# Abstract:


## Development and validation of derivatized chromatographic method to analyze genotoxic impurity Hydrazine Hydrate in Imatinib Mesylate API

Increased concern for impurities present in drug substance and involvement of regulatory body provides threshold to the research of impurity profiling study. Even regulatory body becomes more stringent for impurities in generic products which increase recalls of product from markets. Definition of impurities as per ICH Q3A guidelines “impurity in a drug substance is any component of the drug substance that is not the chemical entity defined as the drug substance” and as per ICH Q3B guidelines “impurity in any component of the drug product that is not the chemical entity defined as the drug substance or an excipient in the drug product”

Imatinib Mesylate is a well-known tyrosine kinase inhibitor used to treat hematological malignancies in patient or malignant sarcomas such as gastrointestinal stromal tumors, chronic myeloid leukemia, acute lymphoblastic leukemia, gastrointestinal stromal tumors.Imatinib mesylate is a specific kind of tyrosine kinase inhibitor in Bcr-Abl+ cell 4-[(4-methylpiperazin-1-yl)methyl]-N-(4-methyl-3-(11) phenyl) benzamide methane sulfonate. Imatinib mesylate is approved for gastrointestinal stroma tumor treatment since 2002.Imatinibmesylate is also used for chronic myelogenous leukemia disease since 2011andapproved by USFDA.

Reduction of nitro group during the synthesis of Imatinib can be achieved by several reducing reagents such as Fe/HCl, SnCl2/HCl, hydrazine hydrate/Raney Ni, hydrazine hydrate/FeCl3/C. The use of Fe/HCl and SnCl2/HCl as reducing agent were not preferred as due to presence of metallic hydroxides emulsion formation occurs during isolation process of imatinib. SnCl2is an expensive reagent and also toxic. In comparison to other reduction process, the reduction with hydrazine hydrate produces harmless byproducts such as nitrogen gas and water. Route of synthesis of imatinib has been described in Fig. 1.

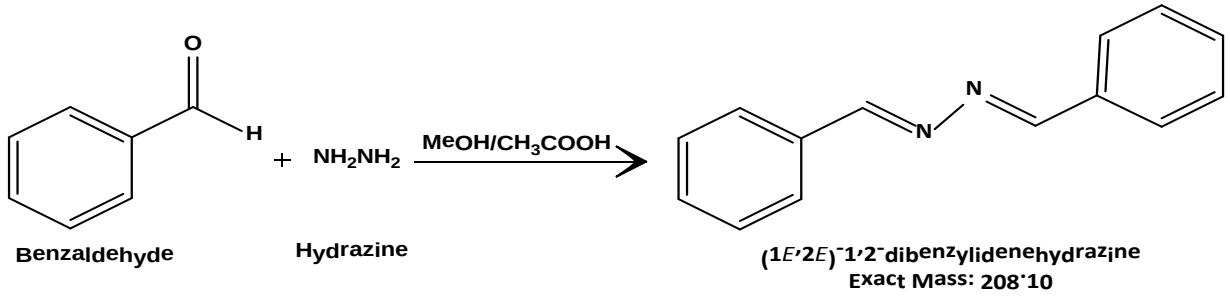



Fig.1. Reaction scheme of Benzaldehyde with Hydrazine in acidic condition for Derivatization

Being polar molecule hydrazine hydrate ($N_2H_4.H_2O$) has no chromophores present in structure which can follow Lambert beer law, thus it is difficult to analyze. The method of quantification was developed by attaching chromophores to hydrazine with derivatization, which helped to increase sensitivity. The derivatization agent should bump conversion of free impurity (hydrazine hydrate) to the derivatized product (1,2-dibenzylidenehydrazine). The absorption of derivatized product should be far away from the absorption of reagents and solvents so the interference should be minimum. For derivatized product 1,2-dibenzylidenehydrazine a strong UV absorption at 300nm is noted which is far away from any interference of solvents and reactive species. A derivatized product 1,2-dibenzylidenehydrazine seems to be highly suitable for the High pressure liquid chromatography due to its high lipophilicity which could help in good mark on retention in C18,C8 and even in C8 columns.

LC-MS chromatogram shows molecular ions at m/z 209.05 ($M^+$+H) confirmed that resultant product was 1,2-dibenzylidenehydrazine.

