide. The mixture was carefully grinded, spread uniformly in a suitable die and submitted in vacuum to a pressure of about 800 MPa (80 kg/cm^2).

Procedure: The infrared absorption spectrum of the above potassium bromide pellet was recorded between 4000 cm^{-1} and 400 cm^{-1} under the specified operational conditions of the instrument.

The IR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid)is represented in Fig 4.6f.

Interpretation:

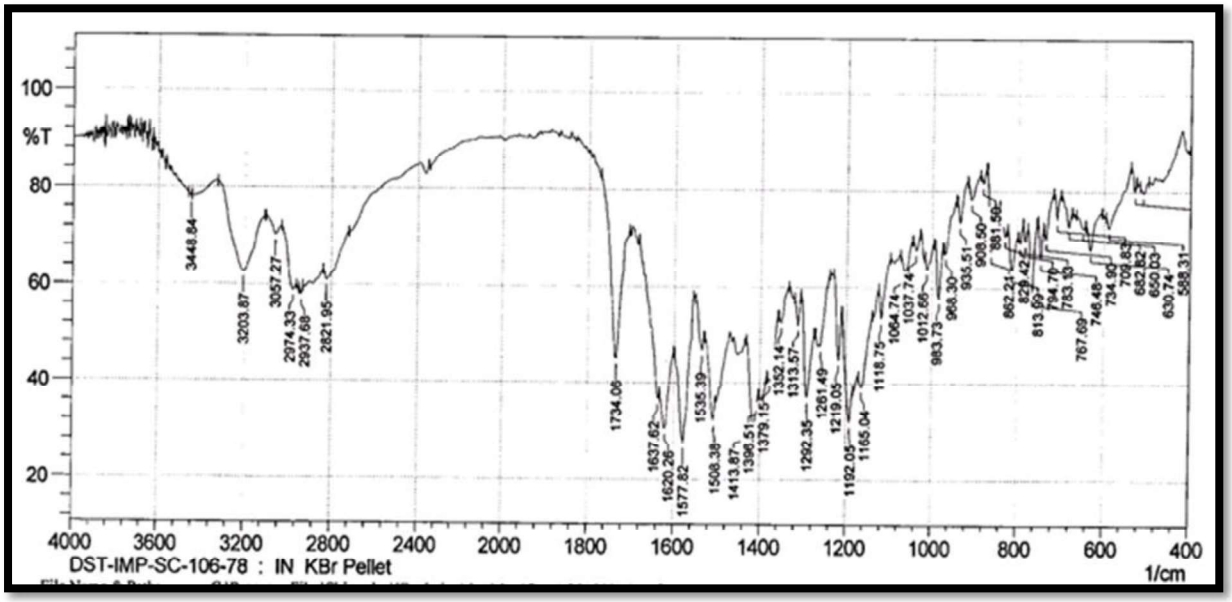


Fig 4.6f IR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

From the recorded IR spectrum, the wave numbers of corresponding groups are shown in Table 4.4d.

Wave number (cm^{-1})	Assignment
3448	-OH Stretching of Hydroxy group
3203	-NH stretching of Amine
3057	-CH Stretching of Aromatic proton
2974	-CH Stretching of $–CH_3$
1620	-C=O stretching of amide
1577	-C=C stretching of aromatic ring
1535	-C=N stretching of aromatic ring
767	-N-H Wagging

Table 4.4d Wave numbers of corresponding groups from the recorded IR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

Conclusion: The data generated from the study of IR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid) is in accordance with the following structure.

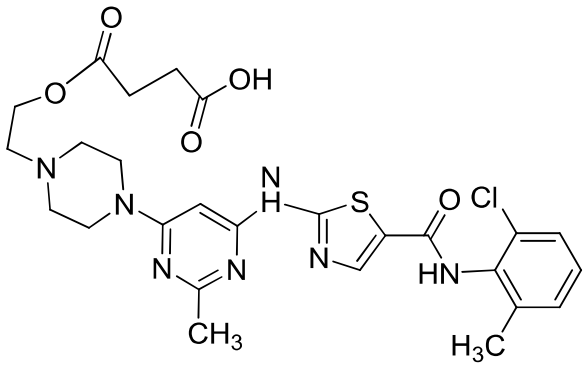


Fig 4.6g Structure elucidation from IR spectrum of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

6. Summary of study: The structural characterization study carried out for 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid using MS, ^{1}H-NMR, ^{13}C-NMR and IR spectroscopy reveals the following structure of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid, which matches with the structure given in literature.

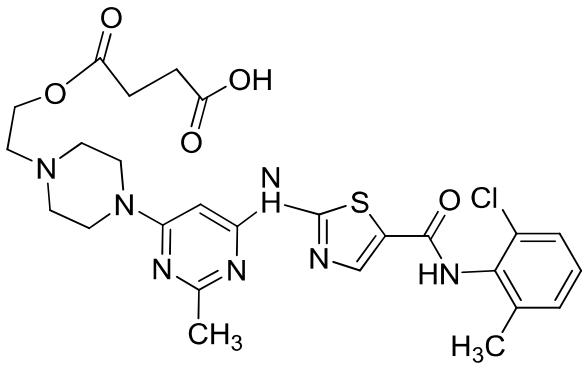


Fig 4.6h structure of 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid

7. Remarks: Therefore compound 4-(2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl) thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethoxy)-4-oxobutanoic acid is qualified as in-house reference standard for intended use.

4.7 Structure Elucidation and Characterization of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide (Impurity 5)

The sample of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide (Synthesized at cadila healthcare ltd., Ahmedabad) as primary reference standard has been analyzed by using following analytical techniques to characterize and conclude the structure.

1. Mass Spectroscopy
2. ^{1}H-NMR Spectroscopy
3. ^{13}C-NMR Spectroscopy
4. IR Spectroscopy

1. Mass Spectroscopy

Instrument used : Quattro Micro Mass

Make : Waters

Test preparation: Test solution of concentration 1 mg/mL of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide in methanol was prepared and used.

Procedure: Mass Spectra has been acquired of the above test solution under the specified conditions of the instrument.

The Mass spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide is represented in Fig 4.7a.

Interpretation:

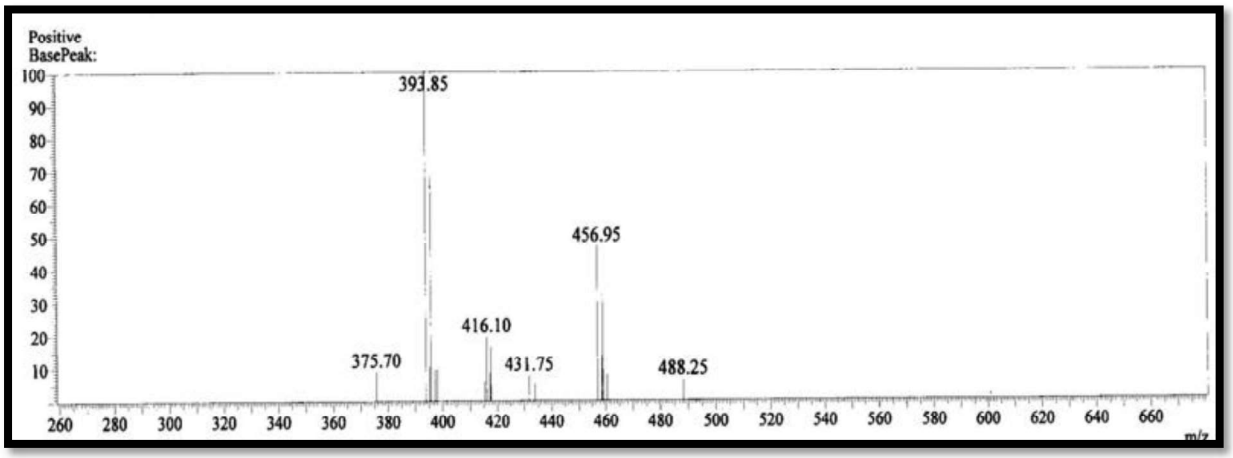


Fig 4.7a Mass spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

From the recorded Mass spectrum, the obtained spectral values are shown in Table 4.5a.

N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide	**Impurity-5**
Molecular Formula	$C_{16}H_{13}Cl_2N_5OS$
Theoretical Molecular Weight	394.28
Mass Fragmentation Peaks	393.85 (M), 396 (M^{+2}), 398 (M^{+4}), 416.1 (M+Na), 456.95 (M+Na+ACN)

Table 4.5a Obtained spectral values from the recorded Mass spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

Discussion: The most abundant positive ion peak is observed at m/e 393.85 indicating molecular ion peak (M) of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide. Peaks observed at m/e 396 (M^{+2}) and 398(M^{+4}) confirmed the presence of two chlorine atoms in the structure.

Conclusion: The positive ion Mass spectral analysis of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide observed at m/e 393.8 (M^{+1}) suggesting the possibility of Molecular formula $C_{16}H_{13}Cl_2N_5OS$ which confirms the theoretical molecular weight of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide.

5. ^{1}H-NMR spectroscopy

Instrument used : AVANCE II 300

Make : BRUKER

Test preparation: About 10 mg of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide was weighed, dissolved in 0.5 ml of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into a clean and dry NMR Tube.

Procedure: ^{1}H-NMRSpectra has been recorded of the above test solution under the specified operational conditions of the instrument.

The ^{1}H-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide is represented in Fig 4.7b.

Interpretation:

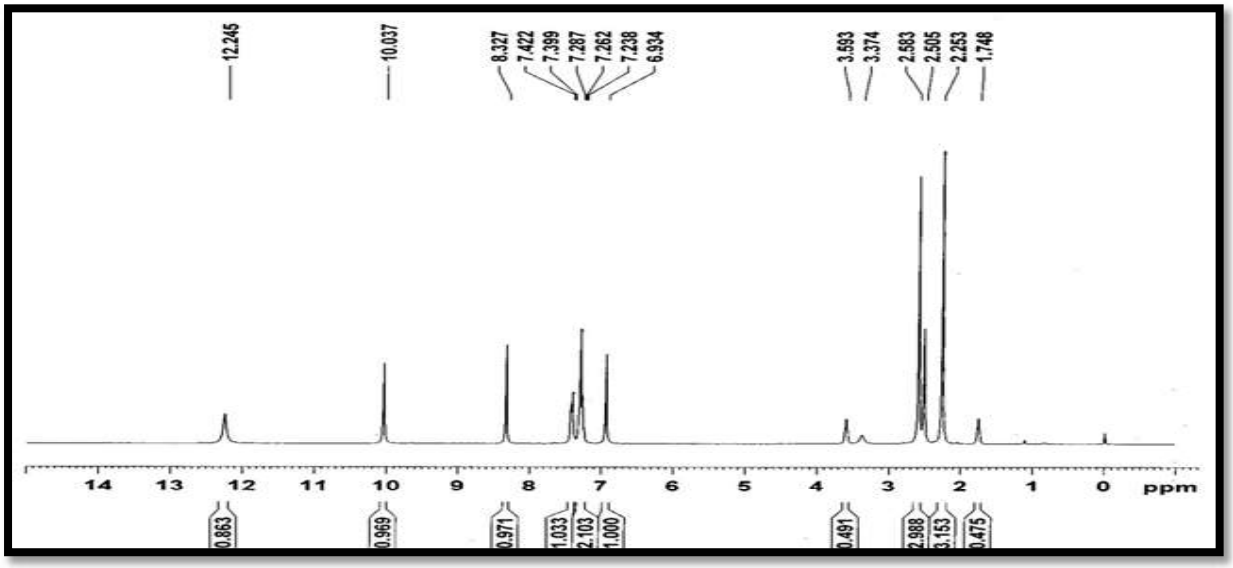


Fig 4.7b ^{1}H-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

From the recorded ^{1}H-NMR spectrum, chemical shifts and the multiplicity of the corresponding protons are shown in Table 4.5b.

Proton No. *	Chemical Shift (δ value in ppm)	Multiplicity	Assigned Proton
A	2.25	Singlet	3H of Methyl group (-CH_3) Aromatic ring
B	2.58	Singlet	3H of Methyl group (-CH_3) Pyrimidine ring
C	6.93	Singlet	1H Pyrimidine ring
d, e	7.23-7.28	Triplet	2H of Aromatic ring
F	7.39-7.42	Doublet	1H of Aromatic ring
G	8.32	Singlet	1H of Thiazole ring
H	10.03	Singlet	1H of Amine group
I	12.24	Singlet	1H of amide proton

* Refer structure for Proton identification.

Table 4.5b Chemical shifts and the multiplicity of the corresponding protons from the recorded ^{1}H-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

Discussion: The ^{1}H-NMR result obtained for N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide is as below:

The protons of methyl group appear at δ 2.25 ppm.

The protons of aromatic ring appear at δ 7.23-8.32 ppm.

The protons of pyrimidine ring appear at δ 6.93 ppm.

The protons of thiazole ring appear at δ 8.32 ppm.

Conclusion: The data generated from the study of ^{1}H-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide is in accordance with the following structure.

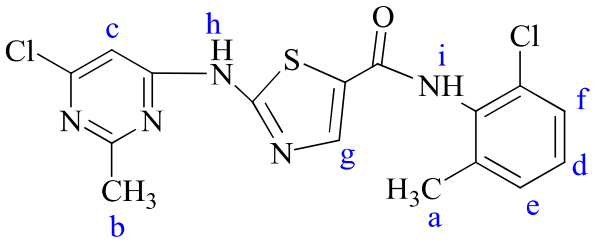


Fig 4.7c Structure elucidation from ^{1}H-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

6. ^{13}C-NMR Spectroscopy

Instrument used : AVANCE II 300

Make : BRUKER

Test preparation: About 40 mg of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide weighed, dissolved in 0.5 mL of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into clean and dry NMR Tube.

Procedure: ^{13}C-NMRSpectra has been recorded of the test solution under the specified operational conditions of the instrument.

The ^{13}C-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide is represented in Fig 4.7d.

Interpretation:

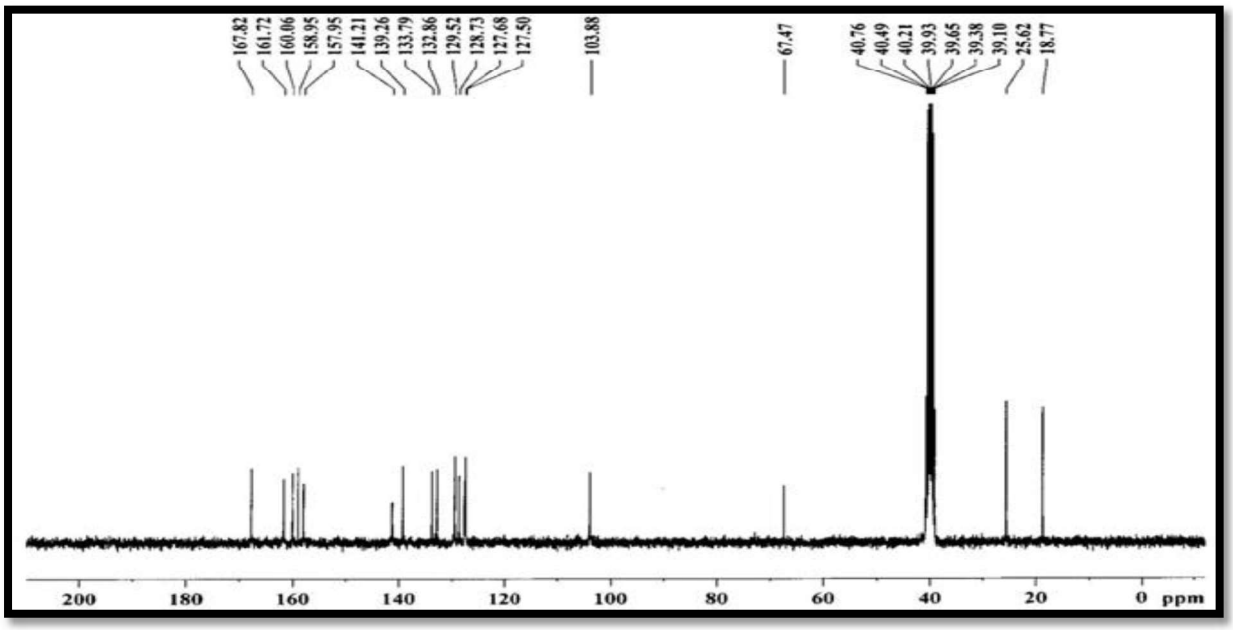


Fig 4.7d ^{13}C-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

From the recorded ^{13}C-NMR spectrum, chemical shifts of corresponding carbons are shown in Table 4.5c.

Carbon No.*	Chemical Shift (δ value in ppm)	Nature of Carbon	Assigned Carbon
A	18.77	Primary	Methyl group (-CH_3)of Aromatic ring
b	25.62	Primary	Methyl group (-CH_3)of pyrimidine ring
c	103.88	Tertiary	Pyrimidine ring
d	127.50	Tertiary	Thiazole ring
e	127.68	Tertiary	Aromatic ring
F	128.73	Tertiary	Aromatic ring
G	129.52	Tertiary	Aromatic ring
h	132.86	Quaternary	Aromatic ring
i	133.79	Quaternary	Aromatic ring
j	139.26	Quaternary	Thiazole ring
k	141.21	Quaternary	Aromatic ring
l	157.95	Quaternary	Thiazole ring
m	158.95	Quaternary	Amide group
n	160.06	Quaternary	Pyrimidine ring
o	161.72	Quaternary	Pyrimidine ring
p	167.82	Quaternary	Pyrimidine ring

* Refer structure for Carbon identification.

Table 4.5c Chemical shifts of corresponding carbons from the recorded ^{13}C-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

Conclusion: The data generated from the study of ^{13}C-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide is in accordance with the following structure.

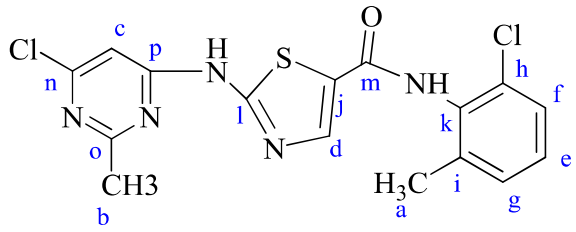


Fig 4.7e Structure elucidation from ^{13}C-NMR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

7. IR Spectroscopy

Instrument used : FT-IR-8300

Make : Shimadzu

Test preparation: About 2 mg of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide triturated with 300 mg of finely powdered and dried potassium bromide. The mixture was carefully grinded, spread uniformly in a suitable die and submitted in vacuum to a pressure of about 800 MPa (80 kg/cm^2).

Procedure: The infrared absorption spectrum of the above potassium bromide pellet was recorded between 4000 cm^{-1} and 400 cm^{-1} under the specified operational conditions of the instrument.

The IR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide is represented in Fig 4.7f.

Interpretation:

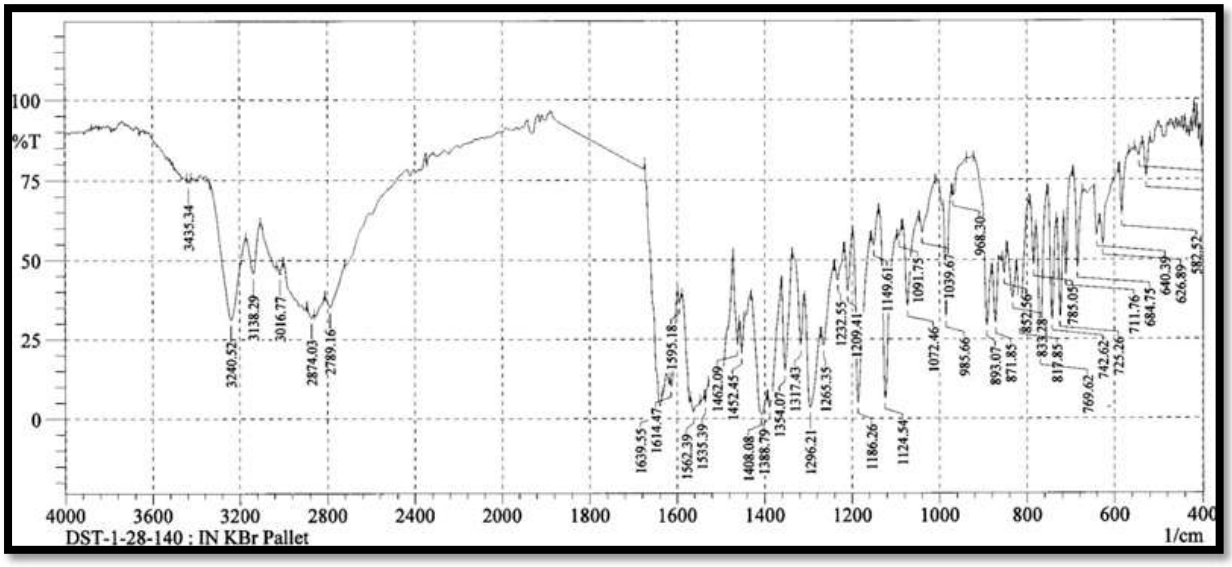


Fig 4.7f IR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

From the recorded IR spectrum, the wave numbers of corresponding groups are shown in Table 4.5d.

