A Validated Stability-Indicative UPLC Method for Nilotinib Hydrochloride for the Determination of Process-Related and Degradation Impurities

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A novel stability-indicating ultra performance liquid chromatographic (UPLC) method has been developed for quantitative determination of nilotinib hydrochloride in active pharmaceutical ingredients along with four impurities (imp-1, imp-2, imp-3 and imp-4). The method is applicable to the quantification of related compounds and assay of nilotinib hydrochloride drug. Efficient chromatographic separation was achieved on a Shim-pack XR-ODS II, 75 × 3.0 mm, 1.8-μm column with a gradient mobile phase combination. Quantification was carried at 260 nm at a flow rate of 0.6 mL min⁻¹. Stress degradation conditions were established for nilotinib hydrochloride by subjecting it to acid, base, oxidation, humidity, thermal and photolysis. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 97.0%. The developed UPLC method was validated according to the International Conference on Harmonisation guidelines for specificity, detection limit, quantitation limit, linearity, accuracy, precision, intermediate precision and robustness. The resolution between nilotinib hydrochloride and four potential impurities is found to be >2.0. Regression analysis shows as r value (correlation coefficient) of >0.999 for nilotinib hydrochloride and four potential impurities.

Materials and Methods

Materials

Methanol HPLC grade and acetonitrile HPLC grade were purchased from Rankem, Mumbai, India. Formic acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide was purchased from Merck, Darmstadt, Germany. HPLC grade water was obtained from Milli-Q water purification system (Millipore, Milford, USA). Samples of nilotinib hydrochloride and its related impurities are received from Hetero labs limited, Hyderabad, India. All impurities and the nilotinib hydrochloride standards are ≥96% purity and individually which contain purity as, nilotinib hydrochloride (99.8%), imp-1 (96.8%), imp-2 (99.1%), imp-3 (97.2%) and imp-4 (98.8%). In addition, HPLC grade acetonitrile and orthophosphoric acid were purchased from Merck, Darmstadt, Germany. High pure water was prepared with the Millipore Milli-Q Plus water purification system.

Equipments

The LC system used for method development (Shimadzu UPLC), forced degradation studies and method validation consisted of a Waters HPLC (Milford, MA, USA) 2695 binary pump with an auto-sampler and a 2996 photodiode array detector (PDA). The output signal is monitored and processed using Empower...
software on a Pentium computer (Digital equipment Co.). Photostability studies are carried out in a photostability chamber (Atlas Suntest CPS+). Thermal stability studies are carried out in a dry hot air oven (Cintex precision hot air oven, Mumbai, India).

**Chromatographic conditions**

A Shim-pack ODS-II 75 × 3.0 mm, 1.8 μm column is used with a mobile phase containing a gradient of solvents A (1.0 mL of orthophosphoric acid in 1000 mL of water) and B (acetone triethylamine). The flow rate of the mobile phase is 0.6 mL/min with a gradient program of 0/0, 5.5/55, 8.5/45, 9.5/0 and 10.5/0 (time (min)/%B). The column temperature is maintained at 40°C and the detection wavelength is set at 260 nm. The injection volume is 4 μL Methanol is used as diluent.

**LC–MS conditions**

The LC–MS system (Waters Micro mass, Quattro micro-API-ESCI) was used for the identification of unknown compounds formed during forced degradation. A Symmetry Shield 100 × 4.6 mm, 2.2-μm column is used as the stationary phase with a mobile phase containing a gradient of solvent A 0.01 M ammonium formate in 1000 mL of water and adjusted to pH 3.0 with formic acid solution) and solvent B (acetone triethylamine). The flow rate of the mobile phase was kept at 0.8 mL min⁻¹ with a gradient program of 0/0, 5.5/55, 8.5/45, 9.5/0 and 10.5/0 (time (min)/%B). The injection volume is 6 μL. The analysis is performed in positive and negative electrospray ionization modes. The capillary and cone voltages are 3.50 kV and 25.00 V, respectively. The extractor and RF lens voltages are 3.0 and 0.3 V, respectively. The source and desolvation temperatures are 120 and 350°C, respectively, and the cone gas flow and desolvation gas flow are 100 and 650 L h⁻¹, respectively.

