A validated Stability-indicating High Performance Liquid Chromatographic Method for Darunavir Ethanolate in Tablet Dosage Form

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ABSTRACT
High performance liquid chromatographic method was developed and validated for the determination of Darunavir Ethanolate in tablet dosage form. The method was carried out on a Phenomenex luna C18 Column (150 × 4.6 mm id, 5µ) maintained at 30oC. The mobile phase consisted of water-acetonitrile (60 + 40, v/v) pumped at a flow rate 1.0 mL/min. Photo diode array detection was at 265 nm. The chromatographic separation was obtained with a retention time of 11.8 min, and the method was linear in the range of 1-30 µg/mL (r² = 0.9997). The specificity and stability indicating capability of the method was proven through forced degradation studies, which also showed that there was no interference of the excipients. The method was validated for linearity, precision, accuracy, robustness, specificity, limit of detection and limit of quantitation. The developed method, after being validated was successively applied to the analysis of tablet formulations. The drug could be effectively separated from different degradation products and hence the method can be used for stability analysis.

Keywords: chromatography, High Performance Liquid Chromatographic, Darunavir, Stability

INTRODUCTION
A novel nonpeptidic human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PI) Darunavir Ethanolate is [(1S,2R)-3-[(4-aminophenyl)sulfonyl][ 2-methylpropyl]amino]-2-hydroxy-1-phenylmethyl) propyl]-carbamic acid (3R,3aS,6aR)-hexahydrofuro[2,3-b] furan-3-yl ester monoethanolate [Figure 1].[1] The molecular weights of darunavir base and Darunavir ethanolate are 547.73 and 593.73 g/mL, respectively. DRV (Darunavir Ethanolate) was active against HIV-1 with PI-resistance mutations and against PI-resistant clinical isolates.[2, 3, 4] This drug is expected to be effective in patients experienced in antiretroviral treatment, such as those carrying HIV-1 strains which are resistant to more than one PI.[5]

From the literature it is revealed that various analytical methods for the determination of DRV have been reported, which include high-performance liquid chromatography (HPLC) with UV detection (HPLC-UV) to determine DRV in tablet dosage form and in human plasma,[5,6]; HPTLC method,[7] a novel LC–ESI–MS method[8]; RP HPLC–MS method for the simultaneous determination of DRV and 11 other antiretroviral agents in plasma of HIV infected patients[9]; LC-tandem MS assay,[10]; and validation of plasma DRV concentrations by the HPLC for PIs[11].
The development of stability indicating assays, using the approach of stress testing as determined by the International Conference on Harmonization (ICH) guidelines [12], is highly recommended for the QC of pharmaceutical formulations[13,14]. Darunavir Ethanolate is commercially available but at the moment is not available in any pharmacopoeia the aim of the present research was to develop and validate a simple stability-indicating high performance liquid chromatographic method for the quantitative analysis of DRV in tablet dosage form.

MATERIALS AND METHODS

Reagents and Chemicals
Darunavir Ethanolate pure powder with 99.96% purity was obtained as a gift sample from Cipla Ltd (Mumbai, India). DRV tablets (300.0 mg/tablet) were purchased from the local market. HPLC grade acetonitrile, methanol and water (Finar chemicals Ltd., Ahmedabad, India) and nylon filter (Millipore Pvt. Ltd., Bangalore, India) were used for study.

Apparatus and Chromatographic Conditions
The method was performed on a Shimadzu LC 2010 C-HT (Kyoto, Japan) HPLC system with autosampler; Phenomenax Luna, C18 column (150 mm x 4.6 mm i.d., 5µ); UV and SPD-M20A Shimadzu PDA detector and rhodyne injector. The peak areas were integrated automatically by computer using a Shimadzu LC Solution software program. All analyses were done at ambient temperature (30°C) using mobile phase water-acetonitrile (60 + 40, v/v). The flow rate was 1.0 mL/min and the injection volume was 20 µL. PDA detection was performed at 265 nm. All weighing were done on analytical balance (Acculab ALC-210.4, Huntingdon Valley, PA). For sonication purpose sonicator (EN 30 US Enertech Fast Clean, Mumbai, India) was used. All solutions were prepared fresh daily.

