LC–MS/MS structural characterization of stress degradation products including the development of a stability indicating assay of Darunavir: An anti-HIV drug

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Darunavir, an anti-HIV drug was subjected to forced degradation under acid, base, thermal and neutral hydrolysis, oxidation and photolysis as prescribed by ICH guidelines. Four major degradation products were formed under acid and base hydrolysis, while stable under neutral and thermal hydrolysis, oxidative and photolysis. The drug and its degradation products were separated on Hiber LiChrospher® 60, RP-select B, C8 column (250 mm × 4.6 mm i.d., 5 μm) using 10 mM ammonium acetate: acetonitrile (52:48, v/v) as mobile phase in an isocratic elution mode by LC. The degradation products were characterized by LC–MS/MS and fragmentation pathways were proposed. The proposed structures of degradation products were confirmed by HRMS and the LC method was validated with respect to specificity, linearity, accuracy, recovery, LOD and LOQ.

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1. Introduction

According to the latest UNAIDS report, about 34 million people are living with human immunodeficiency virus (HIV) worldwide. It also presents the increasing number of new HIV infections, and demand on to the access to antiretroviral therapy. The number of people dying of AIDS-related causes fell to 1.7 million in 2011 from 2.3 million in 2005 [1]. This achievement could be attributed to the advancement of antiretroviral medications. Darunavir (DRV) is one among the most commonly used anti-AIDS drugs in clinic practices. DRV is a prescription medicine approved by FDA for the treatment of HIV infection in adults and children above 3 years [2,3]. It prevents HIV from replicating and lowers the amount of HIV in the blood. It is mostly used in combination with ritonavir and other anti-HIV medicines. It is a non peptidic molecule containing bis-tetrahydrofuran moiety [4,5], which exists in several biologically active natural products viz., aflatoxin, astelotoxin, rhyacophiline, and acmimycin [6].

Literature survey revealed a variety of LC–UV [7,8] and LC–MS [9–12] methods for simultaneous determination of DRV and other antiretroviral agents in blood plasma. Rao et al. reported a RPLC method for quantification of DRV and its unknown impurities in bulk drugs [13]. Simultaneous determination of Darunavir ethanolate and ritonavir by LC was also reported [14]. The content of DRV in different formulations was quantified by HPTLC and RPLC [15–17]. Enantiomers and diastereomers of DRV were separated and determined by LC and CE [18–20]. So far, only one stability indicating assay of DRV was reported in the literature [21]. However, the degradation products were neither characterized nor the fragmentation pathways elucidated. The International Conference on Harmonization (ICH) guidelines [22], suggest stress studies on a drug to establish its inherent stability characteristics not only for identification of degradation products but also understanding the stability of drug molecule. So, it is of great importance to know the complete degradation profile of DRV, which is not yet reported in the literature.

The present manuscript describes (i) forced degradation of DRV under stress conditions like acid, base, neutral, thermal hydrolysis, oxidation and photolysis(ii) optimization of liquid chromatographic condition to separate degradation products, and (iii) structural characterization and elucidation of fragmentation pathways of degradation products by LC–MS/MS and HRMS.

2. Experimental

2.1. Reagents and samples

Analytical grade reagents and HPLC grade solvents were used. Water purified by Millipore synergy (Millipore, France) was used. Ammonium acetate, methanol and acetonitrile were purchased...
from Qualigens Fine Chem (Mumbai, India). Sodium hydroxide, hydrochloric acid and hydrogen peroxide purchased from S.D. Fine Chem Ltd (Mumbai, India). Darunavir drug substance was a gift sample from a local manufacturing unit in Hyderabad, India.

2.2. Instrumentation

The HPLC system consisting of two LC-20AD pumps, SPD-M20A diode array detector, SIL-20AC auto sampler, DGU-20A3 degasser, and CBM-20A system controller (all from Shimadzu, Kyoto, Japan) were used. The chromatographic data were recorded using an HP-Vectra (Hewlett Packed, Waldron, Germany) computer system with LCsolution data acquiring software (Shimadzu, Kyoto, Japan). LC–MS/MS was performed by Agilent 1100 series online ion trap MSD mass spectrometer with ESI source in positive mode equipped with an auto sampler (G1329A), and diode array detector (G1315B) (All from Agilent technologies, Waldbronn, Germany). The data was acquired and processed using LC/MSD trap software 4.2 (Bruker, Waldbronn, Germany). The high resolution mass spectrometry (HRMS) data was acquired using a Q-TOF mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex, USA) equipped with an ESI source. The data acquisition was under the control of Analyst QS software.