**Preparation of standard solutions and sample solutions**

A working standard of nilotinib hydrochloride and its related compounds (0.05 mg/mL) were prepared by appropriate weighing and respective dilution of reference standard and impurities in the diluent to yield a final concentration of 5 μg/mL each for nilotinib hydrochloride, imp-1[3-bromo-5-(trifluoro methyl) aniline], imp-2[4-methyl-1H-imidazol-1-yl]-5-(trifluoromethyl) phenyl]-3(4-pyridinyl)-2-pyrimidinyl[ amino]-benzamides, imp-3[4-methyl-3-(4-pyridine-3-yl) pyrimidin-2-ylamino] benzoic acid] and imp-4[3(4-methyl-1H-imidazole-1-yl)-5-(trifluoromethyl) benzene amine], shown in Figure 1. The drug substance (for degradation) powder equivalent to 100 mg of sample is transferred into a 100-mL volumetric flask, and 10 mL of diluent is added. The flask is attached to a rotary shaker and shaken for 2 min to disperse the powder completely. The mixture is sonicated for 2 min and then diluted to the appropriate volume with diluent to make a solution containing 1.0 mg/mL. The solution is then filtered through a 0.45-μm Nylon 66 membrane filter.

**Results**

**Analytical method validation**

The proposed method was validated as per the ICH guidelines (10, 11).

**Specificity and stress studies**

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (12, 13). The specificity of the developed UPLC method for nilotinib hydrochloride is determined in the presence of its four related impurities (imp-1, imp-2, imp-3 and imp-4) and degradation products. The system suitability results of nilotinib hydrochloride and its impurities are shown in Table I. Forced degradation studies are also performed on nilotinib hydrochloride to provide an indication of the stability-indicating property and specificity of the proposed method. The stress conditions employed for the degradation study included light 1.2 million luxh (carried out as per ICH Q1B Option-1), heat (105°C), humidity (90% RH), acid hydrolysis (1 M HCl heated at 80°C for 8 h), base hydrolysis (0.1 M NaOH heated at 80°C for 1 h) and oxidation (5% H₂O₂ heated at 80°C for 8 h). For heat, humidity and light studies, the samples were exposed for 5 days and 1.2 million lux h, 200 Wh/m², respectively (14). The peak purity of the nilotinib hydrochloride stressed samples is also checked by using the Waters PDA. The purity angle is within the purity threshold limit in all of the stressed samples, demonstrating the homogeneity of the analyte peak. The contents of impurities were calculated for the stress samples against a qualified reference standard. The mass balance (% assay + % sum of all related compounds + % sum of all degradants) is calculated for all the samples. Typical UPLC chromatograms of stress degradation of nilotinib hydrochloride are shown in Figure 2.

**Precision**

Precision of the method is evaluated by carrying out six independent preparations of a test sample of nilotinib hydrochloride against a qualified reference standard. Nilotinib hydrochloride spiked with 0.10% each of imp-1, imp-2, imp-3 and imp-4. The %RSD of the areas of imp-1, imp-2, imp-3 and imp-4 is calculated. The intermediate precision of the method is evaluated using a different analyst, different lot column using, different reagents and instrument located within the same laboratory. The %RSD of nilotinib hydrochloride during the method precision study is within 2.0% and the %RSD values of the area of imp-1, imp-2, imp-3 and imp-4 in the related compounds method precision study are within 5.0%. The %RSD of the results obtained in the intermediate precision study was within 2.0% and the % RSD of the areas of imp-1, imp-2, imp-3 and imp-4 are within 5.0%, revealed the high precision of the method (Table II).

**Detection limit and quantitation limit**

DL and QL for imp-1, imp-2, imp-3 and imp-4 are estimated at a signal-to-noise ratio is 3:1, 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The precision study is also carried out at the QL level by injecting six individual preparations of imp-1, imp-2, imp-3 and imp-4 calculated the %RSD of the areas. The DL and quantitation of nilotinib hydrochloride, imp-1, imp-2, imp-3 and imp-4 for a 4-μL injection volume are given in Table II. The precision at the QL concentration for nilotinib hydrochloride, imp-1, imp-2, imp-3 and imp-4 is <10.0%.

**Linearity and range**

Linearity test solutions for the method are prepared from a stock solution at six concentration levels from QL to 150% of the
Nilotinib hydrochloride and impurities concentration (QL, 50 (0.05%), 80 (0.08%), 100 (0.10%), 120 (0.12%) and 150 (0.15%).

The linear calibration plot for the method is obtained over the tested calibration range (QL—150% level) and the obtained correlation coefficient is >0.999. The results revealed an excellent correlation between the peak area and the analyte concentration. The linear calibration plot for the related compounds method is determined over the calibration range (QL to 0.15% with respect to the analyte concentration) for imp-1, imp-2, imp-3, imp-4 and nilotinib hydrochloride, a correlation coefficient of >0.999 is obtained. Typical Linearity charts and for nilotinib hydrochloride and its impurities (Figure 3). The linearity is checked for the related compounds method over the same concentration range for three consecutive days. The %RSD values of the slope and $y$-intercept of the calibration curves are within 5.0%. These results showed an excellent correlation between the peak areas and concentrations of imp-1, imp-2, imp-3, imp-4.