Wave number (cm^{-1})	Assignment
3240	-NH stretching of amine
3016	-CH stretching of Aromatic ring proton
2874	-CH Stretching of CH_3
1639	-CO stretching of amide
1562	-C=C Stretching of Aromatic ring
1535	-C=N stretching pyrimidine ring
1408	-C=C Stretching of Aromatic ring
1072	-C-Cl Stretching

Table 4.5d Wave numbers of corresponding groups from the recorded IR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

Conclusion: The data generated from the study of IR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide is in accordance with the following structure.

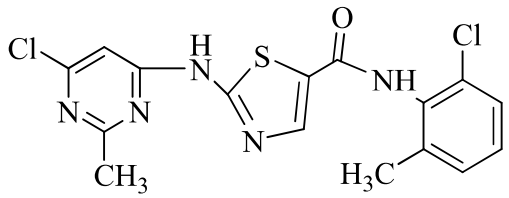


Fig 4.7g Structure elucidation from IR spectrum of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

8. Summary of study: The structural characterization study carried out for N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazolecarboxamide using Mass, ^{1}H-NMR, ^{13}C-NMR and IR spectroscopy reveals the following structure of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide, which matches with the structure given in literature.

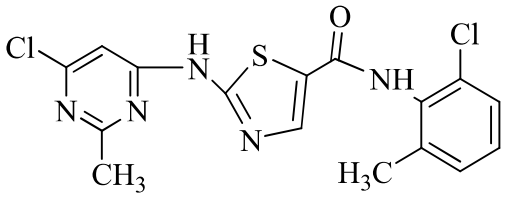


Fig 4.7h structure of N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl) amino]-5-thiazole carboxamide

9. **Remarks:** Therefore compound N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide is qualified as in-house reference standard for intended use.

4.8 Structure Elucidation and Characterization of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate (Impurity 6)

The sample of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate(Synthesized at cadila healthcare ltd., Ahmedabad) as primary reference standard.

1. Mass Spectroscopy

Instrument used : Quattro Micro Mass

Make : Waters

Test preparation: Test solution of concentration 1mg/ml of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetatein methanol was prepared and used.

Procedure: Mass Spectra has been acquired of the above test solution under the specified conditions of the instrument.

The Mass spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetateis represented in Fig 4.8a.

Interpretation:

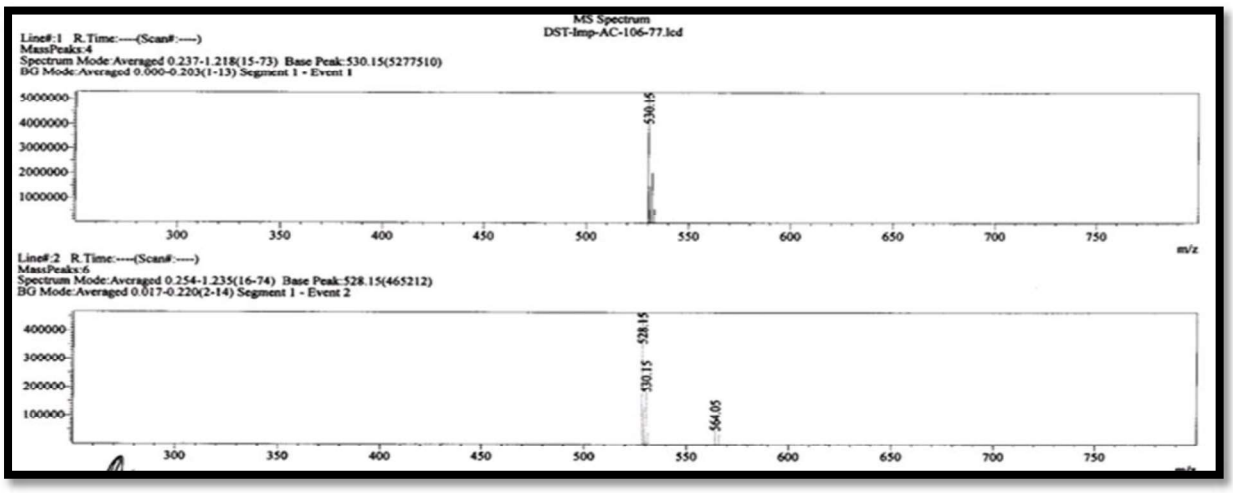


Fig 4.8a Mass spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

From the recorded Mass spectrum, the obtained spectral values are shown in Table 4.6a.

DST-3	**Impurity-6**
Molecular Formula	$C_{24}H_{28}ClN_7O_3S$
Theoretical Molecular Weight	530.04
Mass Fragmentation Peaks	530.15 (M^+)

Table 4.6a Obtained spectral values from the recorded Mass spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

Discussion: The most abundant positive ion peak is observed at m/e 530.15 indicating molecular ion peak (M^+) of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate.

Conclusion: The positive ion Mass spectral analysis of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetateobserved at m/e 530.15(M^+) suggesting the possibility of Molecular formula $C_{22}H_{26}ClN_7O_2S$ which confirms the theoretical molecular weight of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate.

2. ^{1}H-NMR spectroscopy

Instrument used : AVANCE II 400
Make : BRUKER

Test preparation: About 10 mg of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate was weighed, dissolved in 0.5 ml of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into a clean and dry NMR Tube.

Procedure: ^{1}H-NMRSpectra has been recorded of the above test solution under the specified operational conditions of the instrument.

The ^{1}H-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate is represented in Fig 4.8b.

Interpretation:

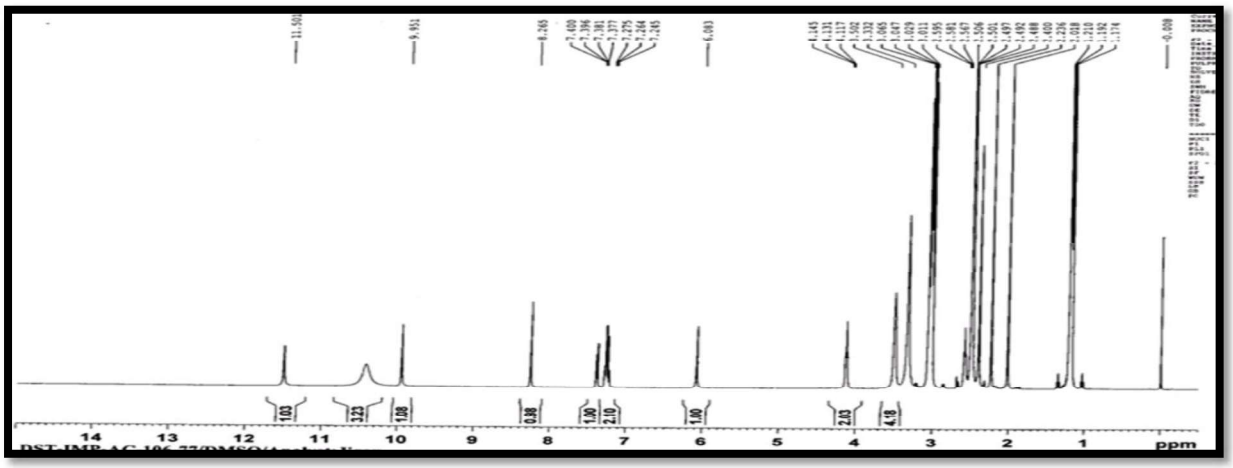


Fig 4.8b ^{1}H-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

From the recorded ^{1}H-NMR spectrum, chemical shifts and the multiplicity of the corresponding protons are shown in Table 4.6b.

Proton No. *	Chemical Shift (δ value in ppm)	Multiplicity	Assigned Proton
A	2.24	Singlet	3H of Methyl group (Ar-CH_3)
b, c	2.40-2.50	Singlet	3H of Methyl group (-CH_3) of pyrimidine & 4H of piperazine ring (-CH_2)
D	2.40-2.50	Multiplet	2H of Ethanol group
e, f	3.50-3.56	Multiplet	2H of Ethanol group & 4H of piperazine ring (-CH_2)

G	4.11-4.14	Triplet	3H of methyl group
H	6.04	Singlet	1H of pyrimidine ring
i, j	7.25-7.27	Triplet	2H of aromatic ring
K	7.37-7.40	Triplet	1H of aromatic ring
L	8.26	Singlet	1H of thiazole group
M	9.95	Singlet	1H of amine group
N	11.50	Singlet	1H of amide group

* Refer structure for Proton identification.

Table 4.6b Chemical shifts and the multiplicity of the corresponding protons from the recorded ^{1}H-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

Discussion: The ^{1}H-NMR result obtained for impurity-6 is as below:

The protons of methyl group appear at δ 2.24 ppm.

The methylene protons of piperazine appear at δ 2.40-2.50 and δ 3.50-3.54 ppm.

The protons of aromatic ring appear at δ 7.25-7.40 ppm.

The protons of thiazole ring appear at δ 8.26 ppm.

Conclusion: The data generated from the study of ^{1}H-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate is in accordance with the following structure.

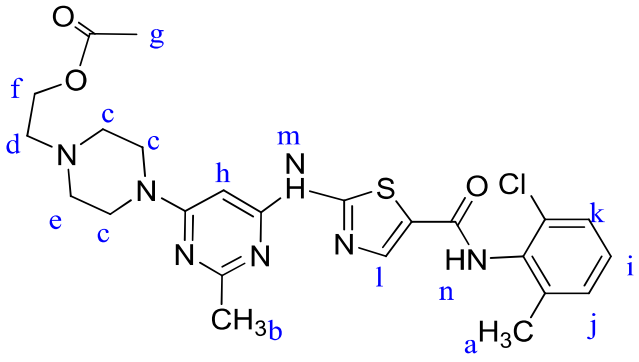


Fig 4.8c Structure elucidation from ^{1}H-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

3. ^{13}C-NMR Spectroscopy

Instrument used : AVANCE II 400

Make : BRUKER

Test preparation: About 40 mg of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate weighed, dissolved in 0.5 mL of deuterated Dimethyl sulphoxide (DMSO-d_6) and transferred into clean and dry NMR Tube.

Procedure: ^{13}C-NMRSpectra has been recorded of the test solution under the specified operational conditions of the instrument.

The ^{13}C-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate is represented in Fig 4.8d.

Interpretation:

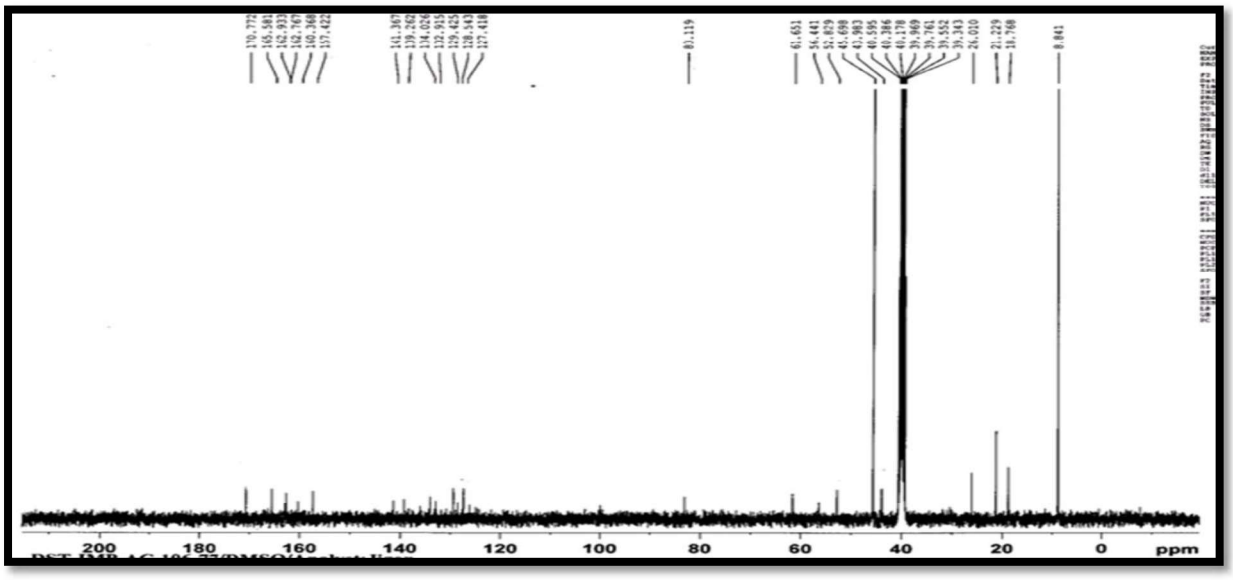


Fig 4.8d^{13}C-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

From the recorded ^{13}C-NMR spectrum, chemical shifts of corresponding carbons are shown in Table 4.6c.

Carbon No.*	Chemical Shift (δ value in ppm)	Nature of Carbon	Assigned Carbon
a	8.841	Primary	$-CH_3$
b	18.76	Primary	$-CH_3$
c	26.01	Secondary	$-CH_2-$
d	43.93	Secondary	$-CH_2$-piperazine ring
e	45.69	Secondary	$-CH_2$-piperazine ring
f	52.82	Secondary	$-CH_2-$
g	56.44	Secondary	$-CH_2-O$
h	61.65	Tertiary	Pyrimidine ring
i	83.11	Tertiary	Thiazole ring
j	126	Tertiary	Aromatic ring
k	127.41	Tertiary	Aromatic ring
l	128.54	Tertiary	Aromatic ring
m	129.42	Quaternary	Aromatic ring
n	132.91	Quaternary	Aromatic ring
o	134.02	Quaternary	Thiazole ring
p	139.26	Quaternary	Aromatic ring
q	141.36	Quaternary	Thiazole ring
r	157.42	Quaternary	Amide Group
s	160.36	Quaternary	Pyrimidine ring
t	162.67	Quaternary	Pyrimidine ring
u	165.58	Quaternary	Pyrimidine ring
v	170.77	Quaternary	Carbonyl group

* Refer structure for Carbon identification.

Table 4.6cChemical shifts of corresponding carbons from the recorded ^{13}C-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

Conclusion: The data generated from the study of ^{13}C-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate is in accordance with the following structure.

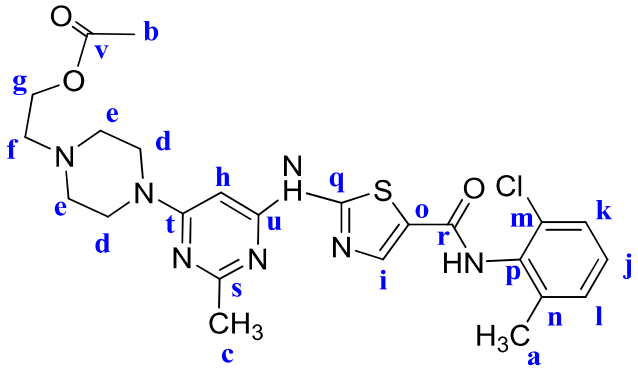


Fig 4.8e Structure elucidation from ^{13}C-NMR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

4. IR Spectroscopy

Instrument used : FT-IR-8300

Make : Shimadzu

Test preparation: About 2 mg of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate triturated with 300 mg of finely powdered and dried potassium bromide. The mixture was carefully grinded, spread uniformly in a suitable die and submitted it in vacuum to a pressure of about 800 MPa (80 kg/cm^2).

Procedure: The infrared absorption spectrum of the above potassium bromide pellet was recorded between 4000 cm^{-1} and 400 cm^{-1} under the specified operational conditions of the instrument.

The IR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetateis represented in Fig 4.8f.

Interpretation:

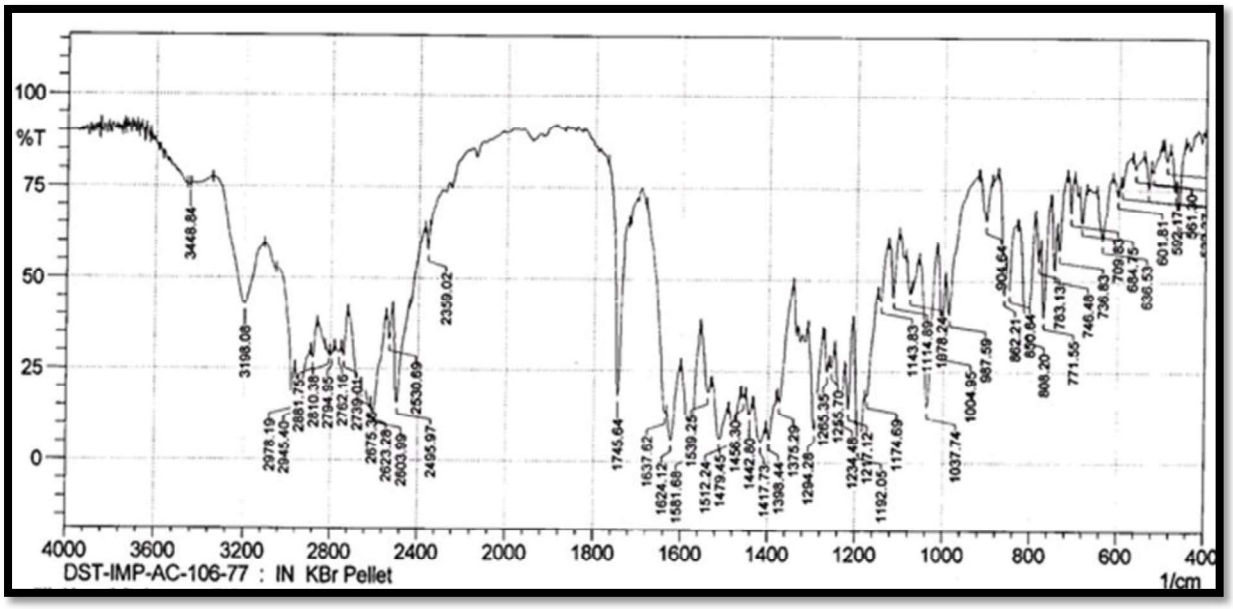


Fig 4.8f IR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

From the recorded IR spectrum, the wave numbers of corresponding groups are shown in Table 4.6d.

Wave number (cm^{-1})	Assignment
3448	-OH Stretching of Hydroxy group
3198	-NH stretching of Amine
2978	-CH Stretching of $–CH_3$
1745	-C=O stretching of acetate
1624	-C=O stretching of amide
1581	-C=C stretching of aromatic ring
1539	-C=N stretching of aromatic ring
771	-N-H Wagging

Table 4.6d Wave numbers of corresponding groups from the recorded IR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

Conclusion: The data generated from the study of IR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate is in accordance with the following structure.

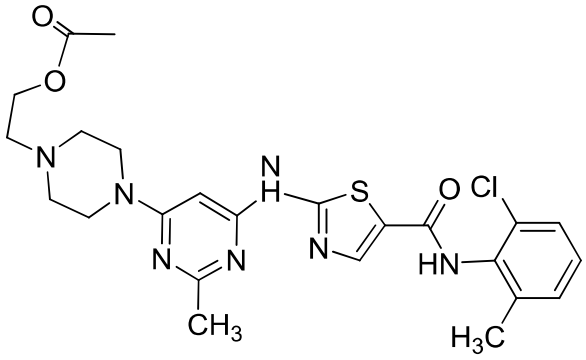


Fig 4.8g Structure elucidation from IR spectrum of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

5. Summary of study: The structural characterization study carried out for 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate) using Mass, ^{1}H-NMR, ^{13}C-NMR and IR spectroscopy reveals the following structure of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate), which matches with the structure given in literature.

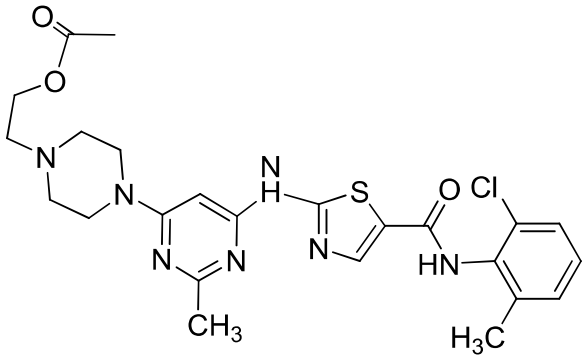


Fig 4.8h structure of 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate

6. Remarks: Based on these studies and results obtained compound 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate) is qualified as in-house reference standard for intended use.