2.3. Forced degradation

Forced degradation of DRV was carried out according to ICH guidelines Q1A (R2) [22]. For each stress condition, the sample was dissolved in 3.0 mL of methanol and remained in solution when acid, base, hydrogen peroxide and water was added to obtain a concentration of 10 mg/10 mL. The DRV was subjected to forced degradation under acidic, basic and neutral conditions by refluxing with each 10.0 mL of 0.5 N HCl, 0.1 N NaOH and water at 75 °C for 48, 48 and 72 h, respectively. Oxidative stress was carried out using 3% and 6% H₂O₂ at room temperature for 48h. The drug was placed in a thermally controlled oven at 75 °C up to 6 days for thermal stress in solid as well as solution forms. For photolytic stress, the drug was exposed to UV light at 320 nm for 60 h. After the specified time intervals, samples were collected and stored in a refrigerator at 5 °C.

2.4. Sample preparation

The stressed samples of acid and base hydrolysis were neutralized with sodium hydroxide and hydrochloric acid, respectively and diluted with mobile phase to obtain 100 μg/mL solutions. Neutral hydrolysis, oxidative stress, thermal hydrolysis and photolytic samples were diluted with mobile phase to obtain 100 μg/mL solutions. All the samples were filtered through 0.22 μm membrane filter before analysis by HPLC.

2.5. Chromatographic conditions

All the samples were analyzed by HPLC on Hiber, LiChrospher® 60, RP-select B, C₈ column (250 mm × 4.6 mm i.d., 5 μm) using methanol (20:80, v/v) as a mobile phase. The peaks corresponding to degradation products did not resolve completely. To achieve an adequate separation, the percentage of organic modifier was gradually decreased to 40%. Ammonium acetate buffer of 10 mM strength was used in place of water. Because, most of the degradation products are not only polar but also ionic in nature, which leads to irregular run time as well as asymmetric peaks. The separation of the DP2 and DP3 was critical, because of similar polarity. The degradation product DP4 has long retention time with broad peak. It was due to the interaction of free amines with silanol groups of the stationary phase. The organic modifier i.e., methanol was replaced with acetonitrile, to reduce broadening of the peaks. The separation was tried using different proportions of acetonitrile and buffer concentrations. Finally, a mobile phase consisting of ammonium acetate buffer: acetonitrile (52:48, v/v/v) at a flow rate of 1.0 mL/min in an isocratic mode and PDA detection at 210 nm, gave good separation of the drug and its degradation products. The optimized LC conditions were extended to LC–MS/MS studies.

2.6. Mass spectrometric conditions

The mass spectra were recorded in positive mode of electro spray ionization (ESI). Nitrogen was the nebulizer and curtain gas. Collision-induced dissociation was achieved by helium as a collision gas. The ion source conditions were set as follows: Dry temperature, 325 °C; nebulizer gas, 60 psi; dry gas, 5.0 mL/min; capillary exit, 113.5 V; capillary current 81.787 nA; corona current 4000 nA; electro multiplier voltage, 2100 V; vaporizer temperature 400 °C and dwell time, 200 ms. The HRMS data was acquired using a Q-TOF mass spectrometer equipped with an ESI source. The typical source conditions were: capillary voltage, 5 kV; declustering potential, 60 V; focusing potential, 220 V; declustering potential-2, 10 V; resolution 10,000 (full-width half-maximum). Ultra high pure nitrogen was used as a curtain and collision gas, whereas zero air was used as a nebulizer. For the collision-induced dissociation (CID) experiments, the precursor ion was selected using the quadrupole and TOF analyzers.

