Development of a high sensitivity RP-HPLC method and stress testing of imatinib mesylate

1. INTRODUCTION
Imatinib mesylate is a tyrosine kinase inhibitor, with excellent activity against chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) and numerous other malignancies. [1] Imatinib acts on and impairs BCR-ABL-mediated transfer of phosphate to its substrates. [2] Thus hampering the rapid growth in tumor cells. HPLC offers a fast and accurate method for the analysis of chemical entity in drug substance and drug products. There were other HPLC methods available for the determination of the imatinib mesylate, however they were unable to detect the drug in nano range. [3, 4] Hence, there is an opportunity to develop the HPLC method which will have high sensitivity at lower concentrations (nano grams). Since, upcoming anticancer formulations highly involve use of nanotechnology, [5] a method for high precision at lower concentration levels is highly desirable. The published ICH guideline entitled “Stability testing of new drug substances and products” mandates stress testing to be performed in order to evaluate the intrinsic stability characteristics of the active pharmaceutical substance. These stress testing include hydrolytic stability, oxidative stability and photolytic stability testing (ICH, Q1A (R2), 2005). [6] An ideal stability indicating analytical method should have high specificity for the pure drug substance in presence of degraded products. In present study we developed a simple, sensitive, accurate RP-HPLC method [7, 8] to detect imatinib mesylate in low concentrations over a broad range of linearity (from nanograms to micrograms). We also evaluated the developed method to be highly specific in detecting the pure drug in presence of degraded products arising from the forced degradation studies; thus, employing the method to investigate the stability of the drug towards different stress conditions.

2. MATERIAL AND METHOD
2.1. Apparatus
The liquid chromatography equipment consisted of a Waters E 2695 separation unit coupled with a fluorescence detector (Waters 2998, Photodiode array detector). The chromatographic separation was achieved on Develosil® ODS-HG-5, Nomula chemical (50 mm×4.6 mm) analytical column.
2.2. Reagents and Materials
HPLC grade solvents, methanol and acetonitrile and sodium dihydrogen orthophosphate dihydrate were purchased from Merck (India). Triethylamine and hydrogen peroxide were purchased from Sigma Aldrich. Sodium hydroxide and hydrochloric acid were obtained from CDH chemicals. MiliQ water was used in all the experiments to prepare aqueous phase.

2.3. Chromatographic conditions
An isocratic elution method was used for the chromatographic separation. The mobile phase consisted of phase A (aqueous) and phase B (organic). Phase A was prepared by dissolving 6 gm of the sodium dihydrogen orthophosphate dihydrate in 1000 mL MiliQ water and the pH was adjusted by triethylamine. Phase B was prepared by mixing methanol and acetonitrile in different ratios. The ratio of phase A to phase B, flow rate, temperature, pH of aqueous phase and the ratio of methanol to acetonitrile were optimized for the development of the chromatographic separation method. Detection of analyte was performed at 267 nm.

2.4. Preparation of standard solution
The stock solution of imatinib mesylate was prepared by dissolving 10 mg in 20 mL of MilliQ water in a 100 mL volumetric flask following a dilution to 100 mL with mobile phase. This solution was then diluted appropriately to prepare different standard solutions from 50 to 10000 ng/mL. The quality control (QC) samples of imatinib at three different levels were prepared at concentrations of low QC (LQC, 100 ng/mL), medium QC (MQC, 6000 ng/mL) and high QC (HQC, 10000 ng/mL).

2.5. Method validation
Validation of the present HPLC method was performed considering various parameters, as required under ICH guideline (ICH, Q2 (R1), 2005). [9]

2.5.1. Linearity
The linearity of the method was confirmed by standard solution of imatinib at different concentrations of analytes within a broad range of 50-50000 ng/mL. Each sample was analyzed in triplicate and the peak area was plotted against the concentration.

2.5.2. Intra and Inter-Day Precision and accuracy
Intra-day (repeatability) and inter-day precision and accuracy of the developed method were determined by six replicate analyses of quality control samples prepared at concentrations of low QC (LQC, 100 ng/mL), medium QC (MQC, 6000 ng/mL) and high QC (HQC, 10000 ng/mL) on the same day and on three consecutive days, respectively. The precision was articulated as the percentage coefficient variation (CV (%)) of measured concentrations for each calibration level, whereas accuracy was calculated as percentage recovery [(Imatinib found/ Imatinib applied) ×100].