4.9 References

1. Jabbour E, Jones D, Kantarjian HM, O'Brien S, Tam C, Koller C, Burger JA, Borthakur G, Wierda WG, Cortes J. Long-term outcome of patients with chronic myeloid leukemia treated with second-generation tyrosine kinase inhibitors after imatinib failure is predicted by the in vitro sensitivity of BCR-ABL kinase domain mutations. Blood, The Journal of the American Society of Hematology. 2009 Sep 3;114(10):2037-43.
2. Ramchandren R, Schiffer CA. Dasatinib in the treatment of imatinib refractory chronic myeloid leukemia. Biologics: targets & therapy. 2009;3:205.
3. Brave M, Goodman V, Kaminskas E, Farrell A, Timmer W, Pope S, et al. Sprycel for chronic myeloid leukemia and Philadelphia chromosome–positive acute lymphoblastic leukemia resistant to or intolerant of imatinib mesylate. Clinical Cancer Research. 2008;14(2):352-9.
4. Kim LC, Rix U, Haura EB. Dasatinib in solid tumors. Expert opinion on investigational drugs. 2010;19(3):415-25.
5. Bonifacio M, Stagno F, Scaffidi L, Krampera M, Di Raimondo F. Management of chronic myeloid leukemia in advanced phase. Frontiers in oncology. 2019;9:1132.
6. Yan R, Yang H, Xu Y. Polymorphs of Dasatinib, preparation methods and pharmaceutical compositions thereof. Google Patents; 2014.
7. Srebalus Barnes CA, Lim A. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. Mass spectrometry reviews. 2007;26(3):370-88.
8. Cooks RG, Ouyang Z, Takats Z, Wiseman JM. Ambient mass spectrometry. Science. 2006;311(5767):1566-70.
9. Andrade FJ, Shelley JT, Wetzel WC, Webb MR, Gamez G, Ray SJ, et al. Atmospheric pressure chemical ionization source. 1. Ionization of compounds in the gas phase. Analytical chemistry. 2008;80(8):2646-53.
10. McLafferty FW, Tureček F, Turecek F. Interpretation of mass spectra: University science books; 1993.
11. Ali SA. Characterization of swift heavy ion induced modification in polymeric materials: Aligarh Muslim University; 2008.
12. Günther H. NMR spectroscopy: basic principles, concepts and applications in chemistry: John Wiley & Sons; 2013. 3rd edition.
13. Jacobsen NE. NMR spectroscopy explained: simplified theory, applications and examples for organic chemistry and structural biology: John Wiley & Sons; 2007.1st edition.

14. Claridge TD. High-resolution NMR techniques in organic chemistry: Elsevier; 2016.

15. Malz F, Jancke H. Validation of quantitative NMR. Journal of pharmaceutical and biomedical analysis. 2005;38(5):813-23.

16. Ernst RR, Anderson WA. Application of Fourier transform spectroscopy to magnetic resonance. Review of Scientific Instruments. 1966;37(1):93-102.

17. Webster GK, Kumar S. Expanding the analytical toolbox: pharmaceutical application of quantitative NMR. ACS Publications; 2014.

18. Dalling DK, Ladner KH, Grant DM, Woolfenden WR. Carbon-13 magnetic resonance. 27. The dependence of chemical shifts on methyl rotational conformations and dynamics in the methylated benzenes and naphthalenes. Journal of the American Chemical Society. 1977;99(22):7142-50.

19. Laude DA, Wilkins CL. Identification of organic mixture components without separation: Quantitative and edited carbon-13 nuclear magnetic resonance spectrometry data for analysis of petroleum distillates. Analytical chemistry. 1986;58(13):2820-4.

20. Tishmack PA, Bugay DE, Byrn SR. Solid-state nuclear magnetic resonance spectroscopy-pharmaceutical applications. Journal of pharmaceutical sciences. 2003;92(3):441-74.

21. Wilson MA. NMR Techniques & Applications in Geochemistry & Soil Chemistry: Elsevier; 2013.

22. Balci M. Basic 1H-and 13C-NMR spectroscopy: Elsevier; 2005.

23. Cen H, He Y. Theory and application of near infrared reflectance spectroscopy in determination of food quality. Trends in Food Science & Technology. 2007;18(2):72-83.

24. Smith BC. Infrared spectral interpretation: a systematic approach: CRC press; 2018.

25. Burns DA, Ciurczak EW. Handbook of near-infrared analysis: CRC press; 2007.

26. Dwivedi SP, Singh KK, Singh NA, Patil AK. U.S. 9,249,134 B2, 2016.

CHAPTER 4a:

DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC METHOD FOR DETERMINATION OF IMPURITIES IN SOLID DISPERSION OF DASATINIB

LIST OF FIGURES

LIST OF TABLES

4a.1 Introduction

The regulatory bodies (1,2) give clear definition about specific impurities, profiling can be termed as “the common name have not provided for analytical activities with an aim of detection, identification and/or elucidationof the structure and quantifying inorganic, organic or even solvent residues present in bulk drugs and pharmaceutical final products." Impurities can be defined in broader term as “a molecule which is the result of degradation of drug substance by oxidation, deamination, proteolysis and many more chemical reactions the result of which could affect stability of drug product over a time” (3). Such impurities should be identified and quantified.

Quality of drug substances in different storage condition having variable temperature and humidity is a base in stability testing that determines the shelf life of any drug substance, which is an integral part of the new drug development process. As per ICH Guideline Q1A (R2), the shelf life of any drug substance is determined by stability studies. The quantification of impurities and Dasatinib API is required to be determined using stability indicating chromatographic method, as suggested by the International Conference on Harmonization (ICH) guidelines (7) and United State Pharmacopoeia (USP) (8). A few methods i.e. High performance liquid chromatography, ultra-high-performance liquid chromatography, LC-MS/MS, HPLC-MS have been reported for quantification of major tyrosine kinase inhibitors for example, Imatinib, Dasatinib and Nilotinib in human plasma (9-20).

Several methods have been reported to determine the process related impurities i.e. impurity 1 and impurity 2 (21-23). Sunil et al. have developed a RP-LC method which only control process related impurities like Imp-3 and Imp-5. However, none of the methods are validated and stability indicating. Thus, a novel robust method for the evaluation of all the process and degradation related impurities of solid dispersion of Dasatinib drug substance is needed.

In the present study we herein report the development and validation of a stability indicating chromatographic method for determination of process and degradation related impurities in solid dispersion of Dasatinib drug substance by evaluating RF values of each impurity as per validation guideline of ICH. The developed method will be of high importance for the commercial production of Dasatinib, an efficient oncological drug, with over $ 2000 million market size.

Therefore, a stability-indicating RP-HPLC method was developed for the quantitative determination of Dasatinib and its six impurities including process and degradation impurities i.e. Imp-1, 2, 3, 4, 5 and 6 (Shown in previous chapter). This method was successfully validated according to the ICH guidelines (validation of analytical procedures: test and methodology Q2). Dasatinib received marketing approval by the European Medicines Agency in November 2006 and was approved by the U.S. Food and Drug Administration in June 2006.

4a.2 Materials and Equipments:

4a.2.1. Materials and Reagents

Dasatinib standards and samples were synthesized in API Division, Cadila Healthcare Ltd. (Ahmedabad, India) and have been characterised as discussed earlier. Route of synthesis for solid dispersion of Dasatinib is described in previous chapter (24). HPLC grade acetonitrile and methanol and analytical grade ammonium acetate, acetic acid solution, hydrogen peroxide solution (30%) were purchased from Merck Specialities Pvt. Ltd. (Mumbai, India). High purity HPLC grade water was prepared by using Millipore Milli-Q plus water purification system, Bradford, PA, USA.

4a.2.2 Equipments:

Table 4a.1 Equipments and instruments used

Equipment/Instrument	Manufacture
Waters HPLC-PDA system	Milford, MA, USA
Prominence Electrospray LC–MS system	Shimadzu, Japan
Analytical balance/ Micro balance	Mettler Toledo, USA
Sonicator	PCI Analytics, India

Detector wavelength was 310 nm on Waters HPLC-PDA system and data processed using Empower 3 software; Version builds 3471. The column used for chromatography was sunniest C18 250 mm, 4.6 mm and 5 µm particle size.

4a.2.3 Preparation of Solutions

Preparation of solid dispersion of Dasatinib

The solid dispersion of Dasatinib was prepared by mixing of Dasatinib with HPMC (Hydroxy propyl methyl cellulose) in the ratio of 70:30 %, w/w (24).

Preparation of diluent

Prepare a mixture of water, acetonitrile and methanol in a ratio of 30:20:50 %v/v/v.

Preparation of solutions

Preparation of standard stock solutions for validation

Dasatinib stock solutions were prepared with a concentration of 1000 µg/ml in DMSO diluent and a stock solution of impurities composite was prepared (a mixture of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-6) at a concentration of 100 µg/ml. For Imp-5, the first stock solution was prepared with DMSO and second stock was prepared at 100 µg/ml with diluent as the concentrations used to prepare the stock are not soluble in diluent.

Preparation of sample solution

Dasatinib test solutions were prepared in a concentration of 1000 µg/mL in diluents, sonicated for 5 min. to dissolve and made up to volume with diluent.

4a.2.4 Generation of stress samples

One lot of Dasatinib Batch No. (DST-4-106-79) synthesized at Cadila Healthcare Ltd. (Ahmedabad, India) was selected for stress testing As per the ICH guideline "stress testing is likely to be carried out on a batch of material". In order to prove the stability-indicating nature and selectivity of the established method, the forced degradation studies were conducted on solid dispersion of Dasatinib drug substance.

Stress studies were performed at a concentration of 1000 µg/ml. Degradation was performed under stress condition of UV light (254 nm), heat (105°C), acid (1.0 N HCl at 60°C), base (1.0 N NaOH at 60°C) and oxidation (3% H_2O_2 at 25°C) to evaluate the capability of the proposed method to separate Dasatinib and all impurities, including process and degradation products. For thermal and photo stress studies, the study period was 24 h, for acid and alkali, the study period was approximately 1h; while for oxidation it was1.5 h. As per the ICH guidelines time period has been selected on basis of 10% to 30% of the degradation. The purity of each peak was checked using PDA detector and the purity angle was found to be less than the purity threshold, directly demonstrated that peak is pure. Mass balance of each condition stressed samples was calculated by addition of %content of Dasatinib + %known impurities + %unknown Impurities in %, w/w.

Peak purity, purity angle and purity threshold:

- Peak Purity is an analysis of absorbance spectra across the peak to determine if they are all similar or there are differences. If there are spectral differences, it implies there are two or more compounds eluting in that chromatographic peak each being spectrally different.
- The term "Purity Angle" and "Purity Threshold" is used for identifying any the co-elution of any other impurity with the analyte.
- The "threshold" means the minimum purity value that means the peak is pure. This value is usually based on a statistical analysis of the noise in the spectra. If the measured value is larger than the threshold, then the peak is considered pure. Otherwise, the peak is considered impure.
- The "Purity angle" is a measure of the spectral heterogeneity of a peak based on the comparison of spectra over the whole peak, using the spectral contrast angle

4a.2.5 Detection of impurity by LC-MS

An electrospray LC-MS system (Shimadzu Prominence HPLC coupled with Triple Quadrupole Mass Spectrometer LCMS-8040 with lab solution software, version 5.72, Japan) was used for identification of degradation impurities formed during stress testing studies. Chromatography was performed on sunniest C18 column packed with 250 mm, 4.6 mm and 5 µm particle size from ChromaNik Technologies Inc. (Made in Japan) using mobile phase A (20 mM ammonium acetate pH 5.0) and mobile phase B (mixture of methanol, buffer and acetonitrile in a ratio of 90:5:5 %, v/v/v) at a flow rate of 1.2 mL/min. The LC gradient program has been applied as per Table 4a.3. The column temperature was maintained at 35°C and Methanol: water: ACN in the ratio of 50:30:20 %, v/v/v was used as a diluent. Injection volume was 15 µL. The analysis was carried out by using electrospray ionization mode (+ve and -ve), with capillary voltage at 3500 V and collision energy at-35 V. Desolvation temperature was maintained at 250°C with a nebulizing gas flow rate of 180 L/h. The LC-MS chromatograms are presented in Fig 4a.1 to 4a.7.

4a.2.6 HPLC instrumentation and working conditions

Chromatographic experiments were performed on a Waters HPLC with photodiode array detector. The detector wavelength was set at 310 nm and data processed using Empower 3 software; Version builds 3471. The column used for chromatography was sunniest C18 250 mm, 4.6 mm and 5 µm particle size. The optimum separation was achieved using a gradient

mode. Mobile phase A was 20 mM ammonium acetate (pH 5.0); Mobile phase B was mixture of methanol, buffer and acetonitrile in a ratio of 90:5:5 (%, v/v/v). The flow rate was 1.2 ml/min. The gradient program used is shown in Table-4a.4. The column temperature was maintained at 35°C. The injection volume was 15 µL. The typical chromatogram of Dasatinib and its six impurities is provided in Fig 4a.9.

4a.2.7Analytical procedure

A 15.0 µL of blank, six replicates of system suitability solution and test sample solution were separately chromatographed. A resolution of not less than 5.0 between impurity-4 and Dasatinib was set as a system suitability requirement in system suitability solution. An 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.

4a.2.8 Method validation

The proposed method has been validated for the determination of related substances in the solid dispersion of Dasatinib by HPLC as per ICH guidelines (25).

Method Validation is required for the following reasons:

- Assuring Quality
- Achieving acceptance of products by the international agencies
- Mandatory requirement for accreditation as per ISO 17025 guidelines
- Mandatory requirement for registration of any pharmaceutical product or Pesticide formulation
- Only validated methods are acceptable for undertaking proficiency testing.
- 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.

4a.3 Result and discussion

4a.3.1 Identification of impurity by LC-MS

Identification of process and degradation related impurities for the oncology drug Dasatinib was done through LC-MS technique. Six impurities were detected in Dasatinib sample which was confirmed and identified through mass spectral analysis.

The positive ion mass spectral analysis of Dasatinib impurity-1 was observed at 444 (M) suggesting the possibility of Molecular formula $C_{20}H_{22}ClN_7OS$, which confirms the theoretical molecular weight of Impurity-1.

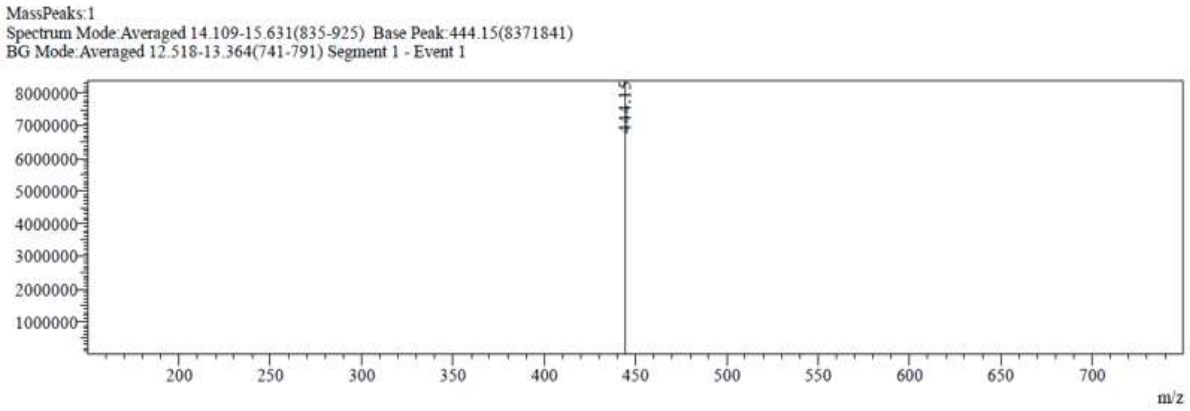


Fig 4a.1 Mass spectra of Impurity-1

The positive ion mass spectral analysis of Dasatinibimpurity-2 was observed at 504.15 (M) suggesting the possibility of Molecular formula $C_{22}H_{26}ClN_7O_3S$, which confirmed the theoretical molecular weight of Impurity-2.

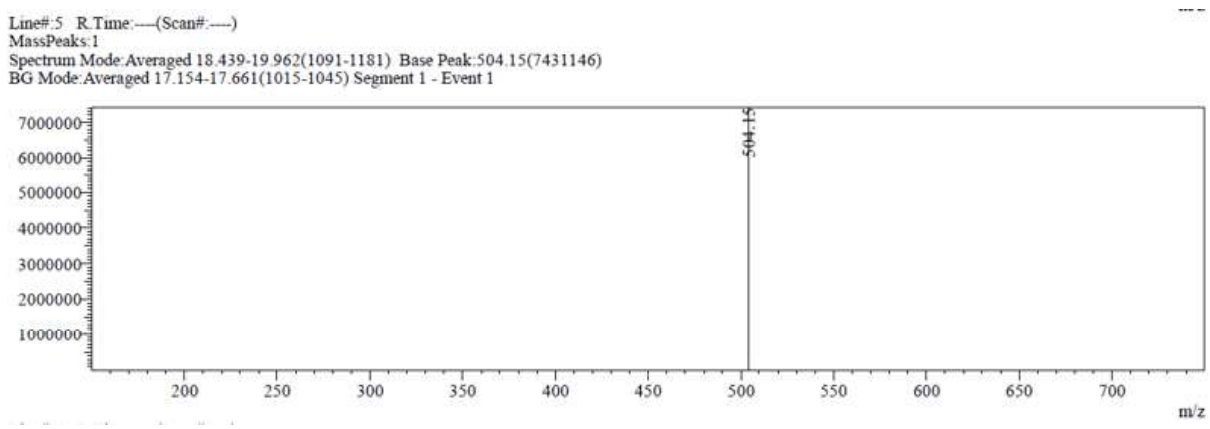


Fig 4a.2 Mass spectra of Impurity-2

The positive ion mass spectral analysis of Dasatinib impurity-3 was observed at 268.05 (M) suggesting the possibility of Molecular formula $C_{11}H_{10}ClN_3OS$, which confirms the theoretical molecular weight of Impurity-3.

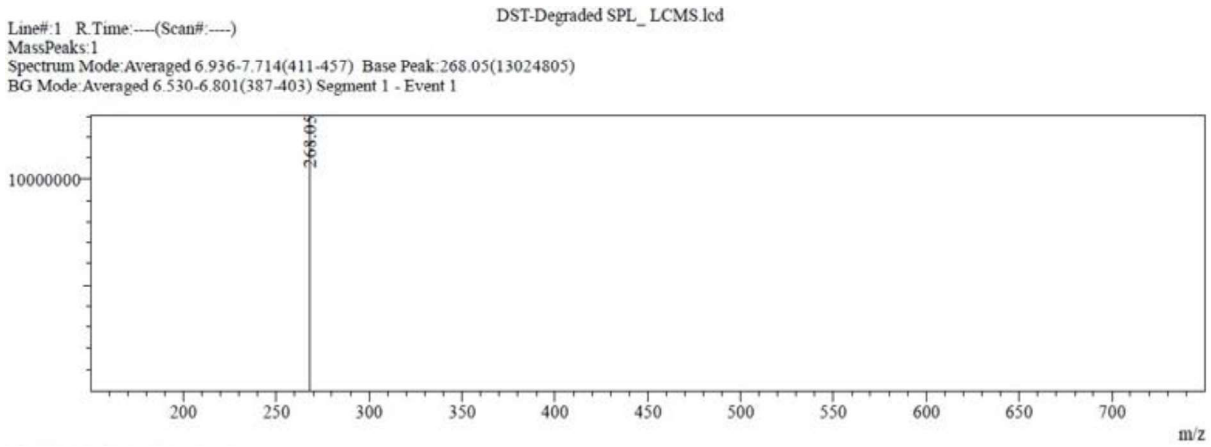


Fig 4a.3 Mass spectra of Impurity-3

The positive ion mass spectral analysis of Dasatinib impurity-4 was observed at 588.15 (M) suggesting the possibility of Molecular formula $C_{26}H_{30}ClN_7O_5S$, which confirms the theoretical molecular weight of Impurity-4.