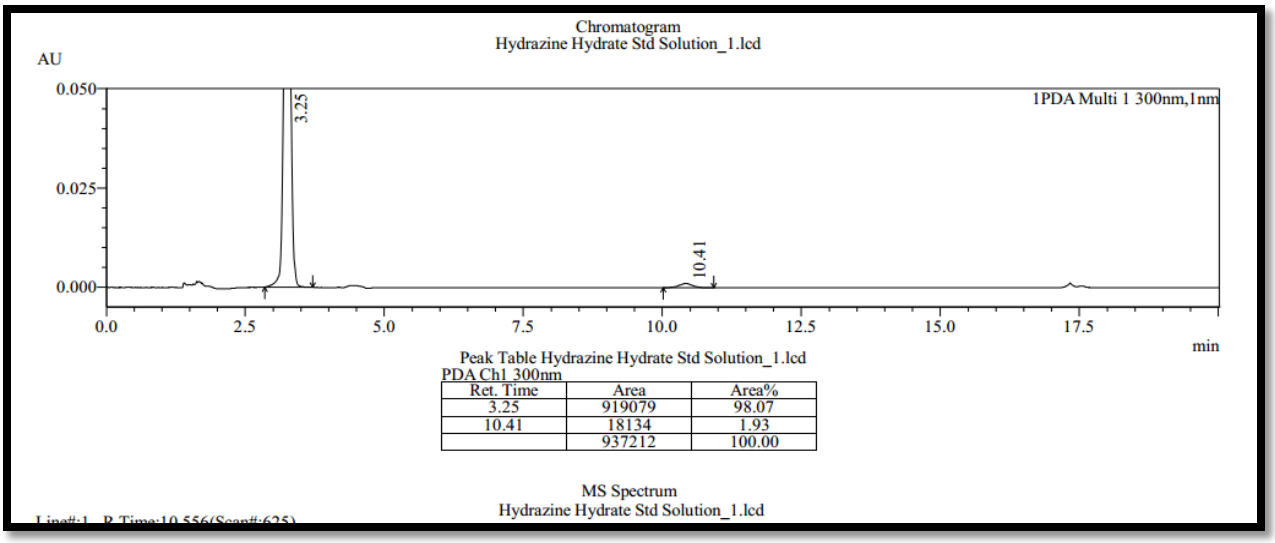



Figure 2. LC-MS chromatogram of derivatized product.

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

Table 1. System suitability criteria

Even the system suitability criteria support the developed analytical method. Recovery studies has been carried out for the determination of the accuracy by analyzing the spiked samples. Known amounts of hydrazine hydrate was spiked in triplicate at four different concentration levels of 0.0040, 0.0075, 0.0150 and 0.0225 µg/g to a previously analyzed Imatinib mesylate drug substance sample. The percentage of recoveries for hydrazine hydrate was calculated. The accuracy and precision was validated on a Imatinib Mesylate spiked with hydrazine hydrate at four concentration levels covering the specified range with 6 replicates for 0.0150 µg/g and 3 replicates for 0.0040, 0.0075 and 0.0225 µg/g. Imatinib mesylate was prepared at a concentration of 8000 µg/g. The percent recovery was calculated by spiking hydrazine in Imatinib Mesylate API. Calculation for accuracy determination is shown in table 2.11. The individual percent recoveries for all preparations were from 97.3-105.2% and the %RSD for all injections was 1.2%.

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

## Development and validation of residual solvent determination by head space gas chromatography in Imatinib Mesylate API

The organic solvents used in the process of manufacturing requires monitoring and control as the solvents are toxic, have no therapeutic importance and affect the quality and stability of drug substances and drug products. Hence they are not desirable in the finished product. Determination of residual solvents becomes a necessary procedure for quality control of drug substances and drug product to meet regulatory guideline and ensure patient safety. Six solvents are required for the synthesis of Imatinib Mesylate i.e. methanol, acetone, dichloromethane, n-hexane, ethyl acetate and pyridine and these should be controlled in final API.

