Development and validation of stability indicating UPLC method for the quantitative determination of related substances in moxifloxacin hydrochloride

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ABSTRACT

The present paper describes the development of a simple, economic and time efficient stability indicating UPLC method for Moxifloxacin HCl in the presence of its impurities and degradation products generated from forced degradation studies. The drug substance was subjected to stress conditions of acid hydrolysis, base hydrolysis, oxidative hydrolysis, photolysis and thermal degradation. The degradation of moxifloxacin hydrochloride was observed under oxidative hydrolysis and base hydrolysis. The drug was found to be stable in all other stress conditions applied. Successful separation of the drug from synthetic impurities and degradation products formed under forced degradation was achieved on a Acquity UPLC BEH C18 column using a mixture of potassium di hydrogen phosphate buffer and methanol (80:20, v/v) as mobile phase in a gradient elution mode. The eluents were monitored at 240 nm. The developed UPLC method was validated with respect to linearity, accuracy, precision, specificity and robustness. It can be used to test the stability samples of moxifloxacin HCl.

KEY WORDS: Moxifloxacin HCl, UPLC, Validation, Stress conditions, Degradation products.

1. INTRODUCTION

Moxifloxacin HCl (1-Cyclopropyl-7-(S,S)-2,8-diazabicyclo(4,3,0)-non-8-yl-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid hydrochloride) is a new fourth generation 8-methoxy fluoro quinolone derivative developed primarily for the treatment of community acquired pneumonia and upper respiratory tract infections. It is active not only against gram-negative pathogens but also against Gram-positive cocci aerobic intracellular bacteria, a typical organisms and anaerobic bacteria [1].

Moxifloxacin is readily absorbed from gastrointestinal (gi) tract. It is 42% protein bound in plasma and penetrates easily into target tissues and fluids. It is mainly metabolized to the N-sulfate and acyl glucuronide. The pharmacokinetics of moxifloxacin were found to be linear within a wide range of single oral and parenthetal doses from 50 to 800 mg. Maximum concentration in plasma is reached after 0.5–4 h (0.29–4.73 mg/L) [2]. Hence, the drug may contain impurities or may get degraded during formulation process and stability testing under accelerated and long term storage conditions. Identification limits must be established for each impurity in accordance to ICH guidelines [3] and if the limit level is exceeded the impurity must be identified and quantified [4,5].

Several HPLC methods have been reported for moxifloxacin determination in its pharmaceutical forms and biological matrices [6–8], none of these were stability indicating methods. Isolation and identification of synthesis-related impurities was dealt with in some communication. Kumar et al. [9] have reported the isolation and structural characterization of four impurities in Moxifloxacin and the impurities are 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[(S,S)-N-methyl-2,8-diazabicyclo(4,3,0)-non-8yl]-4-oxo-3-quinoline carboxylic acid, methyl-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[(S,S)-2,8-diazabicyclo(4,3,0)-non-8-yl]-4-oxo-3-quinoline carboxylate, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-hydroxy-7-[(S,S)-2,8-diazabicyclo(4,3,0)-non-8-yl]-4-oxo-3-quinoline carboxylic acid and 1-cyclopropyl-6,7-difluoro-8-hydroxy-4-oxo-1,4-dihydro-3-quinoline carboxylic acid.

The concern with earlier reported methods did not emphasize with respect to its stability indicating nature. The UPLC technique is a latest and the impurities were separated in a short time. There were no reported methods for Moxifloxacin hydrochloride on UPLC as per the literature search.
2. EXPERIMENTAL

2.1 Chemicals:
Samples of Moxifloxacin HCl and its six impurities namely Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5 and Impurity-6 shown in Figure-1 were received from R&D, Inogent laboratories Private Limited, Hyderabad, India. HPLC grade methanol, AR grade potassium dihydrogen phosphate and trifluoro acetic acid was purchased from Merck, Darmstadt, Germany. High purity water was prepared by using a Millipore (MA 01821, USA) Milli Q plus purification system.

![Figure 1. Molecular structure of Moxifloxacin and Impurities](image)

2.2 Equipment:
The UPLC system used for the method development and validation consisted of gradient pumps from Waters Corporation, Japan, photo diode array detector from Waters Crop., Japan, with auto sampler and auto injector. The UPLC system was equipped with data acquisition and processing software “Empower” Waters Crop., Japan.