2.7. Validation

A stock solution containing 5 mg/5 mL drug was diluted to yield solutions in the concentration range of 50–250 μg/mL to establish linearity and range. The analysis was carried out in triplicate by injecting 5 μL of each solution. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. The intra- and inter-day precisions were established by analyzing 50, 150 and 250 μg/mL of drug solution, three times on the same day and on three consecutive days, respectively. Accuracy was determined by analyzing a known concentration of drug, viz., 50, 150 and 250 μg/mL spiked with stressed sample in triplicate and then determining the percent recovery. The signal to noise ratios were 3:1 and 10:1 for determining LOD and LOQ, respectively.

3. Results and discussion

3.1. Optimization of chromatographic conditions

During the optimization process, preliminary experiments were carried out on Hiber, LiChrospher® 60, RP-select B, C₈ column (250 mm × 4.6 mm i.d., 5 μm) using water: methanol (20:80, v/v) as a mobile phase. The peaks corresponding to degradation products did not resolve completely. To achieve an adequate separation, the percentage of organic modifier was gradually decreased to 40%. Ammonium acetate buffer of 10 mM strength was used in place of water. Because, most of the degradation products are not only polar but also ionic in nature, which leads to irregular run time as well as asymmetric peaks. The separation of the DP2 and DP3 was critical, because of similar polarity. The degradation product DP4 has long retention time with broad peak. It was due to the interaction of free amines with silanol groups of the stationary phase. The organic modifier i.e., methanol was replaced with acetonitrile, to reduce broadening of the peaks. The separation was tried using different proportions of acetonitrile and buffer concentrations. Finally, a mobile phase consisting of ammonium acetate buffer: acetonitrile (52:48, v/v/v) at a flow rate of 1.0 mL/min in an isocratic mode and PDA detection at 210 nm, gave good separation of the drug and its degradation products. The optimized LC conditions were extended to LC–MS/MS studies.

3.2. Validation

The retention time (Rt), relative retention time (RRT), resolution factor (Rₛ) and capacity factor (k¹), LOQ and LOD were calculated and the chromatographic parameters are given in Table 1. The quantitative aspects of the HPLC-PDA method were examined according to ICH guidelines [23]. The developed method was validated with respect to linearity, precision, accuracy, specificity, selectivity, LOD and LOQ. The response for the drug was found to be linear in the investigated concentration range 50–250 μg/mL ($r^2 = 0.999$). Intra-day and inter-day precision data are given in Table 2. The RSD values ranging from 0.21% to 0.79% for intra-day
Table 1
Chromatographic data of DRV and its degradation products.

<table>
<thead>
<tr>
<th>Code</th>
<th>Retention time (Rt) min</th>
<th>Relative retention time (RRT)</th>
<th>Resolution factor (Rs)</th>
<th>Capacity factor (k')</th>
<th>Asymmetry factor (As)</th>
<th>LOQ (μg/mL)</th>
<th>LOD (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP1</td>
<td>7.16</td>
<td>0.79</td>
<td>2.13</td>
<td>2.68</td>
<td>1.08</td>
<td>0.075</td>
<td>0.025</td>
</tr>
<tr>
<td>DRV</td>
<td>8.99</td>
<td>1.00</td>
<td>6.32</td>
<td>3.62</td>
<td>1.05</td>
<td>0.099</td>
<td>0.033</td>
</tr>
<tr>
<td>DP2</td>
<td>9.61</td>
<td>1.06</td>
<td>3.94</td>
<td>1.05</td>
<td>0.13</td>
<td>0.123</td>
<td>0.041</td>
</tr>
<tr>
<td>DP3</td>
<td>10.44</td>
<td>1.16</td>
<td>2.60</td>
<td>4.37</td>
<td>1.04</td>
<td>0.072</td>
<td>0.024</td>
</tr>
<tr>
<td>DP4</td>
<td>14.82</td>
<td>1.64</td>
<td>5.73</td>
<td>8.29</td>
<td>1.02</td>
<td>0.156</td>
<td>0.052</td>
</tr>
</tbody>
</table>

Table 2
Intra-day and inter-day precision data.