| S. No | Name of residual solvent in Imatinib Mesylate | Class of solvent | Permissible daily exposure (PDE) (mg/day) | ICH Limit (ppm) | Density (kg/m3) |
|---|---|---|---|---|---|
| 1 | Methanol | II | 30.0 | 3000 | 791.80 |
| 2 | Acetone | III | 50.0 | 5000 | 791.00 |
| 3 | Dichloromethane | II | 6.0 | 600 | 1326.00 |
| 4 | n-Hexane | II | 2.9 | 290 | 659.1 |
| 5 | Ethyl acetate | III | 50 | 5000 | 897.00 |
| 6 | Pyridine | II | 2.0 | 200 | 981.90 |

Table 2.Solvents and its limits which required in the synthesis of imatinib mesylate

| Solvent name | RT (min) | USP resolution | USP tailing factor | USP theoretical plate | %RSD (n=6) of peak area |
|---|---|---|---|---|---|
| **Methanol** | 4.10 | -- | 1.292 | 20863 | 4.6 |
| **Acetone** | 6.07 | 16.0 | 1.028 | 33847 | 1.9 |
| **Dichloromethane** | 6.92 | 6.3 | 1.065 | 42971 | 2.5 |
| **n-Hexane** | 7.98 | 7.3 | 1.003 | 39627 | 2.1 |
| **Ethyl acetate** | 9.61 | 10.4 | 0.996 | 62912 | 2.3 |
| **Pyridine** | 14.69 | 45.0 | 1.162 | 652171 | 4.3 |

Table 3. System precision and System suitability parameter

The method validation was performed by evaluating specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, intermediate precision, system suitability and method precision of residual solvents as specified in the ICH harmonized tripartite guideline (2005).

| Solvent name | Conc. Range(µg/ml) | | | | | | | Regression Equation (y=ax+b) | Correlation coefficient |
|---|---|---|---|---|---|---|---|---|---|
| | **LOQ** | **30 %** | **50 %** | **80 %** | **100 %** | **120 %** | **150 %** | | |
| **Methanol** | 60.3 | 90.4 | 150.7 | 241.2 | 301.5 | 361.8 | 452.2 | Y=2.8677x-12.273 | 0.9996 |
| **Acetone** | 100.6 | 150.9 | 251.4 | 102.3 | 502.9 | 303.5 | 754.3 | Y=12.01x+57.252 | 0.9997 |
| **DCM** | 15.1 | 22.6 | 37.7 | 60.3 | 75.4 | 90.5 | 113.1 | Y=2.613x+1.6003 | 0.9997 |
| **n-Hexane** | 6.7 | 10.0 | 16.6 | 26.6 | 33.3 | 39.9 | 49.9 | Y=72.831x+4.9268 | 0.9994 |
| **Ethyl acetate** | 99.6 | 149.4 | 249.1 | 398.5 | 498.1 | 597.7 | 747.2 | Y=7.6815x+41.985 | 0.9997 |
| **Pyridine** | 4.8 | 7.2 | 12.0 | 19.3 | 24.1 | 28.9 | 36.1 | Y=2.1041x+0.9255 | 0.9996 |

Table 4. Summary of method validation

Residual solvents analysis was performed on developed and validated method for commercial batch of Imatinib Mesylate API in triplicate. Results are reported in Table 5.

| Solvent name | Batch set-1 | Batch set-2 | Batch set-3 |
|---|---|---|---|
| **Methanol** | 988 ppm | 954 ppm | 928 ppm |
| **Acetone** | 513 ppm | 535 ppm | 522 ppm |
| **Dichloromethane** | ND | ND | ND |
| **n-Hexane** | 0.5 ppm | 0.4 ppm | 0.5 ppm |
| **Ethyl acetate** | 4.1 ppm | 4.2 ppm | 4.5 ppm |
| **Pyridine** | ND | ND | ND |

Table 5. Residual solvents contents in the commercial batch of Imatinib Mesylate API

A selective and sensitive fast static HSGC method has been successfully developed for the determination of methanol, acetone, dichloromethane, n-hexane, ethyl acetate and pyridine in Imatinib Mesylate API through consideration of route of synthesis and solvents nature. The developed method was successfully validated as per regulatory guideline and found to be precise, accurate, linear, robust and specific. Additionally, our method is suitable for analysis of pyridine and other solvents in one single method, which is accurate, precise and linear in presence of sample matrix. However only a limited number of solvents are used in Imatinib Mesylate API, this method may be used to separate the residual solvents present in other drug substances and can be used for routine analysis to monitor in-process drying and in quality control for bulk drug manufacturing. Taken together, our developed HSGC method demonstrated precise, economical and commercially viable quantitative technique for residual solvents determination in Imatinib Mesylate API which will also be advantageous for industrial scale manufacturing.