Table 1

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Resolution</th>
<th>Tailing factor</th>
<th>RT [min]</th>
<th>RRT *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imp-4</td>
<td>–</td>
<td>1.10</td>
<td>1.182</td>
<td>0.30</td>
</tr>
<tr>
<td>Imp-3</td>
<td>8.405</td>
<td>1.12</td>
<td>2.664</td>
<td>0.67</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>6.914</td>
<td>1.39</td>
<td>3.992</td>
<td>1.00</td>
</tr>
<tr>
<td>Imp-2</td>
<td>2.112</td>
<td>1.01</td>
<td>4.162</td>
<td>1.04</td>
</tr>
<tr>
<td>Imp-1</td>
<td>8.665</td>
<td>1.00</td>
<td>6.153</td>
<td>1.54</td>
</tr>
</tbody>
</table>

*RRT: relative retention time.

Figure 1. Chemical structures of nilotinib hydrochloride and its four impurities.
Nilotinib hydrochloride did show the presence of imp-1 at minor concentration, and also contained 0.03% of unknown. Standard addition and recovery experiments are conducted to determine the accuracy of the related compounds method for the quantification of all four impurities (imp-1, imp-2, imp-3 and imp-4) in the drug substance. The study is carried out in triplicate at QL, 0.05, 0.10 and 0.15% of the analyte concentration (1.0 mg/mL). Standard addition and recovery experiments were conducted to determine accuracy of the related substance method for the quantification of all nine impurities in bulk drug samples. The percentage of recoveries for imp-1, imp-2, imp-3 and imp-4 is calculated. The percentage recovery of nilotinib hydrochloride impurities in the drug substances, that is, imp-1, imp-2, imp-3 and imp-4, was ranged from 89.1 to 116.5.

Robustness
To determine the robustness of the developed method, the experimental conditions are altered and the resolution between nilotinib hydrochloride and imp-1, imp-2, imp-3 and imp-4 is evaluated. The flow rate of the mobile phase is 0.6 mL/min. To
study the effect of the flow rate on the resolution, the flow rate is changed by 0.1 units (to 0.5 and 0.7 mL min$^{-1}$).

The effect of the column temperature on the resolution is studied at 35 and 45°C instead of 40°C. In all these varied conditions, the components of the mobile phase remained constant. In all of the deliberately varied chromatographic conditions carried out as described in the Section Robustness (flow rate and column temperature), the resolution between the closely eluting impurities, namely imp-3 and compound, is $>2.0$, illustrating the robustness of the method. The variability of nilotinib hydrochloride and the impurities area response is within ±2% and within ±5%, respectively.

Solution stability and mobile phase stability

The solution stability of nilotinib hydrochloride in the impurities method was carried out by leaving both the sample and reference standard solutions in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions are assaying for in 6-h intervals over the study period. The mobile phase stability was also examined by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 24 h intervals up to 5 days. The prepared mobile phase remained constant during the study period. The %RSD of the nilotinib hydrochloride assay is calculated for the mobile phase and solution stability experiments. The solution stability of nilotinib hydrochloride and its impurities in the related compounds method is carried out by leaving a spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h. The content of imp-1, imp-2, imp-3 and imp-4 is determined at 6 h intervals up to the study period. The mobile phase stability is also investigated for 5 days by injecting the sample solutions for every 24 h interval. The content of imp-1, imp-2, imp-3 and imp-4 is determined in the test solutions. The prepared mobile phase remained constant during the study period. The %RSD of assaying nilotinib hydrochloride during the solution stability and mobile phase stability experiments is within 2%. No significant changes are observed in the content of imp-1, imp-2, imp-3 and imp-4 during the solution stability and mobile phase stability experiments when performed using the related compounds method. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the related compounds determinations are stable up to 48 h. Mobile phase is proved to be stable up to 5 days.