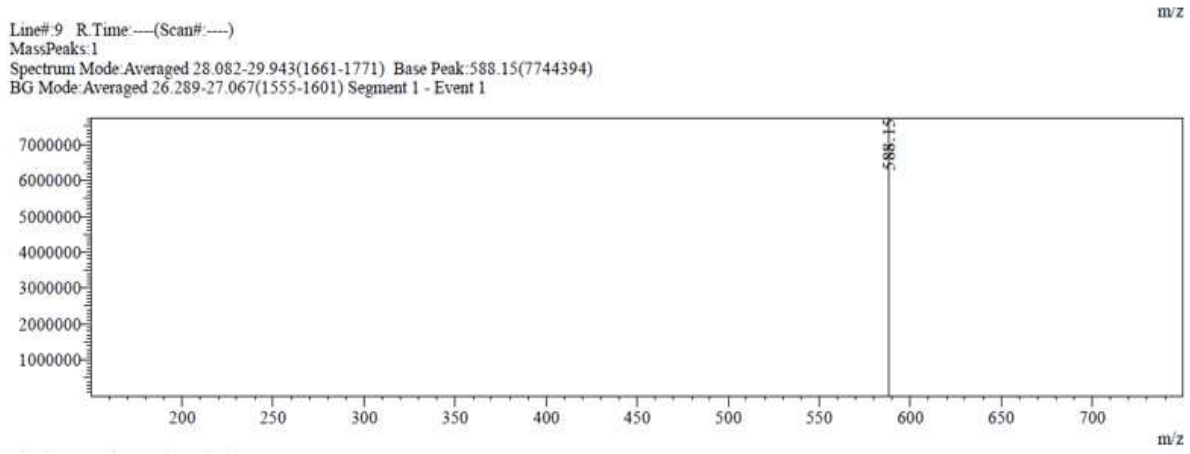


Fig 4a.4 Mass spectra of Impurity-4

The positive ion mass spectral analysis of Dasatinib impurity-5 was observed at 394.05 (M) suggesting the possibility of Molecular formula $C_{16}H_{13}Cl_2N_5OS$, which confirms the theoretical molecular weight of Impurity-5.

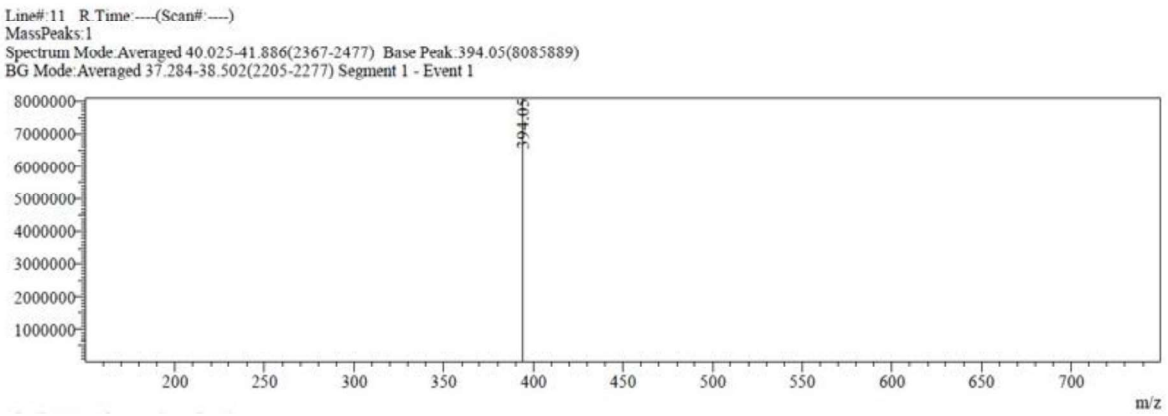


Fig 4a.5 Mass spectra of Impurity-5

The positive ion mass spectral analysis of Dasatinib impurity-6 was observed at 530.20 (M) suggesting the possibility of Molecular formula $C_{24}H_{28}ClN_7O_3S$, which confirms the theoretical molecular weight of Impurity-6.

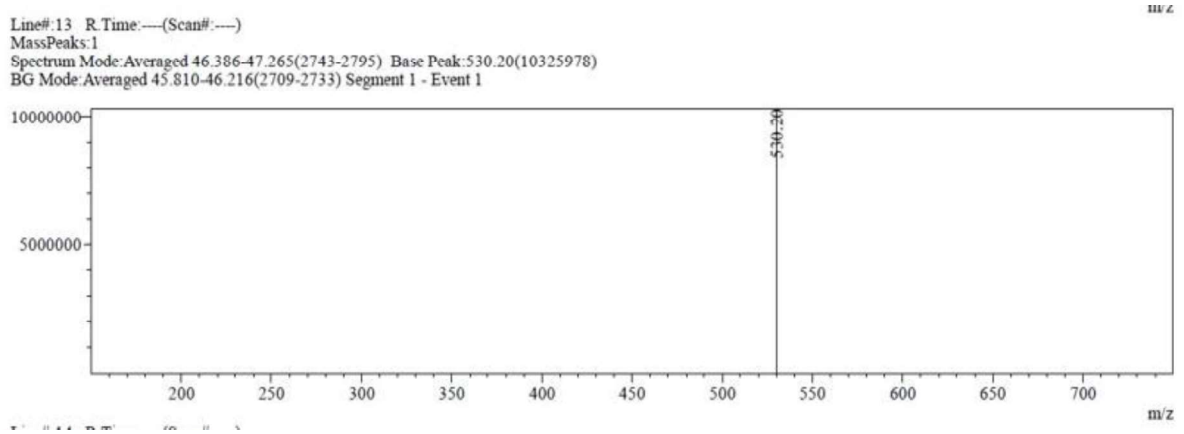


Fig 4a.6 Mass spectra of Impurity-6

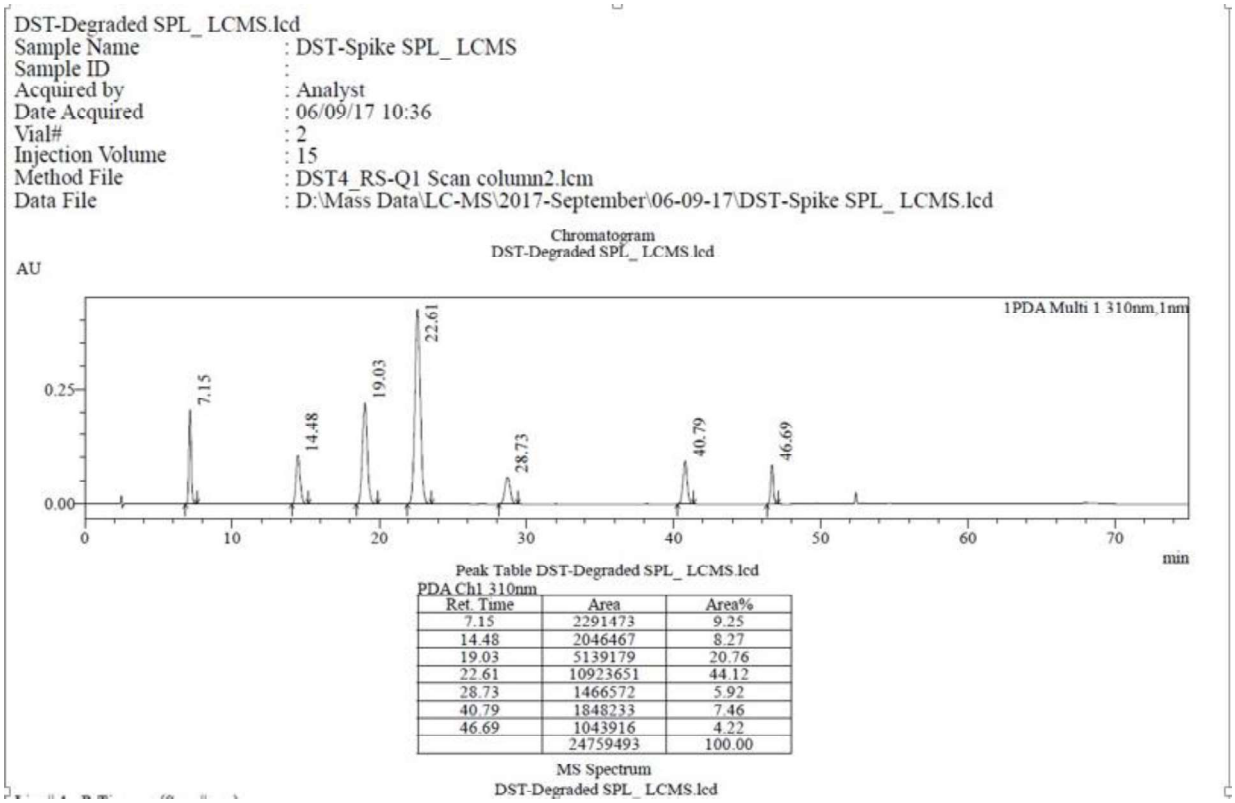


Fig 4a.7 LC spectra in LC-MS for Dasatinib sample spike with impurities

The possible impurities (Imp-1, Imp-2, Imp-3 Imp-4 Imp-5 and Imp-6) were spiked in sample and co-injected with Dasatinib into LCMS to confirm the retention times. All the related substances were well resolved from each other as indicated in fig 4a.7.

4a.3.2 Optimized HPLC parameters

The main criteria for developing chromatographic method was that it must be stability indicating and easy to perform routine analysis in quality control laboratory. The first step for method development was the selection of wavelength. Analysis was performed by using diode array detector for selection of wavelength and to check homogeneity of peaks. The wavelength for analysis and quantification was selected based on UV spectrum of each impurity and analyte peak. Each peak is showing two UV maxima at about 220 nm and 320 nm. The wavelength 310 nm was selected as cross section wavelength for Impurity-3, where absorbance of all analytes were minimum. (26) Reference overlay UV spectra has been provided in Fig 4a.8. For method development, spiked solution of Dasatinib was used. Initially, based on the available literature (27), experiments were done by gradient method using Cosmosil BDS C18 column (100 mm × 4.6 mm, 3.5 µm particle size) with the mobile phase composed of triethyl amine buffer solution pH 6.5 ±0.05 and solvent mixture (methanol, acetonitrile) in 50:50 %, v/v (14).

But in this method, Imp-2 and Imp-4 peaks were merged with Dasatinib peak and Imp-6 peak was split. Hence, a gradient method was investigated using 250 mm × 4.6 mm, column (sun fire C18) of 5 µm particle size and found that only Imp-4 peak was merged with Dasatinib peak.

The effect of pH on the resolution of impurities was investigated ,using buffers at different pH ranging from 4.5 to 7.5 without changing the ionization pH range of Dasatinib i.e. pKa ±1.5 (Dasatinib has a pKa of 10.95 calculated by ACD Chem (SciFinder)), using the same column (28,36). Spike solution was injected with the above mentioned buffers having different pH as mobile phase A and mixture of methanol and acetonitrile in a ratio of 80:20 (%, v/v) as mobile phase B with gradient elution.It was observed that at pH below 4.5 and above 5.5, Imp-4 and Dasatinib merged each other, but separation was achieved for all the six impurities and Dasatinib at pH 5.0±0.5. Hence it was concluded that method is sensitive to pH.It was thus decided to use ammonium acetate-acetic acid buffer of pH 5.0 was thus selected as mobile phase A and methanol: buffer: ACN in the ratio of 90:5:5 %, v/v/v as mobile phase B with a gradient as per Table-4a.4 at a flow rate of 1.2 mL/min. In the above trial, Imp-5 and Imp-6 were eluted in the baseline hump in the applied gradient programme. To overcome this, the gradient was increased to 75 min, 5% buffer was added to mobile phase B and sunniest C18 (250 mm×4.6 mm), 5 µm particle size column was used for resolving Imp-5 and Imp-6. Using these chromatographic conditions, significant separation (>2.0) for all the six impurities and Dasatinib was achieved. The retention time of Dasatinib was 20 min. The typical spike chromatogram of Dasatinib and its impurities is provided in Fig 4a.9. It was confirmed that no blank interference was observed at the retention time of any of the impurities and Dasatinib. LC-MS analysis of impurities was performed as per section 2.5 as instrument condition. All the six impurities were confirmed by LC-MS with a positive and negative mode; the resulting chromatograms are provided in Fig 4a.1 to 4a.7

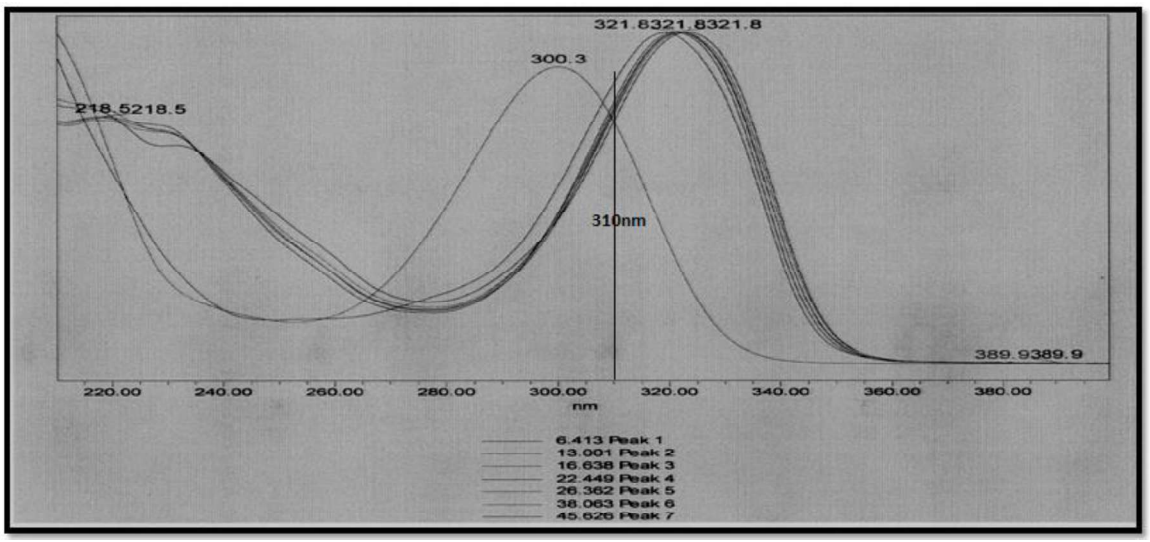


Fig 4a.8 Overlay UV spectra of Dasatinib and its six impurities

Method development trials	Parameter	Outcome
Trial-1	**Column**: Cosmosil BDS C18 column (100 mm × 4.6 mm, 3.5 µm) **Mobile phase** M.P-A : Triethyl amine buffer solution pH 6.5 ±0.05 MP-B: methanol, acetonitrile in 50:50 %, v/v	In this trial, Imp-2 and Imp-4 peaks were merged with Dasatinib Peak and Imp-6 peak was split.
Trial-2	**Column:**Sunfire C18 (250 mm × 4.6 mm, 5 µm) **Mobile phase** M.P-A : Triethyl amine buffer solution pH 6.5 ±0.05 MP-B: methanol, acetonitrile in 50:50 %, v/v	Only Imp-4 peak merged with Dasatinib peak.
Trial-3	**Column:**Sunfire C18 (250 mm × 4.6 mm, 5 µm) **Mobile phase** M.P-A : Ammonium acetate Buffer with pH 7.5 MP-B: methanol, acetonitrile in 80:20 %, v/v	Imp-4 peak merged with Dasatinib peak.

Trial-3	**Column:**Sunfire C18 (250 mm × 4.6 mm, 5 µm) **Mobile phase** M.P-A : Ammonium acetate Buffer with pH 4.0 MP-B: methanol, acetonitrile in 80:20 %, v/v	Imp-4 peak merged with Dasatinib peak It was observed that the buffer with pH range below 4.5 and above 5.5 there is merging of impurity peaks hence impurities are sensitive to pH range.
Trial-4	**Column:**Sunfire C18 (250 mm × 4.6 mm, 5 µm) **Mobile phase** M.P-A : Ammonium acetate-acetic acid buffer of pH 5.0 M.P-B : Methanol: Buffer: ACN in the ratio of 90:5:5 %, v/v/v	All six impurities and Dasatinibwere well separated from each other but impurity 5 and impurity 6 were eluted over gradient hump. Modification in gradient pattern is to be done.
Trial-5	**Column:** Sunniest C18 (250 mm × 4.6 mm, 5 µm) **Mobile phase** M.P-A : Ammonium acetate-acetic acid buffer of pH 5.0 M.P-B : Methanol: Buffer: ACN in the ratio of 90:5:5 %, v/v/v	All the six impurities were well separated and resolution was more than 2.0 between all peaks. Gradient was extended to 75 min. and sunniest column was used to smoothen baseline at retention time of Imp-5 and Imp-6.

Table 4a.2 Method development trials

Table 4a.3 The HPLC method parameters

Parameter	Conditions
HPLC Column	Sunniest C18, 250-mm, 4.6 mm and 5 µm
Mobile Phase	A: 20mM ammonium acetate buffer pH (5.0)
	B: Methanol: Buffer: Acetonitrile (90:5:5 %, v/v/v)
Injection Volume	15 µL
Flow Rate	1.2 mL/min
Column Oven Temp.	35°C
UV Wavelength	310nm
Run Time (min)	75 min

Table 4a.4 Gradient Programme of the method

Time (min)	%Mobile phase-A	%Mobile phase-B
0.01	50	50
23	45	55
42	33	67
50	10	90
65	10	90
68	50	50
75	50	50

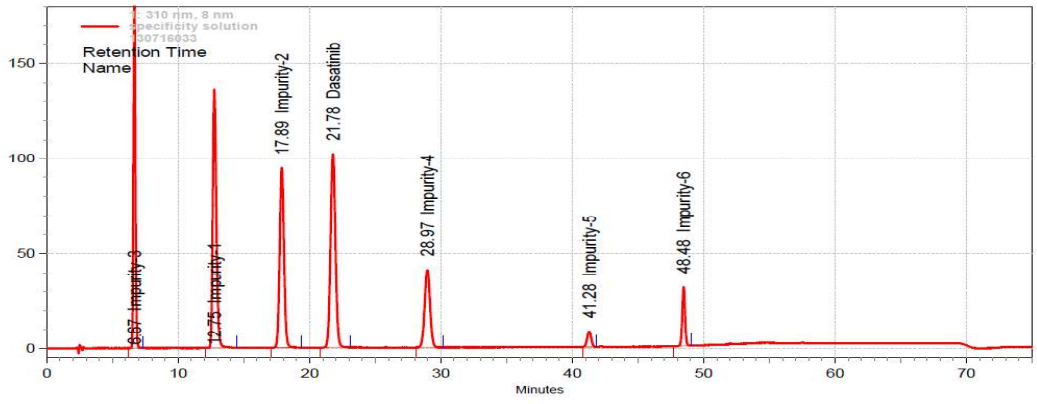


Fig 4a.9 Typical HPLC chromatogram of Dasatinib and its impurities

4a.3.3 System Suitability Criteria:

A suitability of systems can be defined on the basis of results obtained from number of repetitive chromatograms. For the acceptance of column efficiency determined from the analyte peak >5000 theoretical plates, the tailing factor should not be more than 2.0. Besides, % RSD for impurity areas in six replicate injection of system suitability solution was <5.0%. A resolution between any two compounds was >5 in SST (system suitability test) solution. All the system suitability criteria during validation study and batch analysis study were noticed within the acceptable limit as per USP. The results of system suitability are depicted in Table-4a.5.

Imp particulars	RT (min)	RRT	USP resolution	USP tailing factor	USP theoretical plates	%RSD
Imp-3	6.67	0.30	-	1.10	7964	1.22
Imp-1	12.75	0.58	15.37	1.23	10108	0.92
Imp-2	17.89	0.82	9.30	1.04	13629	1.35
Dasatinib	21.78	1.00	6.00	1.04	15084	1.25
Imp-4	28.97	1.33	9.88	1.01	23763	1.65
Imp-5	41.28	1.90	18.35	1.02	79760	1.42
Imp-6	48.48	2.23	14.80	0.98	239541	1.72

Table 4a.5 System Suitability results

4a.3.4 Validation of the method

4a.3.4.1 Selectivity (Specificity)

The terms “specificity” and “selectivity” both give an idea of the reliability of the analytical method. Selectivity refers to the ability of the method to discriminate a particular substance in a complex mixture without interference from other components. On the other hand, specificity can be considered as the ultimate selectivity, i.e. 100% selectivity (or 0% interference).

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc. (29, 30)

The selectivity of analytical method is based on the ability to distinguish response produced by analyte from response of other moieties. The term specificity is usually more preferred and appropriate as there are very few methods that respond to only one analyte. Specificity measures the reliability of measurements in the presence of interferences. (31,32).