Preparation of Standard Solution
Accurately weighed amount of standard DRV (10mg) were transferred to a 10 mL volumetric flask, dissolved and diluted to the mark with methanol to obtain a standard stock solution (1000 µg/mL). An aliquot (1.0 mL) was diluted to 10 mL with water to obtain a working standard solution of DRV (100 µg/mL).

Preparation of Sample Solution
To prepare the sample solution, twenty tablets were accurately weighed and crushed to a fine powder. The accurately weighed powder equivalent to 10.0 mg DRV was transferred to 10 mL volumetric flask and methanol (5.0 mL) was added. The solution was sonicated for 15 min. The flask was allowed to stand at room temperature for 5 min and the volume was diluted to the mark with methanol to obtain the sample stock solution (1000 µg/mL). The solution was filtered and suitably diluted with diluents to obtain sample solution of DRV 10 µg/mL.

Method validation
This optimized HPLC method was validated for the parameters listed in the International Conference on Harmonization (ICH Q2 (R1)) guidelines. [15]

Linearity and range
Seven different concentration levels for DRV were prepared in the range of 1-30 µg/mL. 20 µL of each solution was injected into the HPLC system (n = 6) and mean values of peak areas were plotted against concentrations. The curves were constructed by linear regression with least squares method. The linearity of the proposed method was evaluated by calculating the r², slope and intercept values of the calibration curve.

Precision
The precision of proposed method was evaluated by the intraday and interday repeatability. The repeatability was checked by
repeatedly injecting (n=6) solutions of DRV (10 µg/mL). The intraday and interday precision were determined by measuring the responses 3 times on the same day and on 3 different days for 3 different concentrations of DRV. The precision was expressed as RSD of the responses in each case.

**Accuracy**
To evaluate the accuracy of the proposed method, recovery tests were carried out. Recovery tests were performed by adding known amounts of standard solutions to samples, followed by analysis using the proposed method. The study was done at three different concentration levels (8, 10, 12 µg/mL). The accuracy was calculated as the percentage of the drug recovered from the formulation. Each solution was injected in triplicate and the recovery was calculated by measuring peak areas and fitting these values into the regression equation of the calibration curve.

**Limit of Detection and Limit of Quantitation**
The LOD and LOQ were calculated, as defined by ICH, using the mean values of six independent analytical curves, determined by a linear-regression model, where the factors 3.3 and 10 for the detection and quantitation limits, respectively, were multiplied by the ratio from the SD of the intercept and the slope.

\[
LOD=3.3 \times (\sigma/S), \quad LOQ=10 \times (\sigma /S)
\]

**Robustness**
The robustness of an analytical procedure refers to its ability to remain unaffected by small deliberate variations in method parameters. The robustness was checked by intentional minor modifications in the mobile phase flow rate (±0.1 mL/min) and composition (± 2% in organic phase) and temperature (± 5°C).

**System suitability test parameters**
System suitability parameters were verified with respect to number of theoretical plates, retention time, resolution, tailing factor and % RSD of six replicate injection of DRV (10 µg/mL).

**Specificity**
The specificity of the method was established through the study of resolution factor of the drug peak from the nearest peak and peak purity data of the analyte peaks in forced degradation samples.

**Forced degradation studies**
A stability-indicating method is defined as an analytical method that accurately quantifies the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities. The stability-indicating capability of the method was determined by subjecting a solution to accelerated degradation by acidic, basic, oxidative, thermal and photolytic conditions to evaluate the interference in the quantitation of DRV.