<table>
<thead>
<tr>
<th>Conc. (μg/mL)</th>
<th>Intra-day precision measured conc. (μg/mL), ±SD, RSD(%) (n=5)</th>
<th>Inter-day precision measured conc. (μg/mL), ±SD, RSD(%) (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>49.69 ± 0.5147, 0.79</td>
<td>49.35 ± 0.9224, 0.80</td>
</tr>
<tr>
<td>150</td>
<td>149.66 ± 0.3564, 0.24</td>
<td>149.63 ± 0.5805, 0.39</td>
</tr>
<tr>
<td>250</td>
<td>249.62 ± 0.4150, 0.21</td>
<td>249.43 ± 0.6045, 0.24</td>
</tr>
</tbody>
</table>

n = no. of replicates.

Table 3
Recovery data.

<table>
<thead>
<tr>
<th>Spiked conc. (μg/mL)</th>
<th>Calculated spiked conc. (μg/mL), ±SD, RSD(%) (n=5)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50.13 ± 0.2537, 0.51</td>
<td>100.26</td>
</tr>
<tr>
<td>150</td>
<td>149.60 ± 0.5121, 0.34</td>
<td>99.74</td>
</tr>
<tr>
<td>250</td>
<td>249.71 ± 0.4155, 0.17</td>
<td>99.88</td>
</tr>
</tbody>
</table>

n = no. of replicates.

3.3. Degradation behavior of DRV

The degradation behavior of DRV under various stress conditions was investigated by LC. Fig. 1 shows a typical HPLC chromatogram depicting the separation of degradation products (DP1–DP4) and DRV.

3.3.1. Hydrolysis

Under acid hydrolysis, the drug was degraded more than 60% in 48 h resulting one degradation product DP1. Incase of base hydrolysis three degradation products were formed in 48 h, which were coded as DP2, DP3 and DP4. However, no degradation was observed under neutral hydrolysis.

3.3.2. Oxidation

The drug was stressed under 3% and 6% hydrogen peroxide for 48 h. No degradation products were observed. Hence, the drug was found to be stable under oxidative stress.

3.3.3. Thermal and photolytic degradation

The drug in solid as well as solution forms was kept at 75°C up to 6 days. No significant degradation was observed. The solid and solution forms of the drug remained stable on exposure to UV light at 320 nm for 60 h.

3.4. Mass studies on the drug

The MS2 analysis of the precursor ion (m/z 548) of the drug gave three major fragment ions at m/z 392.2015, 241.1027 and 113.0213. Table 4 lists the HRMS data of DRV, fragment ions and their MS3 data. These were helpful in establishing the origin of each fragment ions and also in understanding the fragmentation pathway of the drug. The most abundant fragment ion at m/z 392 observed in the spectrum was formed by the loss of ([3R,3aS,6aR]-hexahydrofuro[2,3-b]furan-3-yl oxy)x)methylo moiety. In MS3 studies, the m/z 392 molecule again fragmented into three daughter ions of m/z 241, 156 and 57. The ion at m/z 57 was formed by the cleavage of the isopropyl moiety from molecular ion m/z 392. The product ion m/z 241 was formed by the loss of 4-amino-N-isobutyl-N-methylbenzenesulphonamide moiety from parent ion. In MS3 studies, it was again fragmented into two daughter ions of m/z 156 and 57. The third fragmented ion m/z 113 was formed through the McLafferty rearrangement at carbamate site. In MS3 studies the ion at m/z 113 fragmented into daughter ion m/z 69. The fragmentation pathway of DRV is summarized in Fig. 2.

3.5. Characterization of degradation products

All degradation products were subjected to LC-MS/TOF studies in positive ESI mode to establish their fragment profiles and elucidate the structures. Table 5 lists the m/z values of degradation

Table 4
HRMS data of DRV, fragment ions and their MS3 data.