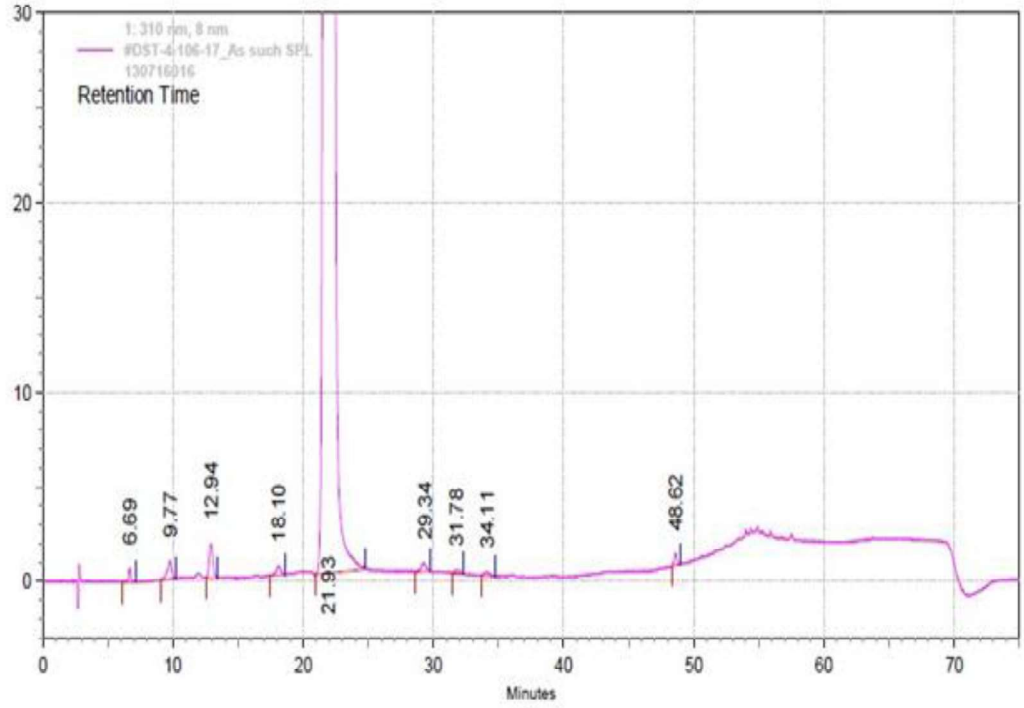
For specificity study, each impurity, Imp-1 to Imp-6, HPMC and Dasatinib were injected separately. Spiked sample was also injected and purity plots were extracted for each impurities in the spiked sample from diode array detector.

Stress testing Studies

A 1000 µg/mL Dasatinib solution was injected for each stress testing study Degradation was not observed when the solid dispersion of Dasatinib was subjected to alkali degradation (1 N NaOH heat at 60°C for 1 h) and acid degradation (1 N HCl heat at 60°C for 1 h) conditions. A 15.0% degradation was observed when the drug was subjected to oxidation (3% H_2O_2 for 1.5h) leading to the formation of Imp- 2, thermal Degradation (105°C approximately 24h) leading to the formation of Imp-4 and Imp-6 and UV degradation (254nm for 24 h) leading to the formation of Imp-1 and Imp-6. The degradation products that were formed during the stress studies were confirmed by co-injecting the standard solution with stressed samples.

Control sample preparation: About 35 mg of solid dispersion of Dasatinib and15 mL diluent were taken into 25 mL volumetric flaskand sonicated for 5 min to dissolve and made up to the mark with diluent. Chromatogram of control sample is shownin Fig 4a.10.

Fig 4a.10 Control sample

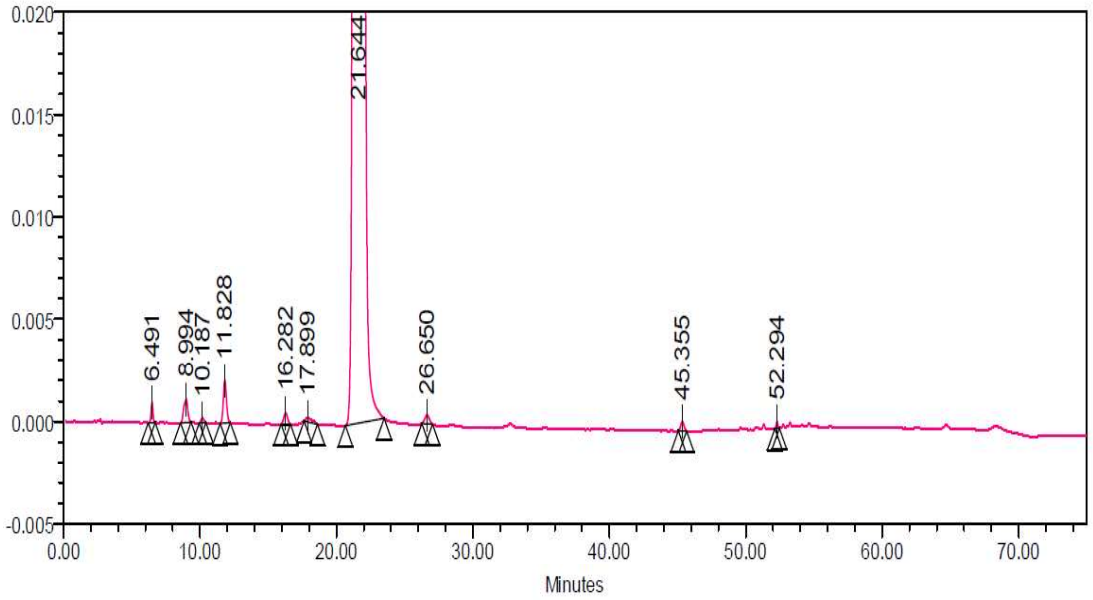


1: 310 nm, 8 nm

Pk #	Retention Time	Area	Area Percent	Name	Relative RT
1	6.69	10449	0.03	Imp-3	0.30
2	9.77	22569	0.05	UI	0.44
3	12.94	35622	0.09	Imp-1	0.59
4	18.10	11295	0.03	Imp-2	0.82
5	21.93	41053650	99.73	Dasatinib	1.00
6	29.34	10770	0.03	Imp-4	1.34
7	31.78	6008	0.01	UI	1.45
8	34.11	7725	0.02	UI	1.56
9	48.62	8188	0.02	Imp-6	2.22
Totals		41166276	100.00		

Acid treated sample preparation: About 35 mg of solid dispersion of Dasatinib and 5 mL of 1N HCLwere taken into 25ml volumetric flask, mixed well and then transfer to beaker for heating 1h at 60°C, allowed to cool at room temperature and neutralized with 1N NaOH and made up to the mark with diluent. Chromatogram of acid treated sample is described in Fig 4a.11.

Fig 4a.11 Acid treated test sample

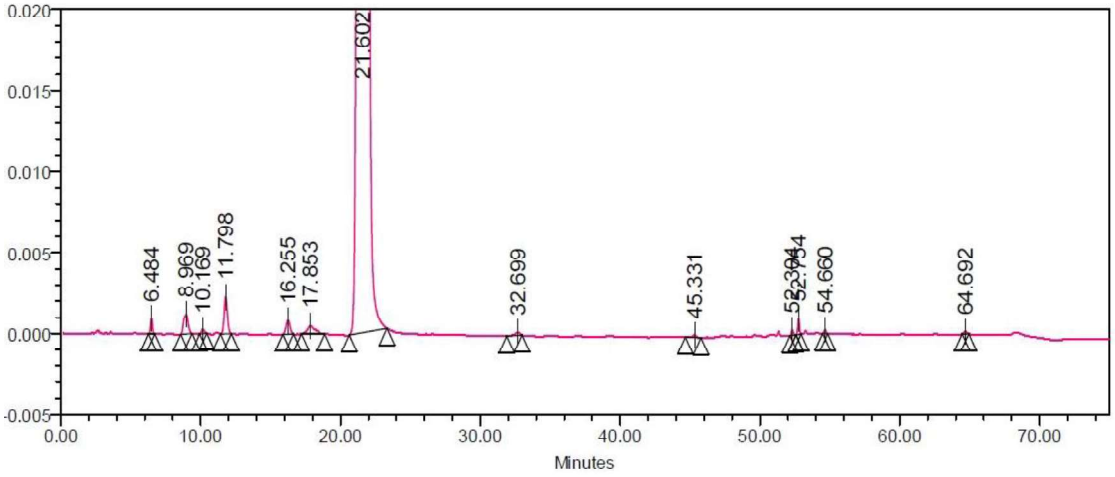


Peak Results

	RT	Area	%Area	Name	Purity1 Angle	Purity1 Threshold
1	6.491	9417	0.02	Imp-3	1.607	1.574
2	8.994	23177	0.06	Peak2	2.325	1.081
3	10.187	3216	0.01	Peak3	3.825	4.718
4	11.828	33811	0.08	Imp-1	0.730	0.718
5	16.282	10097	0.02	Imp-2	1.748	1.885
6	17.899	6941	0.02	Peak6	7.256	4.571
7	21.644	41155854	99.74	Dasatinib	0.590	1.019
8	26.650	9332	0.02	Imp-4	2.322	2.330
9	45.355	7549	0.02	Imp-6	2.459	1.995
10	52.294	2762	0.01	Peak11	1.711	2.616

Alkali treated sample preparation: About 35 mg of solid dispersion of Dasatinib was taken into 25ml volumetric flask with 5ml of 1N NaOH, mixed well and solution was heated for 1h at 60°C, then allowed to cool at room temperature and neutralized with 5 ml of 1N HCL and made up to the mark with diluent. Chromatogram of alkali treated sample is described in Fig 4a.12.

Fig 4a.12 Alkali treated test sample

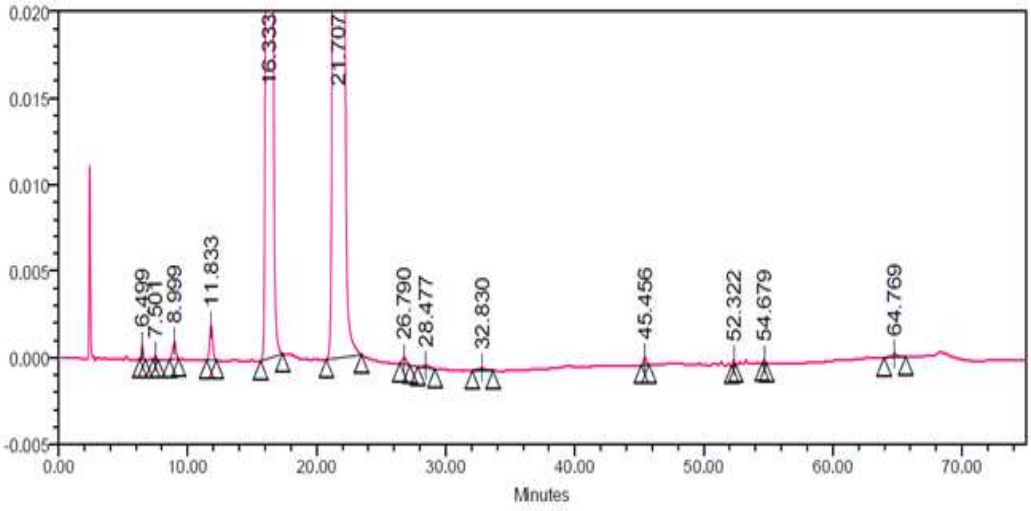


Peak Results

	RT	Area	% Area	Name	Purity1 Angle	Purity1 Threshold
1	6.484	9382	0.02	Imp-3	1.261	1.796
2	8.969	27322	0.07	Peak2	1.888	1.167
3	10.169	3756	0.01	Peak3	5.594	4.770
4	11.798	37487	0.09	Imp-1	0.960	0.778
5	16.255	16450	0.04	Imp-2	1.200	1.581
6	17.853	19779	0.05	Peak6	3.863	2.812
7	21.602	41683110	99.67	Dasatinib	0.608	1.069
8	32.699	4703	0.01	Peak8	7.401	6.780
9	45.331	2829	0.01	Imp-6	21.545	7.846
10	52.304	3165	0.01	Peak10	3.298	3.480
11	52.754	8234	0.02	Peak11	2.326	1.389
12	54.660	2444	0.01	Peak12	2.394	3.963
13	64.692	2113	0.01	Peak13	5.685	9.499

Oxidant treated sample preparation: About 35 mg of solid dispersion of Dasatinib was taken into 25ml volumetric flask with 5ml of 3.0% H_2O_2, mixed well and solution was kept for 1.5h at room temperature, and then made up to the mark with diluent and mixed well. Chromatogram of oxidant treated sample is described in Fig 4a.13.

Fig 4a.13 Oxidantion treated test sample

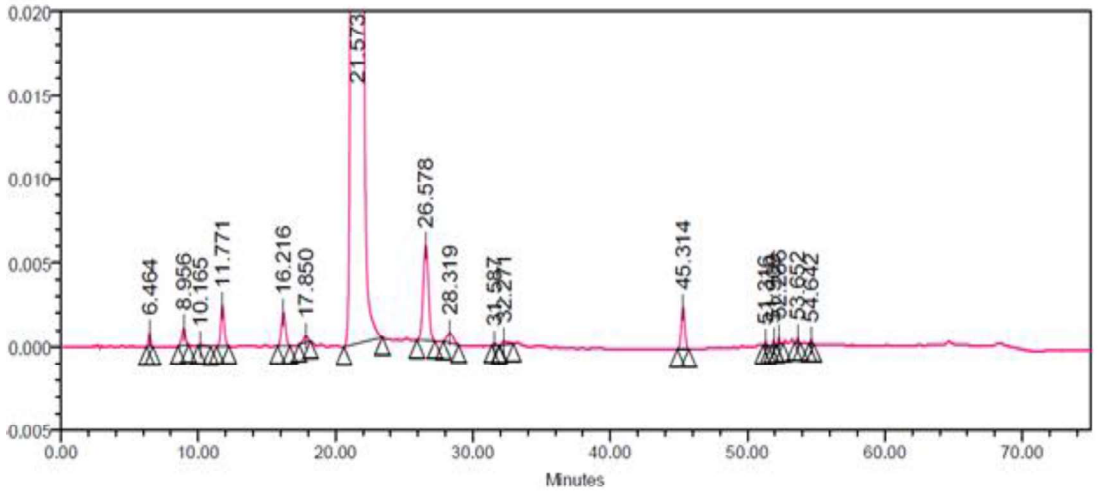


Peak Results

	RT	Area	%Area	Name	Purity1 Angle	Purity1 Threshold
1	6.499	7840	0.02	Imp-3	1.251	1.423
2	7.501	2439	0.01	Peak2	3.823	4.363
3	8.999	18149	0.05	Peak3	1.998	1.115
4	11.833	33677	0.09	Imp-1	0.684	0.664
5	16.333	5522139	14.05	Imp-2	0.079	0.219
6	21.707	33679362	85.70	Dasatinib	0.291	0.522
7	26.790	8746	0.02	Imp-4	2.341	2.302
8	28.477	4920	0.01	Peak8	7.858	6.184
9	32.830	5638	0.01	Peak9	10.165	6.476
10	45.456	7245	0.02	Imp-6	2.667	1.934
11	52.322	2457	0.01	Peak11	2.326	2.740
12	54.679	2211	0.01	Peak12	2.310	3.218
13	64.769	5144	0.01	Peak13	15.408	7.551

Thermal treated sample preparation: About 100 mg of solid dispersion of Dasatinib was taken into petridish and heated at 105°C for 24 h. The Petridish was cooled to room temperature. About 35 mg of solid dispersion of Dasatinib from petridish was transferred to 25 mL volumetric flask and made up to the mark with diluent and mixed well. Chromatogram of thermal treated sample isgiven in Fig 4a.14.

Fig 4a.14 Thermal treated sample

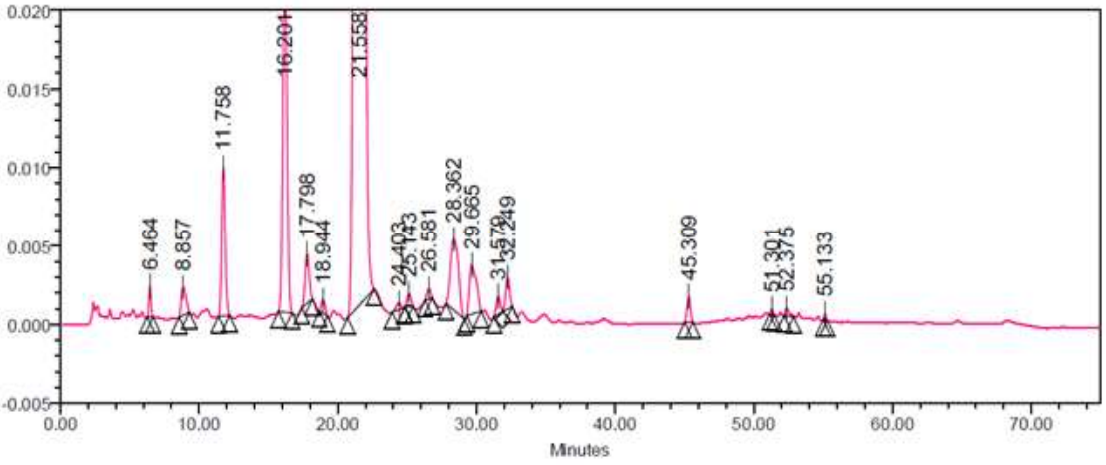


Peak Results

	RT	Area	%Area	Name	Purity1 Angle	Purity1 Threshold
1	6.464	8605	0.02	Imp-3	1.221	1.573
2	8.956	19474	0.05	Peak2	1.595	1.096
3	10.165	2767	0.01	Peak3	13.975	9.749
4	11.771	42378	0.10	Imp-1	0.988	0.612
5	16.216	40855	0.10	Imp-2	0.545	0.643
6	17.850	8280	0.02	Peak6	3.232	2.715
7	21.573	42426727	99.15	Dasatinib	0.631	1.104
8	26.578	153183	0.36	Imp-4	0.263	0.347
9	28.319	20136	0.05	Peak9	2.554	1.546
10	31.587	2099	0.00	Peak10	5.249	6.059
11	32.271	7384	0.02	Peak11	4.048	3.319
12	45.314	45753	0.11	Imp-6	0.524	0.643
13	51.316	2331	0.01	Peak13	2.927	3.268
14	51.909	2341	0.01	Peak14	2.450	3.187
15	52.286	3857	0.01	Peak15	2.911	2.142

UV treated sample preparation: About 35 mg of solid dispersion of Dasatinibwas taken into 25ml volumetric flask and then was kept under UV light at 254nm for 24 h, flask was removed and kept at room temperature to cool, and made up to the mark with diluent. Chromatogram of UV treated sample is described in Fig 4a.15.

Fig 4a.15 UV treated sample



Peak Results

	RT	Area	%Area	Name	Purity1 Angle	Purity1 Threshold
1	6.464	19985	0.05	Imp-3	1.838	1.810
2	8.857	42207	0.11	Peak2	1.622	1.678
3	11.758	167363	0.43	Imp-1	0.259	1.144
4	16.201	474074	1.22	Imp-2	0.116	1.059
5	17.798	64195	0.17	Peak5	0.877	1.446
6	18.944	16505	0.04	Peak6	1.235	2.321
7	21.558	37593717	96.86	Dasatinib	0.417	1.001
8	24.403	10500	0.03	Peak8	3.235	3.787
9	25.143	15668	0.04	Peak9	1.013	2.235
10	26.581	11945	0.03	Imp-4	1.207	2.560
11	28.362	181882	0.47	Peak11	3.572	1.248
12	29.665	108306	0.28	Peak12	7.637	1.233
13	31.579	22825	0.06	Peak13	15.369	1.801
14	32.249	39465	0.10	Peak14	10.626	1.499
15	45.309	27896	0.07	Imp-6	0.692	1.647

Results from stress testing studies are reported in Table 4a.6.

Degradation condition	Time	Temp	Assay (%, w/w)	RS by HPLC % degradation	Mass balance (% assay + % deg. products)	Remarks/ observation
A control sample (untreated)	-	-	100.6	0.27	100.8	NA
HCl, 1.0 N (acid degradation)	1 h	60°C	100.8	0.22	101.0	No significant degradation observed

NaOH, 1.0 N (base degradation)	1 h	60°C	100.9	0.25	101.1	No significant degradation observed
Oxidation by 3.0% H_2O_2	1.5 h	25°C	84.7	12.26	96.9	Imp-2 was formed
Thermally treated	24 h	105°C	98.1	0.68	98.79	Imp-4 and Imp-6 impurities were formed
UV treated (254nm)	24 h	25°C	99.7	0.64	100.4	Imp-1 and Imp-6 impurities were formed

Table 4a.6 Summary of stress testing results.

Degradation condition	Imp-1		Imp-2		Imp-4		Imp-6		Dasatinib	
	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold
Oxidation	-	-	0.079	0.219	-	-	-	-	0.291	0.522
Thermal	-	-	-	-	0.263	0.347	0.524	0.643	0.631	1.104
UV Treated	0.259	1.144	-	-	-	-	0.692	1.647	0.417	1.001

Table 4a.7 Result of peak purity

The analyses were performed against Dasatinib standard solution. The mass balance, calculated for each condition of stressed samples was found to be in the range of 95-105% which confirms that the developed method was stability-indicating. Depending on the chemistry and structure of Dasatinib it may undergo degradation in specific strength of acid, base or peroxide at specific temperature only; hence only some specific conditions influence the degradation of drug substance.