The acidic degradation was induced by storing the solution in 1.0 N hydrochloric acid for 24 hr at room temperature (R.T.), after which the solution was neutralized with base. For basic hydrolysis evaluation, the solution was prepared in 1.0 N sodium hydroxide for 12 hr at R.T. and neutralized with acid. Oxidative degradation was induced by storing the solution in 30% hydrogen peroxide at R.T. for 24 hr protected from light. For the study under thermal condition, drug is heated at 85°C for 24 hr. Photolytic degradation was induced by exposing the drug in a photostability chamber to 1.2 million lux hours of UV for 72 hr. After the procedures, the samples were diluted with the diluents to a final concentration of 10 µg/mL. The stability-indicating capability of the method was established by determining the peak purity of DRV in the degraded samples using a PDA detector.
RESULTS AND DISCUSSION

Optimization of chromatographic conditions
Different mobile phases were tried and the optimum results were obtained with mobile phase consisting of water-acetonitrile (60 + 40, v/v) pumped at a flow rate 1.0 mL/min. The t_R values DRV was observed at 11.8 min. For selection of the best wavelength of detection, a PDA detector was used. The representative chromatogram is given in [Figure 2].

Method Validation Linearity
The response for the drugs was found to be linear in the concentration range of 1-30 µg/mL each for DRV with correlation coefficient of 0.9997. The linear regression equation obtained was y=38428x+9287 [Figure 3]. Table 1 summarizes the linearity range and the linear regression equation for the drug.

Precision
Precision was expressed as the % RSD of the results. The % RSD values for intra-day precision study were <1.0% and for inter-day study were <2.0%, confirming that the method was sufficiently precise. [Table-1]

Accuracy
Excellent recoveries were obtained at each level of added concentrations of 80%, 100%, 120% (n=3). The result obtained indicated the mean recovery of 99.61-100.05%, demonstrating that the method is accurate within the desired range. [Table 2]

LOD and LOQ
The LOD and LOQ values of DRV were found to be 0.77 and 0.232 µg/mL, respectively. [Table 1]

Robustness
There were no significant differences between results obtained by applying the analytical method under established and varied conditions proving the robustness of the method. The % RSD for the measured peak areas using these variations did not exceed 2% thus showing the method to be robust.

System suitability test parameters
The system suitability test parameters like number of theoretical plates, asymmetric factor, resolution and capacity factor are listed in Table 3.

Specificity
The specificity of the method was proved by checking the peak purity of both analyte peaks in forced degradation samples which were close to 1.0 [Figure 4].

Stability of solutions
The stability of standard working solution as well as sample solutions in water was examined and no chromatographic changes were observed within 24 hr at room temperature.

Degradation behavior
Forced degradations are performed to provide indications of the stability-indicating properties of an analytical method, HPLC studies on Darunavir Ethanolate under different stress conditions suggested the following degradation behavior:

Acidic condition
The drug gradually decreased with time in 1.0 N hydrochloric acid after 24 hr at R.T., forming three degradation products at relative retention time (RRT) 0.57, 0.64 and 0.75. The rate of hydrolysis in acid was lesser as compared to that of alkali [Figure 5].

Alkali Degradation
Major degradation product was observed after exposure of drug in 1.0 N sodium hydroxide after 12 hr at R.T., forming degradation product at relative retention time (RRT) 1.18. The rate of hydrolysis in alkali was higher as compared to other conditions [Figure 6].

Oxidative condition

The drug gradually decreased with time on exposure to forming degradation products at RRT 0.35, 0.46 and 0.74. The rate of degradation in this condition was lesser as compared to that of alkali condition [Figure 7].

**Photolytic conditions**
The photolytic study showed that drug was stable to the effect of light. When the drug powder was exposed to sun light for 72 hrs, no decomposition of the drug was seen [Figure 8].

**Thermal conditions**
The study showed that the drug was stable to the effect of temperature. When the drug powder was exposed to dry heat at 85°C for 24 hrs, no decomposition of the drug was seen [Figure 9].

**Analysis of a formulation**

The proposed method was applied for the determination of Darunavir Ethanolate in tablet (Daruvir). The result of these assays were 99.60 ± 0.39 % of the label claim for the formulation. The result of the assay indicates that the method is selective for assay of Darunavir Ethanolate without interference from excipients used in the tablet.

**CONCLUSION**
The study shows that Darunavir Ethanolate is a labile molecule in acid, oxidative and alkali conditions. It is stable to light and dry heat. A stability-indicating method was developed, which separates all the degradation products formed under variety of conditions. The method proved to be simple, accurate, precise, specific and selective. Hence, it is recommended for analysis of the drug and degradation products in stability samples in research and development or by industry/ QC laboratories.