<table>
<thead>
<tr>
<th>[M + H]+</th>
<th>Fragment ions MS2</th>
<th>Best possible molecular formula</th>
<th>Theoretical mass (amu)</th>
<th>Error (ppm)</th>
<th>RDB</th>
<th>MS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>548.2425</td>
<td>–</td>
<td>C27H24N2O12S5</td>
<td>548.2494</td>
<td>-4.30</td>
<td>11.0</td>
<td>–</td>
</tr>
<tr>
<td>392.2015</td>
<td>C27H24N2O12S5</td>
<td>C27H24N2O12S5</td>
<td>392.2012</td>
<td>-2.02</td>
<td>7.5</td>
<td>241.5, 57.3</td>
</tr>
<tr>
<td>241.1027</td>
<td>C27H24N2O12S5</td>
<td>C27H24N2O12S5</td>
<td>241.1035</td>
<td>3.40</td>
<td>9.5</td>
<td>156.2, 57.3</td>
</tr>
<tr>
<td>113.0598</td>
<td>C27H24N2O12S5</td>
<td>C27H24N2O12S5</td>
<td>113.0602</td>
<td>3.72</td>
<td>2.5</td>
<td>69.1</td>
</tr>
</tbody>
</table>

RDB: rings plus double bonds.
products, best possible molecular formula and their major fragmented ions. Fig. 3 shows the fragmentation pattern of degradation products from DP1-DP4. During the acid hydrolysis the drug underwent hydrolysis at carbamate site resulting in the formation of DP1. The accurate m/z value of DP1 was 436.19011 Da. The same was supported even by their elemental composition, calculated from accurate mass value as C21H30N3O5S+. The major fragments of DP1 were 392.2102 (C20H30N3O3S+), 156.0136 (C6H6NO2S+). The hydrolysis of carbamate bond from drug molecule resulted in carboxylic acid group and it underwent dehydration resulting
Fig. 4. Degradation pathways of DRV and its postulated mechanisms.

Fig. 5. The LC–MS/MS spectra of degradation products (DP1–DP4).
in cyclic product DP2 (418.1758). Its suggested chemical formula was C22H30N3O5S+. Similar type of cyclic product was found in base hydrolysis of ritonavir [24]. The fragment ions at m/z 241 (C11H17N+O2S3), 156 (C6H6NO2S+), 92 (C6H6N+) support the structure of the DP2. The carbamate degradation product DP3 (m/z 450.2060) was formed as a result of hydrolysis of the carbamate bridge by attack of more nucleophilic methanolic oxygen on electrophilic CO on carbon carbamate bridge. It was a pseudo-degradation product formed due to the presence of methanol as a diluent. Its chemical formula was C22H30N3O5S+. One of its daughter ions m/z 418 (C11H17N2O3S+) was formed by the loss of methoxide ion from the precursor ion. A similar type of product was also reported in the literature [25]. In case of DP4, the experimental m/z value was 392.2002 Da and its suggested elemental composition could be C22H30N3O5S+. The molecular ion peak formed by loss of CO2 from DP3 during base hydrolysis leads to the formation of free amine product. The combination of the daughter ions m/z 241 and 164 supports the structure of DP4. Degradation pathways of DRV and its postulated mechanisms are shown in the Fig. 4. Fig. 5 shows the LC-MS/MS spectra of DRV and its degradation products (DP1–DP4).

4. Conclusions

Degradation behavior of DRV was explored by exposing it to ICH defined stress conditions. The developed LC method was able to separate DRV and the degradation products (DP1 to DP4) with a minimal resolution of 2.0. Only one degradation product was formed under acid stress, while three were formed under base hydrolysis. The drug was found to be stable under neutral hydrolysis, oxidative stress, thermal stress and photolysis. The degradation products were characterized as (25S,3R)-4-(4-amino-N-isobutylphenylsulfonylamo)-3-hydroxy-1-phenylbutan-2-ylcarboxylic acid (DP1), 4-amino-N-((4S,5R)-4-benzyl-2-oxooxazolidin-5-yl)methyl)-N-isobutylbenzenesulfonyl (DP2), methyl (25S,3R)-4-(4-amino-N-isobutylphenylsulfonylamo)-3-hydroxy-1-phenylbutan-2-ylcarbamate (DP3), 4-amino-N-((2R,3S)-3-amino-2-hydroxy-4-phenylbutyl)-N-isobutylbenzenesulfonylamine (DP4) based on LC/MS and HRMS data. The pathways of fragmentation as well as characterization of degradation products have been reported for the first time. Thus, the developed method could be used not only for evaluating stability indicating assay of DRV but also identification of stress degradation products.

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References