Assay is calculated by using given formula:

$$Assay\left(\%\frac{w}{w}, on\ anhydrous\ basis\right) = \frac{R_u\ x\ W_{std}\ x\ 5\ x\ 25\ x100}{R_s\ x\ 25\ x\ \ 100\ x\ W_{spl}\ x\ 5}\ X\ \frac{(P - W_{std})}{(100\ -\ W_{spl}\ )}$$

Where,

R_u =Peak area of Dasatinib peak from sample solution

R_s =Avg peak area of Dasatinib from standard solution

W_{std} =Weight of Dasatinib standard in mg.

W_{spl}= weight of sample in mg.

W_{spl}= Percent water content of sample (%w/w)

W_{std}= Percent water content of standard (%w/w)

P = Potency of Dasatinib standard (%w/w, on anhydrous basis)

Peak purity was checked in the degraded sample and calculations of purity angle and purity threshold for the known impurity are reported in Table 4a.7. The table shows that purity angle is less than purity threshold for known impurity peak formed due to degradation.

4a.3.4.2 LOD and LOQ (Limit of Detection and Limit of Quantification)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. It is a limit test that specifies whether or not an analyte is above or below a certain value.
Several approaches for determining the detection limit are possible, depending on whether the procedure is non-instrumental or instrumental (33).

Based on Visual Evaluation Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis

of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected (22).

Based on Signal-to-Noise this approach can only be applied to analytical procedures which exhibit baseline noise. A signal-to-noise ratio between 3:1 or 2:1 is generally considered acceptable for estimating the detection limit (22).

Based on the Standard Deviation of the Response and the Slope- The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\,\sigma}{S}$$

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve

Quantitation limit: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. (22, 33)

The quantitation limit is a parameter for quantitative assay of low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

Based on Visual Evaluation Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

Based on Signal-to-Noise: A signal-to-noise ratio of 10:1 is generally considered acceptable for estimating the quantization limit. (22, 33)

Based on the Standard Deviation of the Response and the Slope the quantization limit (QL) may be expressed as:

$$QL = \frac{10\,\sigma}{S}$$

Where,
σ = the standard deviation of the response
S = the slope of the calibration curve

Sensitivity of the method was proved by establishing the limit of detection (LOD) and limit of quantization (LOQ) for Dasatinib 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 Dasatinib spiked with Impurities (Impurity-1 to imp-6) at LOQ level and by calculating % recoveries of all impurities content.

Table 4a.8 LOD and LOQ data

Imp particulars	LOD (µg/ mL)	LOQ (µg/ mL)
Imp-3	0.06	0.21
Imp-1	0.07	0.23
Imp-2	0.04	0.15
Dasatinib	0.05	0.18
Imp-4	0.07	0.21
Imp-5	0.08	0.25
Imp-6	0.15	0.30

4a.3.4.3 Linearity and range:

Linearity:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included (34, 35).

- ✓ Demonstrate across the entire range of the analytical procedure.
- ✓ A minimum of five concentrations is recommended.

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The specified range is normally derived from linearity studies and depends on the intended application of the procedure. The following minimum specified ranges should be considered: (34, 35)

- For the assay of a drug substance or a finished (drug) product: normally from 90 to 110 percent of the test concentration;
- For content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;
- For dissolution testing: +/-20 % over the specified range
- For the determination of an impurity: from the reporting level of an impurity to 150% of the specification.

The linearity of peak areas versus different concentrations was evaluated for Dasatinib and all the related substances using seven levels ranging from LOQ to 150% (20, 30, 50, 80, 100, 120 and 150%) with respect to the specification level of impurities. Linearity of method was calculated at seven level ranging from LOQ to 150% (i.e. 0.30, 0.45, 0.75, 1.20, 1.50, 1.80 and 2.25 µg/mL) to the specification level of 0.15%, while For drug substance calibration curve was obtained for Dasatinib in the concentrations ranging from LOQ to 150% (i.e. 0.20, 0.30, 0.50, 0.80, 1.00, 1.20 and 1.50 µg/mL) to the specification level of 0.10%.

To establish linearity of the method, solutions were prepared by diluting the Dasatinib and impurities second stock solution having 8ppm of each analytes to obtain the required concentrations at six different levels ranging from LOQ to 150% all the linearity curves are shown in Figure 4a.16 to 4a.23. The correlation coefficient, slope and y-intercept of the calibration curve were calculated which are shown in Table 4a.11.The method has been demonstrated to be linear in a range of 20% to 150% level.

Table 4a.9 Linearity stock solutions

Standard Details							
	Imp-3	**Imp-1**	**Imp-2**	**Dasatinib**	**Imp-4**	**Imp-5**	**Imp-6**
Potency (%)	99.90	99.90	99.90	67.5	99.9	99.9	94.86
Wt. Taken (mg)	5.37	4.9	4.97	5.4	5.16	5.4	4.8
Dilution (ml)-**Stock-1**	25	25	25	25	25	100	25
Volume taken (ml)	2	2	2	2	2	8	2
Dilution (ml)-**Stock-2**	50	50	50	50	50	50	50

Table 4a.10 Preparation of Linearity solutions

Sample ID	Level	Volume taken from Stock-2 (mL)	Dilution (mL)
Linearity solution-1	20%	1	25
Linearity solution-2	30%	1.5	25
Linearity solution-3	50%	2.5	25
Linearity solution-4	80%	4	25
Linearity solution-5	100%	5	25
Linearity solution-6	120%	6	25
Linearity solution-7	150%	7.5	25

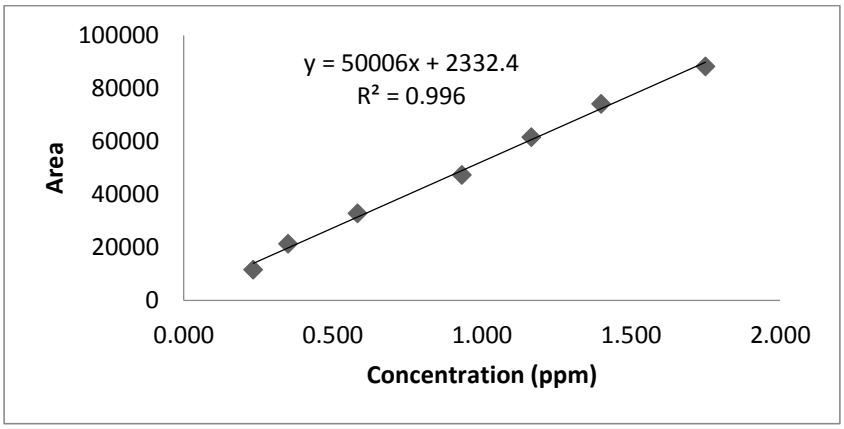


Fig 4a.16 Linearity curve of Dasatinib

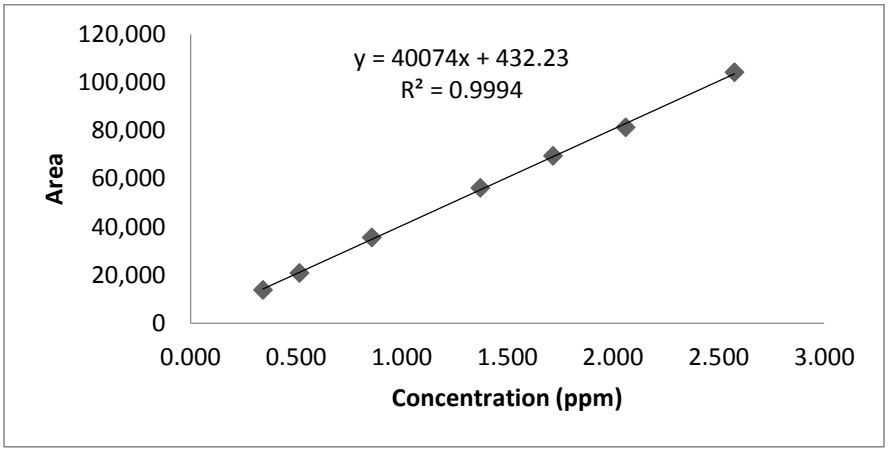


Fig 4a.17 Linearity curve of Impurity-3

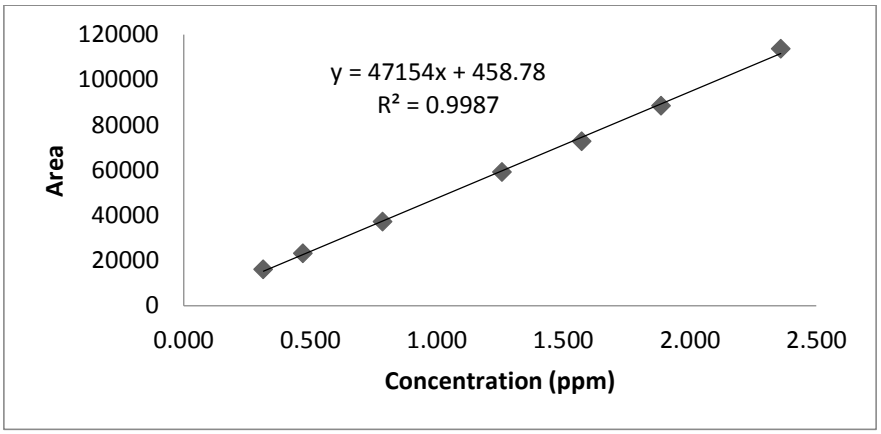


Fig 4a.18 Linearity curve of Impurity-1

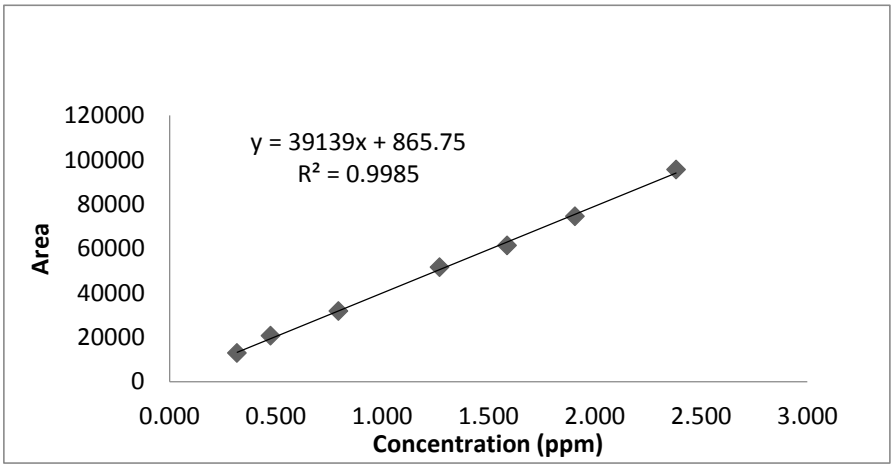


Fig 4a.19 Linearity curve of Impurity-2

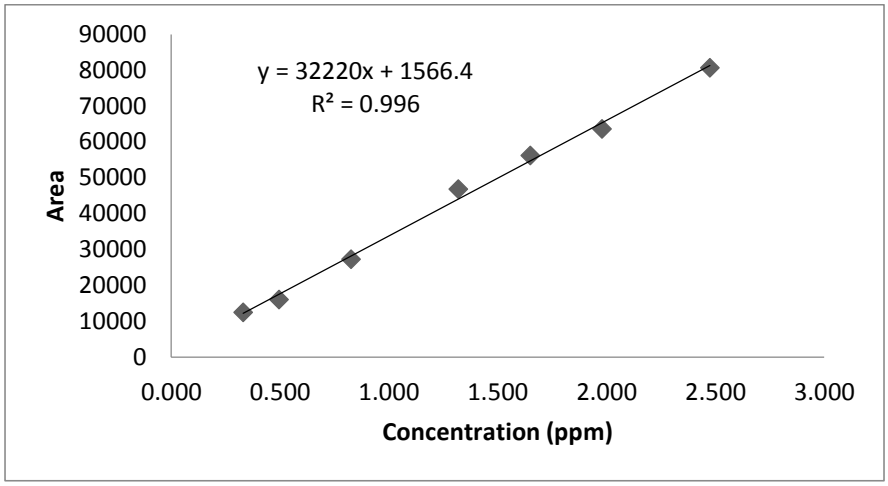


Fig 4a.20 Linearity curve of Impurity-4

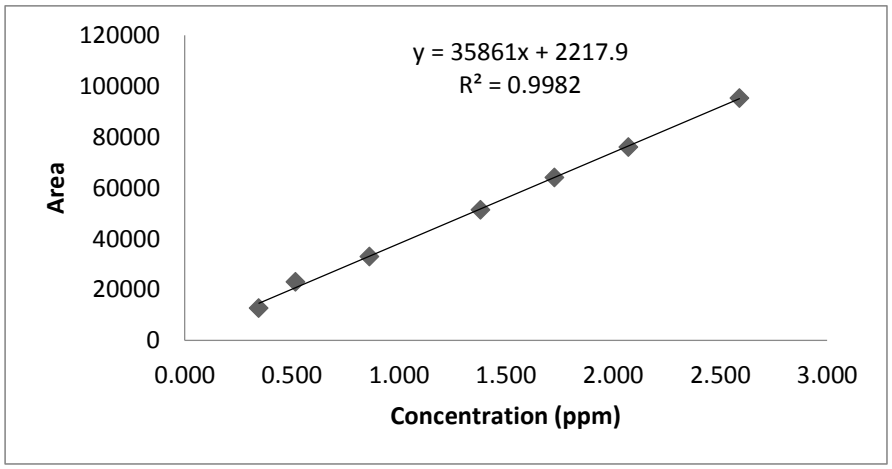


Fig 4a.21 Linearity curve of Impurity-5

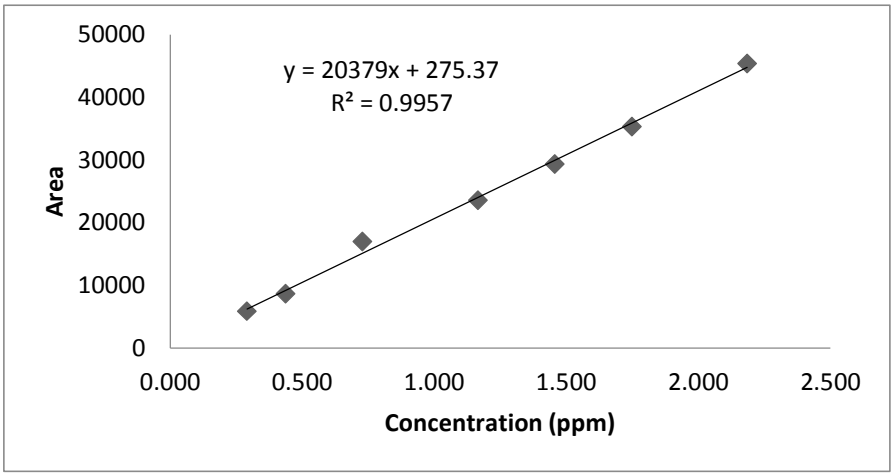


Fig4a.22 Linearity curve of Impurity-6

The slope, correlation coefficient and y-intercept values are reported in Table 4a.11 which indicated that the method under study was linear.

Table 4a.11 Correlation coefficient, slope and y-intercept of the calibration curve

Component	Regression equation (y)		R^2 value	RRF (relative response factor)
	Slope (b)	Intercept (a)		
Imp-3	40074	432.2	0.999	1.25
Imp-1	47154	458.7	0.998	1.05
Imp-2	39139	865.7	0.998	1.28
Dasatinib	50006	2332	0.996	1.00
Imp-4	32220	1566	0.996	1.55
Imp-5	35861	2217	0.998	1.42
Imp-6	20379	275.3	0.995	2.48

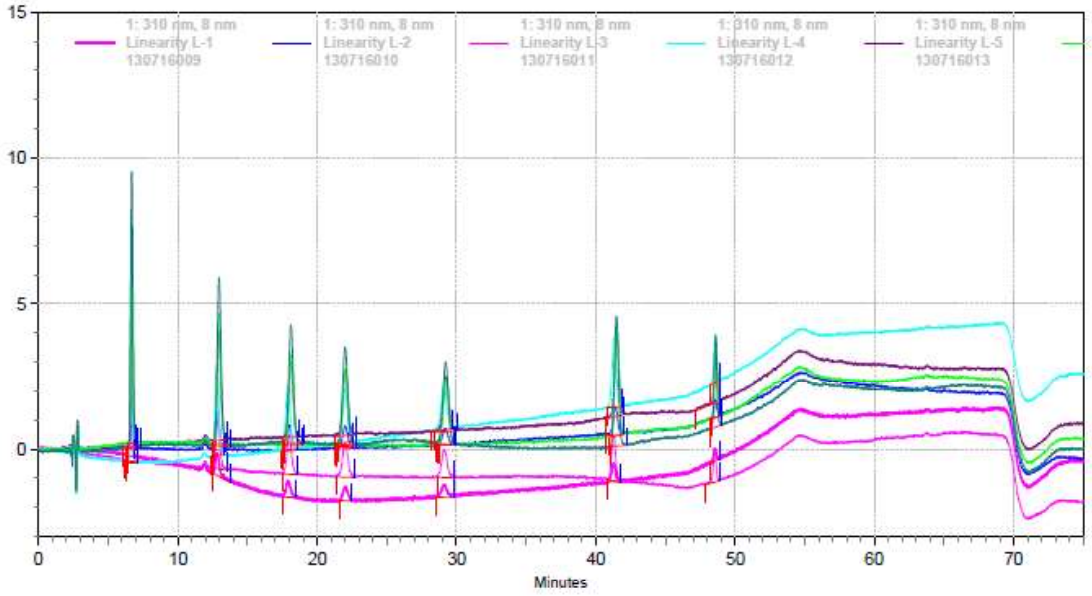


Fig 4a.23 Linearity overlay graph of Dasatinib and all impurities

3.3.4.4 Precision:

System precision for related substances determination was verified by injecting standard preparation of Dasatinib which was analyzed six times.

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same

homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Repeatability should be assessed using:

a) Minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or

b) Minimum of 6 determinations at 100% of the test concentration.

Intermediate precision

Intermediate precision expresses within-laboratory variations: different days, different analysts, different equipment, etc. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. As per ICH Q2 (R1) typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually (24). The use of an experimental design (matrix) is encouraged.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology). Reproducibility is assessed by means of an inter-laboratory trial.

Methodology

System precision

System precision determination was performed by injecting limit level standard preparation (1ppm) of Dasatinib which was analyzed six times and RSD of all six injections was not exceeding 5%.