2.5.3. Robustness
Robustness of the developed method was evaluated by analyzing a sample with known concentration (6000 ng/mL) by altering both flow rate condition (0.8, 1 and 1.2 mL/min) as well as temperature condition (20, 25 and 30°C).

2.5.4. Ruggedness
Ruggedness was performed by analyst variation. A concentration of 6000 ng/mL of imatinib mesylate was analysed by two different analysts. Six injections were done by each analyst and the results are reported in the form of % recovery.

2.5.5. Limit of detection and Limit of quantification
LOD and LOQ values proclaim the sensitivity of the analytical method. LOD is the lowest detectable concentration of the analyte, and LOQ is the lowest quantifiable concentration.

The LOD and LOQ were calculated from the calibration curve by using the following formulae:

LOD = 3.3 × σ/S  
LOQ = 10 × σ/S  

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

2.6. Forced degradation studies
ICH guidelines require forced decomposition studies to be conducted under various stress conditions and separation of the pure drug from its degradants for stability indicating assay method (ICH, Q6A, 1999).
For the forced degradation studies, aqueous stock solution of imatinib mesylate at the concentration of 10 μg/mL was prepared. The acid and base induced degradations of the drug were performed separately by transferring 2 mL of aqueous solution of the imatinib mesylate (10 μg/mL) to a 10 mL amber colored volumetric flasks and added with 8 mL of 0.1 M HCl and 0.1 M NaOH, respectively. Both the flasks were sealed and refluxed at 80°C for 30 min. The oxidative degradation was executed by transferring 2 mL of the aqueous solution of imatinib mesylate (10 μg/mL) to 10 mL amber colored volumetric flask and 8 mL of H₂O₂ (20%, v/v) was added. The flask was sealed and heated under reflux at 80°C for 30 min. UV-degradation was carried out as per option 2 of Q1B in ICH guidelines (ICH, Q1B, 1996). Briefly, 10 mL of the aqueous solution of imatinib mesylate (10 μg/mL) was transferred in a transparent volumetric flask and kept at UV light cabinet (Thermo-lab, India) followed by exposure to radiation at 320–400 nm for 8 h at 25°C. All the samples forced degradation were prepared for analysis by diluting to 15000 ng/ml with the mobile phase. All the solutions were filtered through a 0.22 μm nylon membrane filter, before analyzing the solution into the chromatographic system. UV spectra of the degraded products were also obtained from the peaks by PDA detector.

3. RESULTS AND DISCUSSION

3.1. Method development

The chromatographic conditions were optimized with an aim to develop a reverse-phase HPLC stability-indicating assay method for imatinib mesylate. As there was no extraction procedure involved, internal standard was not used for the development of the method. Among different flow rates, 1 mL/min was considered to be the best for achieving a smaller retention time (RT) and avoiding tailing. Methanol and acetonitrile mixed in a ratio of 6:4 and phase A to phase B ratio of 55:45 were found to be the optimized conditions for the chromatographic separation in the developed method. A pH of 8 for the phase A (aqueous phase) was observed to be good for the separation. One reason for such observation might be the lower aqueous solubility of imatinib mesylate at alkaline pH. Thus, the pH 8 of the mobile phase contributed to the faster elusion and shorter RT. A RT of 7 min was achieved by the method with good a sharp peak of the analyte with required symmetry and lack of tailing (Fig 1). The optimized conditions for the developed method are shown in table 1.

3.2 Method validation

The correlation coefficients (R² = 0.9992) of the calibration plots reflects good linearity in the range of 50–50000 ng/mL (Fig. 2). The regression equation for the calibration plot was y= 45.68x + 42.11 (n = 3). No significant difference was observed in the slopes of calibration plots prepared at different times (ANOVA, P > 0.05). The intra-day precision was found to be ≤0.128 % (n= 6) and interday precision over three successive days was calculated as ≤0.168 % (n=6). Intra-day and inter-day accuracy were found to be 99.89–99.99 % and 99.84–100.01 %, respectively. The low CV% and good accuracy values for the developed method (Table 2) proclaimed the superiority of the developed method. HPLC method was found to be robust as the % RSD resulting from variation of various method parameters was less than 1 %, except in the case of variation of flow rate which resulted in % RSD of 2.99. Therefore, flow rate of mobile phase should carefully be controlled at 1 ml/min. LOD and LOQ obtained for the HPLC method were 0.61 ng/ml and 1.87 ng/ml respectively. These values demonstrate a high level of sensitivity in lower concentrations.