2.3 Preparation of Standard solutions:
A stock solution of moxifloxacin hydrochloride was prepared by dissolving appropriate amount of substance in diluent. Standard solutions of 0.5 mg/mL were prepared from the above stock solutions for the determination of related substance. Stock solutions of impurities (mixture of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6) at 0.5 mg/mL were also prepared with diluent.

2.4 Chromatographic Conditions:
The Chromatographic separation was achieved on an Acquity UPLC BEH C18, 100mm X 2.1 mm ID with 1.7 microns particle size with gradient elution of %B 0/20,3/20,9/80,12.01/20 and 15/20. Mobile phase-A consists of 10 mM aqueous potassium dihydrogen phosphate pH adjusted to 2.5 with diluted TFA and methanol (80:20, v/v) and Mobile phase-B consists of methanol and water (80:20 v/v) used as
a mobile phases. The mobile phases were filtered through nylon membrane (pore size 0.22 µm) and degassed by using vacuum pump and sonicate for 15 minutes prior to use. The flow rate of mobile phase was 0.3 mL/min. The column temperature was maintained at 40°C and wave length was monitored at 240 nm. The injection volume was 1µL. The standard and the test dilutions were prepared in methanol.

2.5 Validation of the method:

2.5.1 Specificity:
Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed UPLC method for moxifloxacin hydrochloride was carried out in the presence of its impurities namely Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6. Stress studies were performed for moxifloxacin hydrochloride bulk drug to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of UV light (254 nm), heat (105 °C), acid (0.5 N HCl), base (0.5 N NaOH), oxidation (3.0 % degradation was attempted to stress conditions of UV light (254 nm), cating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of UV light (254 nm), heat (105 °C), acid (0.5 N HCl), base (0.5 N NaOH), oxidation (3.0 % degradation was attempted to stress conditions of UV light (254 nm), heat (105 °C), acid (0.5 N HCl), base (0.5 N NaOH), oxidation (3.0 % degradation was attempted to stress conditions of UV light (254 nm), heat (105 °C), acid (0.5 N HCl), base (0.5 N NaOH), oxidation (3.0 %

2.5.2 Precision:
The precision of the related substances method was checked by injecting six individual preparations of moxifloxacin hydrochloride (0.5mg/mL) spiked with 0.3 % of imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 with respect to moxifloxacin hydrochloride analyte concentration. % RSD of area for each Imp -1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 was calculated. The intermediate precision of the method was also evaluated using different analyst and different instrument in the same laboratory.

2.5.3 Limit of detection (LOD) and Limit of Quantification (LOQ):
The limit of detection and limit of quantification were determined at a signal to noise of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six individual preparations of Imp -1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 and then calculated the % RSD of the peak area.

2.5.4 Linearity:
Linearity test solutions for the related substance method were prepared by dilution of stock solution to the required concentrations. The solutions were prepared at six concentration levels from 0.05 % to 0.3 % of specification level of impurities namely Imp -1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 (0.05%, 0.1%, 0.15%, 0.2%, 0.25% and 0.3%). Above test were carried out of three consecutive days in the same concentration range for related substances method. The % RSD value for the Slope and Y-intercept of the calibration curve was calculated.

2.5.5 Accuracy:
The accuracy study of impurities was carried out in triplicate at 50%, 100% and 150 % of specification level (0.2%) to the moxifloxacin HCl analyte concentration. The percentages of recoveries for impurities were calculated from the slope and Y- Intercept of the calibration curve.

2.5.6 Robustness
To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between Moxifloxacin Hydrochloride, Imp -1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 was recorded. The effect of the methanol ratio in mobile phase preparation studied on resolution by varying by -5 to + 5 %, while other mobile phase components were held constant as stated in Chromatographic conditions. The column temperature was varied by -5 to +5°C and flow rate of the mobile phase varied from – 0.1 to +0.1 mL/ min.

2.5.7 Solution stability and mobile phase stability:
The solution stability of moxifloxacin hydrochloride in the assay method was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature for 48 hrs. The same sample solutions were assayed for 6 hrs interval up to the study period. The mobile phase stability was also carried out by assaying the freshly prepared sample solution against freshly prepared reference standard solution for 6 hrs interval up to 48 hrs. Mobile phase prepared was kept constant during the study period. The % RSD for the assay of Moxifloxacin Hydrochloride was calculated during mobile phase and solution stability experiment. The solution stability of Moxifloxacin Hydrochloride and its impurities in the related substance method was carried out by leaving spiked sample solution in tightly capped volumetric flasks at room temperature for 48 hrs. Content of Imp -1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 were checked in the test solutions.