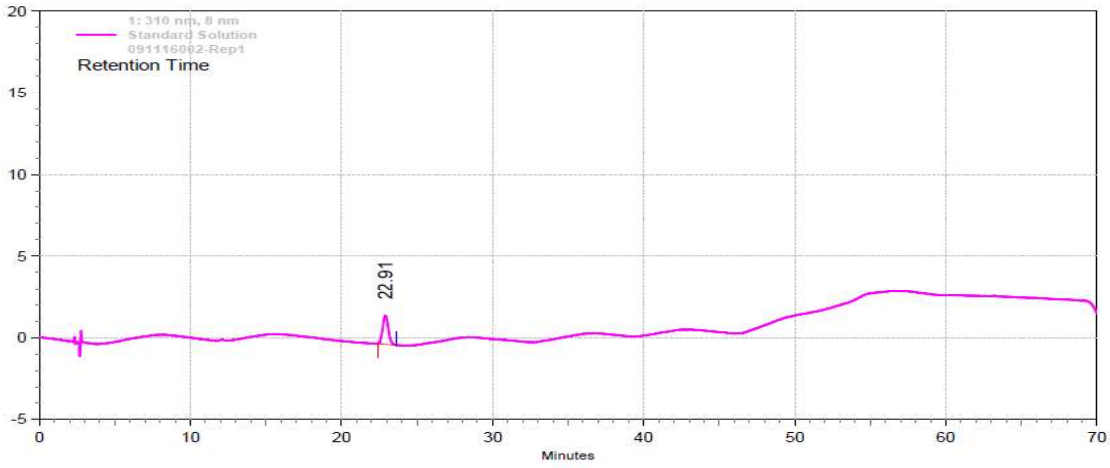


Fig 4a.24 Standard preparation injection-1

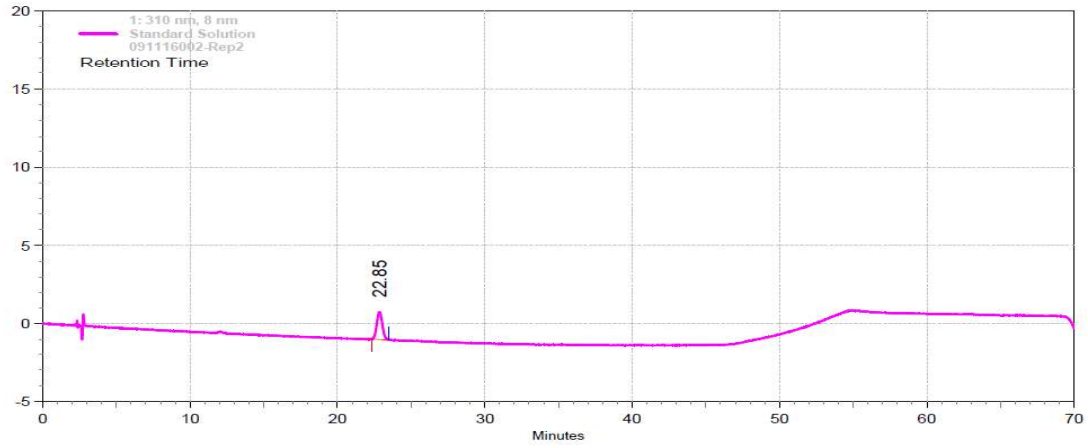


Fig 4a.25 Standard preparation injection-2

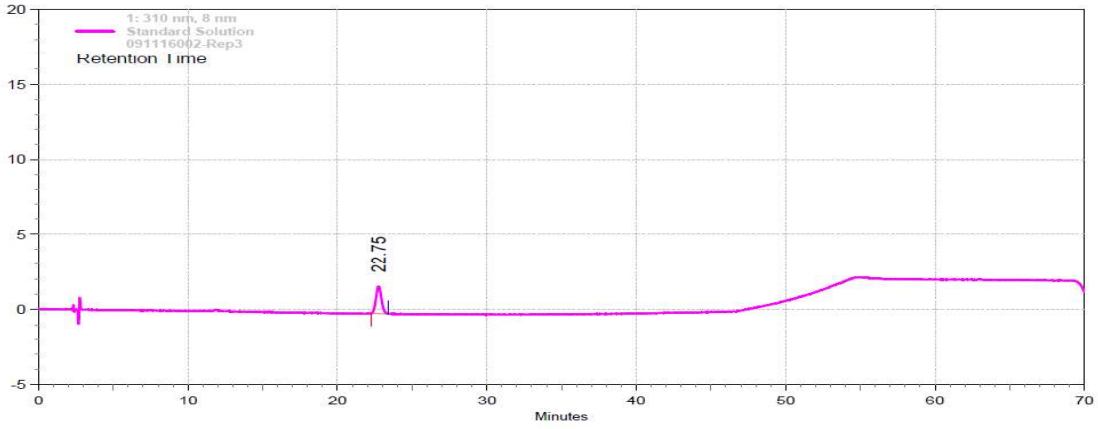


Fig 4a.26 Standard preparation injection-3

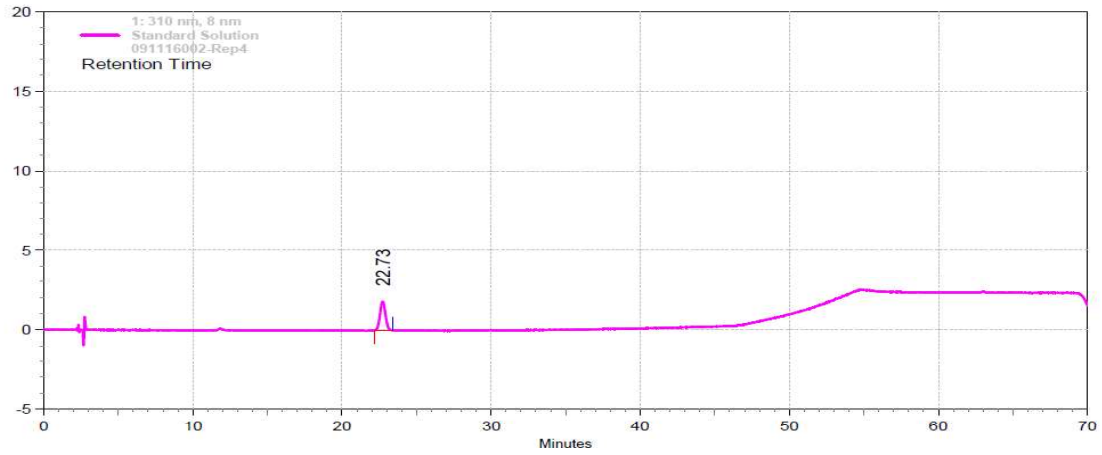


Fig 4a.27 Standard preparation injection-4

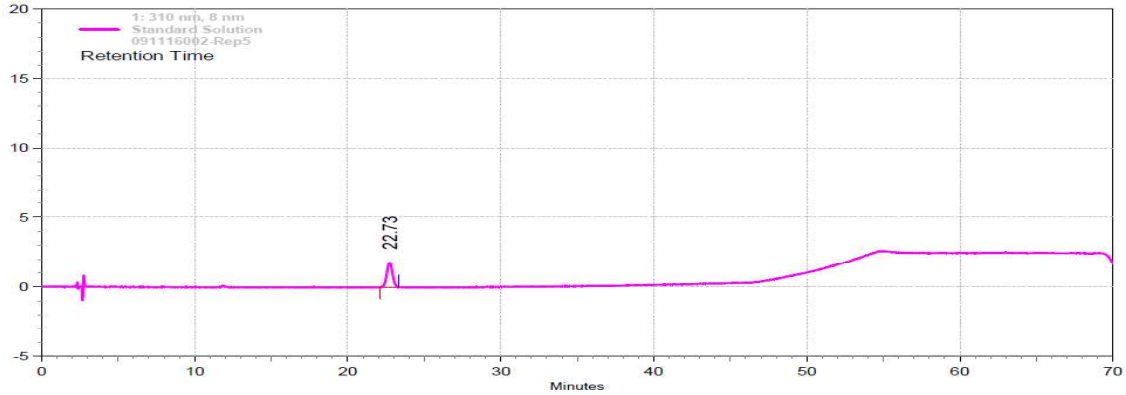


Fig 4a.28 Standard preparation injection-5

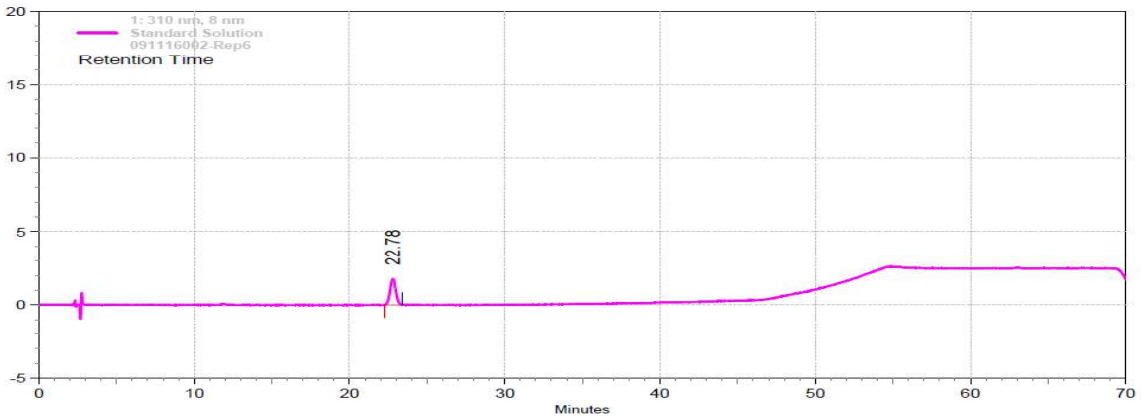


Fig 4a.29 Standard preparation injection-6

Method precision

The precision of a method is determined by assaying aliquots of a homogeneous sample to be able to calculate statistically significant estimates of standard deviation or relative standard deviation (coefficient of variation). Assays should be of samples that have gone through the

entire analytical procedure from sample preparation through final analysis. A minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or minimum of six determinations at 100% of the test or target concentration is recommended. The Fig 4a.30 to 4a.35 represents chromatograms of method precision.

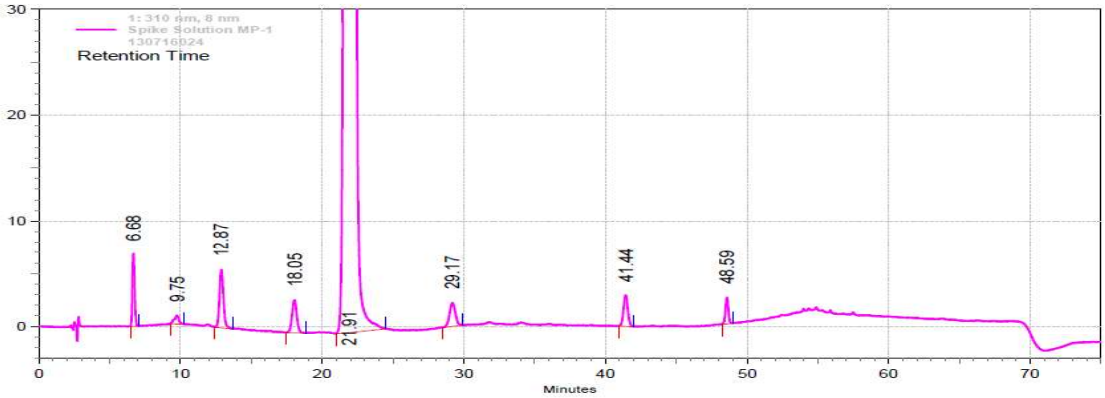


Fig 4a.30 Method Precision set-1

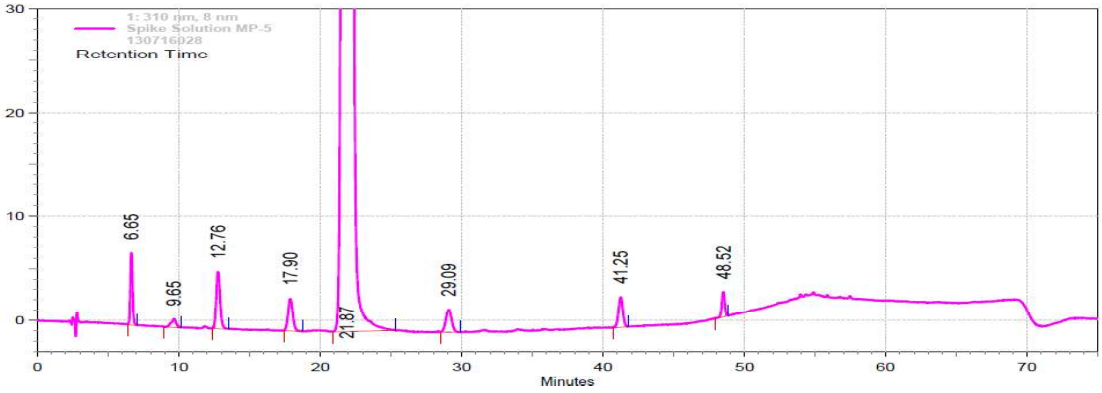


Fig 4a.31 Method Precision set-2

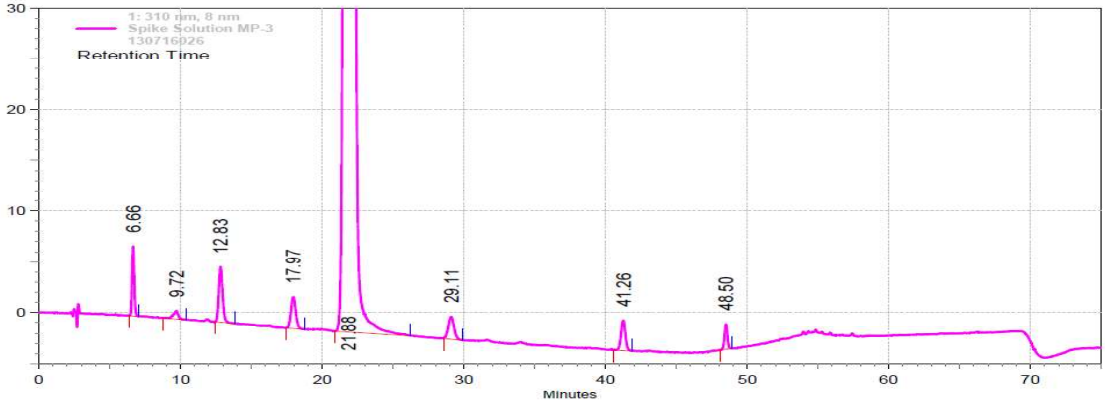


Fig 4a.32 Method Precision set-3

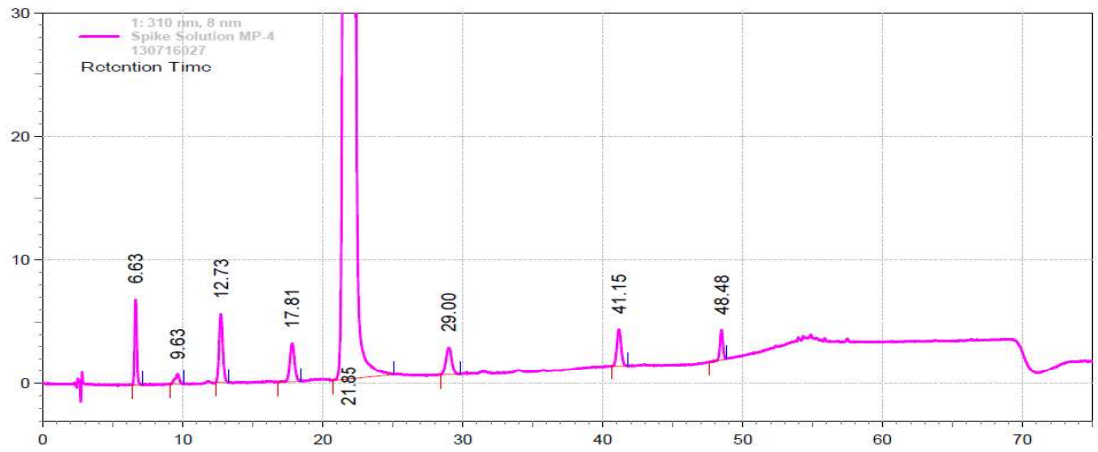


Fig4a.33 Method Precision set-4

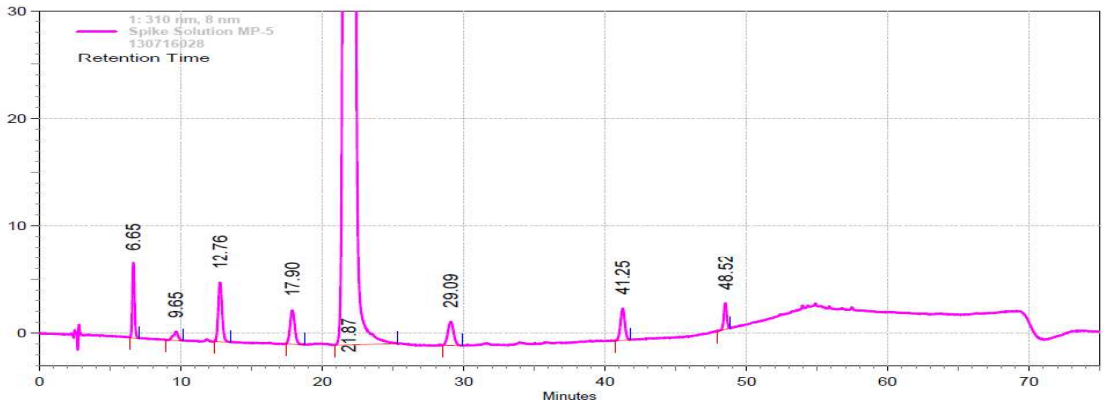


Fig 4a.34 Method Precision set-5

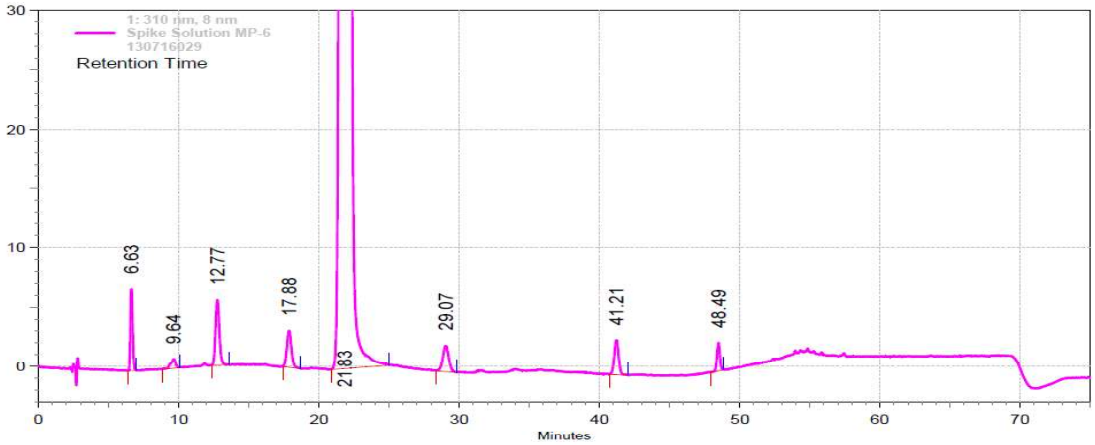


Fig 4a.35 Method Precision set-6

Table 4a.12 Method Precision of Impurity-3

Sr. No.	Levels	Area	Amount Added (ppm)	Amount Found (ppm)	Amount recovered (ppm)	Recovery (%)
1	Control sample	9117	0.000	0.024	-0.024	
2	Method precision Prep.-1	74170	0.172	0.196	0.172	100.0
3	Method precision Prep.-2	76218	0.172	0.203	0.179	104.1
4	Method precision Prep.-3	74090	0.172	0.198	0.174	101.1
5	Method precision Prep.-4	74899	0.172	0.201	0.177	103.0
6	Method precision Prep.-5	75273	0.172	0.202	0.178	103.3
7	Method precision Prep.-6	74110	0.172	0.200	0.176	102.1
			Overall Mean			**102.28**
			Overall SD			1.524
			Overall RSD (%)			**1.49**

Table 4a.13 Method Precision of Impurity-1

Sr. No.	Levels	Area	Amount Added (ppm)	Amount Found (ppm)	Amount recovered (ppm)	Recovery (%)
1	Control sample	32089	0.000	0.072	-0.072	
2	Method precision Prep.-1	108471	0.157	0.241	0.169	107.3
3	Method precision Prep.-2	105676	0.157	0.237	0.165	104.6
4	Method precision Prep.-3	105658	0.157	0.237	0.165	105.0
5	Method precision Prep.-4	102449	0.157	0.231	0.159	101.2
6	Method precision Prep.-5	104796	0.157	0.236	0.164	104.2
7	Method precision Prep.-6	105193	0.157	0.238	0.166	105.6
				Overall Mean		**104.64**
				Overall SD		1.995
				Overall RSD (%)		**1.91**

Table 4a.14 Method Precision of Impurity-2

Sr. No.	Levels	Area	Amount Added (ppm)	Amount Found (ppm)	Amount recovered (ppm)	Recovery (%)
1	Control sample	11791	0.000	0.032	-0.032	
2	Method precision Prep.-1	73878	0.159	0.200	0.168	105.5
3	Method precision Prep.-2	73021	0.159	0.199	0.167	105.1
4	Method precision Prep.-3	72783	0.159	0.199	0.167	105.0
5	Method precision Prep.-4	75004	0.159	0.207	0.174	109.6
6	Method precision Prep.-5	72843	0.159	0.200	0.168	105.5
7	Method precision Prep.-6	72524	0.159	0.200	0.168	105.6
				Overall Mean		**106.06**
				Overall SD		1.744
				Overall RSD (%)		**1.64**

Table 4a.15 Method Precision of Impurity-4

Sr. No.	Levels	Area	Amount Added (ppm)	Amount Found (ppm)	Amount recovered (ppm)	Recovery (%)
1	Control sample	12875	0.000	0.043	-0.043	
2	Method precision Prep.-1	63764	0.165	0.209	0.166	100.8
3	Method precision Prep.-2	62620	0.165	0.207	0.164	99.6
4	Method precision Prep.-3	63103	0.165	0.209	0.167	100.9
5	Method precision Prep.-4	63817	0.165	0.213	0.170	103.0
6	Method precision Prep.-5	62620	0.165	0.208	0.166	100.3
7	Method precision Prep.-6	66375	0.165	0.222	0.179	108.6
				Overall Mean		**102.19**
				Overall SD		3.342
				Overall RSD (%)		**3.27**

Table 4a.16 Method Precision of Impurity-5

Sr. No.	Levels	Area	Amount Added (ppm)	Amount Found (ppm)	Amount recovered (ppm)	Recovery (%)
1	Control sample	0	0.000	0.000	0.000	
2	Method precision Prep.-1	63324	0.196	0.190	0.190	97.2
3	Method precision Prep.-2	65256	0.196	0.198	0.198	101.0
4	Method precision Prep.-3	65002	0.196	0.198	0.198	100.8
5	Method precision Prep.-4	64227	0.196	0.196	0.196	100.2
6	Method precision Prep.-5	64171	0.196	0.196	0.196	99.8
7	Method precision Prep.-6	64319	0.196	0.197	0.197	100.6
				Overall Mean		**99.94**
				Overall SD		1.425
				Overall RSD (%)		**1.43**

Table-3.17: Method Precision of Impurity-6

Sr. No.	Levels	Area	Amount Added (ppm)	Amount Found (ppm)	Amount recovered (ppm)	Recovery (%)
1	Control sample	7857	0.000	0.042	-0.042	
2	Method precision Prep.-1	36500	0.154	0.192	0.150	97.6
3	Method precision Prep.-2	35864	0.154	0.190	0.148	96.4
4	Method precision Prep.-3	35031	0.154	0.186	0.144	93.9
5	Method precision Prep.-4	37750	0.154	0.201	0.160	104.0
6	Method precision Prep.-5	35774	0.154	0.190	0.149	96.8
7	Method precision Prep.-6	35110	0.154	0.188	0.146	95.2
				Overall Mean		**97.31**
				Overall SD		3.529
				Overall RSD (%)		**3.63**

Repeatability of the method was checked by analyzing six replicate samples of 1000 µg/g Dasatinib spiked with impurities (at 100% level). The %RSD was calculated for Impurities for its content. In the studies %RSD of peak areas for all the impurities were less than 5.0%. All the graphs for the method precision are shown in figures4a.30 to 4a.35.