3.3 Forced degradation studies

The results from forced degradation studies were conclusive of the high specificity of the method. The method was capable of separating degradation products in the presence of pure drug. Well-separated analyte and degradation peaks were obtained in all the degradation experiments such as acid degradation, alkaline degradation, oxidative degradation and photo...
degradation, respectively (Fig. 3). The drug under investigation was found to be resistant to any photo and alkaline degradation. However, degradation of the drug was observed on oxidation and acidic reflux. Approximately 53% of the drug was degraded on acidic reflux for 30 min. Only one degradant peak was observed for acidic degradation (RT= 5.62 min). However, multiple degradant products were obtained on oxidative stress (RT= 1.64, 2.86, 3.24, 3.63 min). Although, peak 3.24 min and 3.63 min were in close proximity, they were found to be sharp and well distinguishable (Fig. 3). Moreover, all the degradant peaks were away from drug peak and highly specific. 54 % of the drug got degraded by oxidative stress, establishing the drug to be oxidation labile at extreme conditions.

4. CONCLUSION
The developed method was found to be linear over a broad range. It was found to good on the parameters of precision, accuracy, robustness and ruggedness. Very low CV% suggested that the developed method was highly precise in analysis of imatinib mesylate. Furthermore, the method can be employed for the investigation of degradants under various stress conditions such as alkaline, acidic, oxidative and photolytic stress. The drug was found to be oxidative and acidic stress. The method was found to be highly specific in determining the pure drug along with the other degradation products.

| Table 1: Chromatographic conditions for the optimized method |
| Column | Developsil® ODS-HG-5, Nomula chemical (50 mm×4.6 mm) analytical column |
| pH of buffer (phase A) | 8 |
| Methanol: acetonitril (phase B) | 6:4 |
| Mobile phase | Phase A: phase B (55:45) |
| Flow rate | 1 mL/min |
| Wavelength | 267 nm |
| Retention time | 7 min |

| Table 2: Precision and accuracy data (n=6) |
| Parameters | Applied (ng/mL) | Found (ng) ± SD | Precision (CV, %) | Accuracy (%) |
| Intra-day precision | | | | |
| 100 (LQC) | 99.89 ± 0.128 | 0.128 | 99.89 |
| 6000 (MQC) | 5998.99 ± 0.867 | 0.0145 | 99.98 |
| 10000 (HQC) | 9998.95 ± 1.441 | 0.0144 | 99.99 |
| Inter-day precision | | | | |
| 100 (LQC) | 99.84 ± 0.168 | 0.168 | 99.84 |
| 6000 (MQC) | 5998.96 ± 0.877 | 0.0146 | 99.98 |
| 10000 (HQC) | 10000.93 ± 1.471 | 0.0147 | 100.01 |

| Table 3: Forced degradation studies |
| Exposure conditions | Applied (ng/mL) | Imatinib found (ng) | CV (%) | Remaining (%) | RT value for degradants |
| Acid degradation | 15000 | 7080 ±164.22 | 2.31 | 47.20±1.20 | 5.62 |
| Alkaline degradation | 15000 | 14700 ± 182.41 | 1.24 | 98.00 ± 0.81 | - |
| Photo degradation | 15000 | 14811 ± 183.45 | 1.24 | 98.74 ± 0.78 | - |
| Oxidative degradation | 15000 | 6923± 105.76 | 1.53 | 46.1± 0.88 | 1.64, 2.86, 3.24, 3.63 |
Figure 1: HPLC chromatogram of imatinib mesylate by developed method

Figure 2: Calibration curve of imatinib mesylate

Figure 3: Chromatograms obtained for degradation products and imatinib mesylate samples
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REFERENCES

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