3 RESULTS AND DISCUSSION

3.1 Optimization of Chromatographic conditions
The main objective of Chromatographic method is to separate Moxifloxacin Hydrochloride from Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6. Impurities were co-eluted using different stationary phases such as C8, Cyno and Phenyl as well as different mobile phases. The chromatographic separation was achieved on an Acquity UPLC BEH
C18, 100mm X 2.1 mm ID with 1.7 microns particle size with gradient elution of %B 0/20,3/20,9/80,12.01/20 and 15/20. Mobile phase-A consists of aqueous potassium dihydrogen phosphate pH adjusted to 2.5 with TFA (10mM) and methanol (80:20) (v/v) and Mobile phase-B consists of methanol and water (80:20) used as a mobile phases. The flow rate of the mobile phase was 0.3 mL / min, at 40 °C column temperature, the peak shape of the Moxifloxacin Hydrochloride was found to be symmetrical. In optimized chromatographic conditions of Moxifloxacin Hydrochloride, Imp -1, Imp-2, Imp-3,Imp-4,Imp-5 and Imp-6 were separated with resolution greater than 2, typical retention times were about 4.11, 3.58, 5.23, 5.63, 7.60, 8.96 and 9.32 minutes respectively (Fig 2).

![Graph a) Blank](image1)

![Graph b) Moxifloxacin hydrochloride sample](image2)
The system suitability results are given in Table-1 and developed UPLC method was found to specific for Moxifloxacin Hydrochloride and its six impurities namely Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6. Find the above typical chromatograms of a) blank, b) Moxifloxacin hydrochloride sample and c) Impurity blend solution shown in Figure-2.

Table-1: System suitability Results

<table>
<thead>
<tr>
<th>System suitability</th>
<th>Moxifloxacin</th>
<th>Imp-1</th>
<th>Imp-2</th>
<th>Imp-3</th>
<th>Imp-4</th>
<th>Imp-5</th>
<th>Imp-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>R_t</td>
<td>4.11</td>
<td>3.58</td>
<td>5.23</td>
<td>5.63</td>
<td>7.6</td>
<td>8.96</td>
<td>9.32</td>
</tr>
<tr>
<td>RR_t</td>
<td>1</td>
<td>0.88</td>
<td>1.27</td>
<td>1.37</td>
<td>1.85</td>
<td>2.18</td>
<td>2.27</td>
</tr>
<tr>
<td>R_s</td>
<td>2.81</td>
<td>-</td>
<td>6.04</td>
<td>3.93</td>
<td>21.33</td>
<td>15.28</td>
<td>4.16</td>
</tr>
<tr>
<td>T</td>
<td>1.4</td>
<td>0.99</td>
<td>0.92</td>
<td>1.02</td>
<td>1.02</td>
<td>1</td>
<td>1.03</td>
</tr>
<tr>
<td>N</td>
<td>3214</td>
<td>16938</td>
<td>36234</td>
<td>57081</td>
<td>105396</td>
<td>165542</td>
<td>186120</td>
</tr>
</tbody>
</table>

R_t: Retention time; RR_t: Relative retention time; R_s: Resolution; T: USP tailing factor; N: Theoretical plate

3.2 Results of forced degradation studies:
Degradation was not observed in moxifloxacin HCl sample when subjected to stress conditions like light, heat and acid hydrolysis. Moxifloxacin HCl was degraded to unknown under oxidative hydrolysis, in base hydrolysis moxifloxacin HCl was degraded to unknown Impurity. Peak purity test results confirmed that the moxifloxacin Peak is homogenous and pure in all the analyzed stress samples. The assay of moxifloxacin HCl is unaffected in the presence of all impurities and its degradation products confirm the stability indicating power of the method. The summary of forced degradation studies is given in Table 2.