4a.3.4.5 Accuracy:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Accuracy should be established across the specified range of the analytical procedure.

Recommended data: - Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

Methodology

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.

Quantification of impurities

- Analyze samples (drug substance or drug product) spiked with known amounts of impurities.

Table 4a.18 Accuracy Calculation for Impurity-3

Accuracy Calculation for Impurity-3							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			(ppm)	**(ppm)**	**(ppm)**	**(%)**	**(%)**
1	Control sample	9117	0.000	0.024	-0.024		
2	Level-(150%) Sample Prep.-1	110362	0.255	0.297	0.272	106.8	
3	Level-(150%) Sample Prep.-1	110947	0.255	0.297	0.272	106.7	**106.2**
4	Level-(150%) Sample Prep.-1	110180	0.255	0.292	0.268	105.0	
5	Level-(100%) Sample Prep.-1	74170	0.170	0.196	0.172	101.0	
6	Level-(100%) Sample Prep.-2	76218	0.170	0.203	0.179	105.2	**102.8**
7	Level-(100%) Sample Prep.-3	74090	0.170	0.198	0.174	102.2	
11	Level-(50%) Sample Prep.-1	42594	0.085	0.114	0.089	104.9	
12	Level-(50%) Sample Prep.-2	40701	0.085	0.108	0.083	97.9	**101.6**
13	Level-(50%) Sample Prep.-3	42182	0.085	0.111	0.087	102.1	
14	Level-(25%) Sample Prep.-1	22494	0.034	0.061	0.036	106.2	
15	Level-(25%) Sample Prep.-2	21320	0.034	0.057	0.032	94.5	**98.8**
16	Level-(25%) Sample Prep.-3	21520	0.034	0.057	0.033	95.6	
				Overall Mean		**102.34**	
				Overall SD		4.289	
				Overall RSD (%)		**4.19**	

Table 4a.19 Accuracy Calculation for Impurity-1

Accuracy Calculation for Impurity-1							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			(ppm)	**(ppm)**	**(ppm)**	**(%)**	**(%)**
1	Control sample	32089	0.000	0.072	-0.072		
2	Level-(150%) Sample Prep.-1	145334	0.234	0.328	0.256	109.6	
3	Level-(150%) Sample Prep.-1	146697	0.234	0.330	0.257	110.1	**109.4**
4	Level-(150%) Sample Prep.-1	146086	0.234	0.325	0.253	108.4	
5	Level-(100%) Sample Prep.-1	108471	0.156	0.241	0.169	108.4	
6	Level-(100%) Sample Prep.-2	105676	0.156	0.237	0.165	105.6	**106.7**
7	Level-(100%) Sample Prep.-3	105658	0.156	0.237	0.165	106.0	
11	Level-(50%) Sample Prep.-1	68484	0.078	0.153	0.081	104.4	
12	Level-(50%) Sample Prep.-2	66858	0.078	0.149	0.076	98.1	**99.4**
13	Level-(50%) Sample Prep.-3	66160	0.078	0.147	0.075	95.6	
14	Level-(25%) Sample Prep.-1	44630	0.031	0.101	0.029	92.2	
15	Level-(25%) Sample Prep.-2	46534	0.031	0.104	0.032	101.3	**92.1**
16	Level-(25%) Sample Prep.-3	44057	0.031	0.098	0.026	82.7	
					Overall Mean		**101.88**
					Overall SD		8.334
					Overall RSD (%)		**8.18**

Table 4a.20 Accuracy Calculation for Impurity-2

Accuracy Calculation for Impurity-2							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			(ppm)	**(ppm)**	**(ppm)**	**(%)**	**(%)**
1	Control sample	11791	0.000	0.032	-0.032		
2	Level-(150%) Sample Prep.-1	104729	0.236	0.288	0.256	108.5	
3	Level-(150%) Sample Prep.-1	106355	0.236	0.291	0.259	109.7	**108.6**
4	Level-(150%) Sample Prep.-1	105619	0.236	0.287	0.255	107.8	
5	Level-(100%) Sample Prep.-1	73878	0.157	0.200	0.168	106.6	
6	Level-(100%) Sample Prep.-2	73021	0.157	0.199	0.167	106.2	**106.3**
7	Level-(100%) Sample Prep.-3	72783	0.157	0.199	0.167	106.1	
11	Level-(50%) Sample Prep.-1	43624	0.079	0.119	0.087	110.3	
12	Level-(50%) Sample Prep.-2	42453	0.079	0.115	0.083	105.0	**106.5**
13	Level-(50%) Sample Prep.-3	42314	0.079	0.114	0.082	104.2	
14	Level-(25%) Sample Prep.-1	24769	0.031	0.068	0.036	114.1	
15	Level-(25%) Sample Prep.-2	24269	0.031	0.066	0.034	106.8	**106.8**
16	Level-(25%) Sample Prep.-3	23485	0.031	0.064	0.031	99.5	
					Overall Mean		**107.05**
					Overall SD		3.589
					Overall RSD (%)		**3.35**

Table 4a.21 Accuracy Calculation for Impurity-4

Accuracy Calculation for Impurity-4							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			(ppm)	**(ppm)**	**(ppm)**	**(%)**	**(%)**
1	Control sample	12875	0.000	0.043	-0.043		
2	Level-(150%) Sample Prep.-1	93521	0.245	0.312	0.269	109.8	
3	Level-(150%) Sample Prep.-1	91701	0.245	0.304	0.261	106.6	**106.7**
4	Level-(150%) Sample Prep.-1	90231	0.245	0.297	0.254	103.6	
5	Level-(100%) Sample Prep.-1	63764	0.163	0.209	0.166	101.8	
6	Level-(100%) Sample Prep.-2	62620	0.163	0.207	0.164	100.6	**101.4**
7	Level-(100%) Sample Prep.-3	63103	0.163	0.209	0.167	101.9	
11	Level-(50%) Sample Prep.-1	39427	0.082	0.130	0.088	107.3	
12	Level-(50%) Sample Prep.-2	40469	0.082	0.133	0.090	110.2	**108.6**
13	Level-(50%) Sample Prep.-3	40132	0.082	0.131	0.089	108.4	
14	Level-(25%) Sample Prep.-1	23119	0.033	0.077	0.034	105.2	
15	Level-(25%) Sample Prep.-2	24118	0.033	0.079	0.037	112.0	**100.9**
16	Level-(25%) Sample Prep.-3	21540	0.033	0.071	0.028	85.5	
					Overall Mean		**104.41**
					Overall SD		6.979
					Overall RSD (%)		**6.68**

Table 4a.22 Accuracy Calculation for Impurity-5

Accuracy Calculation for Impurity-5							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			(% w/w)	**(% w/w)**	**(% w/w)**	**(%)**	**(%)**
1	Control sample	0	0.000	0.000	0.000		
2	Level-(150%) Sample Prep.-1	98587	0.291	0.301	0.301	103.6	
3	Level-(150%) Sample Prep.-1	99673	0.291	0.303	0.303	104.1	**103.5**
4	Level-(150%) Sample Prep.-1	99076	0.291	0.299	0.299	102.7	
5	Level-(100%) Sample Prep.-1	63324	0.194	0.190	0.190	98.1	
6	Level-(100%) Sample Prep.-2	65256	0.194	0.198	0.198	102.0	**100.7**
7	Level-(100%) Sample Prep.-3	65002	0.194	0.198	0.198	101.9	
11	Level-(50%) Sample Prep.-1	33956	0.097	0.103	0.103	106.1	
12	Level-(50%) Sample Prep.-2	35054	0.097	0.105	0.105	108.7	**104.4**
13	Level-(50%) Sample Prep.-3	31838	0.097	0.095	0.095	98.4	
14	Level-(25%) Sample Prep.-1	13914	0.039	0.043	0.043	109.6	
15	Level-(25%) Sample Prep.-2	14136	0.039	0.043	0.043	109.8	**108.6**
16	Level-(25%) Sample Prep.-3	13705	0.039	0.041	0.041	106.2	
					Overall Mean		**104.27**
					Overall SD		3.958
					Overall RSD (%)		**3.80**

Table 4a.23 Accuracy Calculation for Impurity-6

Accuracy Calculation for Impurity-6							
Sr. No.	**Levels**	**Area**	**Amount Added**	**Amount Found**	**Amount recovered**	**Recovery**	**Mean recovery**
			(% w/w)	**(% w/w)**	**(% w/w)**	**(%)**	**(%)**
1	Control sample	7857	0.000	0.042	-0.042		
2	Level-(150%) Sample Prep.-1	48748	0.228	0.260	0.218	95.8	
3	Level-(150%) Sample Prep.-1	51756	0.228	0.275	0.233	102.1	**97.7**
4	Level-(150%) Sample Prep.-1	49199	0.228	0.259	0.217	95.2	
5	Level-(100%) Sample Prep.-1	36500	0.152	0.192	0.150	98.5	
6	Level-(100%) Sample Prep.-2	35864	0.152	0.190	0.148	97.4	**96.9**
7	Level-(100%) Sample Prep.-3	35031	0.152	0.186	0.144	94.8	
11	Level-(50%) Sample Prep.-1	21429	0.076	0.113	0.072	94.3	
12	Level-(50%) Sample Prep.-2	21867	0.076	0.115	0.073	96.1	**95.9**
13	Level-(50%) Sample Prep.-3	22106	0.076	0.116	0.074	97.3	
14	Level-(25%) Sample Prep.-1	13959	0.030	0.074	0.033	107.8	
15	Level-(25%) Sample Prep.-2	12886	0.030	0.068	0.026	85.9	**100.8**
16	Level-(25%) Sample Prep.-3	14234	0.030	0.075	0.033	108.5	
				Overall Mean			**97.82**
				Overall SD			6.122
				Overall RSD (%)			**6.26**

4a.3.4.6 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. We have performed robustness study as per ICH guideline of analytical method validation, which covers most of the critical variables of analytical method. To demonstrate the robustness of developed method spiked sample was analyzed by altered chromatographic conditions (flow rate, pH and column temperature).No significant difference in quantification and resolution confirmed that the developed method is robust. Results of robustness study are reported in Table 4a.24.

Table 4a.24 Summary of Robustness Data

Parameter/variation	USP resolution						
	Imp-3	Imp-1	Imp-2	Dasatinib	Imp-4	Imp-5	Imp-6
As such conditions	5.6	15.37	9.30	6.00	9.88	18.35	14.80
Flow rate (mL/min)							
a. 1.15	5.7	15.8	9.8	6.2	10.1	18.9	15.2
b. 1.25	5.4	15.2	9.1	5.8	9.4	18.0	14.5
Temp. (°C)							
c. 32	5.6	15.7	9.8	6.3	10.0	18.7	15.0
d. 38	5.3	15.1	9.0	5.6	9.2	17.9	14.8
Buffer pH							
c. 4.9	5.2	15.3	9.3	5.9	10.8	18.0	14.7
d. 5.1	5.5	15.3	9.8	6.0	7.0	18.1	15.2

4a.3.5 Application of method:

- The developed method is capable for quantitative analysis of potential impurities present in bulk drug and in pharmaceutical dosage form of Dasatinib.
- Method validation is performed as per guidelines provided by ICH.

- As HPLC is involved in method of detection, method can be performed easily at academic and industrial level due to very cheap solvents and easily available agents are used during method development.
- Forced degradation study & its importance in analytical method isdiscussed.
- Reproducibility of method is very high so it can be performed at any facility with basic instrumentation requirements so the method is applicable for routine analysis for determination of impurities in Dasatinib bulk drug and formulation in Quality Control Labs.

4a.4 Conclusion:

The developed analytical method presented in chapter presents a rapid, simple, precise, robust, accurate and selective gradient RP-LC method that separates the Dasatinib and all of its impurities and degradation products with good resolution.

The process and degradation related impurities, which were present in Dasatinib sample were identified by LC-MS and characterized using MS, FT-IR, ^{1}HNMR, ^{13}CNMR spectral data which are shown in chapter 2.

The developed method was validated to ensure the compliance in accordance with ICH guidelines. This method can be used for routine testing and stability analysis in quality control laboratories for related substances of Dasatinib in bulk and pharmaceutical formulation.

4a.5 References

1. Guideline IHT, editor. Impurities in new drug substances Q3A (R2). Proceedings of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland; 2006.
2. Ahuja S, Alsante KM. Handbook of isolation and characterization of impurities in pharmaceuticals: Academic press. 2003.
3. Ali MA. Chronic Myeloid leukemia in the Era of tyrosine kinase inhibitors: An evolving paradigm of Molecular Targeted therapy. Molecular Diagnosis and Theory. 2016; 20:315-333
4. Miura M, Therapeutic Drug monitoring of Imatinib, Nilotinib and Dasatinib for Patients with Chronic Myeloid Leukemia. Biological and Pharmaceutical bulletin. 2015; 38:645-654
5. Drug Bank: Dasatinib (DB01254).
6. ICH Q1A (R2), Stability Testing of New Drug Substances and Products, 2003.
7. The United States Pharmacopoeia 39th ed., US Pharmacopoeia Convention, MD. 2017.
8. Zeng, J, Cai HL, Jiang ZP, Wang Q, Zhu Y, Xu P, Zhao XL. A validated UPLC-MS/MS method for simultaneous determination of imatinib, Dasatinib and nilotinib in human plasma. Journal of Pharmaceutical Analysis. 2017; 7:374-380
9. Pirro E, De Francia S, De Martino F, Fava C, Ulisciani S, Cambrin G, Racca S, Saglio G, Di Carlo FJ. A New HPLC-UV validated Method for Therapeutic Drug Monitoring of Tyrosine Kinase Inhibitors in Leukemia Patients. Chromatography Science. 2011; 49(10):753-757
10. Gonzalez AG, Taraba L, Hranicek J, Kozlik P, Coufal PJ. Determination of Dasatinib in the tablet dosage form by ultra-high-performance liquid chromatograph2y, capillary zone electrophoresis, and sequential injection analysis. Separation Science and technology. 2017; 40:400-406
11. Prinesh P, Gananadhamu S, Veeraraghavan, S, Rambabu A, Kanthi Kiran VS, Swaroop Kumar VVS. Validated LC-MS/MS method for simultaneous determination of Dasatinib and Sitagliptin in rat plasma and its application to pharmacokinetic study. Analytical Methods. 2014; 6:433-439

12. D'Avolio A, Simiele M, De Francia S, Ariaudo A, Baietto L, Cusato J, Fava C, Saglio G, Di Carlo F, Di Perri GJ.HPLC-MS method for the simultaneous quantification of the antileukemia drugs imatinib, Dasatinib and nilotinib in human peripheral blood mononuclear cell (PBMC). Journal of pharmaceutical and biomedical analysis. 2012; 59:109-116
13. Lankheet N, Hillebrand M, Rosing H, Schellens J, Beijnen J, Huitema A.Method development and validation for the quantification of Dasatinib, erlotinib, gefitinib, imatinib, lapatinib, nilotinib, sorafenib and sunitinib in human plasma by liquid chromatography coupled with tandem mass spectrometry. Biomedical Chromatography. 2013; 27(4):466-476
14. Hesham K, Motiur RAF M, Mohammed K. Dasatinib, Profiles of Drug Substances, Excipients and Related Methodology. 2014; 39(4):205-237.
15. De Francia S, D'Avolio A, De Martino F, Pirro E, Baietto L, Siccardi M, Simiele M, Racca S, Saglio G, Di Carlo F, Di PerriGJ.New HPLC-MS method for the simultaneous quantification of the antileukemia drugs imatinib, Dasatinib, and nilotinib in human plasma.Journal of Chromatography B, Analytical technologies in the biomedical and life science. 2009; 877: 1721-1726
16. Andriamanana I, Gana I, Duretz B, Hulin AJ.Simultaneous analysis of anticancer agents bortezomib, imatinib, nilotinib, Dasatinib, erlotinib, lapatinib, sorafenib, sunitinib and vandetanib in human plasma using LC/MS/MS Journal of Chromatography B, Analytical technologies in the biomedical and life science. 2013; 926:83-91
17. Catarina J, Victor D. Redox mechanism, Spectrophotometrical characterization and Voltammetric determination in serum samples of Kinase inhibitor and anticancer drug Dasatinib. Journal of Electroanalytical Chemistry. 2015; 752:47-53
18. Couchman L, Birch M, Ireland R, Corrigan A, Wickramasinghe S, Josephs D, Spicer J, Flanagan R. An automated method for the measurement of a range of tyrosine kinase inhibitors in human plasma or serum using turbulent flow liquid chromatography-tandem mass spectrometry.Anal. Bioanal. Chem. 2012, 403, 1685-1695.
19. Gotze, L.; Hegele, A.; Metzelder, S.; Renz, H.; Nockher, W. Development and Clinical application of a LC-MS/MS method for simultaneous determination of various tyrosine kinase inhibitor in human plasma. Clinical Chimica Acta. 2012; 413:43-149.
20. Balaji N, Sultana S, Trace level determination and quantification of potential genotoxic impurities in Dasatinib drug substance by UHPLC/infinity LC. International Journal of Pharmaceutical science. 2016;8(10): 209-216.

21. Sunil L, Sajid S, Dhramveer Singh S, NishikumarN, RadhiahChe R, NurulSyazwani N, MohdZulfadli M. Rapid and Sensitive RP-HPLC Method for Determination of Potential Genotoxic Impurity in Dasatinib Drug Substance.International Journal of Chemical Science. 2017; 15(4): 177.
22. Sunil L, Dhramveer Singh S, Nishikumar N, RadhiahChe R, Sajid Syed S.Rapid and Sensitive RP-HPLC Method for Determination of Potential Genotoxic Impurity in Dasatinib Drug Substance. International Journal of Science and Research. 2017; 6(11):51-58
23. Dwivedi SP, Singh KK, Singh NA, Patil A. U.S. 9,249,134 B2, Cadila healthcare ltd, Ahmedabad, India. 2016.
24. ICH Q2 (R1) Validation of Analytical Procedures: Text and Methodology, 2005.
25. "High Performance Liquid Chromatography (HPLC) in the pharmaceutical analysis", 2014, http://www.forumsci.co.il/HPLC/WEBPharm_Review/HPLC_pharma_SST.html
26. Neue UD. HPLC Columns. Theory, Technology, and Practice John Wiley & Sons, New York. 1997; 926-936.
27. Ghulam AS. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonization. Journal of Chromatography A. 2003; 987(1):57-66
28. Persson BA, Vessman J, McDowall R. Is your method specific or just selective? LC GC. 1998; 16(6):556-60
29. Bakshi M and Singh S. Guidance on Conduct of Stress Tests to Determine Inherent Stability of Drugs. Pharmaceutical Technology. 2000; 1-9.
30. Bakshi M and Singh S, Development of validated stability-indicating assay Methods-critical review. Journal of Pharmaceutical and biomedical analysis 2002; 1011-1040.
31. Stability Testing of Pharmaceutical Products in a Global environment, Dec 2014, www.who.int/medicines/areas/quality.../RAJ2006WHOStability.pdf
32. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. The clinical biochemist reviews. 2008;29(1):S49.
33. Swartz ME, Krull IS. Analytical method development and validation. CRC Press Book.1997; (1)1:96.
34. The United States Pharmacopeia (USP), Validation of Compendial Methods, 32 (USP 32), Section < 1225 > (2009).

35. Snyder, L, Kirkland, J. Journal of Practical HPLC Method Development. John Wiley and Sons, New York, 1997; 2:184-185.