## Characterization of related impurities present in Dasatinib

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.

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.

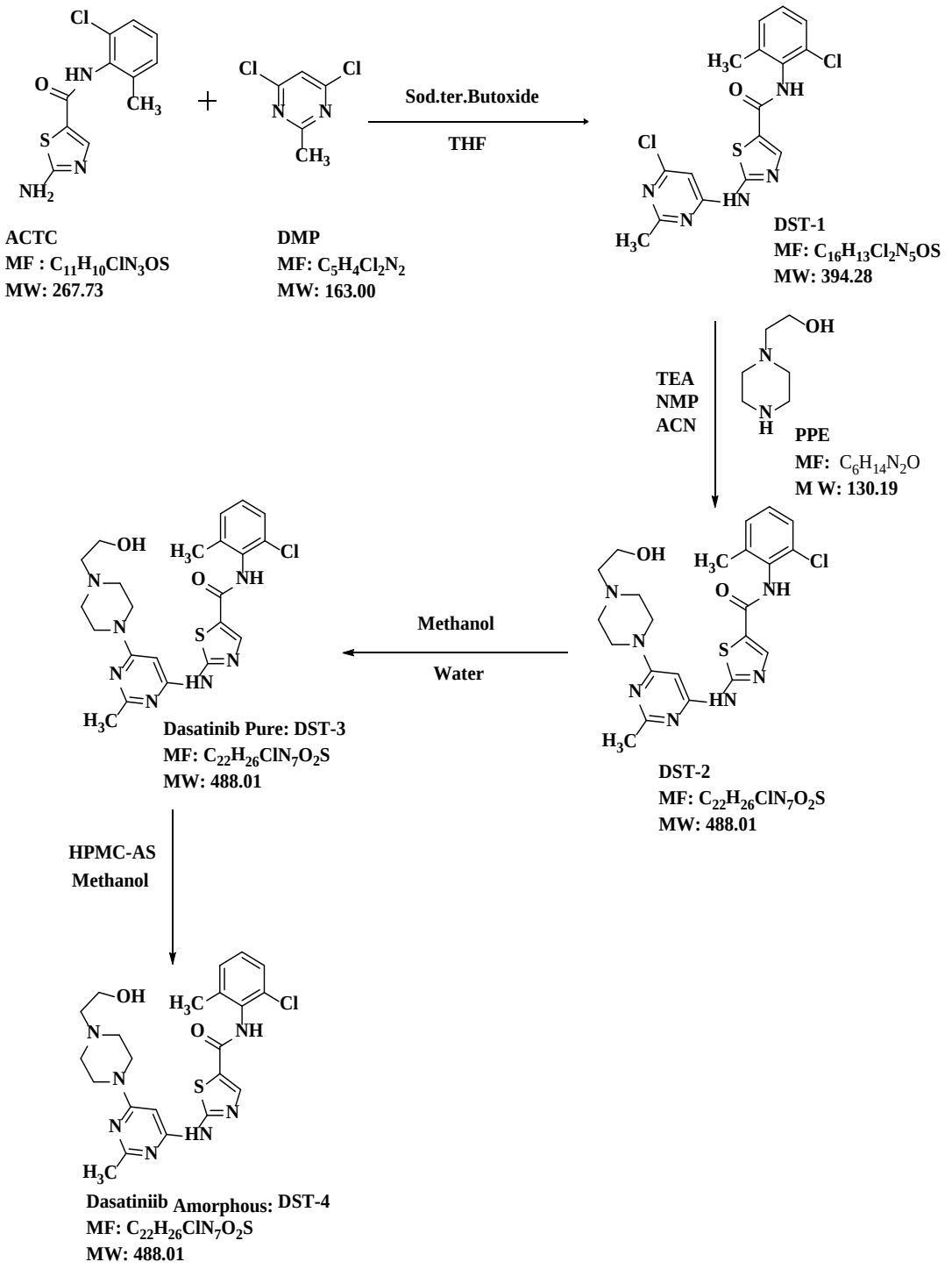
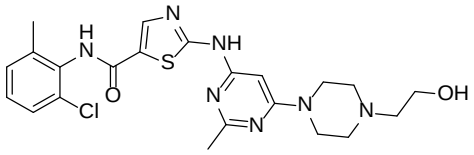
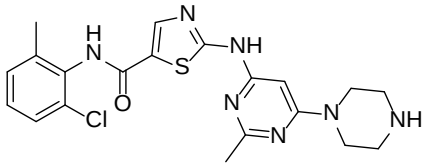
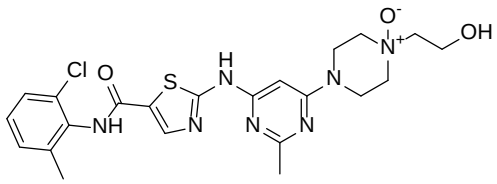
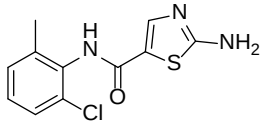
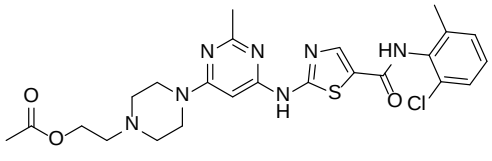