Table-2: Summary of forced degradation

<table>
<thead>
<tr>
<th>Stress conditions</th>
<th>Time (h)</th>
<th>Purity</th>
<th>Total Impurities</th>
<th>Peak Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>99.87%</td>
<td>0.13%</td>
<td>Pass</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>12</td>
<td>99.82%</td>
<td>0.18%</td>
<td>Pass</td>
</tr>
<tr>
<td>Base hydrolysis</td>
<td>12</td>
<td>99.31%</td>
<td>0.69%</td>
<td>Pass</td>
</tr>
<tr>
<td>Oxidation(6%H2O2)</td>
<td>12</td>
<td>98.71%</td>
<td>1.29%</td>
<td>Pass</td>
</tr>
<tr>
<td>Water, 70°C</td>
<td>12</td>
<td>99.83%</td>
<td>0.17%</td>
<td>Pass</td>
</tr>
<tr>
<td>Thermal 105°C</td>
<td>7 days</td>
<td>99.86%</td>
<td>0.14%</td>
<td>Pass</td>
</tr>
<tr>
<td>Visible</td>
<td>7 days</td>
<td>99.81%</td>
<td>0.19%</td>
<td>Pass</td>
</tr>
</tbody>
</table>

3.3 Precision:
The %RSD for the area of Imp -1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 in related substances method precision study was within 4.2% confirming good precision of the method.

3.4 Limit of detection (LOD) and Limit of Quantification (LOQ):
The limit of detection of all impurities namely Imp -1, Imp-2, Imp-3,Imp-4,Imp-5 and Imp-6 were achieved 0.007%,0.012%,0.018%, n0.0012%,0.025% and 0.028% for 1 µL injection volume. The limit of quantification of all impurities namely Imp -1, Imp-2, Imp-3,Imp-4,Imp-5 and Imp-6 are 0.022%,0.036%,0.055%,0.038%,0.077% and 0.084% for 1µL injection volume. The precision at the LOQ concentrations for Imp -1, Imp-2, Imp-3,Imp-4,Imp-5 and Imp-6 were below 4.3%.
3.5 Linearity
Linear calibration plot for the related substances method was obtained over the calibration ranges tested i.e. 0.05% to 0.3% for impurity Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6. The correlation coefficient obtained greater than 0.998. Linearity was checked for the related substances method over the same concentration ranges for 3 consecutive days. The %RSD values of the Slope and Y-intercept of calibration curve were 3.2 and 2.8 respectively. The above results shows that an excellent correlation existed between the peaks and the concentrations of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, and Imp-6. Results shown in below Table-3.

### Table-3: Linearity data of Impurities

<table>
<thead>
<tr>
<th>Name</th>
<th>Imp-1</th>
<th>Imp-2</th>
<th>Imp-3</th>
<th>Imp-4</th>
<th>Imp-5</th>
<th>Imp-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y Intercept</td>
<td>-96</td>
<td>31</td>
<td>-99</td>
<td>151</td>
<td>-316</td>
<td>-19</td>
</tr>
<tr>
<td>Slope</td>
<td>16372</td>
<td>24900</td>
<td>18808</td>
<td>39765</td>
<td>43468</td>
<td>32475</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.9998</td>
<td>0.9995</td>
<td>0.998</td>
<td>0.9991</td>
<td>0.999</td>
<td>0.998</td>
</tr>
</tbody>
</table>

3.6 Accuracy
The percentage recoveries of all six impurities in moxifloxacin hydrochloride samples varied from 98.1-108.5%.

3.7 Robustness
In all the deliberate varied chromatographic conditions (flow rate, composition of organic solvent & column temperature) the resolution between critical pair, i.e. Moxifloxacin HCl and Imp-1 was greater than 2.5, illustrating the robustness of the method.

3.8 Solution stability and Mobile phase stability
The % RSD of related substances of Moxifloxacin Hydrochloride during solution stability experiments No significant change were observed in the content of impurities namely Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and Imp-6 during the solution stability and mobile phase stability experiments when performed using the related substance method. The solution stability and mobile phase stability experiment data confirms that the sample solution and mobile phase used during the related substance determination were stable for 48 hrs.

CONCLUSION
The UPLC method developed for quantitative and related substance determination of moxifloxacin hydrochloride is linear, accurate, precise, rapid and specific. The method was fully validated showing satisfactory data for all method validation parameters tested. The developed method is stability indicating and can be conveniently used by quality control department to determine the related substance and assay in regular moxifloxacin hydrochloride production samples and also stability samples. The UPLC technique is a latest and the impurities were separated in a short time.

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REFERENCES:

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