Discussion

Method development and optimization

The main objective of the chromatographic method was to separate imp-1, imp-2, imp-3 and imp-4, nilotinib hydrochloride and the generated degradation products from the analyte peak during stress studies. Impurities and degradation products are co-eluted by using different stationary phases, such as C18 with various mobile phases and organic modifiers, including acetonitrile and methanol, in the mobile phase. Impurities and nilotinib hydrochloride solutions are prepared in diluent at a concentration of 0.01% and scanned in UV–visible spectrometer; all the four impurities and nilotinib hydrochloride are having UV maxima at around 260 nm which is selected for the method development purpose. In order to separate nilotinib hydrochloride and impurities, optimization started with acetonitrile–buffer (adjusted to pH 2 using orthophosphoric acid) in proportion of 20:80 (v/v) on Shim-pack XR-ODSII 75 × 3.0 mm, on which nilotinib hydrochloride is retained with poor peak shape and no separation is observed between impurities, later on by changing ratio of acetonitrile buffer (adjusted to pH 3 using orthophosphoric acid) in proportion of 30:70 (v/v) on Shim-pack XR-ODSII 75 × 3.0 mm, nilotinib hydrochloride is retained with peak tailing, no separation between impurities. Further development is continued acetonitrile buffer (adjusted to pH 3 using orthophosphoric acid) in proportion of 20:80 (v/v) on Hypersil BDS C8 100 × 4.6 mm, 3.0 μm column nilotinib hydrochloride is tailed with asymmetry of 3.5. Different gradient programs are investigated and satisfactory results are obtained when a gradient program with a flow rate of the mobile phase at 0.6 mL/min and a gradient program of 0/0, 5.5/55, 8.5/45, 9.5/0 and 10.5/0 (time (min)/%B) is used. The column used with the said satisfactory conditions is Shim-pack ODS-II 75 × 3.0 mm, 1.8 μm column.

Results of forced degradation studies

Degradation is not observed in nilotinib hydrochloride stressed samples subjected to light, humidity and heat. Significant degradation of the drug substance and product is detected under base
table I

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>% of Ti</th>
<th>Imp-1</th>
<th>Imp-2</th>
<th>Imp-3</th>
<th>Imp-4</th>
<th>% assay of active substance</th>
<th>Mass balance a</th>
</tr>
</thead>
<tbody>
<tr>
<td>As it is sample</td>
<td>0.10</td>
<td>0.02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>99.7</td>
<td>99.8</td>
</tr>
<tr>
<td>Kept the flask at 80°C for 8 h by adding the 10 mL of 1 M HCl on shaking water bath</td>
<td>1.40</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
<td>0.70</td>
<td>0.20</td>
<td>97.2</td>
</tr>
<tr>
<td>Kept the flask at 80°C for 1 h by adding the 10 mL of 0.1 M NaOH on shaking water bath</td>
<td>18.30</td>
<td>0.02</td>
<td>ND</td>
<td>12.5</td>
<td>3.1</td>
<td>80.5</td>
<td>98.6</td>
</tr>
<tr>
<td>Kept the flask at 80°C for 8 h by adding the 10 mL of 5% H₂O₂ on shaking water bath</td>
<td>3.50</td>
<td>0.01</td>
<td>ND</td>
<td>0.03</td>
<td>0.01</td>
<td>95.6</td>
<td>99.1</td>
</tr>
<tr>
<td>Exposed for thermal degradation at 105°C for about 5 days</td>
<td>0.10</td>
<td>0.02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>99.6</td>
<td>99.7</td>
</tr>
<tr>
<td>Exposed to photo light both for about 1.2 million lux h and 200 Wh/m² in photostability chamber</td>
<td>0.15</td>
<td>0.02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>99.5</td>
<td>99.7</td>
</tr>
<tr>
<td>Exposed to humidity at 25°C, 90%RH for about 5 days</td>
<td>0.12</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>99.7</td>
<td>99.8</td>
</tr>
</tbody>
</table>

ND, not detected; Ti, total impurities.

*% assay + % sum of all related compounds + % sum all degradants.

Identification of major degradation product (imp-3 and imp-4) formed in base hydrolysis and oxidative (stress conditions)

An LC–MS study was carried out to determine the m/z value of the major degradation product formed under base hydrolysis using a Waters Micro mass, Quattro micro-API-ESCI liquid chromatography mass spectrometer. Method conditions are described in LC–mass spectrum conditions. The degraded impurities in the test sample are identified with m/z values and the same are confirmed with pure impurities.

Conclusion

The degradation conditions of nilotinib hydrochloride are established as per the ICH recommendations. The UPLC method developed and used for stress studies also fit for quantitative, related compounds and assay determination of nilotinib hydrochloride. The behavior of nilotinib hydrochloride under various stress conditions was studied and observed that all the degradation products and process impurities are well separated from the drug substance demonstrates the stability-indicating power of the method. The method is validated as per the ICH recommendations. The developed UPLC method is stability indicating which can be used to carry out the analysis of nilotinib hydrochloride in routine analysis of production samples and stability samples.

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References

10. ICH, Q2 (R1); Validation of analytical procedures: text and methodology, In International Conference on Harmonization, Geneva; (2005).