Figure 3. Route of synthesis of Dasatinib

| | |
|---|---|
| 1. | 2-amino-n-(2-chloro-6-methylphenyl)thiazole-5-carboxamide |
| 2. | N-(2-chloro-6-methylphenyl)-2-((2-methyl-6-(piperazin-1-yl) pyrimidin-4-yl) amino) thiazole-5-carboxamide. |
| 3. | 4-(6-((5-((2-chloro-6-methylphenyl) carbamoyl) thiazol-2-yl) amino)-2- |

| | |
|---|---|
| | methylpyrimidin-4-yl)-1-(2-hydroxyethyl) piperazine 1-oxide. |
| 4. | 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. | 2-(4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate |
| 6. | N-(2-chloro-6-methylphenyl)-2-[(6-chloro-2-methyl-4-pyrimidinyl)amino]-5-thiazole carboxamide |

Table 6. list of impurities present in dasatinib

| Sr. No | Impurity particulars | Chemical structure | IUPAC name | Source |
|---|---|---|---|---|
| 1 | Dasatinib |  | N-(2-chloro-6-methylphenyl)-2-((6-(4-(2-hydroxyethyl) piperazin-1-yl)-2-methylpyrimidin-4-yl) amino) thiazole-5-carboxamide. | Target API |
| 2 | Imp-1 |  | N-(2-chloro-6-methylphenyl)-2-((2-methyl-6-(piperazin-1-yl) pyrimidin-4-yl) amino) thiazole-5-carboxamide. | Degradation impurity |
| 3 | Imp-2 |  | 4-(6-((5-((2-chloro-6-methylphenyl) carbamoyl) thiazol-2-yl) amino)-2-methylpyrimidin-4-yl)-1-(2-hydroxyethyl) piperazine 1-oxide. | Degradation impurity |
| 4 | Imp-3 |  | 2-amino-N-(2-chloro-6-methylphenyl) thiazole-5-carboxamide | Key Starting Material |
| 5 | Imp-4 |  | 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 | Degradation impurity due to Hydroxy Propyl Methyl Cellulose |

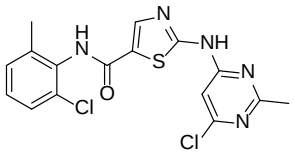
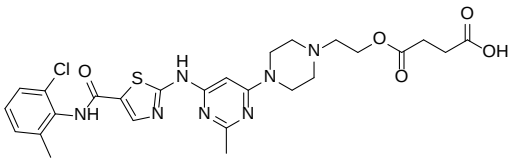
| | | | | |
|---|---|---|---|---|
| 6 | Imp-5 |  | 2-((6-chloro-2-methylpyrimidin-4-yl)amino)-N-(2-chloro-6-methylphenyl) thiazole-5-carboxamide | Intermediate |
| 7 | Imp-6 |  | 2-(4-(6-((5-((2-chloro-6-methylphenyl) carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazin-1-yl)ethyl acetate | Degradation impurity due to Hydroxy Propyl Methyl Cellulose |

Table 7. Chemical structure and IUPAC name of the characterized dasatinib impurity

All the six impurities present in Dasatinib are identified and characterized by Mass Spectroscopy, 1H-NMR spectroscopy, 13C-NMR Spectroscopy and IR Spectroscopy.