**TABLES:**
Table-1 Summary of validation parameters for the proposed method

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HPLC method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity</td>
<td>1-30 µg/mL</td>
</tr>
<tr>
<td><strong>Linear regression equation</strong>*</td>
<td></td>
</tr>
<tr>
<td>Intercept (c)</td>
<td>9287</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>38428</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9997</td>
</tr>
<tr>
<td>LOD</td>
<td>0.077 µg/mL</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.232 µg/mL</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td></td>
</tr>
<tr>
<td>Intraday (n=3 ), %</td>
<td>0.38</td>
</tr>
<tr>
<td>Interday (n=3 ), %</td>
<td>0.97</td>
</tr>
<tr>
<td>Repeatability of injection (n=6 ), %</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* y=mx+c
† LOD – Limit of Detection; LOQ – Limit of Quantitation; RSD – Relative Standard Deviation
Table-2 Accuracy data (recovery studies)

<table>
<thead>
<tr>
<th>Amt taken, µg/mL</th>
<th>Amt added, µg/mL</th>
<th>Total amt found, µg/mL</th>
<th>Recovery, %*</th>
<th>RSD, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8</td>
<td>17.93</td>
<td>99.61</td>
<td>0.31</td>
</tr>
<tr>
<td>10</td>
<td>20.01</td>
<td>100.05</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>21.97</td>
<td>99.86</td>
<td>0.99</td>
<td></td>
</tr>
</tbody>
</table>

*Average of six determinations

Table-3 System Suitability Parameters

<table>
<thead>
<tr>
<th>Parameters (n=6)</th>
<th>DRV mean ± SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates</td>
<td>7254 ± 34.19</td>
<td>0.45</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>11.89 ± 0.103</td>
<td>0.87</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>1.06 ± 0.0072</td>
<td>0.68</td>
</tr>
<tr>
<td>Resolution</td>
<td>12.58 ± 0.101</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table-4 Analysis of the formulation

<table>
<thead>
<tr>
<th>Labeled amt, mg/tablet</th>
<th>Amt found, mg/tablet*</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>298.79</td>
<td>99.60 ± 0.39</td>
</tr>
</tbody>
</table>

*Average of six determinations

Table-5 Result of Degradation Study

<table>
<thead>
<tr>
<th>Stress conditions</th>
<th>% Degradation</th>
<th>RRT (min)</th>
<th>Peakpurity index (DRV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>As such</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>1 N HCl, 24 hr at R.T.</td>
<td>12.89%</td>
<td>DP-A 0.57</td>
<td>0.9999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DP-B 0.64</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DP-C 0.75</td>
<td></td>
</tr>
<tr>
<td>1 N NaOH, 12 hr at R.T.</td>
<td>25.51%</td>
<td>DP-D 1.18</td>
<td>0.9999</td>
</tr>
<tr>
<td>30% H2O2, 24 hr at R.T.</td>
<td>19.51%</td>
<td>DP-E 0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DP-F 0.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DP-C 0.74</td>
<td></td>
</tr>
<tr>
<td>Thermal 85°C for 24 hours</td>
<td>negligible</td>
<td>-</td>
<td>0.9995</td>
</tr>
<tr>
<td>Photolytic, 72 hours</td>
<td>negligible</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

* RRT – Relative Retention Time

FIGURES:
Figure 1: Chemical Structure of Darunavir Ethanolate
Figure 2: Representative chromatogram of DRV (10 µg/mL)

Figure 3: Calibration data for analysis of Darunavir ethanolate

\[ y = 38428x + 9287. \quad R^2 = 0.9997 \]

Figure 4: Peak purity spectra of Darunavir ethanolate
Figure 5: Chromatogram showing degradation in acidic condition (1.0 N HCl)

Figure 6: Chromatogram showing degradation in alkaline condition (1.0 N NaOH)

Figure 7: Chromatogram showing degradation in oxidative condition

Figure 8: Chromatogram after exposure of drug to photolytic condition
REFERENCES