## Development and validation of chromatographic method for determination of impurities in solid dispersion of Dasatinib

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 Response Factor (RF) values of each impurity as per validation guideline of ICH. The developed method was validated for the determination of related substances in the solid dispersion of dasatinib by HPLC as per ICH guidelines. The limit of detection (LOD) and limit of quantification (LOQ) for dasatinib and all six impurities were determined by signal to noise ratio of 3:1 and 10:1 respectively.

| 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 |

Table 8. Summary of method validation

Peak purity was checked in the degraded sample and calculate purity angle and purity threshold for the known impurity are reported in Table 9. This table shows that purity angle is less than purity threshold for known impurity peak formed due to degradation.

| 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 9.Peak purity results

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.

| 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 10. Summary of stress studies

An accurate, selective and sensitive gradient RP-HPLC method has been developed and validated as per regulatory guideline for the determination of process and degradation related impurities for the oncology drug, Dasatinib.In addition, this method is cost effective as there is no need to inject expensive impurities standard solution during method validation. Taken together, developed RP-HPLC method demonstrated precise, economical and commercially viable quantitative determination of Dasatinib impurities which will also be useful for industrial scale manufacturing.

## CHAPTER 5:Impurity profiling of novel 2-thio imidazole derivative ZY12201 -an antidiabetic agent.

A wide range of structurally diverse modulators of TGR5 have been reported in the literature by various pharmaceutical companies. Most of these reported TGR5 agonists, however, possess insufficient potency and/or lack metabolic stability. This thesis describes the impurity profiling of 2-((2-(4-(1H-imidazol-1-yl)phenoxy)ethyl) thio)-5-(2-(3,4-dimethoxyphenyl) propan-2-yl)-1-(4-fluoro phenyl)-1H-imidazole (6g), a potent, selective, and orally efficacious TGR5 agonist.

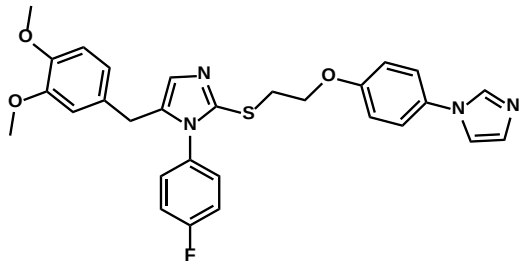



Figure 4. Structure of novel compound ZY12201

Degradation pathways of novel compound ZY12201has been developed to recognize the chemical properties of drug substance. From force degradation study we can elucidate the structure of degradation products along with resolution of stability-related problems. From these type of studies we can produce more stable formulations of drugs and it also helps in determination of expiry date of drugs. To generate a degradation profile similar to that of what would be observed in a formal stability study under ICH conditions.

| 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% H2O2 | 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 11. summary of force degradation studies

Peak purity is a comparison of the reference standard to the API in the sample stressed by forced degradation (thus specificity). In essence you are showing that no impurity (related substance) is eluting underneath the main API peak in HPLC. Peak threshold is used as a parameter for determining peak purity in HPLC. For the acceptance of the peak purity, angle should be less than a purity threshold.

| Degradation condition | Acid treated | Alkali treated | Oxidation treated | Photolytic treated | Thermal treated |
|---|---|---|---|---|---|
| Purity angle | 3.467 | 3.557 | 0.702 | 1.855 | 1.512 |
| Purity threshold | 5.716 | 5.390 | 1.344 | 3.001 | 2.450 |

Table 12. Peak purity table

Identification of degradation related impurities for the ZY12201 was done in oxidation treated sample through LC-MS technique. One impurity was detected in ZY12201 oxidation treated sample which was confirmed and identified through mass spectral analysis.

The positive ion mass spectral analysis of impurity-1 was observed at 575 (M) suggesting the possibility of Molecular formula $C_{31}H_{31}FN_4O_4S$, which confirms the theoretical molecular weight of Impurity-1.

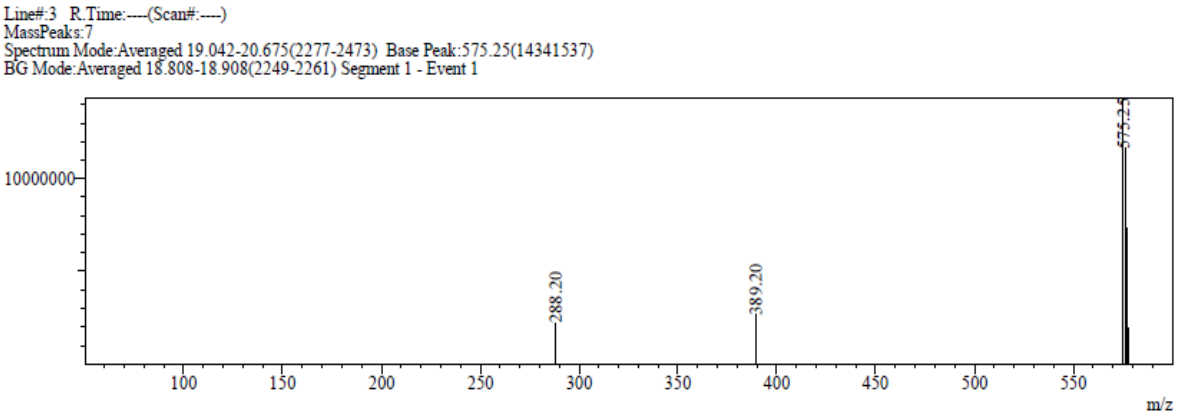



Figure 5. Mass spectra of degradation impurity

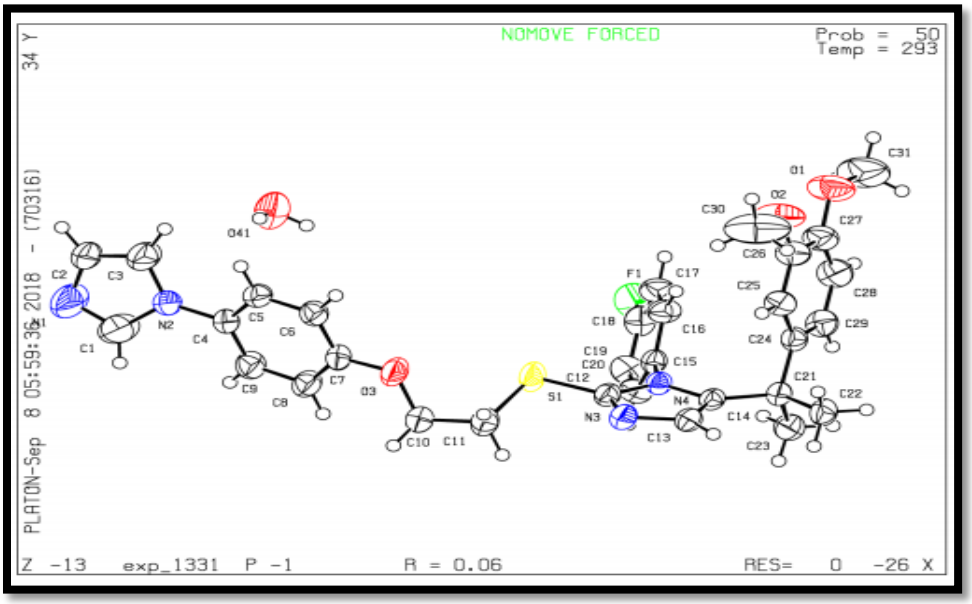



Figure 6.ORTEP Image of ZY12201

From force degradation study of novel compound ZY12201 can elucidate the structure of degradation products along with resolution of stability-related problems. From these types of studies we can produce more stable formulations of drugs and it also helps in determination of expiry date of drugs. Stability studies can be useful in formulation selection, packaging selection and storage conditions for the products. The developed analytical method is rapid, simple, precise, robust, accurate and selective gradient RP-LC method that separates the ZY12201 and its impurities and degradation products with good resolution. The developed method was validated to ensure the compliance in accordance with ICH guidelines. The developed method

can be used for routine testing and stability analysis in quality control laboratories to check purity of ZY12201 in bulk and pharmaceutical formulation.

Overall work presented have produced a very useful result and on the verge of the industrial application.